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Myelin Proteomics: Molecular Anatomy of an Insulating Sheath

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Abstract Fast-transmitting vertebrate axons are electrically insulated with multiple layers of nonconductive plasma membrane of glial cell origin, termed myelin. The myelin membrane is dominated by lipids, and its protein composition has historically been viewed to be of very low complexity. In this review, we discuss an updated reference compendium of 342 proteins associated with central nervous system myelin that represents a valuable resource for analyzing myelin biogenesis and white matter homeostasis. Cataloging the myelin proteome has been made possible by technical advances in the separation and mass spectrometric detection of proteins, also referred to as proteomics. This led to the identification of a large number of novel myelin-associated proteins, many of which represent low abundant components involved in catalytic activities, the cytoskeleton, vesicular trafficking, or cell adhesion. By mass spectrometry-based quantification, proteolipid protein and myelin basic protein constitute 17% and 8% of total myelin protein, respectively, suggesting that their abundance was previously overestimated. As the biochemical profile of myelin-associated proteins is highly reproducible, differential proteome analyses can be applied to material isolated from patients or animal models of myelin-related diseases such as multiple sclerosis and leukodystrophies.

Keyword Oligodendrocyte · Leukodystrophy · Myelin · Internode · Proteome · Proteomics · Cytoskeleton · Neurodegeneration · Proteolipid protein · Myelin basic protein

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Introduction

Neuronal signal propagation in vertebrates is sped up by the electrical insulation of axons with an ensheathing, specialized glial plasma membrane: myelin. Myelination of axons reduces their transverse capacitance and increases their transverse resistance [1]. Insulation is achieved by the multilayered arrangement of the myelin membrane (Fig. 1) and its special molecular composition, mainly its very high lipid content. In myelinated axons, action potentials are restricted to periodically spaced small segments spared from coverage with myelin, termed the nodes of Ranvier [2]. In the central nervous system (CNS), any individual oligodendrocyte myelinates up to 50 axon segments, termed internodes [3]. Oligodendrocyte precursor cell division, migration, and regular alignment along the axons have been recently visualized *in vivo* in

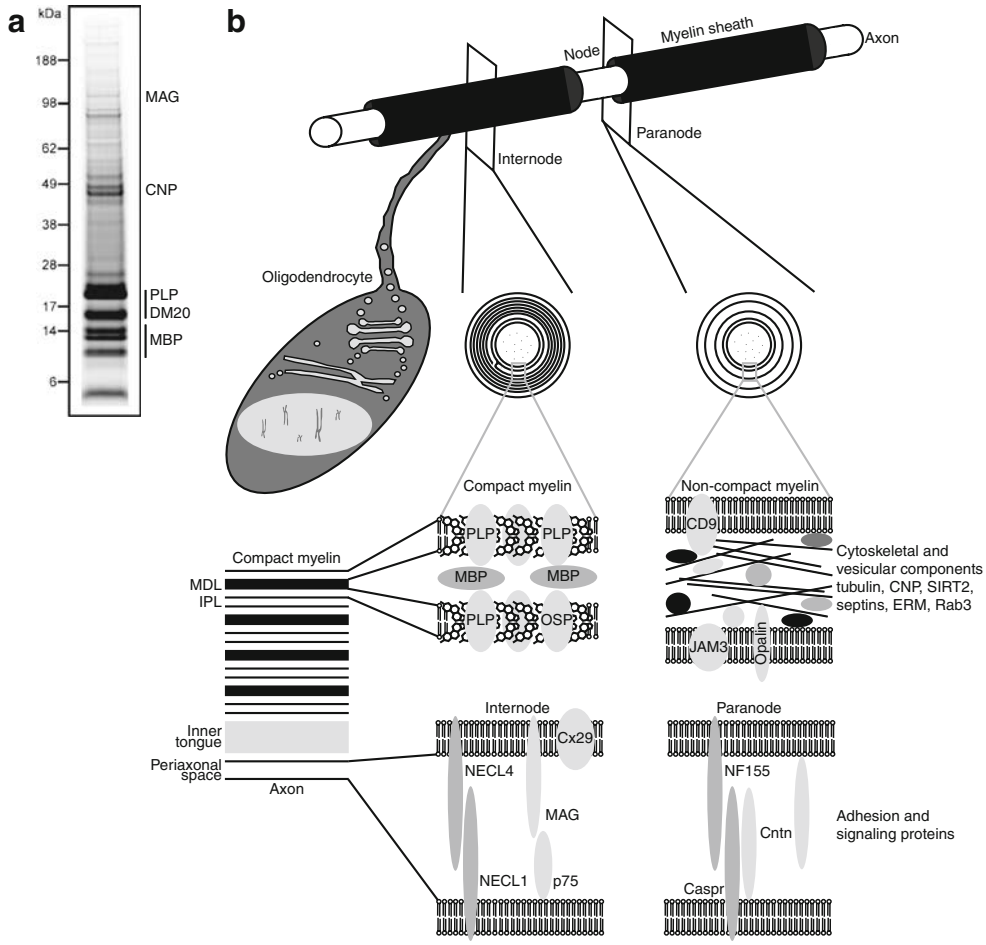

Fig. 1 CNS myelin. **a** Purified mouse brain myelin was one-dimensionally separated in a 4–12% Bis–Tris gradient gel using a morpholineethanesulfonic acid buffer system. Proteins were visualized by colloidal Coomassie staining. Bands constituted by abundant myelin proteins are annotated. **b** Schematic depiction of an oligodendrocyte myelinating an axon, cross-sections in the internodal and paranodal segments, and subcellular localization of myelin proteins. Structural proteins of compact myelin (*middle*), cytoskeletal and vesicular proteins located in uncompacted regions (*right*), and adhesion proteins mediating association with the axon (*bottom*) are shown. *CNP* 2′,3′-cyclic nucleotide phosphodiesterase, *Cntn* contactin, *Caspr* contactin-associated protein, *Cx29* connexin 29 kDa, *DM20* small splice isoform of PLP, *ERM* ezrin, radixin, moesin, *IPL* intraperiod line, *JAM3* junctional adhesion molecule 3, *MAG* myelin-associated glycoprotein, *MBP* myelin basic protein, *MDL* major dense line, *Necl* nectin-like protein, *NF155* neurofascin 155 kDa, *OSP* oligodendrocyte-specific protein/claudin-11, *PLP* proteolipid protein, *Rab3* Ras-related protein Rab3, *SIRT2* sirtuin 2

zebrafish [4], which today complement rodents as an important model organism for myelin research [5–8]. Myelin formation proceeds with outgrowth and retraction of glial cell processes, target axon recognition, stabilization of cellular contacts, rapid biosynthesis and trafficking of lipid and protein constituents of the myelin membrane, and its organization as a multilayered structure around the axon [9, 10]. Once myelinated, axons become dependent on glial support [11]. Some of the molecules involved in myelin development and function are known but a detailed molecular picture has not been gained yet.

That CNS myelin is important for normal sensation, cognition, and motor function is obvious considering that myelin-related disorders often affect humans lethally. Besides the inflammatory demyelinating disease multiple sclerosis [12], there are genetically inherited disorders that affect CNS myelin, collectively termed leukodystrophies [13]. This heterogeneous group of diseases is characterized by the loss of motoric, sensory, and mental capabilities and the susceptibility to seizures. A detailed knowledge of the molecular expression profiles of oligodendrocytes and myelin will be crucial to understand the pathomechanisms of white matter diseases. For example, the mRNAs [14–16] and proteins expressed in cultivated oligodendrocytes [17] and oligodendroglial exosomes [18] have been recently examined. This review focuses on systematic analyses of the molecular composition of mammalian CNS myelin, while no such compendium of

peripheral nervous system (PNS) myelin proteins has been published yet. Proteomics approaches to myelin provide a valuable resource to understand its biogenesis, function, and pathology. Although only a few comparative studies have been reported to date, novel insights into the molecular basis of myelin-related diseases are beginning to emerge.

A Myelin-Enriched Fraction from the Central Nervous System

A comparatively simple method is available for the isolation of a myelin-enriched fraction from the CNS. Biochemically, myelin is defined as the lightweight membranous material accumulating at the interface between 0.32 and 0.85 M sucrose after sequential ultracentrifugation combined with osmotic shocks [19, 20]. The most commonly used protocol starts from brain homogenate contained in 0.32 M sucrose as the top layer, "spinning-down" myelin to accumulate at the interface with the bottom 0.85 M sucrose layer. One valuable modification is "floating-up" of myelin starting from brain homogenate contained in a more concentrated sucrose solution as the bottom layer (0.85, 1.2, 1.44, or 2 M). During ultracentrifugation, myelin also accumulates at the interface between the upper 0.85 and 0.32 M sucrose layers, while other fractions of interest assemble at higher sucrose concentrations. This method allows the simultaneous isolation of other brain fractions such as rough microsomes [21] or axogliosomes [22, 23]. The lightweight fraction from the interphase between 0.32 and 0.85 M sucrose is the most frequently used one for biochemical and proteomic experiments. This fraction is enriched in the most abundant proteins of compact myelin, proteolipid protein (PLP), and myelin basic protein (MBP), and as revealed by electron microscopy, mainly contains multilamellar membranes with a periodicity comparable to that of myelin in native or perfused brains [24, 25]. However, we suggest to term this fraction "myelin-enriched" rather than "compact myelin", as it also contains proteins from the noncompacted cytosolic channels in myelin (i.e., adaxonal and paranodal myelin) and proteins associated with the axonal membrane. Myelin purification is very reproducible across different laboratories, even when applied to different species (e.g., mouse–rat) or to mutant mice with altered myelin protein or lipid composition, such as $Cnp^{Cre/+}$*$Fdft^{flox/flox}$ [26], $Ugt3a1^{null}$ [27], $Arsa^{null}$ [28], and Plp^{null} [29] (see below). Thus, the method has proven to be very robust, explaining why the original protocol from the early 1970s is still in common use. It is generally assumed that myelin purification relies on its special lipid content and composition.

Myelin Lipids

The molecular composition of myelin differs from other plasma membranes in that it contains 70–75% of its dry weight as lipid, unusually high compared to other eukaryotic plasma membranes. Also, its molar ratio of lipids with approximately 2:2:1:1 for cholesterol/phospholipid/galactolipid/plasmalogen [30, 31] distinguishes myelin from other cellular membranes. The abundance of cholesterol within a membrane affects its biophysical properties, including fluidity and curving [32]. Cholesterol has earlier been identified as unusually enriched in myelin and constitutes 24–28% of the total myelin lipids [19]. That the cellular cholesterol supply is rate-limiting for myelin membrane biogenesis has been shown in mice lacking squalene synthase (also termed farnesyl diphosphate farnesyl transferase [FDFT]) exclusively in myelinating glia [26]. FDFT mediates a crucial step of cholesterol biosynthesis. CNS myelination is severely delayed in $Cnp^{Cre/+}$*$Fdft1^{flox/flox}$ mice, and that any myelin made in these mice is likely due to compensatory cholesterol uptake from other cells [26].

The biophysical properties of myelin are also influenced by its unusually high concentration of the galactolipids galactosylceramide (GalC), its sulfated form 3-O-sulfogalactosylceramide (SGalC), and their hydroxylated forms GalC-OH and SGalC-OH. Together, they add up to 20–26% of total myelin lipids. Myelination is moderately delayed in mice lacking UDP-galactose:ceramide galactosyltransferase (Ugt3a1), an enzyme required for galactolipid synthesis. Additionally, impaired glia–axonal interactions at the paranodes were observed [27, 33, 34]. Paranodal disruption was at least partly due to the lack of SGalC and hydroxylated galactolipids, since the long-term integrity of the sodium channel domain of the nodes of Ranvier was also impaired in mice lacking galactosylceramide-3-O-sulfotransferase (Gal3st1), the enzyme converting GalC into SGalC [35–37], and late onset myelin degeneration was also reported for mice lacking fatty acid 2-hydroxylase (Fa2h), the enzyme hydroxylating GalC and SGalC [38]. Absence of functional arylsulfatase A (ARSA), the enzyme degrading SGalC, causes metachromatic leukodystrophy (MLD), illustrating that a regulated galactolipid metabolism is required for long-term integrity of the white matter. SGalC accumulation and many pathological features of MLD are modeled in $Arsa^{null}$ mice and in transgenic mice overexpressing Ugt3a1 or Gal3st1 in neurons or oligodendrocytes [28, 39, 40]. Sulfatide metabolism with respect to myelin and MLD pathology was recently reviewed [41].

Also, the plasmalogen class of phospholipids is associated with white matter disease. Plasmalogens are ether-linked (as opposed to ester-linked) phospholipids, the main species being ethanolamine–plasmalogen. They are ubiquitous structural components of mammalian cell membranes

and amount to 12–15% of total myelin lipid [19] and, when processed by plasmalogen-selective phospholipase A2, give rise to the second messengers arachidonic acid and eicosanoids [42]. At low concentrations, these metabolites have trophic effects, but at high levels, they are cytotoxic and may induce inflammation [43]. The reactivity of the alkenyl ether bond makes plasmalogens more susceptible to oxidative reactions than their fatty acid ester analogs. Thus, myelin plasmalogens may act as endogenous antioxidants protecting cells from oxidative stress [44]. Disrupted activity of peroxisomal plasmalogen synthesizing enzymes results in peroxisomal biogenesis disorders such as rhizomelic chondrodysplasia punctata (RCDP) in which hypomyelination of the optic nerve has been observed. Decreased plasmalogen levels [45, 46] and increased levels of reactive oxygen species [47, 48] may also contribute to the demyelination in X-linked adrenoleukodystrophy caused by the mutated peroxisomal transporter ABCD1, suggesting that a normal plasmalogen metabolism may prevent peroxisomal- and myelin-related disease. Mice lacking dihydroxyacetonephosphate acyltransferase (DAPAT) model several aspects of the RCDP pathology, including optic nerve hypoplasia [49]. Interestingly, the association of flotillin-1 and contactin with plasmalogen-deficient brain membrane microdomains was diminished in DAPAT^{null} mice [49], suggesting that the local concentration of membrane lipids dictates the association of particular proteins.

Association of Myelin Lipids and Proteins

Cholesterol assembles with galactolipids and plasmalogens within the plane of the membrane, but how they are enriched to the levels found in myelin is unknown. It has been suggested that lipids are targeted to future myelin membrane by their association with myelin-bound proteins [9]. SGalC appears to be an example to the contrary. SGalC is associated with myelin and lymphocyte protein (MAL) [50]. Lack of SGalC and lack of MAL lead to similar paranodal malformation [35, 51]. The subcellular trafficking of MAL, as well as its abundance in myelin, is determined by SgalC [28], whereas SGalC abundance is not altered in Mal^{null} myelin [51]. It is likely that other myelin proteins are also incorporated into the sheath by attachment with future myelin membrane because of its special lipid composition. Thus, whether myelin proteins dictate the fate of lipids or vice versa may not be generalized. It appears likely that the association of both molecule classes results in each other's control of abundance and trafficking.

That myelin lipids and proteins are closely associated was suggested earlier after the characterization of two types of protein fractions isolated from the white matter based on their resistance to aqueous or organic solvents or to enzymatic proteolysis. One fraction behaved as a lipid with regard to its solubility and was termed PLP [52, 53]. PLP was later identified to be the most abundant protein of mammalian CNS myelin. It has a high affinity to phospholipids and cholesterol [54–56], and impaired interactions of mutant PLP with membrane lipids are a likely key step in the molecular pathogenesis of the leukodystrophy Pelizaeus–Merzbacher disease [57]. The other fraction, termed trypsin-resistant protein residue, was insoluble in organic solvent and attached to the membrane lipid phosphatidylinositol phosphate [58, 59]. The application of extraction methods by Folch became commonly used to categorize myelin proteins according to their biophysical properties.

More recently, the myelin-enriched brain fraction has been chemically subfractionated by differential detergent extraction at low temperatures, resulting in distinct non-identical but overlapping assemblies of myelin-associated proteins and lipids that were suggested to represent myelin subcompartments [60, 61]. Cholesterol- and galactolipid-rich membrane microdomains (also referred to as "lipid rafts") have been suggested to deliver myelin proteins to the plasma membrane [62–64]. The relevance of applying the analysis of biochemical characteristics established for membrane microdomains to such a large structure as myelin has remained debated. However, it is widely accepted now that lipid-associated cell signaling molecules, such as the protein tyrosine kinase fyn, have central roles in myelination [65, 66].

In oligodendroglial processes, fyn is activated by axonal signals via integrin alpha6beta1 [67]. Among other fyn substrates [68, 69], the protein translation repressor heterogeneous nuclear ribonucleoprotein (hnRNP) A2 upon phosphorylation is released from its binding site in the 3′ UTR of mRNA encoding MBP [70], the second-most abundant myelin protein. hnRNP A2 binding represses translation during the translocation of MBP mRNA to distal sites of the cell [71] where newly translated MBP is directly incorporated into the extending oligodendroglial process [21, 72]. It is generally assumed that MBP mediates the adhesion of the cytoplasmic surfaces between the individual layers of compact myelin [73] via binding of its many basic residues with the negatively charged headgroups of membrane lipids. Indeed, membrane association of MBP is controlled by the membrane lipid phosphatidylinositol-(4,5)-bisphosphate [74–76]. For over 30 years, it has been known that MBP is highly heterogeneous due to alternative splicing and multiple post-translational modifications (PTMs) [77]. More recently, modern mass spectrometric techniques have been used to compare the PTMs of MBP from normal and multiple sclerosis brains with respect to

methylation, phosphorylation, and arginine deimination [78]. PTM alterations affect charge, conformation, and hydrogen bonding of MBP, which may modulate its affinity to the myelin membrane and play a role in myelin compaction and in the pathogenesis of demyelinating diseases. MBP is the only myelin protein that has been shown to be essential for myelin formation, as became obvious with the analysis of the natural mouse mutant *shiverer* and the rat mutant *long evans shaker* [79, 80], which are severely hypomyelinated. Interestingly, mice lacking fyn are also hypomyelinated [81, 82], likely due to affected translational regulation of MBP expression [70, 83]. Together, a multitude of factors affects mRNA transcription and transport, translation at axonal contact sites, or membrane binding of MBP, and we speculate that several myelin proteins with yet unidentified roles affect MBP abundance and function.

Systematic Analysis of the CNS Myelin Protein Composition

The relative abundance of myelin proteins has previously been calculated based on their binding to Buffalo black [84], Fast green [85], or Coomassie blue [86] after separation in one-dimensional (1D) sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE). In these measurements, a small number of proteins was determined to be extraordinarily abundant in CNS myelin. PLP and its smaller splice isoform DM20 accounted for 30–45% of total myelin protein, two of the four MBP splice isoforms for 22–35%, 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP) for 4–15%, and all remaining proteins for 5–25% [19, 85, 87, 88]. Similarly, PNS myelin is also dominated by two proteins, myelin protein zero (MPZ, P0) and MBP, which have been estimated to account for 50–70% and 15%, respectively [89]. In comparison, the most abundant proteins in a brain fraction enriched for synaptic vesicles are synaptobrevin 2 and synaptophysin, which constitute 8% and 10% of the total synaptic vesicle proteins, respectively, as revealed by quantitative immunoblotting [90]. How and why myelin proteins are enriched to their unusual relative abundance is unclear, considering that PLP and CNP are not essential for the formation of normal amounts of CNS myelin [29, 91, 92].

Various proteomic techniques have been applied towards the systematic protein composition analysis of the myelin-enriched fraction. Traditionally, first insights into proteomes of subcellular structures often come from two-dimensional (2D) protein maps generated by utilizing isoelectric focusing (IEF) with immobilized pH gradients in the first and SDS-PAGE in the second dimension (2D-IEF/SDS-PAGE) (Fig. 2a). Proteins of interest are then excised from the gel, proteolytically digested *in situ*, and finally, identified by mass spectrometry (MS) [93]. Due to its high resolving power, 2D-IEF/SDS-PAGE can be routinely applied for profiling of proteins from complex mixtures and, as protein integrity is retained, also leads to information on protein abundance and processing [94]. However, major shortcomings of 2D-IEF/SDS-PAGE concern a limited dynamic range, the display of basic and hydrophobic proteins, and—most importantly—the under-representation of membrane proteins. As myelin is dominated by MBP (a highly basic protein) and PLP (a hydrophobic tetraspan protein), incremental improvements in 2D-IEF/SDS-PAGE technology were required before the first 2D mapping of myelin was presented [95]. By using the zwitterionic detergent amidosulfobetaine-14 (ASB-14) instead of the most commonly used 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) [96], it was possible to solubilize myelin proteins much more effectively and to identify 98 proteins (91 by MS and seven by immunoblotting) in the myelin-enriched fraction from mouse CNS [95]. This crucial effect of the solubilization conditions is further underscored by two more recent 2D-IEF/SDS-PAGE mapping studies of similar input material. Thirty-eight myelin-associated proteins were identified in one study after CHAPS solubilization [97], but 131 proteins were identified in another study with ASB-14 [25]. Thus, at least in the presence of appropriate detergents, myelin can now be considered as well accessible by 2D-IEF/SDS-PAGE, which not only facilitates protein cataloging but also paves the way for differential myelin proteomics on the basis of the 2D differential fluorescence intensity gel electrophoresis technology (2D-DIGE, see below). It is important to note that all conventional 2D mapping approaches mentioned above failed to appropriately display relatively abundant transmembrane myelin marker proteins such as PLP, myelin-associated glycoprotein (MAG) [98], myelin oligodendrocyte glycoprotein (MOG) [99], tetraspanin 2 [100], M6B [101], or oligodendrocyte-specific protein (OSP/claudin-11) [102–104]. A potential remedy is to perform the first dimension separation as nonequilibrium pH gradient electrophoresis for the 2D mapping of myelin proteins [105]. However, although this method appeared promising particularly for displaying highly basic proteins, it did not get as popular as 2D-IEF/SDS-PAGE with immobilized pH gradients, mainly due to limitations in reproducibility and resolution.

More complete proteome coverage while retaining the benefits of displaying intact proteins can be reached by the additional use of alternative 2D gel systems. Here, the charge-dependent separation in the first dimension (i.e., the IEF) is replaced by a size-dependent separation in the presence of cationic detergents such as 16-benzyldimethyl-*n*-hexadecylammonium chloride (16-BAC; Fig. 2b) [106]

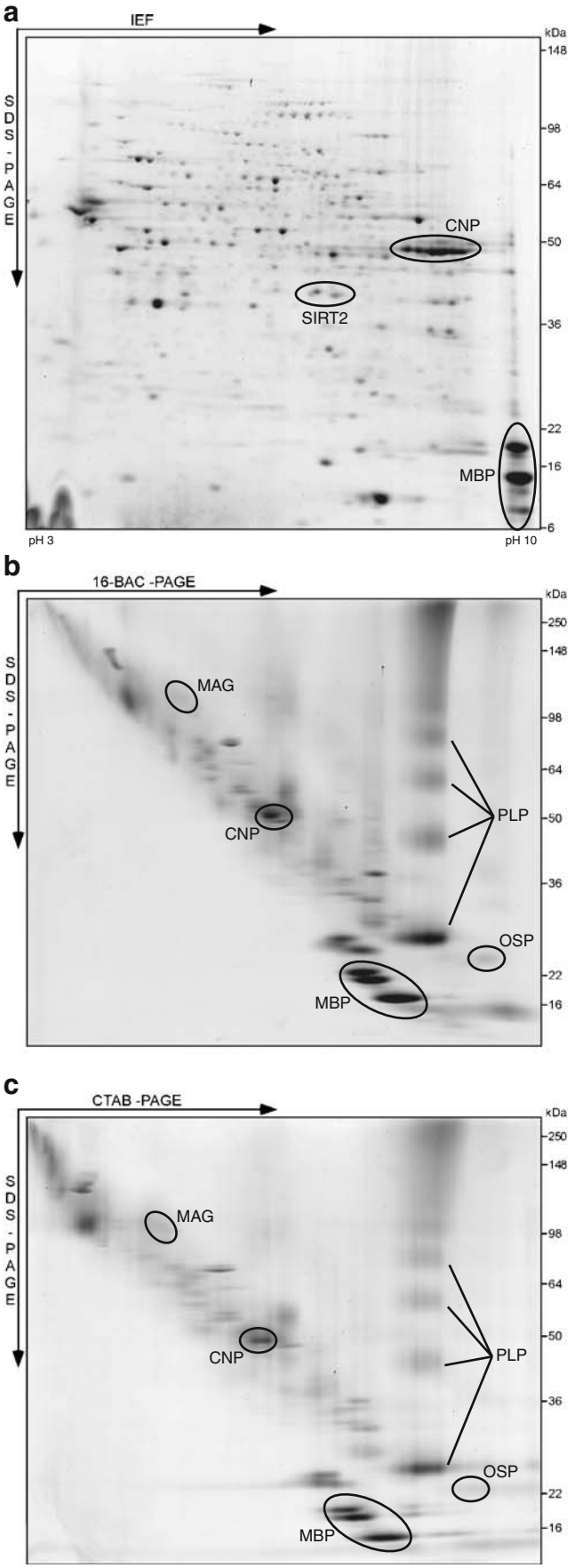

Fig. 2 Gel-based myelin proteome maps. Purified mouse brain myelin was two-dimensionally separated in different gel systems. Proteins were visualized by colloidal Coomassie staining, and spots constituted by selected myelin proteins are indicated. **a** 2D-IEF/SDS-PAGE with IEF in a nonlinear pH gradient (pH 3–10) as the first and gradient SDS-PAGE (8–16% acrylamide) as the second dimension. To improve resolution, myelin was delipidated and precipitated by a methanol/chloroform treatment prior to IEF [25]. **b** 2D-16-BAC/SDS-PAGE with separation in a 16-BAC gel (10% acrylamide) as the first and gradient SDS-PAGE (8–16% acrylamide) as the second dimension. **c** 2D-CTAB/SDS-PAGE with separation in a CTAB gel (10% acrylamide) as the first and gradient SDS-PAGE (8–16% acrylamide) as the second dimension. To deplete soluble and membrane-associated proteins, myelin was subjected to a multistep wash procedure before separation [25]. 16-BAC and CTAB resulted in similar spot patterns. 2D-IEF/SDS-PAGE provides good resolution but basic, hydrophobic, and transmembrane proteins are under-represented. 2D-16-BAC/SDS-PAGE and 2D-CTAB/SDS-PAGE lead to efficient representation of basic, hydrophobic, and transmembrane proteins but have a lower resolution since separation occurs by protein size in both dimensions

or cetyltrimethylammonium bromide (CTAB; Fig. 2c) [107]. Due to the similar separation principle in both dimensions, proteins are typically dispersed along a diagonal rather than distributed over the entire gel area. Accordingly, these gel systems have a lower resolution compared to 2D-IEF/SDS-PAGE, but can resolve highly basic and even membrane-spanning proteins [108]. Application of 2D-16-BAC/SDS-PAGE to mouse CNS myelin resulted in the identification of 62 proteins and readily enabled displaying of the transmembrane myelin proteins PLP, MAG, MOG, and OSP/claudin-11 [25]. Thus, the combination of 2D-IEF/SDS-PAGE and 2D-16-BAC/SDS-PAGE has, so far, yielded the most comprehensive gel-based proteome compendium of mouse CNS myelin, consisting of 162 nonredundant proteins [25]. Further technical refinements of the method were established in a recent systematic evaluation of five different cationic detergents for the 2D gel electrophoresis of myelin proteins. Here, 16-BAC was the most effective agent for the separation of myelin proteins in the first dimension, while CTAB was most effective for their solubilization [109, 110]. As resolution improves, 2D gel electrophoresis with cationic detergents may be combined with the DIGE technology as a future tool for monitoring abundance changes of highly basic and membrane-spanning myelin proteins [111].

To overcome the limitations of gel-based proteomic methods, in particular those of 2D-IEF/SDS-PAGE, gel-free techniques, commonly referred to as shotgun approaches, have emerged in recent years [93, 112]. Here, separation at the level of intact proteins is omitted and the protein preparation is proteolytically digested at the expense of information related to protein integrity, such as protein size and charge. Separation takes place at the level of proteolytic

peptides before interfacing with MS. The tremendous complexity of such peptide mixtures requires a high resolving power and is, therefore, often addressed by the application of 2D liquid chromatography (2D-LC), usually consisting of strong cation exchange in the first and reversed-phase chromatography in the second dimension. In the first application of shotgun proteomics to the myelin-enriched fraction from the mouse CNS [97], 93 proteins were identified resulting—by combination with 2D-IEF/SDS-PAGE (see above)—in a myelin proteome compendium consisting of 103 proteins. The application of a similar shotgun approach to a myelin-enriched fraction from rat CNS led to the identification of 97 myelin proteins [23]. Both shotgun approaches yielded quite a high overlap of approximately 50% with the so far most comprehensive gel-based library [25] and contained transmembrane myelin proteins such as PLP, MAG, and MOG. highly abundant PLP and MBP due to limitations concerning gel separation and/or protein staining.

We conclude that modern LC-MS-based approaches—though technically more demanding than gel-based studies—appear to be appropriate for tackling the myelin proteome as they cover several orders of magnitude of protein abundance and detect highly basic, hydrophobic, and membrane-spanning proteins. This tackles the bias towards certain protein classes, which is the major shortcoming particularly of 2D-IEF/SDS-PAGE (Fig. 4c). Moreover, LC-MS-based approaches enable the gel- and label-free quantification of proteins from complex mixtures, which allowed for the systematic reassignment of protein abundance in CNS myelin (see above). Finally, they require only low amounts of sample, which is of special relevance for the proteome analysis of myelin purified from hypomyelinated model animals or human brain autopsy material.

Relative Abundance of Myelin Proteins

To understand myelin biogenesis and pathology, a comprehensive knowledge of the proteins associated with myelin is a prerequisite. We have confirmed and expanded the previous myelin protein compendia by applying nanoscale 1D ultra performance liquid chromatography (1D-UP-LC) separation coupled to detection with a quadrupole time-of-flight (QTOF) mass spectrometer (Tenzer et al., unpublished). Data were acquired by LC-MS using an alternating low (MS) and elevated (MS^E) collision energy mode of acquisition (LC-MS^E), which allows simultaneous identification and label-free relative quantification of the proteins in the sample [113–115]. The identified peptides were annotated to a total of 294 myelin-associated proteins (Table 1) based on a minimum of two peptides per protein with an effective false-positive rate of <0.2%. They showed a very good overlap of 141 proteins that were also detected in previous myelin proteome analyses and included several established myelin markers (Table 1 and Fig. 3). We have calculated the relative abundance of the myelin-associated proteins based on the average intensity of the three most abundant peptides per protein. In the few cases where only two peptides were identified, their average intensity was used. Strikingly, PLP, MBP, and CNP constituted only 17%, 8%, and 4% of the total myelin-associated proteins, respectively (Fig. 4). All previously known myelin proteins together constituted 35%, while newly identified myelin-associated proteins accounted for 65%. These quantifications take into question previous estimates based on conventional techniques (Fig. 4b and see above). We suggest that the complexity of myelin protein composition has been overlooked because low abundant proteins did not constitute significant bands on gels when compared to the

Technical Limitations

How pure is the myelin-enriched fraction? Myelin-associated proteins are defined as proteins in the myelin-enriched fraction since all studies have operationally defined the term “myelin protein” without systematic experimental verification. Although the identification of new myelin proteins by more than one study and the detection of established myelin markers increase confidence, some of these proteins may only have copurified with myelin. The high dynamic range of LC-MS^E leads to the new identification of proteins as myelin-associated, but also to the false-positive identification of contaminants. These mainly stem from copurifying mitochondria and synaptic vesicles. In reverse, proteomic compendia of mammalian brain mitochondria [116] or synaptic vesicles [90] include classical myelin proteins such as PLP, MBP, MOBP, and MAG. Notwithstanding that some of these proteins may have a dual localization, cross-contamination occurs likely due to similar floatation properties in sucrose or Percoll gradients and can only be excluded once improved separation protocols become available. Proteins of the axonal plasma membrane, such as potassium channels or Na^+/K^+-ATPases, have also been detected in the myelin fraction, which can be explained by the tight linkage of the membranes via adhesion proteins, sometimes referred to as the myelin–axolemma complex [24]. Indeed, some adhesion complexes are present in the myelin-enriched fraction, such as the glial neurofascin (NF155) and contactin and their axonal partner contactin associated protein 1 (Caspr) [117–120] and the glial nectin-like protein Necl4 and its axonal counterpart Necl1 [121–124]. Importantly, myelin proteome analysis also revealed novel candidate proteins to mediate intracellular or intercellular

Table 1 The CNS myelin proteome

Protein name	ID	Gene	Reference
A: Known myelin proteins			
CD81	P35762	Cd81	E
CD9	P40240	Cd9	ND
Claudin 11, OSP	Q60771	Cldn11	B,S,T,E
CNP	P16330	Cnp	W,B,S,R,T,E
Contactin 1	P12960	Cntn1	B,S,R,T,E
Ermin	Q5EBJ4	Ermn	E
Ezrin	P26040	Ezr	W,T,E
Glycoprotein M6B	P35803	Gpm6b	E
Myelin and lymphocyte protein	O09198	Mal	ND, T (blot)
Myelin-associated glycoprotein	P20917	Mag	B,S,R,E
Myelin basic protein	P04370	Mbp	W,B,S,V,R,E
Myelin oligodendrocyte glycoprotein	Q61885	Mog	B,S,R,E
Myelin protein zero, P0	P27573	Mpz	R
Myelin proteolipid protein	P60202	Plp1	B,S,R,T,E
Myelin/oligodendrocyte basic protein	Q9D2P8	Mobp	E
Necl1, Ig superfamily member 4b	Q99N28	Cadm3	S
Necl4, Ig superfamily member 4c	Q8R464	Cadm4	S,E
Neural cell adhesion molecule 1	P13595	Ncam1	W,S,R,T,E
Neurofascin	Q810U3	Nfasc	B,R,E
Oligodendrocyte myelin glycoprotein	Q63912	Omg	ND
Opalin, TMP10	Q7M750	Opalin	R,E
Plasmolipin	Q9DCU2	Pllp	E
Ras-related protein Rab 3A	P63011	Rab3a	E
Ras-related protein Rab 3C	P62823	Rab3c	E
Sirtuin 2	Q8VDQ8	Sirt2	W,S,V,R,T,E
Tetraspanin 2	Q922J6	Tspan2	E
B: Newly identified myelin-associated proteins			
14-3-3 protein beta	Q9CQV8	Ywhab	E
14-3-3 protein epsilon	P62259	Ywhae	S,R,E
14-3-3 protein eta	P68510	Ywhah	E
14-3-3 protein gamma	P61982	Ywhag	W,V,R,T,E
14-3-3 protein sigma, stratifin	O70456	Sfn	E
14-3-3 protein theta	P68254	Ywhaq	E
14-3-3 protein zeta delta	P63101	Ywhaz	S,R,E
Actin α cardiac muscle 1	P68033	Actc1	E
Actin α1	P68134	Acta1	E
Actin α	P62737	Acta2	R,E
Actin β	P60710	Actb	W,S,V,R,T,E
Actin γ1	P63260	Actg1	B,E
Actin γ2	P63268	Actg2	E
Acyl-CoA thioesterase 7	Q91V12	Acot7	R,E
ADAM 23	Q9R1V7	Adam23	E
Adenylate cyclase associated 1	P40124	Cap1	T
ADP ribosylation factor 1	P84078	Arf1	S,T,E
ADP ribosylation factor 2	Q8BSL7	Arf2	E
ADP ribosylation factor 3	P61205	Arf3	E
ADP ribosylation factor 4	P61750	Arf4	E
ADP ribosylation factor 5	P84084	Arf5	E
ADP ribosylation factor 6	P62331	Arf6	W,E
Aldehyde dehydrogenase 1A1	P24549	Aldh1a1	E
Aldolase A, fructose-bisphosphate	P05064	Aldoa	W,S,V,R,T,E
Aldolase C, fructose bisphosphate	P05063	Aldoc	R,T,E
Amphiphysin 2, bridging integrator 1	O08539	Bin1	E
Anillin	Q8K298	Anln	R,E
Annexin A2	P07356	Anxa2	E
Annexin A6	P14824	Anxa6	R,T
Argininosuccinate synthase 1	P16460	Ass1	B,E
α-Synuclein	O55042	Snca	E
Band 4.1 like protein 3	Q9WV92	Epb4.1l3	E
Brain acid soluble protein 1, NAP22	Q91XV3	Basp1	S,E
Breast carcinoma amplified seq 1	Q80YN3	Bcas1	S,E
β-Synuclein	Q91ZZ3	Sncb	E
Ca^{++} ATPase 1	Q3TSK3	Atp2b1	E
Ca^{++} ATPase 2	Q9R0K7	Atp2b2	E
Ca^{++} ATPase 3	Q0VF55	Atp2b3	E
Ca^{++} ATPase 4	Q6Q476	Atp2b4	E
Calmodulin CaM	P62204	Calm3	S,V,E
Calnexin	P35564	Canx	B,R
Calpain 5	O08688	Capn5	T
CaM kinase IIα	P11798	Camk2a	E
CaM kinase IIβ	P28652	Camk2b	E
CaM kinase IIδ	Q6PHZ2	Camk2d	E
CaM kinase IIγ	Q923T9	Camk2g	E
Cannabinoid receptor interacting 1	Q5M8N0	Cnrip1	W,E
Carbonic anhydrase 2	P00920	Car2	W,S,T,E
CD47, integrin signal transducer	Q61735	Cd47	E
CD82	P40237	Cd82	E
CDGSH iron sulfur domain 1	Q91WS0	Cisd1	E
Cell cycle exit and neuronal diff.	Q9JKC6	Cend1	E
Cell division control protein 42	P60766	Cdc42	W,E
Centractin α	P61164	Actr1a	W
Choline transporter CD92	Q6X893	Slc44a1	E
Clathrin heavy chain	Q68FD5	Cltc	B,R,E
Cofilin 1	P18760	Cfl1	S,V,T,E
Cofilin 2	P45591	Cfl2	E
Contactin associated protein 1	O54991	Cntnap1	B,E
Coronin 1C	Q9WUM4	Coro1c	E
Creatine kinase brain	Q04447	Ckb	W,S,V,R,T,E
Crystallin α2	P23927	Cryab	W,S,T,E
Cyclophilin A	P17742	Ppia	W,S,V,E
Cysteine and glycine rich protein 1	P97315	Csrp1	E
Cytokeratin 1	P04104	Krt1	E
Cytokeratin 1B	Q6IFZ6	Krt77	E

Table 1 (continued)

Protein name	ID	Gene	Reference
Cytokeratin 5	Q922U2	Krt5	E
Cytokeratin 6A	P50446	Krt6a	E
Cytokeratin 6G	Q9R0H5	Krt71	E
Cytokeratin 10	P02535	Krt10	R,E
Cytokeratin 16	Q9Z2K1	Krt16	E
Desmin	P31001	Des	E
Destrin	Q9R0P5	Dstn	E
Dihydropyrimidinase-like 1, CRMP1	P97427	Crmp1	E
Dihydropyrimidinase-like 2, CRMP2	O08553	Dpysl2	W,B,S,V,R,T,E
Dihydropyrimidinase-like 3, CRMP4	Q62188	Dpysl3	E
Dihydropyrimidinase-like 4, CRMP3	O35098	Dpysl4	E
Dipeptidylpeptidase 6	Q9Z218	Dpp6	T
Down syndrome cell adhesion like 1	Q8R4B4	Dscaml1	E
Dynactin 2	Q99KJ8	Dctn2	V
Dynamin 1	P39053	Dnm1	W,B,R,T,E
Dynamin 2	P39054	Dnm2	E
Dynamin 3	Q8BZ98	Dnm3	R
Dynein heavy chain	Q9JHU4	Dync1h1	R
Ectonucleotide pyrophosphatase 6	Q8BGN3	Enpp6	E
EH domain containing protein 1	Q9WVK4	Ehd1	B,S,T,E
EH domain containing protein 3	Q9QXY6	Ehd3	B,E
EH domain containing protein 4	Q9EQP2	Ehd4	E
Elongation factor 1α1	P10126	Eef1a1	W,B,S,R,E
Elongation factor 1α2	P62631	Eef1a2	W,B,E
Elongation factor 1β	O70251	Eef1b2	T
Elongation factor 2	P58252	Eef2	T
Endonuclease domain containing 1	Q8C522	Endod1	E
Enolase 1, non-neuronal	P17182	Eno1	W,B,S,V,T,E
Enolase 2, neuronal	P17183	Eno2	W,S,V,T,E
Enolase 3, muscle	P21550	Eno3	E
Fascin	Q61553	Fscn1	W,E
Fatty acid synthase	P19096	Fasn	R
FK506 binding protein 1a	P26883	Fkbp1a	S,E
Flotillin 1	O08917	Flot1	ND, T (blot)
G protein α transducing 1	P20612	Gnat1	E
G protein α transducing 2	P50149	Gnat2	E
G protein α transducing 3	Q3V3I2	Gnat3	E
G protein α11	P21278	Gna11	E
G protein α14	P30677	Gna14	E
G protein αI1	B2RSH2	Gnai1	E
G protein αI2	P08752	Gnai2	E
G protein αI3	Q9DC51	Gnai3	E
G protein αO1	P18872	Gnao1	S,T,E
G protein αO2	P18873	Gna0	B,T,E
G protein αq	P21279	Gnaq	T,E

Table 1 (continued)

Protein name	ID	Gene	Reference
G protein αS	P63094	Gnas	S,E
G protein αS olfactory	Q8CGK7	Gnal	E
G protein β1	P62874	Gnb1	W,S,V,T,E
G protein β2	P62880	Gnb2	W,V,R,E
G protein β3	Q61011	Gnb3	E
G protein β4	P29387	Gnb4	W,E
G protein β5	P62881	Gnb5	W
G protein γ12	Q9DAS9	Gng12	E
GAPDH	P16858	Gapdh	W,S,V,T,E
GAPDH sperm	Q64467	Gapdhs	E
Gelsolin	P13020	Gsn	V,R,T
Glial fibrillary acidic protein	P03995	Gfap	W,B
Glucose-6-phosphate isomerase	P06745	Gpi1	B,R,E
Glutamate oxaloacetate transaminase	P05201	Got1	E
Glutamate transporter GLAST	P56564	Slc1a3	E
Glutamate transporter GLT1	P43006	Slc1a2	R,E
Glutamine synthetase	P15105	Glul	W,S,V,T,E
Glutathione S transferase micros. 3	Q9CPU4	Mgst3	E
Glutathione S transferase Mu1	P10649	Gstm1	W,E
Glutathione S transferase Mu2	P15626	Gstm2	E
Glutathione S transferase Mu6	O35660	Gstm6	E
Glutathione S transferase P1	P19157	Gstp1	S,V,E
Glutathione S transferase P2	P46425	Gstp2	T
Growth associated protein 43	P06837	Gap43	T
GTPase Ran	P62827	Ran	E
H^+/K^+ ATPase α1	Q64436	Atp4a	E
H^+/K^+ ATPase α2	Q9Z1W8	Atp12a	E
Heat shock 70 kDa protein 1A	Q61696	Hspa1a	E
Heat shock 70 kDa protein 1B	P17879	Hspa1b	R,E
Heat shock 70 kDa protein 1L	P16627	Hspa1l	E
Heat shock 70 kDa protein 2	P17156	Hspa2	W,B,E
Heat shock 70 kDa protein 4	Q61316	Hspa4	T
Heat shock 70 kDa protein 5	P20029	Hspa5	W,T,E
Heat shock 70 kDa protein 8	P63017	Hspa8	W,B,S,V,R,T,E
Heat shock 70 kDa protein 12A	Q8K0U4	Hspa12a	E
Heat shock protein 90 kDa αA1	P07901	Hsp90aa1	B,E
Heat shock protein 90 kDa αB1	P11499	Hsp90ab1	T,E
Hexokinase 1	P17710	Hk1	T,E
Hexokinase 2	O08528	Hk2	E
Ig superfamily member 8, EWI-2	Q8R366	Igsf8	B,S,R,E
Internexin α, Neurofilament 66 kDa	P46660	Ina	W,B,V,R,T,E
Junctional adhesion molecule C	Q9D8B7	Jam3	S,E
K^+ channel A1	P16388	Kcna1	E
K^+ channel A2	P63141	Kcna2	E
K^+ channel A3	P16390	Kcna3	E
K^+ channel B2	P62482	Kcnab2	E

Table 1 (continued)

Protein name	ID	Gene	Reference
Lactate dehydrogenase A	P06151	Ldha	T,E
Lactate dehydrogenase B	P16125	Ldhb	W,T,E
Lactate dehydrogenase C	P00342	Ldhc	E
Leucine rich repeat containing 57	Q9D1G5	Lrrc57	E
Leucine rich repeat LGI 3	Q8K406	Lgi3	E
Limbic system associated membrane	Q8BLK3	Lsamp	S,E
Lymphocyte antigen 6H	Q9WUC3	Ly6h	E
Macrophage migration inhibitory factor	P34884	Mif	W,S,E
Malate dehydrogenase	P14152	Mdh1	W,S,V,T,E
MARCKS related protein	P28667	Marcksl1	S
Microtubule associated protein 1B	P14873	Mtap1b	E
Microtubule associated protein 6	Q7TSJ2	Mtap6	E
Microtubule associated protein tau	P10637	Mapt	E
Mitogen activated protein kinase 1	P63085	Mapk1	E
Moesin	P26041	Msn	W,E
Munc 18, syntaxin binding protein 1	O08599	Stxbp1	B,R,T,E
Myosin Id	Q5SYD0	Myo1d	B,R,E
Na^+/K^+ ATPase α1	Q8VDN2	Atp1a1	B,S,R,E
Na^+/K^+ ATPase α2	Q6PIE5	Atp1a2	B,R,E
Na^+/K^+ ATPase α3	Q6PIC6	Atp1a3	B,R,E
Na^+/K^+ ATPase α4	Q9WV27	Atp1a4	E
Na^+/K^+ ATPase β1	P14094	Atp1b1	B,S,R,E
Na^+/K^+ ATPase β3	P97370	Atp1b3	E
$Na^+/K^+/Cl^-$ cotransporter	P55012	Slc12a2	E
N-ethylmaleimide sensitive fusion	P46460	Nsf	W,B,R,T,E
Neurocalcin δ	Q91X97	Ncald	S
Neurofilament H	P19246	Nefh	W,B,E
Neurofilament L	P08551	Nefl	W,B,V,R,E
Neurofilament M	P08553	Nefm	B,R,E
Neuroligin 1	Q99K10	Nlgn1	T
Neurotrimin	Q99PJ0	Hnt	E
N-myc downstream regulated	Q62433	Ndrg1	W,S,V,T,E
Nucleoside diphosphate kinase A	P15532	Nme1	W,S,T,E
Nucleoside diphosphate kinase B	Q01768	Nme2	W,S,T,E
Parkinson disease protein 7	Q99LX0	Park7	E
Peroxiredoxin 1	P35700	Prdx1	W,V,R,T,E
Peroxiredoxin 2	Q61171	Prdx2	W,V,E
Peroxiredoxin 5	P99029	Prdx5	S,E
Phosphatidylethanolamine binding 1	P70296	Pebp1	W,V,E
Phosphatidylinositol transfer α	P53810	Pitpna	W
Phosphofructokinase 1	P47857	Pfkm	E
Phosphoglycerate dehydrogenase	Q61753	Phgdh	W
Phosphoglycerate kinase 1	P09411	Pgk1	S,V,T,E
Phosphoglycerate kinase 2	P09041	Pgk2	E
Phosphoglycerate mutase 1	Q9DBJ1	Pgam1	W,S,T,E

Table 1 (continued)

Protein name	ID	Gene	Reference
Phospholipase Cβ1	Q9Z1B3	Plcb1	W,T,E
Phosphoserine aminotransferase	Q99K85	Psat1	R
Prion protein	P04925	Prnp	E
Prion protein dublet	Q9QYT9	Prnd	E
Programmed cell death 6 interacting	Q9WU78	Pdcd6ip	W
Prohibitin	P67778	Phb	W,B,E
Prohibitin 2	O35129	Phb2	E
Protein arginine deiminase 2	Q08642	Padi2	E
Protein disulfide isomerase A3	P27773	Pdia3	W,T
Protein kinase Cγ	P63318	Prkcc	E
Pyruvate kinase isozyme M2	P52480	Pkm2	W,S,V,T,E
Quinoid dihydropteridine reductase	Q8BVI4	Qdpr	E
Rab 1A	P62821	Rab1	E
Rab 1B	Q9D1G1	Rab1b	E
Rab 2A	P53994	Rab2a	R,E
Rab 2B	P59279	Rab2b	E
Rab 3B	Q9CZT8	Rab3b	E
Rab 3D	P35276	Rab3d	E
Rab 4A	P56371	Rab4a	E
Rab 4B	Q91ZR1	Rab4b	E
Rab 5C	P35278	Rab5c	E
Rab 7A	P51150	Rab7	R
Rab 8A	P55258	Rab8a	E
Rab 8B	P61028	Rab8b	E
Rab 10	P61027	Rab10	S,E
Rab 12	P35283	Rab12	E
Rab 13	Q9DD03	Rab13	E
Rab 14	Q91V41	Rab14	E
Rab 15	Q8K386	Rab15	E
Rab 18	P35293	Rab18	E
Rab 26	Q504M8	Rab26	E
Rab 30	Q923S9	Rab30	E
Rab 35	Q6PHN9	Rab35	E
Rab 37	Q9JKM7	Rab37	E
Rab 39B	Q8BHC1	Rab39b	E
Rab 43	Q8CG50	Rab43	E
Rab GDP dissociation inhibitor α	P50396	Gdi1	W,S,R,T,E
Rab GDP dissociation inhibitor β	Q61598	Gdi2	W,T,E
Rac1	P63001	Rac1	S,R,E
Rac2	Q05144	Rac2	E
Rac3	P60764	Rac3	E
Radixin	P26043	Rdx	W,E
Ras-related protein Ral A	P63321	Rala	B,E
Ras-related protein Ral B	Q9JIW9	Ralb	E
Ras-related protein Rap 1A	P62835	Rap1a	W,S,R,T,E
Ras-related protein Rap 1B	Q99JI6	Rap1b	E
Ras-related protein Rap 2a	Q80ZJ1	Rap2a	R
Reticulon 3	Q9ES97	Rtn3	R

Table 1 (continued)

Protein name	ID	Gene	Reference
Rho GDP dissociation inhibitor 1	Q99PT1	Arhgdia	V,T
RhoA	Q9QUI0	Rhoa	E
RhoB	P62746	Rhob	T,E
RhoC	Q62159	Rhoc	E
RhoG	P84096	Rhog	E
S-100β	P50114	S100b	R
Septin 2	P42208	Sept2	W,B,S,T,E
Septin 4	P28661	Sept4	W,E
Septin 7	O55131	Sept7	W,B,S,R,T,E
Septin 8	Q8CHH9	Sept8	W,B,S,V,R,T,E
Septin 11	Q8C1B7	Sept11	E
Sideroflexin 3	Q91V61	Sfxn3	E
Soluble NSF attachment protein α	Q9DB05	Napa	W
Soluble NSF attachment protein β	P28663	Napb	W,E
Soluble NSF attachment protein γ	Q9CWZ7	Napg	W
Spectrin α2	P16546	Spna2	B,T,E
Spectrin β2	Q62261	Spnb2	R,E
Stress induced phosphoprotein 1	Q60864	Stip1	W,T
Superoxide dismutase	P08228	Sod1	W,S
Synapsin 1	O88935	Syn1	W,E
Synapsin 2	Q64332	Syn2	W,E
Synaptic vesicle membrane protein	Q62465	Vat1	R,T
Synaptobrevin 2	P63044	Vamp2	E
Synaptobrevin 3	P63024	Vamp3	E
Synaptophysin	Q62277	Syp	E
Synaptosomal associated protein 23	O09044	Snap23	E
Synaptosomal associated protein 25	P60879	Snap25	W,S,V,R,E
Synaptotagmin 1	P46096	Syt1	E
Synaptotagmin 5	Q9R0N5	Syt5	E
Syndapin 1	Q61644	Pacsin1	W,E
Syntaxin 1A	O35526	Stx1a	E
Syntaxin 1B	P61264	Stx1b	S,R,E
T-complex 1α	P11983	Tcp1	W
T-complex 1β	P80314	Cct2	W
T-complex 1δ	P80315	Cct4	R
T-complex 1ε	P80316	Cct5	W
T-complex 1γ	P80318	Cct3	W
Thy 1 membrane glycoprotein	P01831	Thy1	W,S,R,E
Transgelin 3	Q9R1Q8	Tagln3	W,E
Transitional ER ATPase	Q01853	Vcp	W,T,E
Transketolase	P40142	Tkt	W,B,S,T,E
Triosephosphate isomerase	P17751	Tpi1	S,E
Tubulin α1A	P68369	Tuba1a	W,B,R,E
Tubulin α1B	P05213	Tuba1b	W,S,V,T,E
Tubulin α1C	P68373	Tuba1c	E
Tubulin α3A	P05214	Tuba3a	E
Tubulin α4A	P68368	Tuba4a	E

Table 1 (continued)

Protein name	ID	Gene	Reference
Tubulin α8	Q9JJZ2	Tuba8	E
Tubulin β2A	Q7TMM9	Tubb2a	T,E
Tubulin β2B	Q9CWF2	Tubb2b	E
Tubulin β2C	P68372	Tubb2c	W,B,S,R,E
Tubulin β3	Q9ERD7	Tubb3	E
Tubulin β4	Q9D6F9	Tubb4	W,B,S,V,R,E
Tubulin β5	P99024	Tubb5	E
Tubulin β6	Q922F4	Tubb6	R,E
Tubulin polymerization promoting	Q7TQD2	Tppp	W,E
Tubulin polymerization promoting 3	Q9CRB6	Tppp3	S,E
Ubiquitin	P62991	Ub	W,S,E
Ubiquitin activating enzyme E1	Q02053	Uba1	T
Ubiquitin C-terminal hydrolase L1	Q9R0P9	Uchl1	W,T,E
Vacuolar ATP synthase A	P50516	Atp6v1a	W,E
Vacuolar ATP synthase B, brain	P62814	Atp6v1b2	W,E
Vacuolar ATP synthase C	Q9Z1G3	Atp6v1c1	T,E
Vacuolar ATP synthase E1	P50518	Atp6v1e1	T,E
Vimentin	P20152	Vim	E
Visinin like protein 1	P62761	Vsnl1	S,R,E
Visinin like protein 3	P62748	Hpcal1	S
WD repeat protein 1	O88342	Wdr1	W

Proteins identified in purified CNS myelin by MS

ID Swissprot or Trembl accession, *Gene* official NCBI Entrez gene name, *Reference* and method of detection, *T* 2D-IEF/SDS-PAGE or immunoblotting [95], *V* 2D-IEF/SDS-PAGE [97], *W* 2D-IEF/SDS-PAGE [25], *B* 2D-16-BAC/SDS-PAGE [25], *R* shotgun [23], *S* shotgun [97], *E* LC-MS^E (Tenzer et al., unpublished), *ND* not detected by MS

adhesion, such as the immunoglobulin domain superfamily protein Igsf8, also termed EWI-2 [23]. Igsf8 is associated with the myelin tetraspanins CD9 and CD81 and regulates integrin function, at least *in vitro* [125, 126], but its function *in vivo* remains to be shown. The experimental validation or falsification of newly identified myelin-associated proteins will be a matter of the systematic application of histological techniques, provided that reliable antibodies are available.

How many proteins can be considered true myelin proteins? Though proteomic compendia aim at completeness, the number can only be guessed at this time. As the dynamic range of current MS-based protein identification schemes is in the range of three to five orders of magnitude, detection of infrequent proteins remains a challenge. Additionally, some technical impediments remain. The myelin proteins CD9 [127, 128], oligodendrocyte myelin

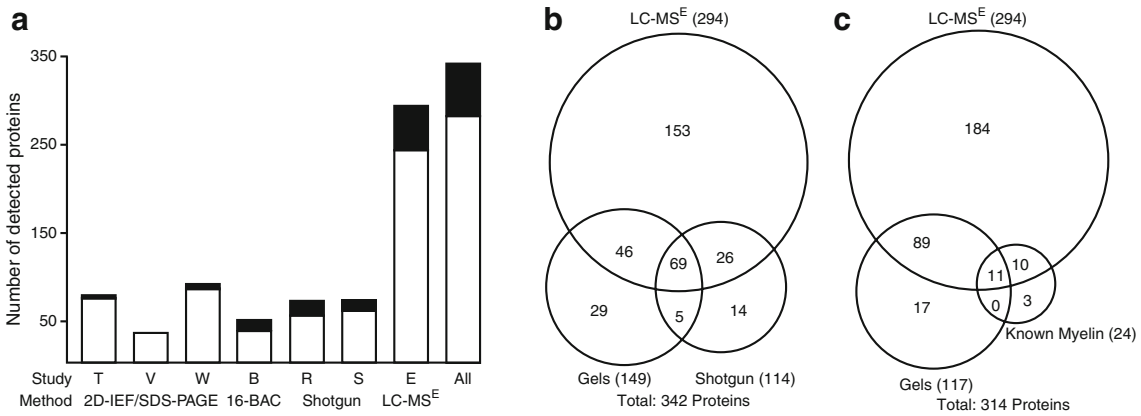

Fig. 3 Assembling a compendium of myelin proteins. **a** The number of proteins identified by MS in different approaches to the CNS myelin proteome is plotted. The total number of myelin-associated proteins is unknown. Transmembrane proteins (*black*) have been categorized based on prior experimental studies or have been predicted using TMHMM and Phobius software. Proteins associated with mitochondria, which copurify with myelin, were omitted. *T* 2D-IEF/SDS-PAGE [95], *V* 2D-IEF/SDS-PAGE [97], *W* 2D-IEF/SDS-PAGE [25], *B* 2D-16-BAC/SDS-PAGE [25], *R* shotgun [23], *S* shotgun [97], *E* LC-MS^E (Tenzer et al., unpublished). **b** Venn diagram comparing the number of myelin-associated proteins identified by MS after gel separation [25, 95, 97], previous gel-free shotgun approaches by LC/LC-MS/MS [23, 97], with those identified by LC-MS^E (Tenzer et al., unpublished). Note the high overlap of proteins identified independent of the technique used. **c** Venn diagram showing our own experience with the identification of myelin-associated proteins by MS after combined 2D-IEF/SDS-PAGE and 2D-16-BAC/SDS-PAGE separation [25] or by LC-MS^E with known myelin proteins according to the literature

glycoprotein [22, 129], and MAL [51] have not yet been detected by proteomic approaches, and the appearance of MAL in one catalog [95] is due to the additional use of immunoblotting. Its nondetectability illustrates the limitations of proteome analysis. MAL is a very hydrophobic protein with four transmembrane domains and very small cytoplasmic and extracellular domains and is, therefore, hardly accessible by MS-based identification. Apart from the membrane-spanning peptides not visible in proteomic approaches, complete tryptic digest of MAL results in only four theoretically detectable peptides: one of 120 amino acids (which is too long for identification by MS), two of two amino acids each (too short to provide useful sequence information), and one of 29 amino acids, which is, in principle, appropriate for identification. However, to obtain a reasonable level of confidence for protein identification, the detection of two peptides per protein is usually set as a prerequisite in the algorithms. This suggests that all proteome approaches requiring protease cleavage have an inherent bias against very small polypeptides or proteins with an unusual cleavage site pattern. In future experiments, the lack of suitable trypsin cleavage sites may be circumvented by the use of endopeptidases with different specificities (e.g., GluC or AspN), although they create proteolytic peptides lacking a basic C-terminal amino acid and are difficult to sequence [130]. This suggests that the detection of more myelin-associated proteins is not just a matter of higher resolving power but also of other technical refinements.

Newly Identified Myelin-Associated Proteins

The compendium of proteins identified in the myelin-enriched brain fraction represents a valuable reference for myelin research. The proteins are candidates for performing important functions in myelin biogenesis and integrity, molecular interactions between myelinating glia and neighboring cells, and white matter homeostasis. By gene ontology terms (http://david.abcc.ncifcrf.gov), many myelin-associated proteins are implicated in catalytic activities (48%), the cytoskeleton (20%), protein transport (21%), vesicular trafficking (6.8%), cell adhesion (6.3%), phospholipid binding (4.2%), or glycolysis/gluconeogenesis (5.1%). Among the recently identified myelin proteins, some were first and others subsequently detected using proteomic approaches. They include proteins of quite various anticipated functions, such as the NAD^+-dependent deacetylase sirtuin 2 (SIRT2, see below), cytoskeletal proteins of the septin family [23, 25, 131], and ermin [132], regulators of intracellular vesicle transport in the secretory pathway, such as cdc42 and Rac1 [133], Rab3A, and other Rab-GTPases [134, 135], the paranodal transmembrane glycoprotein Opalin/TMEM10 with a suggested signaling or adhesive function [136–138], the nucleoside diphosphate kinases NM23A and NM23B [95], and a protein particularly abundant in the CNS myelin of teleost fish, the 36K protein, also termed short-chain dehydrogenase/reductase (SDR family) member 12 (DHRS12) [139]. Some of these are quite abundant myelin proteins as judged both by the spots

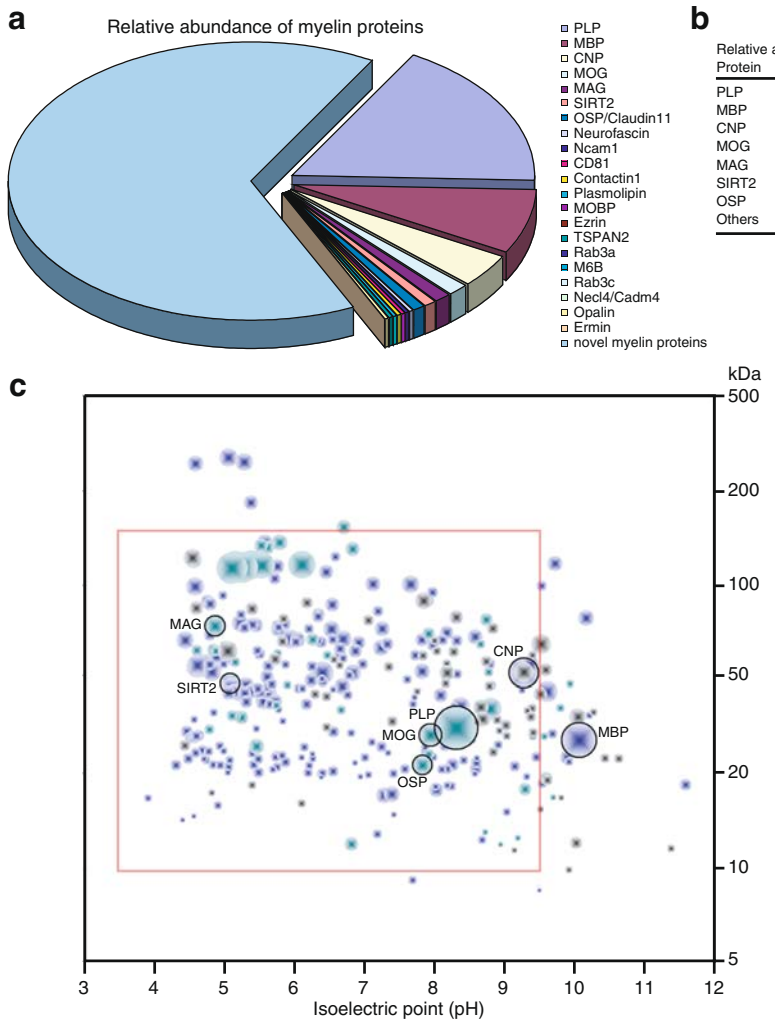

Fig. 4 Relative abundance of myelin proteins. **a** The abundance of known myelin proteins was determined by LC-MS^E. Note that known myelin proteins constitute less than 50% of the total myelin protein. Mitochondrial proteins were not considered. **b** Comparison of myelin protein abundance as quantified by LC-MS^E with previous estimates based on band intensity after 1D-PAGE and various protein staining techniques [19, 85, 87, 88]. Note that the abundance of PLP and MBP was previously overestimated because low abundant proteins did not constitute significant bands due to limitations in the resolving power of the 1D gels and in the dynamic range of protein staining. **c** Simulated 2D map of myelin-associated proteins identified by LC-MS^E. Proteins are indicated as *dots* at their molecular weight and isoelectric point as predicted from the amino acid sequence. The size of each dot reflects the relative abundance as determined by LC-MS^E. Myelin-associated proteins without transmembrane domains are shown in *blue* and transmembrane proteins in *green*, the latter being usually under-represented or absent from conventional 2D gels. Mitochondrial proteins are shown in *gray*. The *red frame* indicates the portion of proteins that can be reproducibly displayed by 2D-IEF/SDS-PAGE (see Fig. 2a)

constituted on 2D gels and LC-based quantification, and the challenge to establish their functions *in vivo* promises a deepened understanding of myelin. Besides, novel myelin proteins are candidates to cause (when mutated), enhance, or ameliorate white matter disease, such as leukodystrophies.

Differential Myelin Proteome Analysis in Myelin-Related Disease

The proteomic comparison of myelin from human patients or animal models with that of respective controls is a powerful approach towards the identification of secondary molecular changes that may contribute to the pathogenesis of myelin-related disease. Such a differential approach has first been applied to myelin purified from PLP^{null} mice [25], which provide a genuine model for spastic paraplegia (SPG-2) in humans, a comparatively mild variant of the leukodystrophy Pelizaeus–Merzbacher Disease with progressive axonal degeneration in the presence of normal amounts of CNS myelin [29, 140]. In that study, 2D-DIGE [141] was used to screen for candidate proteins that could be involved in the oligodendroglial failure to support the long-term integrity of myelinated axons. Three distinct proteins of the cytoskeletal

septin family were found to be reduced, and the deacetylase SIRT2 was virtually absent from PLP^{null} myelin. SIRT2 is an abundant myelin protein in the CNS and the PNS [23, 25, 142] and regulates microtubule dynamics during oligodendrocyte development [143]. Whether acetylated α-tubulin is a relevant substrate of SIRT2 *in vivo* remains to be shown. Similar to PLP^{null} mice, CNP^{null} mice are also normally myelinated but develop length-dependent axonal loss [92, 144]. It is intriguing that CNP also modulates microtubule dynamics [145, 146]. Taken together, spatiotemporal control of microtubule stability in oligodendrocytes (by SIRT2, CNP, and likely other factors) seems critical for normal axon–glia interaction.

Acetylation is a reversible post-translational modification of numerous mammalian proteins [147, 148], and all acetylated myelin proteins (α-tubulin, MBP, MOG, and several nonidentified proteins of lower abundance) are candidate substrates for SIRT2 [25]. In oligodendrocytes and myelin, SIRT2 activation upon increased axonal NAD^+ levels may remove acetyl residues from myelin-associated proteins with consequences for their net charge and function. Interestingly, SIRT2 has been recently shown to interact with 14-3-3 beta and gamma [149], which are myelin-associated as revealed by proteome analysis (Table 1). Their interaction is strengthened by the serine/threonine kinase AKT [149], which is a central signaling molecule for CNS myelination [150]. 14-3-3 proteins have been implicated in membrane protein transport, exocytosis [151], and stress response [152], but their function in myelin has not yet been investigated. 14-3-3 proteins are homologs of the *C. elegans* partitioning-defective polarity protein Par5 and bind to the tight junction-associated Par3 [153, 154], which is required for establishing polarity prior to myelination, at least by Schwann cells in the PNS [155]. To determine whether SIRT2, 14-3-3 proteins, Par-proteins, protein kinases, and tight junctions indeed interact in myelinating glia will be an important topic of future investigation. We speculate that the competence of oligodendrocytes to dynamically react to NAD^+ level changes in white matter tracts is required for their role in maintaining long-term axonal integrity.

With the objective to identify novel therapeutic targets for the treatment of multiple sclerosis, a systematic proteomic profiling of tissue samples from three brain lesions affected to various degrees (acute plaque, chronic active plaque, and chronic plaque) has recently been performed [156]. Material from the respective lesion type was collected by laser-capture microdissection and extracted proteins were separated by 1D gel electrophoresis followed by mass spectrometric protein identification. Unexpectedly, five coagulation proteins, including tissue factor and protein C inhibitor, were only present in chronic active plaque characterized by concomitant inflammation and degeneration, a finding that provided new insights in the relationship between the coagulation cascade and inflammation. Most importantly, administration of inhibitors to tissue factor (i.e., hirudin) and protein C inhibitor (i.e., activated protein C [aPC]) indeed ameliorated the disease phenotype in experimental autoimmune encephalomyelitis, a model of multiple sclerosis. The anti-inflammatory treatment with engineered aPC variants may develop into an alternative route to a therapy of multiple sclerosis. Together, differential proteome analysis has identified secondary molecular changes that contribute to understanding the pathogenesis of myelin-related disease and support the design of rational treatment strategies.

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