Published in final edited form as:

Anal Chem. 2013 August 6; 85(15): 7191–7196. doi:10.1021/ac400902h.

MALDI imaging and *in situ* identification of integral membrane proteins from rat brain tissue sections

Joshua J. Nicklay¹, Glenn A. Harris¹, Kevin L. Schey^{1,5}, and Richard M. Caprioli^{1,2,3,4,*}

¹Mass Spectrometry Research Center, Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

²Department of Chemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

³Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

⁴Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

⁵Department of Ophthalmology and Visual Sciences, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

Abstract

Transmembrane proteins are greatly underrepresented in data generated by imaging mass spectrometry (IMS) because of analytical challenges related to their size and solubility. Here we present the first example of MALDI IMS of two highly modified multi-transmembrane domain proteins, myelin proteolipid protein (PLP, 30 kDa) and DM-20 (26 kDa), from various regions of rat brain, namely the cerebrum, cerebellum, and medulla. We utilize a novel tissue pre-treatment aimed at transmembrane protein enrichment to show the *in situ* distribution of fatty acylation of these proteins, particularly of post-translational palmitoylation. Additionally, we demonstrate the utility of protease-encapsulated hydrogels for spatially localized on-tissue protein digestion and peptide extraction for subsequent direct coupling to LC-MS/MS for protein identification.

Keywords

MALDI imaging; imaging mass spectrometry; IMS; matrix assisted laser desorption ionization; myelin; proteolipid protein; PLP; DM-20; palmitoylation; palmitic acid; transmembrane protein; hydrogel; protein extraction

Introduction

MALDI Imaging Mass Spectrometry (IMS) is a powerful technique for analyzing the spatial distribution of biologically relevant molecules directly within tissues.¹ The majority of work to date in MALDI IMS applications has largely focused on small molecule pharmaceuticals and metabolites,^{2–4} lipids,^{5,6} peptides,⁷ and small, abundant, soluble proteins.^{8,9} In contrast to the major advances in IMS regarding these molecular classes, imaging of large (>30,000 Da), post-translationally modified and/or hydrophobic proteins, particularly transmembrane proteins, remains a difficult task. Aside from a few reports of transmembrane protein imaging, namely the membrane channel aquaporin 0 within the lens and the G protein-

*Corresponding Author: r.caprioli@vanderbilt.edu.

Supporting Information Available: This material is available free of charge via the Internet at <http://pubs.acs.org>.

coupled receptor opsin within the retina, this important class of biomolecules remains under-represented in the literature.¹⁰

The primary structure of transmembrane proteins introduces a number of difficulties that complicate their direct, *in situ* analysis by MALDI IMS. Specifically, the numerous hydrophobic domains that transverse the lipid bilayer result in a lack of solubility in solvent systems traditionally used during the matrix application procedures for imaging experiments. Analysis of these molecules requires tissue sample pre-treatment that disrupts the lipid bilayer in combination with a solvent system that efficiently extracts and solubilizes the proteins for efficient co-crystallization with deposited matrices. Solubilization is typically accomplished by the addition of organic solvents,^{11,12} detergents,¹³ chaotropes,¹⁴ or high pH buffer salts.^{15,16} Additionally, the complex nature of tissue sections introduces dynamic range limitations whereby smaller, soluble proteins are more readily extracted, ionized and detected relative to larger, less soluble transmembrane proteins.¹⁰ Therefore extensive tissue washing is necessary to reduce the contribution of soluble proteins to the resulting mass spectra. All of these combined factors necessitate the development of novel methodologies for tissue treatment from sectioning to final analysis.

As a major tissue component of brain tissue, myelin is a compact multilayered sheath composed of lipid and protein that electrically insulates the axons of nerve cells and allows for nerve impulse propagation. Alterations to the normal chemical components of myelin (demyelination) and the inability to properly form myelin (dysmyelination) have been implicated in a number of neurodegenerative diseases, including multiple sclerosis.^{17,18} Previous work has imaged the distribution of myelin basic protein within myelinated regions of mouse cerebrum via MALDI.¹⁹ However, to date there have been no reports of the *in situ* localization of myelin proteolipid protein (PLP), the most abundant protein (>50%) of CNS myelin, nor its splice variant, DM-20.²⁰ Both proteins contain four transmembrane domains and are known to be post-translationally modified by fatty acylation, particularly by palmitic acid, stearic acid and oleic acid moieties.²¹ These modifications have been shown to be critical for overall compaction of the myelin sheath and alterations to their normal distribution have been shown to play a role in certain neurodegenerative diseases.²²

This study is the first to reveal the spatial distribution of myelin proteolipid protein and its splice variant, DM-20, within the CNS. Additionally, this study measures the *in situ* distribution of fatty acylations on each protein species. MALDI IMS of these highly important modified proteins required a novel approach to tissue pre-treatment that we anticipate will assist in future analyses of transmembrane proteins from other tissue types.

In this study, we have utilized novel methodologies focused on the selective enrichment of membrane proteins *in situ* directly on tissue sections for the purpose of MALDI IMS. This approach involves immediate disruption of the lipid bilayer upon sample mounting by solubilizing specific, relatively polar lipid classes with methanol; extensive washing with water to minimize the resulting detector contribution of abundant, soluble proteins; and organic solvent-based extraction for protein/matrix co-crystallization. Our work also includes an approach for on-tissue, spatially defined protein identification utilizing novel trypsin-encapsulated hydrogel micro-reaction networks.²³

Materials

HPLC grade methanol, acetonitrile, acetone and chloroform were purchased from Fisher Scientific (Fairlawn, NJ). Sinapinic acid, alpha-cyano-4-hydroxycinnamic acid (CHCA), trifluoroacetic acid (TFA), formic acid, hexafluoroisopropanol (HFIP), protease inhibitor tablets, sucrose, Tris-HCl, dithiothreitol, ethylenediaminetetraacetic acid (EDTA),

diethylether, ammonium persulfate (APS), sodium dodecyl sulfate (SDS) and ammonium bicarbonate were all purchased from Sigma-Aldrich (St. Louis, MO). Sephadex LH-20 was purchased from GE Healthcare (Piscataway, NJ). Acrylamide/bis-acrylamide and tetramethylethylenediamine (TEMED) were purchased from Bio-Rad Life Sciences (Hercules, CA). Trypsin was purchased from Promega (Madison, WI). Octyl-beta-glucoside was purchased from Thermo Fisher Scientific (Rockford, IL). All water was purified through a Millipore Milli-Q system (Billerica, MA).

Methods

Tissue Sectioning and Pre-treatment

Brains from Sprague-Dawley rats were collected and stored at -80°C prior to sectioning. Coronal sections (6 μm thickness) were taken at -20°C at multiple depths through the cerebrum and the cerebellum/medulla on a cryostat (Leica Microsystems Inc., Bannockburn, IL). Tissue sections were mounted on gold plated MALDI targets (Applied Biosystems, Foster City, CA) using a modified methanol soft-landing technique.¹⁰ A thin layer of room temperature methanol was applied to the target plate and, prior to evaporation, the tissue slice was transferred to the plate using a small paintbrush. Mounted tissue sections were placed in a desiccator for at least 30 minutes. Tissue sections were then washed 11x by pipetting deionized water ($\sim 300\mu\text{L}$) onto the tissue, which remained for one minute without agitation prior to aspiration. Tissue sections were desiccated for 15 minutes between washes. The final wash consisted of a one minute, 1% acetonitrile wash with aspiration immediately followed by a 30 second deionized water wash, aspiration, and desiccation.

A saturated matrix solution of sinapinic acid (20 mg/mL) dissolved in 90% acetonitrile/0.25% TFA/0.1% HFIP was applied to tissue sections using a Portrait 630 acoustic reagent multispotter (Labcyte, Inc., Sunnyvale, CA). A total of forty droplets of $\sim 160\text{ pL}$ each were applied to each raster spot across the entire tissue one droplet at a time with a diameter of 150 μm and 300 μm center-to-center spacing. Samples were then placed into in a desiccator in the dark until analysis.

MALDI Imaging Mass Spectrometry

Images were acquired on an Autoflex Speed TOF/TOF (Bruker Daltonics, Bremen, Germany) operated in positive, linear mode at a lateral resolution of 300 μm . Each pixel consisted of a sum of 500 spectra acquired over a diameter of 150 μm using an in-house modified laser system whereby the original SmartBeam spatial light modulator was removed, resulting in a clean Gaussian-shaped laser beam, produced by the original, frequency-tripled Nd:YAG laser at 355 nm. Additionally, the standard focusing lens in the sample chamber was replaced with a precisely aligned aspheric lens. As a result, a focused laser beam spot of 5 μm in diameter was achieved on the target. Images were visualized in FlexImaging v 2.1. The instrument was calibrated prior to every image acquisition using a standard mix of proteins including bovine insulin, equine cytochrome c, equine apomyoglobin, and bovine trypsinogen spotted onto a serial tissue section mounted on the same plate.

In situ Hydrogel-based Protein Digestion

Circular, trypsin-encapsulated hydrogel micro-reaction networks (3 mm in diameter) were utilized for *in situ* protein digestion using modifications to a previously described protocol noted below.²³ Specifically acrylamide was used to form the hydrogel in place of alginate. A solution of 7.5% acrylamide made from 30% 37.5:1 acrylamide:bis-acrylamide, 0.5 M Tris-HCl (pH 6.8), and deionized water was degassed for 30 minutes. TEMED and 10% APS were added to 5 mL acrylamide, and the mixture was quickly stirred and poured into a 50

mm pyrex dish. Upon polymerization, a 3 mm tissue biopsy punch was used to produce the hydrogels. Hydrogels were dried down completely in a Speedvac (Thermo Scientific, Waltham, MA) and frozen at -80°C prior to usage. Hydrogels were re-swelled in a solution of 1 $\mu\text{g}/\mu\text{L}$ trypsin for 30 minutes and manually placed on the tissue region of interest. Enzymatic digestion proceeded in a humidity chamber at 50°C for 4 hours. All tissue sections underwent identical sample pre-treatment as the imaged tissue sections to remove soluble proteins. Upon digest completion, the gel was manually removed from the tissue section for peptide extraction.

LC-MS/MS Identification of Hydrogel-extracted Peptides

Extraction of peptides from the gels proceeded through the repeated shrinking and swelling (3x each) in minimal volumes (30 μL) of organic (50% acetonitrile/5% formic acid) and aqueous (100 mM ammonium bicarbonate) solvents.²⁴ A final treatment consisted of 100% acetonitrile. The pooled peptides were dried down and analyzed by LC-MS/MS.

Peptides were reconstituted in 100 μL 0.1% formic acid and bomb-loaded onto a self-packed pre-column (4 cm x 0.1 mm, Jupiter 5 μm , 300 Å, C18) (Phenomenex, Torrance, CA) fritted with an M520 inline microfilter union (IDEX, Lake Forest, IL). Following equilibration, this column was attached to a self-packed analytical column (20 cm x 0.1 mm, Jupiter 3 μm , 300 Å, C18) (Phenomenex, Torrance, CA) equipped with a laser-pulled, 1 μm nanospray emitter tip (P-2000, Sutter Instruments, Novato, CA) and coupled directly to an LTQ (Thermo Scientific, Waltham, MA).

Peptides were eluted over 75 minutes at 500 nL/min on a nanoAcquity UPLC system (Waters, Manchester, UK) using the following gradient: initial flow of 98% solvent A (0.1% formic acid), 2 % solvent B (acetonitrile, 0.1% formic acid), ramped to 25% B over 45 minutes, ramped to 90% B over 15 minutes, held for 5 minutes, ramped down to 2% over 2 minutes and held for 8 minutes. Throughout the entire run, a full scan was taken followed by five data-dependent MS/MS scans with dynamic exclusion enabled with a repeat count of 1, a repeat duration of 30 seconds and an exclusion duration of 60 seconds. The resulting MS/MS spectra were searched via SEQUEST against a rat database (Uniprot taxonomy ID 10116) using Thermo Proteome Discoverer 1.4 software (Thermo Fisher Scientific, San Jose, CA). The Percolator node within Proteome Discoverer was used for peptide confidence filtering and scoring.²⁵ Identifications were filtered at the peptide level to achieve a 1% false discovery rate. Proteins were filtered to two unique peptides and were automatically grouped by similarity. All MS/MS spectra from PLP/DM-20 peptides were manually verified.

PLP/DM-20 Purification

Rat brain myelin was isolated from 3 brains (3.6 g total) by the method described by Larocca and Norton.²⁶ Frozen brains were diced and homogenized using a Dounce homogenizer in a 320 mM sucrose solution containing 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, and 1 mM DTT and layered over a similar solution containing 800 mM sucrose. Samples were centrifuged at 104,000 x g for 60 minutes at 4°C . Myelin was collected from the interface and underwent osmotic shock by the following steps: myelin was re-homogenized in a similar Tris-buffered solution as above but without sucrose. Samples were centrifuged at 116,000 x g for 30 minutes at 4°C . The supernatant was discarded and the pellets were resuspended in Tris-buffered solution (without sucrose) and re-homogenized. Samples were centrifuged at 12,000 x g for 15 minutes at 4°C . The supernatant was discarded and the pellets were again resuspended in Tris-buffered solution and centrifuged at 12,000 x g for 15 minutes at 4°C . The resulting pellets were combined in 300 mM sucrose

solution and the entire process was repeated once. The final pellets were dried down and stored at -80°C until further processing.

PLP and DM-20 were purified from isolated myelin following a modified Folch method.^{27–29} A fraction of purified myelin (24 mg) was reconstituted in 8 mL 2:1 chloroform:methanol (v/v) with vigorous pipetting and vortexing. 400 μL deionized water was added and the mixture was centrifuged at $1,000 \times g$ for 30 minutes at 4°C . The aqueous layer was discarded and the intermediate layer was re-extracted with a minimal volume of 3:47:48 chloroform:methanol:water (v/v/v). The upper and intermediate layers were discarded, and 2.7 mL methanol was added to the organic layer. The solution was evaporated under nitrogen while slowly adding chloroform until all methanol was removed. The solution was then evaporated to dryness.

Eight mL diethyl ether was added to the sample and the mixture was shaken at 4°C for 2 hours. Proteins were pelleted by centrifugation at $1,000 \times g$ for 30 minutes at 4°C and resolubilized in 1% SDS. An aliquot was taken for a BCA assay and SDS-PAGE to confirm protein contents. SDS removal was performed via five acetone precipitations at -20°C with centrifugation at $13,000 \times g$. After the final spin, the pellet was dried under nitrogen and stored at -80°C .

MALDI Profiling of Intact PLP/DM-20

An aliquot of purified, acetone-precipitated PLP/DM-20 was reconstituted in 90% acetonitrile/0.25 % TFA/10 mM n-octylglucoside. An aliquot was mixed 1:1 (v/v) with 10 mg/mL CHCA in 90% acetonitrile/0.25% TFA/1% HFIP. The resulting spectra were the average of 6000 laser shots acquired from m/z 20,000 – 40,000 on an Autoflex Speed TOF/TOF operated in positive, linear mode. Data was analyzed in FlexAnalysis v 3.3.

LC-MS/MS Identification of Purified PLP/DM-20

An aliquot of purified, acetone-precipitated PLP/DM-20 was reconstituted in 1% SDS and separated on a precast, NuPAGE Novex 10% Bis-Tris gel and stained. Bands within the 25–35 kDa range were excised and diced into small pieces before undergoing in-gel tryptic digestion and extraction.²⁴ Peptide extracts were dried down in a Speedvac and kept at -80°C prior to further analysis. LC-MS/MS analyses proceeded as described above.

Results and Discussion

MALDI-based detection of membrane proteins directly from tissue requires the use of non-traditional sample preparation approaches for enhanced enrichment and solubilization. Specifically, spotting matrix compounds within high percentages of organic solvents is necessary for membrane protein analysis. This inevitably results in a tradeoff between spatial resolution and sensitivity. Due to the low surface tension of the matrix solution, the individual matrix spots were on average 150 μm in diameter on tissue, thus limiting the maximum attainable spatial resolution. Sampling with a 5 μm laser with multiple laser shots over the entirety of each 150 μm spot ensured adequate laser fluence to readily desorb the molecules of interest and measure them with satisfactory signal-to-noise ratios.

MALDI IMS of rat cerebrum following the protocol described above produced mass spectra containing a unique series of equally distributed, high molecular weight proteins localized near 27.1 kDa and 31.4 kDa (Figure 1). Based on the mass differences between consecutive peaks, we hypothesized that this distribution was representative of multiple, post-translational fatty acylation events. Specifically, we noted peaks differing by roughly 238 Da, indicative of palmitoylation, and roughly 264/266 Da, indicative of oleic acid/stearic acid additions; however, due to the relatively large peak width at this m/z , we were unable to

distinguish between the latter two fatty acid modifications. MALDI IMS of rat cerebellum and medulla under the same conditions revealed an identical distribution of masses.

MALDI IMS images for one of the more abundant proteins from coronal sections of cerebrum, cerebellum and medulla are shown in Figure 2. For all brain regions analyzed, images of each observed m/z species display an identical spatial distribution (Supplemental Figure 1). Specifically, within the cerebrum, all ions of interest localize to heavily myelinated regions, particularly the corpus callosum, the caudate-putamen, and the region of the septal nuclei. Within the cerebellum, all ions localize to the branching, myelinated structures of the arbor vitae. Within the medulla, all ions are abundantly and homogeneously distributed throughout the entirety of the tissue, indicative of the bundles of myelinated axons passing through this structure.

Given the enrichment of these molecular species within white matter regions throughout both the forebrain and hindbrain, we sought to determine whether these modified proteins were components of myelin. We approached initial identification of these proteins utilizing recently developed, hydrogel-based micro-reaction chamber technique for spatially localized on-tissue protein digestion and peptide extraction. Based on the homogenous distribution of these proteins throughout the medulla, we performed digestion and extraction on a single, 12 μm -thick section. Upon peptide extraction and subsequent LC-MS/MS, we identified over 50 protein groups with high confidence (1% FDR), including multiple proteins larger than 100 kDa, cytoskeletal proteins, and transmembrane proteins (Table 1). One of the detected proteins was myelin proteolipid protein (PLP, 30 kDa). PLP is known to be post-translationally palmitoylated, and is the major protein species within myelin (Supplemental Figure 2).²⁰ These post-translationally modified forms of PLP and its splice variant, DM-20 have molecular weights consistent with proteins shown in Figure 1. Solution-based digestion (non-hydrogel-mediated) of an entire 12 μm -thick medulla tissue section in 0.1% Triton X-100 revealed similar protein identifications (Supplemental Table 1).

Due to uncertainties in assigning identities to MALDI-imaged proteins based on LCMS/MS peptide information from non-enriched tissue homogenates, we isolated PLP and DM-20 intact directly from rat brain. First enriching for myelin from whole brain and then utilizing chloroform:methanol treatment to specifically isolate PLP and DM-20, we successfully recovered the proteins of interest, as confirmed by SDS-PAGE and subsequent in-gel digestion, extraction, and LC-MS/MS. Spotting these proteins intact for MALDI analysis revealed two broad, high mass protein signals localized at 28,000 and 32,280 separated by 4,280 Da (spectra not shown). This difference is equal to the difference between the most abundant forms of PLP and DM-20 within the spectra shown in Figure 1. We surmised that the observed peak shifting and broadening following intact isolation is a result of the adduction of multiple detergent molecules to each protein.³⁰

Table 2 shows the proposed identifications of the modified forms of DM-20 and PLP observed within our imaging analyses. The most abundant form of DM-20 centered at m/z 27119 contains a likely N-terminal myristic acid group (210 Da, predicted based on observed mass as well as the known consensus sequence for myristylation), a single palmitic acid group (210 Da), and two oleic acid (264 Da) or stearic acid (266 Da) modifications (a difference of 2 Da between these two modifications cannot be discerned at this m/z).³¹ The most abundant form of PLP centered at m/z 31400 contains a likely N-terminal myristic acid group, three palmitic acid groups, as well as two oleic acid or stearic acid modifications. Given the broadness of the observed peaks, we cannot exclude the possibility of multiple combinations of oleic acid and stearic acid existing on PLP/DM-20. All masses agree to within less than 0.04% error of the theoretical masses.

Previous work has identified Cys 5, 6, 9, 106, 138 and 140 of PLP and Cys 5, 6, 9, and 106 of DM-20 as sites of fatty acylation.³² DM-20 lacks 35 residues predicted to reside on the cytoplasmic side of the lipid bilayer, including two cysteine residues (Cys 138 and 140) that are believed to be sites of palmitoylation.³³ Based on the masses observed within our imaging experiments, we noted that the difference in modification states between the most abundant forms of DM-20 and PLP consists of two palmitoylations. This supports previous work that suggests both cysteines present in PLP, but not present in DM-20, are likely sites of palmitoylation.³² However, further sequence analysis needs to be performed to verify this finding.

PLP is the major protein of CNS myelin and has been the source of extensive research over the past 60 years due to its critical structural role within myelinated nerve fibers. Previous immunohistochemical studies have localized PLP to the anterior commissure of the cerebrum, the medial longitudinal fasciculus of the brainstem, and cerebellar white matter.³⁴ However, no immunohistochemical work has separately analyzed the spatial distribution of both PLP and DM-20. Additionally, despite being heavily modified, no work has attempted to describe the spatial distribution of the variously modified forms of PLP and DM-20, nor has previous work determined whether these modifications affect the localization and cellular targeting of either of these proteins. Imaging mass spectrometry is uniquely suited to overcome these issues and is able to identify and determine the *in situ* distribution of the various forms of these proteins within tissue simultaneously and within a single experiment.

Our findings show that, at the spatial resolution utilized, the presence of post-translational fatty acids does not affect the distribution of these protein species within the brain regions analyzed. This agrees with previous work that describes the role of these modifications in allowing for enhanced compaction of myelin sheaths and intercellular adhesion..^{22,35} With further refinements to sample preparation to limit solvent spreading we hope to achieve spatial resolutions that are closer to the limits of our current laser system. We expect this will further elucidate the biological roles of differential fatty acylation of PLP/DM-20.

Conclusions

We have presented a novel sample preparation procedure for MALDI IMS of transmembrane proteins directly from tissue. The utility of this new technique was demonstrated by assessing the spatial distribution of myelin proteolipid protein (PLP) and DM-20, two highly abundant proteins within myelin of the central nervous system, throughout various regions of rat brain. Additionally, we have shown the *in situ* distribution of post-translational fatty acylations of both PLP and DM-20, providing the first MALDI MS images of transmembrane proteins within brain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding

This project was supported by a grant from the National Institute of General Medical Sciences (5 P41 GM103391-02), formerly the National Center for Research Resources (5P41RR031461-01).

References

1. Caprioli RM, Farmer TB, Gile J. Anal Chem. 1997; 69:4751–4760. [PubMed: 9406525]

2. Cornett DS, Frappier SL, Caprioli RM. *Anal Chem.* 2008; 80:5648–5653. [PubMed: 18564854]
3. Reyzer ML, Hsieh Y, Ng K, Korfmacher WA, Caprioli RM. *J Mass Spectrom.* 2003; 38:1081–1092. [PubMed: 14595858]
4. Stoeckli M, Staab D, Schweitzer A. *Int J Mass Spectrom.* 2007; 260:195–202.
5. Manna JD, Reyzer ML, Latham JC, Weaver CD, Marnett LJ, Caprioli RM. *Anal Chem.* 2011; 83:6683–6688. [PubMed: 21770391]
6. Burnum KE, Cornett DS, Puolitaival SM, Milne SB, Myers DS, Tranguch S, Brown HA, Dey SK, Caprioli RM. *J Lipid Res.* 2009; 50:2290–2298. [PubMed: 19429885]
7. Groseclose MR, Andersson M, Hardesty WM, Caprioli RM. *J Mass Spectrom.* 2007; 42:254–262. [PubMed: 17230433]
8. Seeley EH, Oppenheimer SR, Mi D, Chaurand P, Caprioli RM. *J Am Soc Mass Spectrom.* 2008; 19:1069–1077. [PubMed: 18472274]
9. Seeley EH, Caprioli RM. *Proc Natl Acad Sci U S A.* 2008; 105:18126. [PubMed: 18776051]
10. Grey AC, Chaurand P, Caprioli RM, Schey KL. *J Proteome Res.* 2009; 8:3278–3283. [PubMed: 19326924]
11. Patra M, Salonen E, Terama E, Vattulainen I, Faller R, Lee BW, Holopainen J, Karttunen M. *Biophys J.* 2006; 90:1121–1135. [PubMed: 16326895]
12. Martosella J, Zolotarjova N, Liu H, Moyer SC, Perkins PD, Boyes BE. *J Proteome Res.* 2006; 5:1301–1312. [PubMed: 16739982]
13. Ruth MC, Old WM, Emrick MA, Meyer-Arendt K, Aveline-Wolf LD, Pierce KG, Mendoza AM, Sevinsky JR, Hamady M, Knight RD, Resing KA, Ahn NG. *J Proteome Res.* 2006; 5:709–719. [PubMed: 16512687]
14. Rabilloud T, Adessi C, Giraudel A, Lunardi J. *Electrophoresis.* 1997; 18:307–316. [PubMed: 9150907]
15. Pedersen SK, Harry JL, Sebastian L, Baker J, Traini MD, McCarthy JT, Manoharan A, Wilkins MR, Gooley AA, Righetti PG, Packer NH, Williams KL, Herbert BR. *J Proteome Res.* 2003; 2:303–311. [PubMed: 12814269]
16. Wu CC, MacCoss MJ, Howell KE, Yates JR. *Nat Biotechnol.* 2003; 21:532–538. [PubMed: 12692561]
17. Love S. *J Clin Pathol.* 2006; 59:1151–1159. [PubMed: 17071802]
18. Schiffmann R, van der Knaap MS. *Curr Opin Neurol.* 2004; 17:187–192. [PubMed: 15021247]
19. Ceuppens R, Dumont D, Van Brussel L, Van de Plas B, Daniels R, Noben JP, Verhaert P, Van der Gucht E, Robben J, Clerens S. *Int J Mass Spectrom.* 2007; 260:185–194.
20. Bizzozero OA, Leyba J, Nunez DJ. *J Biol Chem.* 1992; 267:7886–7894. [PubMed: 1560018]
21. Tetzloff SU, Bizzozero OA. *J Biol Chem.* 1998; 273:279–285. [PubMed: 9417076]
22. Bizzozero OA, Bixler HA, Davis JD, Espinosa A, Messier AM. *J Neurochem.* 2001; 76:1129–1141. [PubMed: 11181833]
23. Harris GA, Nicklay JJ, Caprioli RM. *Anal Chem.* 2013; 85:2717–2723. [PubMed: 23402265]
24. Shevchenko A, Wilm M, Vorm O, Mann M. *Anal Chem.* 1996; 68:850–858. [PubMed: 8779443]
25. Kall L, Canterbury JD, Weston J, Noble WS, MacCoss MJ. *Nat Methods.* 2007; 4:923–925. [PubMed: 17952086]
26. Larocca JN, Norton WT. *Curr Protoc Cell Biol.* 2007; 33:3.25.1–3.25.19.
27. Folch J, Lees M, Sloane Stanley GH. *J Biol Chem.* 1957; 226:497–509. [PubMed: 13428781]
28. Bizzozero OA, Malkoski SP, Mobarak C, Bixler HA, Evans JE. *J Neurochem.* 2002; 81:636–645. [PubMed: 12065672]
29. Fannon AM, Mastronardi FG, Moscarello MA. *Neurochem Res.* 1994; 19:1005–1012. [PubMed: 7528345]
30. Rosinke B, Strupat K, Hillenkamp F, Rosenbusch J, Dencher N, Kruger U, Galla HJ. *J Mass Spectrom.* 1995; 30:1462–1468.
31. Farazi TA, Waksman G, Gordon JI. *J Biol Chem.* 2001; 276:39501–39504. [PubMed: 11527981]
32. Weimbs T, Stoffel W. *Biochemistry.* 1992; 31:12289–12296. [PubMed: 1281423]
33. Greer JM, Lees MB. *Int J Biochem Cell Biol.* 2002; 34:211–215. [PubMed: 11849988]

34. Agrawal HC, Hartman B, Shearer W, Kalmbach S, Margolis F. J Neurochem. 1977; 28:495–508. [PubMed: 404395]
35. Resh MD. Biochim Biophys Acta. 1999; 1451:1–16. [PubMed: 10446384]

Figure 1.

Averaged MALDI mass spectrum (m/z 5,000 – 50,000) over a single, 6 μm -thick section of cerebellum and medulla following transmembrane protein enrichment. Inset shows an expanded view from m/z 25,000 – 35,000. Mass differences between consecutive peaks correlate with multiple fatty acylations. Reported m/z values are from the center of the top of the peak.

Figure 2.

Ion images for the protein species at m/z 26,860 within cerebrum (left) and cerebellum/medulla (right). Exclusive localization to white matter regions of brain, particularly to the corpus callosum (Cc), caudate-putamen (Cp), and septal nucleus (Sn) of the cerebrum and the arbor vitae (Arb) of the cerebellum and the entire medulla (Med), is apparent.

Top 20 most abundant proteins (based on number of assigned spectra) identified from a single, 12 μ m-thick section of medulla following hydrogel-based tryptic digestion and extraction and LC-MS/MS. Reported protein scores were provided by the Percolator node within Proteome Discoverer.

Table 1

Protein Name	UniProt Accession Number	Molecular Weight (kDa)	# Assigned Spectra	# Detected Peptides	Protein Score	Cellular Component
Tubulin beta-2C	G3V7C6	49.8	27	17	98.51	cytoskeleton
Tubulin beta-2A	P85108	49.9	22	16	82.96	cytoskeleton
Spectrin alpha	E9PSZ3	284.9	22	21	62.37	cytoskeleton
Tubulin beta-3	Q4QRB4	50.4	22	14	77.34	cytoskeleton
Myelin Proteolipid Protein (PLP/DM-20)	P60203	30.1	19	3	52.06	membrane
Na(+)/K(+) ATPase alpha-2 subunit	P06686	112.1	19	14	68.21	membrane
Na(+)/K(+) ATPase alpha-3 subunit	P06687	111.6	19	13	69.10	membrane
Myelin basic protein S	P02688	18.5	14	7	41.31	membrane
Na(+)/K(+) ATPase alpha-1 subunit	P06685	113.0	13	10	49.31	membrane
Tubulin alpha-1A	P68370	50.1	12	9	41.68	cytoskeleton
Actin	P60711	41.7	11	6	33.90	cytoskeleton
ATP synthase subunit beta	G3V6D3	56.3	10	9	38.59	membrane
ATP synthase subunit alpha	P15999	59.7	10	9	30.56	membrane
Neurofilament light polypeptide	P19527	61.3	9	9	28.77	cytoskeleton
Glial fibrillary acidic protein	P47819	48.8	8	8	27.16	cytoskeleton
Spectrin beta	G3V6S0	273.9	7	7	22.65	cytoskeleton
2,3 -cyclic-nucleotide 3 -phosphodiesterase	P13233	47.2	7	6	22.66	membrane
Heat shock protein 90-alpha	P82995	84.8	7	7	22.13	cytoplasm
Glyceraldehyde-3-phosphate dehydrogenase	D3ZGY4	35.8	6	6	25.02	cytoplasm
Clathrin heavy chain	F1M779	191.4	6	6	19.54	membrane

Table 2

Protein identifications and modification states for all major species shown in Figure 1. Reported theoretical masses take into account all possible combinations of stearic acid and oleic acid additions. All observed masses are within 10 Da (<0.04% error) of theoretical masses.

Observed Mass (Da)	Theoretical Mass (Da)	Protein Assignment	Modifications
26,860	26853/26855	DM-20	1 myristic + 1 oleic/stearic + 1 palmitic
27,119	27117/27119/27121	DM-20	1 myristic + 2 oleic/stearic + 1 palmitic
27,345	27355/27357/27359	DM-20	1 myristic + 2 oleic/stearic + 2 palmitic
27,598	27594/27596/27598	DM-20	1 myristic + 2 oleic/stearic + 3 palmitic
30,922	30921/30923/30925	PLP	1 myristic + 2 oleic/stearic + 1 palmitic
31,159	31159/31161/31163	PLP	1 myristic + 2 oleic/stearic + 2 palmitic
31,400	31397/31399/31401	PLP	1 myristic + 2 oleic/stearic + 3 palmitic
31,660	31662/31664/31666/31668	PLP	1 myristic + 3 oleic/stearic + 3 palmitic