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Comprehensive Analysis of the Mouse Brain Proteome Sampled in Mass Spectrometry Imaging

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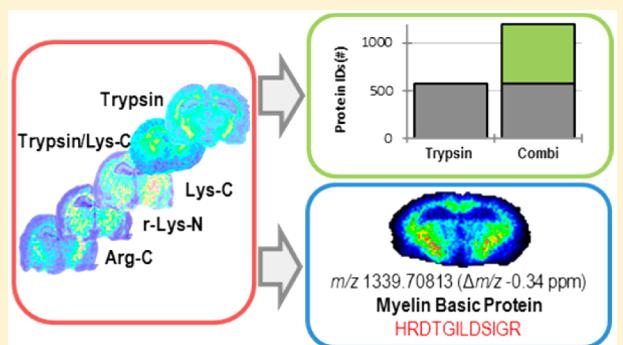
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Supporting Information

ABSTRACT: On-tissue enzymatic digestion is performed in mass spectrometry imaging (MSI) experiments to access larger proteins and to assign protein identities. Most on-tissue digestion MSI studies have focused on method development rather than identifying the molecular features observed. Herein, we report a comprehensive study of the mouse brain proteome sampled by MSI. Using complementary proteases, we were able to identify 5337 peptides in the matrix-assisted laser desorption/ionization (MALDI) matrix, corresponding to 1198 proteins. 630 of these peptides, corresponding to 280 proteins, could be assigned to peaks in MSI data sets. Gene ontology and pathway analyses revealed that many of the proteins are involved in neurodegenerative disorders, such as Alzheimer's, Parkinson's, and Huntington's disease.



Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry imaging (MSI) is an analytical technique in which the distributions of hundreds of biomolecular ions can be recorded directly from tissue sections.¹ It allows the simultaneous and untargeted investigation of many molecular classes, including pharmaceuticals, metabolites, lipids, peptides, and proteins.² One of the principal application areas of MALDI-MSI is the investigation of the molecular content of pathological tissue samples in order to find biomarkers or provide insights into the molecular mechanisms underlying a disorder. The discovery of putative biomarkers has often focused on proteins since the results can then be independently validated using immunohistochemistry.^{3–5}

To increase proteome coverage, aid protein identification, and enable the analysis of formalin-fixed paraffin embedded (FFPE) tissues, the enzymatic protein digestion performed in classic bottom-up proteomics has been adapted for MALDI-MSI.^{6–8} Protein identification directly from the tissue section can be performed by MS/MS analysis of the proteolytic peptides but remains challenging due to the very high complexity of the peptide mixture generated after digestion.⁹ Instead, the peptide identities are commonly assigned to those previously identified via extraction of proteolytic peptides followed by LC-MS/MS analysis.^{10,11}

As in bottom-up LC-MS/MS based proteomics, on-tissue digestion is most often performed using trypsin; its cleavage specificity, C-terminal of arginine and lysine, results in proteolytic peptides that have an intrinsic positive charge on the C-terminus, thus enhancing their detection by positive-ion MS.^{12,13} A crucial difference is that MALDI-MS is biased toward the detection of Arg-terminated peptides.¹⁴ The suppression of Lys C-terminated tryptic peptides in MALDI-MS results in an undesired loss of sequence information and proteome coverage. This is likely to be exacerbated in MALDI-MSI because of the absence of any explicit peptide separation.

The use of multiple proteases has been shown to have a beneficial effect on proteome and sequence coverage in both LC-ESI-MS and LC-MALDI-MS based studies.^{15–18} A recent study indicated that similar complementarities might be obtained in on-tissue digestion MALDI-MSI experiments: a combination of trypsin, pepsin, and elastase was shown to have a positive effect on protein sequence coverage.¹⁹ However, since pepsin and elastase do not cleave basic amino acids, many of the proteolytic peptides will not be detected with high sensitivity. To maintain high detection sensitivity, it is essential

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that the proteases cleave the most basic amino acids, i.e., Lys and Arg. While these are the same residues cleaved by trypsin, previous LC-MS/MS results have already demonstrated that the different sequence and conformation specificities of Lys-C, Arg-C, and Lys-N can improve proteome and sequence coverage by reducing the number of missed cleavages.¹³

Here, we have investigated the degree of increased proteome coverage that may be obtained in MALDI-MSI by using a similar complementary cohort of enzymes, specifically (i) trypsin, (ii) Lys-C, (iii) recombinant Lys-N (r-Lys-N), (iv) Arg-C, and (v) a mixture of trypsin and Lys-C (trypsin/Lys-C).

■ EXPERIMENTAL SECTION

Chemicals and Reagents. All chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) except ethanol (Merck, Darmstadt, Germany). All proteases were purchased from Promega (Madison, USA) except r-Lys-N (U-Protein Express BV, Utrecht, The Netherlands).

Sample Collection. Three month-old, male, C57BL/6J mice were sacrificed by cervical dislocation. The brains were excised, flash-frozen on dry ice, and stored at -80°C until analysis. Twelve- μm thick coronal tissue sections were obtained using a cryostat microtome (Leica Microsystems, Wetzlar, Germany) at -12°C . The sections were thaw-mounted onto poly-L-lysine coated indium–tin–oxide (ITO) glass slides (Bruker Daltonics, Bremen, Germany) and stored at -80°C . All experiments were approved by the Animal Experiment Ethics Committee of Leiden University Medical Center.

Tissue Preparation. The tissue sections mounted on ITO slides were collected from -80°C storage and equilibrated to room temperature in a freeze-dryer for 30 min. Thereafter, all tissues were washed as follows: (i) submerge in 70% ethanol for 30 s; (ii) submerge in 96% ethanol for 30 s; (iii) 10 short dips in deionized water; (iv) submerge in 70% ethanol for 30 s; (v) submerge in 96% ethanol for 30 s.²⁰ Finally, the sections were dried in a vacuum desiccator for 15 min.

On-Tissue Disulfide Bond Reduction. Tissue sections were covered with 4 layers of 2 mM TCEP (in deionized water) using the SunCollect automatic sprayer (SunChrom, Friedrichsdorf, Germany). The flow rate for the TCEP application was set to 10 $\mu\text{L}/\text{min}$, which resulted in the application of 360 nmol TCEP/ cm^2 . Detailed information on the SunCollect settings can be found in Figure S-1 (Supporting Information). The total incubation time at 23°C (room temperature) was 30 min (including ca. 10 min spraying time).

On-Tissue Enzymatic Digestion. All proteases were dissolved in deionized water to a final concentration of 0.02 $\mu\text{g}/\mu\text{L}$. HEPES containing buffers for Lys-C and r-Lys-N were exchanged with deionized water using a 10 kDa molecular weight cut off (MWCO) spin filter (Merck Millipore, Billerica, MA, USA). Arg-C was dissolved in 2 mM DTT to activate the enzyme. Five layers of proteolytic enzymes were applied using the SunCollect automatic sprayer at a flow rate of 5 $\mu\text{L}/\text{min}$. This resulted in the application of 7.5 μg enzyme/ cm^2 (Figure S-1, Supporting Information). The tissues were incubated for 18 h at 37°C in a saturated air chamber (50% methanol in deionized water). Finally, MALDI matrix was applied using the SunCollect sprayer. For the MALDI-TOF-MSI analyses, 5 mg/mL α -cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile (ACN) and 0.3% trifluoroacetic acid (TFA) was applied. For MALDI MSI experiments performed with the MALDI-FTICR mass spectrometer, it was found that the CHCA matrix produced excessive matrix clusters. Accordingly for MALDI-

FTICR-MSI analyses, 50 mg/mL 2,5-dihydroxybenzoic acid (DHB) in 50% ACN and 0.1% TFA was used as matrix.

MALDI-TOF Imaging. MALDI-TOF-MSI was performed on an UltrafileXtreme MALDI-TOF/TOF (Bruker Daltonics) in positive-ion reflectron mode, using 500 laser shots per spot and 100 μm pixel size. Data was acquired in a *m/z* range from 800 to 3000 Da. Data acquisition, preprocessing, and visualization were performed using the flex software package by Bruker Daltonics (flexControl 3.4; flexAnalysis 3.4; flexImaging 3.0).

MALDI-FTICR Imaging. MALDI-FTICR-MSI was performed on a 9.4T SolariX XR mass spectrometer (Bruker Daltonics) in positive-ion mode, using 250 laser shots per spot and 150 μm pixel size. Data was acquired in a *m/z* range from 600 to 3500 Da with a 512k data point transient (1.1 s duration) and an estimated resolution of 200.000 at *m/z* 400 Da. Data acquisition was performed using ftmsControl (Bruker Daltonics), and visualizations were obtained from flexImaging 4.0 (Bruker Daltonics).

Data Analysis MALDI-TOF-MSI. Regions of interest (ROIs) containing the full area of the coronal section were selected in flexImaging and 500 random spectra from within each ROI extracted into ClinProTools 3.0 (build 22, Bruker Daltonics). The spectra underwent smoothing and baseline subtraction (Top Hat algorithm), total-ion-count normalization, and peak picking at different signal-to-noise ratios. The peak lists were exported to Excel 2010 for further calculations.

Peptide Extraction. Consecutive tissue sections were prepared for MALDI-MSI using the same sample preparation protocol. Instead of MALDI-MSI analysis, the proteolytic peptides were extracted from the matrix coating using a series of solvents with increasing organic content: (i) 10 μL of 0.1% TFA (repeat 4 times); (ii) 10 μL of 50% ACN/0.1% TFA (repeat 4 times); (iii) 90% ACN/0.1% TFA (repeat 4 times).²⁰ Extracts were combined, dried, and resuspended in 0.1% TFA and then cleaned with Omix C18 tips (Agilent). The purified extracts were dried and stored at -20°C until LC-ESI-MS/MS analysis.

LC-MS/MS Analysis. Peptide extracts were analyzed using an Easy nLC1000 (Thermo, Bremen, Germany) coupled to a Q-Exactive mass spectrometer (Thermo). Fractions were injected onto a homemade precolumn (100 μm \times 15 mm; Reprosil-Pur C18-AQ 3 μm , Dr. Maisch, Ammerbuch, Germany) and eluted via a homemade analytical column (15 cm \times 50 μm ; Reprosil-Pur C18-AQ 3 μm). The gradient was 0% to 30% solvent B (90% ACN/0.1% FA) in 120 min. The analytical column was drawn to a tip of \sim 5 μm and acted as the electrospray needle of the MS source. The Q-Exactive mass spectrometer was operated in top10-mode. Parameters were as follows: full scan, resolution 17 500, AGC target 3 000 000, max fill time 20 ms; MS/MS, resolution 35 000, AGC target 1 000 000, max fill time 60 ms, intensity threshold 17400. Apex trigger was set to 1–5 s, and allowed charges were 1–5.

LC-ESI-MS/MS Database Search. Peptide and protein identifications were extracted from the SwissProt database using the Mascot server. Up to two missed cleavages were allowed, and methionine oxidation was set as a variable modification. Peptide assignments were made with a tolerance of 10 ppm. MS/MS fragment tolerance was 20 mmu. Protein identifications were assigned on the basis of a minimum of one confident peptide at 1% false discovery rate (FDR). Only peptides and proteins with red bold notation were included in

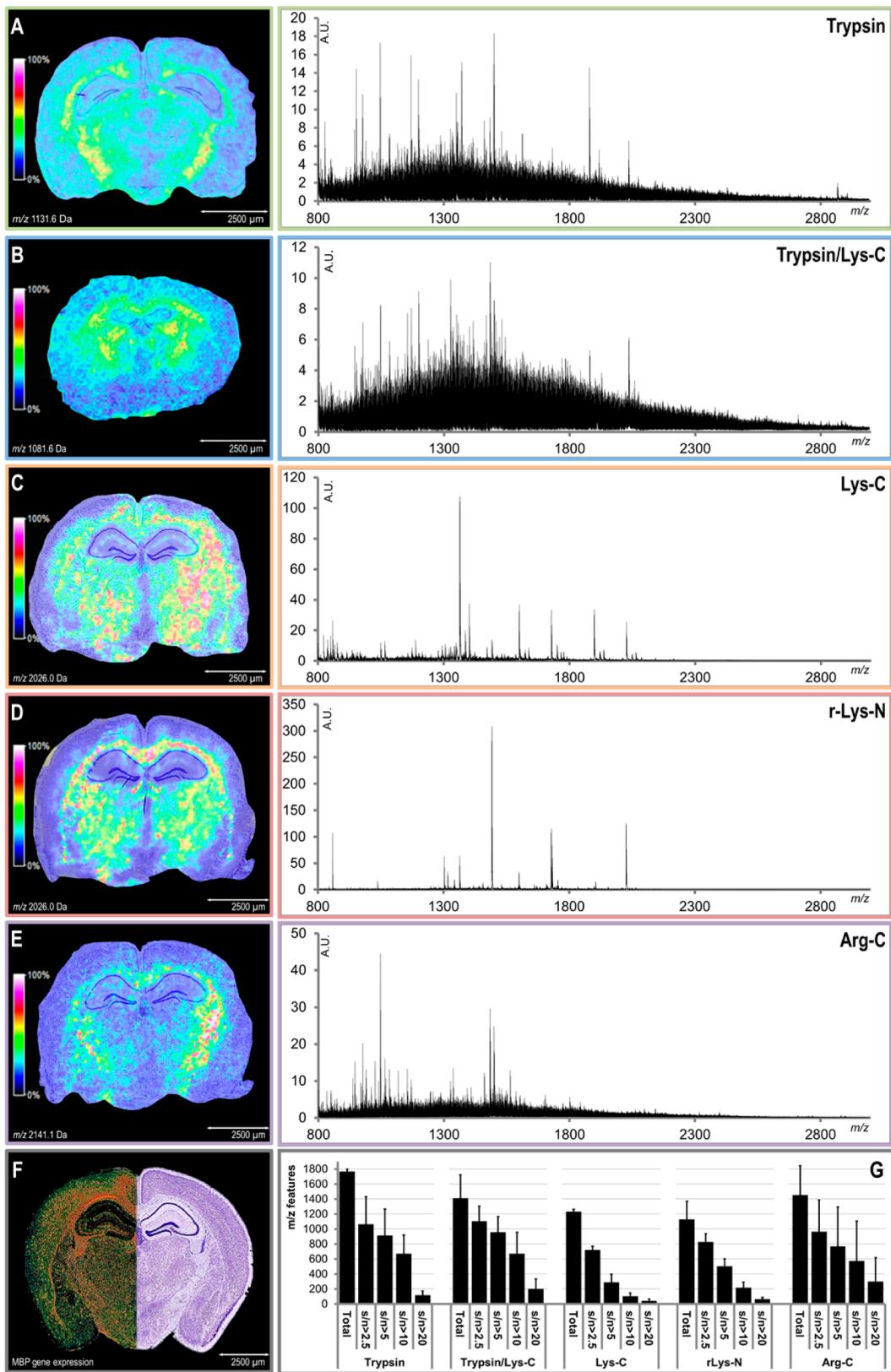


Figure 1. Overview of the on-tissue-digestion MALDI-MSI data. Myelin basic protein peptide ion distributions and average mass spectra for the digested tissue sections; (A) $m/z = 1131.6$ (TTHYGSLPK); (B) $m/z = 1081.6$ (FFSGDRGAPK); (C) $m/z = 2026.0$ (SQHGRTQDENPVVHFFK); (D) $m/z = 2026.0$ (KSQHGRTQDENPVVHFF); (E) $m/z = 2141.1$ (TQDENPVVHFFKNIVTPR); (F) mouse brain Nissl histologic staining and MBP gene expression data from the Allen Brain Atlas; (G) average number ($n = 4$) of extracted m/z features with different signal-to-noise ratios.

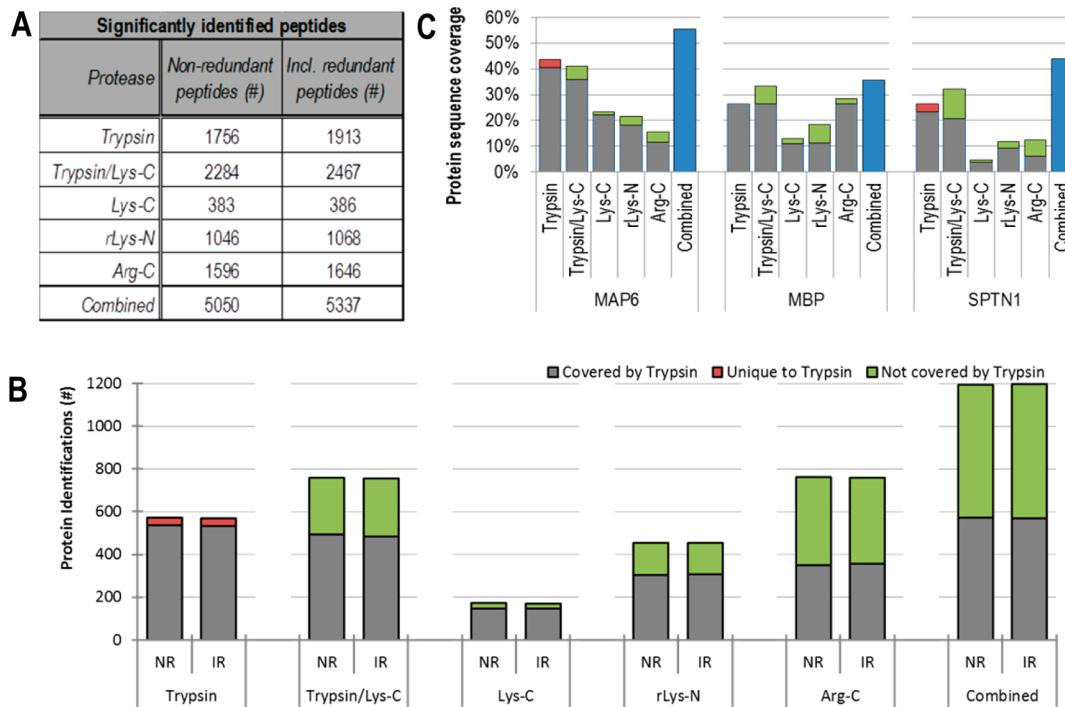


Figure 2. Analysis of the on-tissue digested matrix proteome. (A) Number of peptides identified for each protease above the significant threshold. Confident nonredundant peptides required a red bold notation in Mascot and a 1% FDR. Mascot score thresholds were ≥ 30 for trypsin and trypsin/Lys-C and ≥ 25 for Lys-C, r-Lys-N, and Arg-C. Addition of redundant peptides was achieved by removing the red bold requirement. (B) Number of proteins identified for each protease. Confident nonredundant (NR) protein identifications were assigned on the basis of 1 or more significant peptides, a red bold notation in Mascot and 1% FDR. Mascot score thresholds were ≥ 30 for trypsin and trypsin/Lys-C and ≥ 25 for Lys-C, r-Lys-N, and Arg-C. Addition of redundant proteins (IR) was achieved by removing the red bold requirement. (C) A comparison of sequence coverage for microtubule associated protein 6 (MAP6), myelin basic protein (MBP), and spectrin-1 (SPTN1). The blue bars represent the total sequence coverage after combining the separate measurements.

the final lists of nonredundant peptides. For trypsin and trypsin/Lys-C peptides and proteins, a Mascot significance score ≥ 30 was used; for Lys-C, r-Lys-N, and Arg-C, the threshold score was ≥ 25 .

Gene Ontology and Pathway Analysis. A list containing the Uniprot accession numbers of the identified proteins was uploaded into the STRAP software tool (v1.1.0.0, Boston University School of Medicine).²¹ The KEGG pathway analysis (PA) was performed by uploading the list in the online STRING 9.1 tool.²² As *p*-value correction, the “FDR correction” option was applied.

Data Analysis MALDI-FTICR-MSI. Average spectra from the MALDI-FTICR-MSI data sets were exported into CSV format and loaded into mMass (<http://www.mmass.org>).²³ Peak picking was performed on peaks with $S/N \geq 5$ and intensity ≥ 1500 A.U. The peak lists were then deisotoped with a maximum charge of 3+ and an isotope mass tolerance of 0.05 Da.

MALDI-FTICR-MSI Peptide Identity Assignment. MALDI-FTICR-MSI peak lists and Mascot database search results from the LC-ESI-MS/MS analyses were exported to Excel 2010. The MSI peaks were assigned to identified peptides on the basis of a mass tolerance of 20 ppm.

RESULTS

On-tissue digestion is used to increase proteome coverage and to assign identities to the peaks detected by MALDI-MSI. In this study, we investigated whether additional arginine and lysine proteolytic enzymes, with different cleavage specificities, could further improve on-tissue digestion MALDI-MSI by

increasing sequence and proteome coverage. MALDI-MSI was combined with LC-MS/MS analyses of on-tissue digestion matrix extracts to compare the proteolytic peptides produced by the enzymes trypsin, Lys-C, trypsin/Lys-C, r-Lys-N, and Arg-C and to determine which proteolytic peptides were detected by MALDI-MSI.

MALDI-TOF-MSI: Image Comparison. To assess the effects of sample preparation (Figure S-2, Supporting Information) for the different enzymes, ion distributions for proteolytic peptides from the same protein were compared with each other and with the gene expression data from the Allen Mouse Brain Atlas (<http://www.brain-map.org>). Figure 1 shows example mass spectra and MSI images obtained from coronal sections of a C57BL/6J mouse subject to on-tissue digestion with the different proteases. The images are of proteolytic peptides originating from myelin basic protein (MBP), assigned to confidently identified peptides from the LC-ESI-MS/MS analysis of matrix extracts. The mass resolution of the UltraflexTreme MALDI-TOF/TOF used for these experiments is insufficient to resolve the isobaric ions produced by on-tissue digestion, and so, the images may include contributions from unresolved isobaric ions. The distributions of the MBP proteolytic peptides were consistent with the MBP gene expression images contained in the Allen Brain Atlas, Figure 1F, indicating that any isobaric ions were minor contributors and that no artifacts resulted from the on-tissue digestion procedure.

It is important to note that the quality of the MSI images was poor when the enzymes were applied using the buffer system recommended by the manufacturers (e.g., ammonium bicar-

bonate; HEPES; Tris-HCl), with no visualization of clear anatomical structures for different m/z values (Figure S-3, Supporting Information). For this reason, all enzymes, except Arg-C, were dissolved in MQ-water (pH 6.5–7).

MALDI-TOF-MSI: Comparison of Average Spectra. The average mass spectra obtained by MALDI-MSI analysis for both trypsin (Figure 1A) and trypsin/Lys-C (Figure 1B) digestions presented a curved mass spectral profile that has already been described in several publications.^{5–8} The profile reflects the very high complexity of the resulting proteolytic peptide mixtures as well as the inability of the MALDI-TOF/TOF analyzer to fully resolve all peaks. Proteolytic digestions using proteases with cleavage specificity for single amino acids, such as Lys-C, r-Lys-N, or Arg-C, yielded a smaller number of peptides. As can be seen in Figure 1C–E, when Lys-C, r-Lys-N, or Arg-C were used, the spectra exhibited a flatter baseline and peaks with higher absolute intensity. Figure 1G shows that digestion with trypsin yielded a higher total number of m/z features than digestions using proteases with single site cleavage specificity. However, on-tissue digestion with Arg-C presented the largest number of high intensity m/z features, $S/N > 20$, consistent with the previously reported bias of MALDI toward Arg-terminated tryptic peptides. Note: the number of m/z features reported in Figure 1G should be considered a low estimate because of the inability of the reflectron-TOF mass analyzer to resolve isobaric ions.²⁴

Peptide and Protein Identification. To further investigate the dependence of the on-tissue digestion MALDI-MSI experiment on the protease, the proteolytic peptides extracted from the tissue section, following deposition and crystallization of the matrix, were analyzed by LC-ESI-MS/MS (using consecutive tissue sections that had undergone identical sample preparation). The analysis of the matrix extracts of the five protease preparations resulted in a total of 5050 nonredundant peptides (Supplementary Excel File, Supporting Information), over 2.8 times the number obtained from the matrix extract of the trypsin digested tissue. When including redundant peptides, the total number increased to 5337 (Supplementary Excel File, Supporting Information), which was also 2.8 times the number of peptides identified with trypsin alone (Figure 2A). The relatively low number of peptides identified after digestion with Lys-C and r-Lys-N was unexpected but confirmed by repeat experiments. This observation is due to the enzyme buffer exchange performed for both enzymes. To achieve optimal digestion conditions, proteases are usually dissolved in specific buffer systems containing additives, such as chelating agents, salts, and sugars. The buffers for both Lys-C and r-Lys-N contained 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), a compound known to cause signal suppression in MALDI-MS above a certain concentration threshold.^{25,26} This effect is demonstrated in Figure S-3, Supporting Information, which compares the average spectra obtained after on-tissue digestion MALDI-MSI with Lys-C dissolved in the manufacturer's recommended buffer (50 mM HEPES (pH 8.0), 10 mM EDTA, and 5 mg/mL raffinose) and the average spectra obtained after digestion with the same enzyme dissolved in MQ-water after buffer exchange. The latter spectrum shows an increase of more than 2-fold in absolute intensity, but the number of peptides is significantly lower than would be expected on the basis of previous LC-ESI-MS/MS experiments.

Similar results were achieved at the protein level (Figure 2B). The combined data of all on-tissue digestion experiments resulted in a total of 1194 confidently identified nonredundant

proteins. When redundant proteins were included, a total of 1198 proteins were identified of which 52.2% had not been previously identified in the matrix extract of the trypsin-digested tissue section. Thirty seven proteins, including redundant proteins, were identified exclusively in the tryptic digests, corresponding to 3.1% of the total number of identified proteins. The largest gain in proteome coverage was achieved using Arg-C: 22.7% of the total number of identified proteins originated exclusively from this extract. A subset of only 96 proteins was identified in all matrix extracts, which corresponds to 8.0% of the total number of identified proteins.

Besides the increase in proteome coverage, individual protein sequence coverage also gained from combining the data obtained with multiple proteases (Figure 2C). For instance, analysis of spectrin-1 (SPTN1; 2472 amino acid residues; MW = 284 kDa; Uniprot entry: P16546), a highly abundant protein in mouse brain that is involved cytoskeletal structure,²⁷ resulted in a sequence coverage of 26.3% from the matrix extract of the trypsin digested tissue section. By combining the data from all enzymes, the sequence coverage for SPTN1 increased to 44%. Similar trends were observed for other proteins as shown in Figure 2C.

To test whether the proteome coverage could be improved by reducing protein disulfide bonds before enzymatic digestion, a series of experiments was performed in which on-tissue enzymatic digestion was preceded by on-tissue disulfide bond reduction using 2 mM TCEP (in MQ-water), a compound that was previously shown to be compatible with MALDI-MS.^{28,29} The ion distributions from the reduced tissues were of similar quality as the ones obtained from nonreduced tissues, indicating that the TCEP reduction did not affect the localization of the peptides in the tissue (data not shown). The gain in proteome coverage for each protease was determined by comparing protein identifications from both nonreduced and reduced data sets (Figure 3). For r-Lys-N, the TCEP-reduced protein extract resulted in the identification of an additional 142 proteins, which were not previously identified in the nonreduced sample. A small gain in proteome coverage was also observed in the extract of trypsin (32 additional

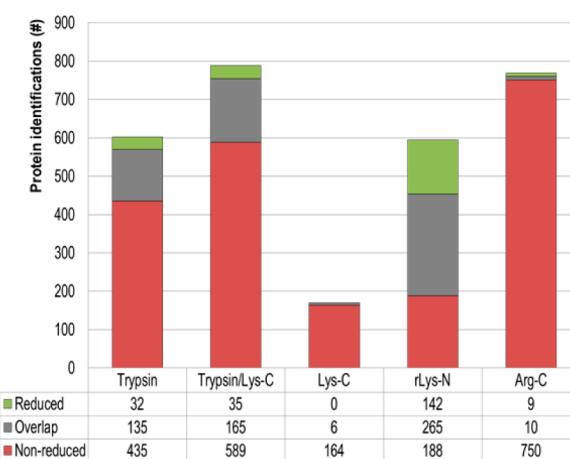


Figure 3. Comparison of confident unique protein identifications obtained from matrix extracts taken from tissue sections treated with on-tissue digestion (red) and tissue sections treated with on-tissue disulfide bond reduction followed by on-tissue digestion (green). Proteins occurring in both experiments were designated as overlap (gray).

identified proteins) and trypsin/Lys-C (35 additional identified proteins).

Gene Ontology Analysis of the Matrix Proteome. The combined data from the digested matrix proteomes was submitted to a gene ontology analysis. The results show that the matrix proteome contained mostly cytoplasmic proteins (48.7%), followed by nuclear proteins (14.9%) and membrane proteins (16.3%) (Figure S-4, Supporting Information). These results are in accordance with previous data by Maier et al.,¹¹ which focused on the intact proteins sampled by MALDI-MSI.

A KEGG pathway analysis, Figure S-5 (Supporting Information), revealed that many of the proteins in the digested matrix proteome play a role in metabolism: among the most significant pathways are oxidative phosphorylation, the Krebs cycle, glycolysis, and pyruvate metabolism. Protein pathways involved in neurodegenerative disorders (e.g., Alzheimer's disease, Parkinson's disease, and Huntington's disease) as well as in long-term depression were identified among the most significant hits.

MALDI-FTICR-MSI: Linking MSI to LC-MS/MS. To resolve isobaric peptide ions and assign identities, the on-tissue digestion experiments were repeated with a 9.4T MALDI-FTICR. For trypsin digestion, 156 peptides, corresponding to 100 proteins (Table 1) and 8.2% of the peptides identified by

Table 1. Results of the Peptide Identity Assignment on the MALDI-FTICR-MSI Data

protease	assigned peptides incl. redundant peptides total (#)	corresponding proteins total (#)
trypsin	156	100
trypsin/ Lys-C	242	133
Lys-C	67	40
r-Lys-N	135	86
Arg-C	152	115

LC-MS/MS, could be assigned to peaks in the MSI data set. For the other proteases, the numbers were as follows: (i) trypsin/Lys-C 9.8%; (ii) Lys-C 17.4%; (iii) r-Lys-N 12.6%; (iv) Arg-C 9.2%. The combined list of assigned peptides for the five proteases corresponded to 280 proteins (an increase of 180 proteins if compared to just trypsin alone).

For several proteins, more than one peptide was assigned, which allowed for comparison of the peptide distribution within one tissue section or between tissue sections that were digested with different proteases. Figure 4 shows several examples of MBP proteolytic fragments that were assigned on the basis of the described methodology. The peptide distributions were verified using the gene expression profile for the *Mbp* gene obtained from the Allen Brain Atlas (Figure 1F). More examples are presented in Figure S-6 (Supporting Information).

■ DISCUSSION

One of the most comprehensive studies regarding the proteome sampled by MALDI-MSI identified 1400 proteins from diverse human tissues.¹¹ It was based on the extraction of intact proteins from tissues coated with MALDI matrix, the matrix proteome, followed by separation by gel electrophoresis and in-gel trypsin digestion. Despite the valuable information collected, in terms of the proteins ultimately accessible by MSI, the published data does not allow a direct comparison with

MALDI-MSI because most identified proteins are substantially larger than those usually detected by MALDI-MSI.^{24,30} Instead, the identification of the proteins analyzed by MALDI-MSI is often performed using on-tissue enzymatic digestion, in which the MALDI-MSI data is assigned, on the basis of accurate mass, to LC-MS/MS data from tissue extracts.^{11,31,32} Digestion with different enzymes has been shown to increase proteome coverage in bottom-up proteomics.^{15–18} Similarly, Enthaler et al.¹⁹ demonstrated that the combination of trypsin, pepsin, and elastase increased protein sequence coverage in MALDI-MSI. The increased sequence coverage follows from the high number of cleavage sites; elastase cleaves at the C-terminus of small hydrophobic amino acids, G, S, I, L, A, and V³³ and pepsin at the C-terminus of amino acids, P, Y, W, and L³⁴ but at the expense of very complex MSI data sets containing many isobaric ions.

Instead, we investigated whether the different activities of additional enzymes that cleave at the basic amino acids (to maintain MS detection sensitivity) can also aid MALDI-MSI. The enzymes trypsin, Lys-C, trypsin/Lys-C, r-Lys-N, and Arg-C were investigated. A standard digestion protocol was first established using trypsin. Once similar results were reproducibly obtained, including those from different animals, the method was adapted for the other proteases by changing the solution conditions (minimal changes to the spraying and incubation conditions). The success of the different proteases further demonstrates the robustness of the method (see Figure S-1, Supporting Information, for spray method). In this paper, we have focused on the improvement in proteome coverage that may be obtained in on-tissue digestion MALDI-MSI by combining the results from different proteases; to ensure comparability, the data presented are obtained from a single animal.

All enzymes were diluted to the same concentration and sprayed over the tissue with the same method. The presence of salts and buffers in the protease solutions of Lys-C and r-Lys-N led to poor matrix crystallization, low signal intensity, and noisy MSI images (Figure S-3, Supporting Information). A buffer exchange with MQ-water (pH 6.5–7) improved the quality of the MS images, but the low number of peptide and protein identifications indicated that the lack of salts and buffers adversely affected enzymatic activity (Figure 2). Still, the combination of proteases greatly increased the number of detected peptides and proteins; the 5337 peptides and 1198 proteins represent an increase of 179% and 110%, respectively, as compared to trypsin alone (1913 peptides, 570 proteins).

Unlike LC-MS/MS based protein identification, MSI of proteolytic peptides does not differentiate unique peptides from nonunique peptides and all peptides are detected in the same mass spectrum. Consequently, the data sets will contain nonunique peptides as well as many isobaric ions. Both of these characteristics frustrate efforts to identify proteolytic peptides directly from tissue using MS/MS.⁹ The use of multiple enzymes for on-tissue digestion has the advantage of producing different peptides from the same protein that can be used to confirm its distribution in the tissue sample (Figures 1 and 4).

To connect the confidently identified peptides and proteins from the matrix extracts to the on-tissue digestion MALDI-MSI experiments, high mass resolution MALDI-MSI data was acquired on a 9.4T MALDI-FTICR instrument. Between 8.2% and 17.4% of the peptides identified by LC-MS/MS could be assigned to peaks in the high mass resolution MALDI-

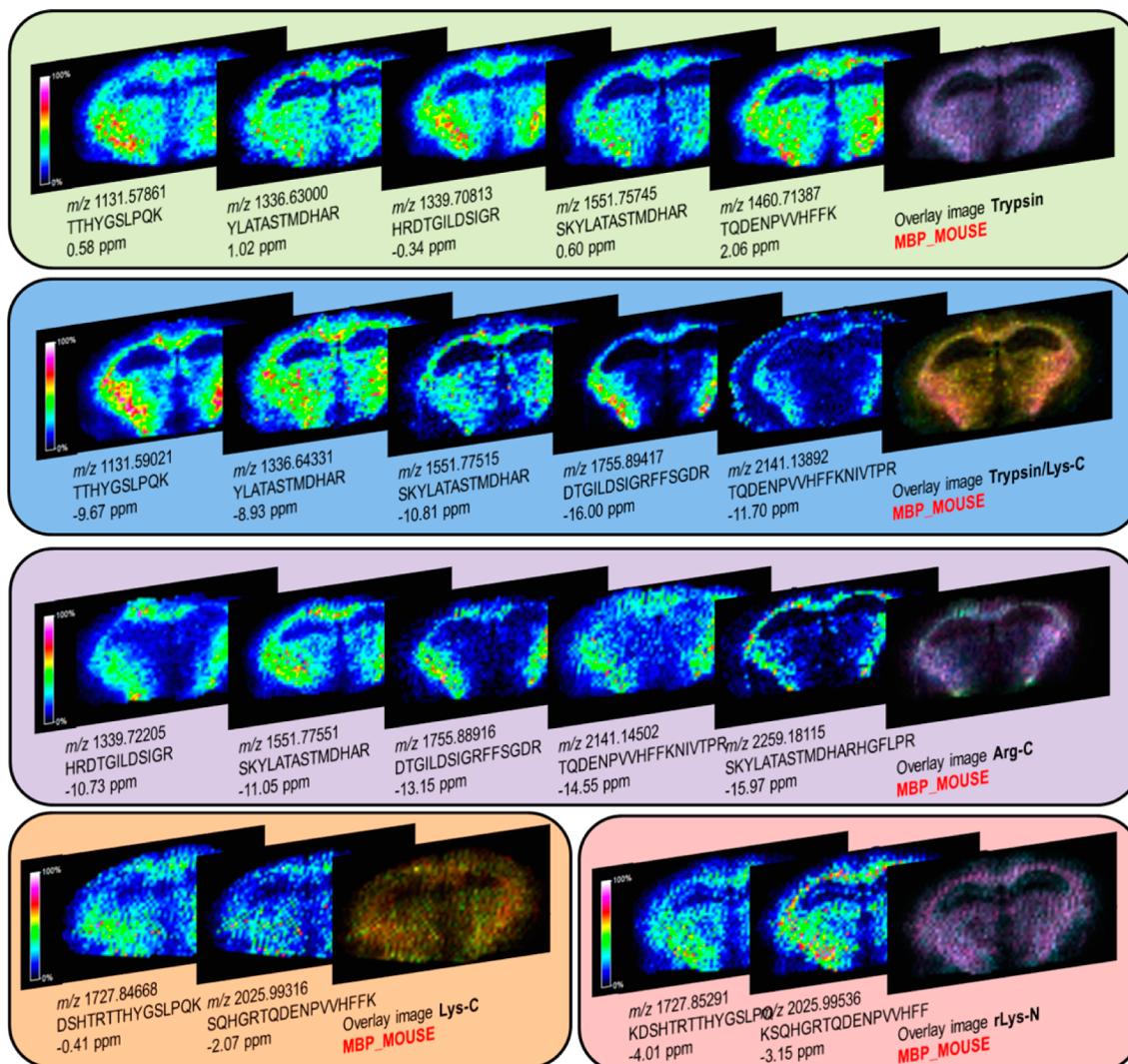


Figure 4. Ion distributions representing MBP peptides obtained from MALDI-FTICR-MSI were assigned to peptides identified by LC-ESI-MS/MS. The images were obtained from the MALDI-FTICR-MSI data sets of (i) trypsin (green box), (ii) trypsin/Lys-C (blue box), (iii) Arg-C (purple box), (iv) Lys-C (orange box), and (v) r-Lys-N (red box). For each of the single ion images (represented by the jet intensity scale), the corresponding sequence and measurement error are presented.

MSI data sets (Table 1). The combined total number of 633 peptides originated from 280 proteins (Supplementary Excel File, Supporting Information). A previous publication by Schober et al.²⁴ reports a similar approach where over 1100 peptides were first identified from a mouse brain tissue homogenate, of which 13% were assigned to m/z features in a high mass resolution MALDI-MSI data set, and corresponded to 101 proteins. The Schober paper used extensive peptide fractionation to increase protein identification rates and analyzed the entire tissue's proteome. In contrast, the results reported here utilized no fractionation and only analyzed the matrix proteome. Without the fractionation step, 140 peptides were identified in the LC-MS/MS analysis of which 60 could be assigned to m/z features in the MALDI-MSI data set, corresponding to 38 proteins.²⁴ Here, using complementary enzymes for on-tissue digestion and LC-MS/MS of the matrix peptides (without any fractionation step), we could assign more than 600 peptides, corresponding to 280 proteins.

The peptide assignments were based on a mass error tolerance of ± 20 ppm. The cyclotron frequency of an ion inside an ICR cell, and therefore the accurate calibration of an FTICR

instrument, is dependent on the magnetic field strength as well as the local electric field. Accordingly, the calibration is dependent on the number of ions in the cell, which in MALDI MS varies from shot-to-shot and in MALDI-MSI from pixel to pixel. Consequently, there are slight mass deviations in all pixels, resulting in broader peaks in the average mass spectrum. Peak picking and peptide identity assignment were based on the average, nonaligned mass spectrum, and therefore, a higher mass tolerance was applied. However, the consensus between the images obtained for different peptides, utilizing different proteases, provides additional corroboration.

The LC-MS/MS data reported here of MALDI matrix extracts also act as a guide to the proteins/peptides that may be analyzed via MALDI-MSI. It has previously been shown that MSI detected only a fraction of those detected by LC-MS/MS analysis of tissue extracts.¹⁰ A single 100 μm pixel analyses the equivalent of ≈ 12 cells (assuming average cell size of 20 μm and tissue thickness of 10 μm) and is analyzed without any explicit purification and separation step. To be detected by MALDI-MSI, the peptides are required to be present in the MALDI matrix and present at sufficiently high levels. While we

have demonstrated an increase in proteome coverage, the number remains a fraction of the tissue's total proteome, because of the sample-volume-limited nature of the analysis and the need for matrix incorporation of the peptides. The FTICR mass spectrometer used here and the Orbitrap used previously²⁴ are characteristically very high dynamic range mass analyzers. To significantly increase the number of peptides detected by MSI will require significant increases in the charge capacity of these ion trap mass analyzers (while maintaining performance) in order for the lower level peptides to be above the detection threshold.

Among the 280 assigned proteins detected here were several histones. Histones are highly implicated in cancer progression through their role in determining chromatin structure and gene accessibility. Proteolytic fragments of histones have been previously reported in cancerous tissues analyzed by on-tissue-digestion MALDI-MSI.^{35,36} Several peptides were assigned to subunits of mitochondrial ATPases (AT1A1, AT1A2, AT1A3, AT2B1, AT2B2, ATPA, ATPSH, and ATP5J). These trans-membrane proteins are known to be involved in a multitude of neurological disorders, e.g., familial hemiplegic migraine^{37,38} and Alzheimer's disease.^{39,40} These results were confirmed by the GO analysis and KEGG pathway analysis which show that proteins involved in several metabolic pathways and neurological disorders are well represented in the digested matrix proteome (Figures S-4 and S-5, Supporting Information).

CONCLUSIONS

This work reports the peptides identified from the on-tissue-digestion matrix proteome as well as the peptides detected by ultrahigh mass resolution MALDI-MSI, for multiple enzymes, both valuable sources of information for researchers applying on-tissue digestion MSI. The results demonstrate the ability to increase proteome coverage by using different enzymes and indicate that on-tissue digestion MALDI-MSI analysis of the mouse brain can be applied in a wide range of disease related, biomarker discovery or other neuroscientific research applications.

It should be noted that we have been deliberately conservative with the thresholds used in the assignments (>0.6% base peak). It is known that FTICR and Orbitrap mass spectrometry can span a very wide dynamic range, and more peptides could have been assigned if a lower threshold was used. However, at lower thresholds, many peaks were associated with noisy images and the greatly increased number of peaks is accompanied by an increased risk of misassignment. It is expected that, with additional improvements in dynamic range, sensitivity, and on-tissue MS/MS based verification of peptide identities, even more peptides will be assignable and the application potential of MALDI-MSI will be increased further.

ASSOCIATED CONTENT

Supporting Information

Settings for the SunChrom SunCollect, additional MSI images of assigned proteolytic peptides, and extensive lists of both identified peptides in the MALDI matrix and assigned peptides from the high-mass resolution analyses. This material is available free of charge via the Internet at <http://pubs.acs.org/>.

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Notes

The authors declare no competing financial interest.

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REFERENCES

- (1) Caprioli, R.; Farmer, T.; Gile, J. *Anal. Chem.* **1997**, *69*, 4751–4760.
- (2) McDonnell, L.; Heeren, R. *Mass Spectrom. Rev.* **2007**, *26*, 606–643.
- (3) Balluff, B.; Elsner, M.; Kowarsch, A.; Rauser, S.; Meding, S.; Schuhmacher, C.; Feith, M.; Herrmann, K.; Röcken, C.; Schmid, R. M.; Höfler, H.; Walch, A.; Ebert, M. P. *J. Proteome Res.* **2010**, *9*, 6317–6322.
- (4) Meding, S.; Nitsche, U.; Balluff, B.; Elsner, M.; Rauser, S.; Schöne, C.; Nipp, M.; Maak, M.; Feith, M.; Ebert, M. P.; Friess, H.; Langer, R.; Höfler, H.; Zitzelsberger, H.; Rosenberg, R.; Walch, A. *J. Proteome Res.* **2012**, *11*, 1996–2003.
- (5) Cazares, L. H.; Troyer, D. A.; Wang, B.; Drake, R. R.; Semmes, O. *J. Anal. Bioanal. Chem.* **2011**, *401*, 17–27.
- (6) Shuichi, S.; Masaru, F.; Katsuhiko, I.; Yoshikazu, Y.; Mitsutoshi, S. *Surf. Interface Anal.* **2006**, *38*, 1712–1714.
- (7) Lemaire, R.; Desmons, A.; Tabet, J.; Day, R.; Salzet, M.; Fournier, I. *J. Proteome Res.* **2007**, *6*, 1295–1305.
- (8) Groseclose, M.; Massion, P.; Chaurand, P.; Caprioli, R. *Proteomics* **2008**, *8*, 3715–3724.
- (9) Houel, S.; Abernathy, R.; Renganathan, K.; Meyer-Arendt, K.; Ahn, N. G.; Old, W. M. *J. Proteome Res.* **2010**, *9*, 4152–4160.
- (10) Schober, Y.; Schramm, T.; Spengler, B.; Römpf, A. *Rapid Commun. Mass Spectrom.* **2011**, *25*, 2475–2483.
- (11) Maier, S.; Hahne, H.; Gholami, A.; Balluff, B.; Meding, S.; Schoene, C.; Walch, A.; Kuster, B. *Mol. Cell. Proteomics* **2013**, *12*, 2901–2910.
- (12) Vandermarliere, E.; Mueller, M.; Martens, L. *Mass Spectrom. Rev.* **2013**, *32*, 453–465.
- (13) Brownridge, P.; Beynon, R. *Methods* **2011**, *54*, 351–360.
- (14) Krause, E.; Wenschuh, H.; Jungblut, P. R. *Anal. Chem.* **1999**, *71*, 4160–4165.
- (15) Choudhary, G.; Wu, S.-L.; Shieh, P.; Hancock, W. *J. Proteome Res.* **2003**, *2*, 59–67.
- (16) Hohmann, L.; Sherwood, C.; Eastham, A.; Peterson, A.; Eng, J.; Eddes, J.; Shteynberg, D.; Martin, D. *J. Proteome Res.* **2009**, *8*, 1415–1422.
- (17) Wa, C.; Cerny, R.; Hage, D. *Anal. Biochem.* **2006**, *349*, 229–241.
- (18) Gatlin, C.; Eng, J.; Cross, S.; Detter, J.; Yates, J. *Anal. Chem.* **2000**, *72*, 757–763.
- (19) Enthaler, B.; Trusch, M.; Fischer, M.; Rapp, C.; Pruns, J.; Vietzke, J.-P. *Anal. Bioanal. Chem.* **2013**, *405*, 1159–1170.
- (20) Enthaler, B.; Bussmann, T.; Pruns, J.; Rapp, C.; Fischer, M.; Vietzke, J.-P. *Rapid Commun. Mass Spectrom.* **2013**, *27*, 878–884.

- (21) Bhatia, V.; Perlman, D.; Costello, C.; McComb, M. *Anal. Chem.* **2009**, *81*, 9819–9823.
- (22) Jensen, L.; Kuhn, M.; Stark, M.; Chaffron, S.; Creevey, C.; Muller, J.; Doerks, T.; Julien, P.; Roth, A.; Simonovic, M.; Bork, P.; von Mering, C. *Nucleic Acids Res.* **2009**, *37*, D412–D416.
- (23) Strohalm, M.; Hassman, M.; Košata, B.; Kodíček, M. *Rapid Commun. Mass Spectrom.* **2008**, *22*, 905–908.
- (24) Schober, Y.; Guenther, S.; Spengler, B.; Römpf, A. *Rapid Commun. Mass Spectrom.* **2012**, *26*, 1141–1146.
- (25) Amini, A.; Dormady, S. J.; Riggs, L.; Regnier, F. E. *J. Chromatogr., A* **2000**, *894*, 345–355.
- (26) Signor, L.; Boeri Erba, E. *J. Vis. Exp.* **2013**, *79*, No. e50635.
- (27) Zagon, I. S.; Higbee, R.; Riederer, B. M.; Goodman, S. R. *J. Neurosci.* **1986**, *6*, 2977–2986.
- (28) Fischer, W.; Rivier, J.; Craig, A. *Rapid Commun. Mass Spectrom.* **1993**, *7*, 225–228.
- (29) Kulak, N. A.; Pichler, G.; Paron, I.; Nagaraj, N.; Mann, M. *Nat. Methods* **2014**, *11*, 319–324.
- (30) McDonnell, L. A.; Walch, A.; Stoeckli, M.; Corthals, G. L. *J. Proteome Res.* **2014**, *13*, 1138–1142.
- (31) Groseclose, M.; Andersson, M.; Hardesty, W.; Caprioli, R. *J. Mass Spectrom.* **2007**, *42*, 254–262.
- (32) Gustafsson, O. J.; Eddes, J. S.; Meding, S.; McColl, S. R.; Oehler, M. K.; Hoffmann, P. *Rapid Commun. Mass Spectrom.* **2013**, *27*, 655–670.
- (33) Rietschel, B.; Arrey, T. N.; Meyer, B.; Bornemann, S.; Schuerken, M.; Karas, M.; Poetsch, A. *Mol. Cell. Proteomics* **2008**, *8*, 1029–1043.
- (34) János, M.; Ferenc, T.; József, T. *Biomolecules* **2013**, *3*, 923–942.
- (35) Djidja, M.-C. C.; Claude, E.; Snel, M. F.; Scriven, P.; Francese, S.; Carolan, V.; Clench, M. R. *J. Proteome Res.* **2009**, *8*, 4876–4884.
- (36) Djidja, M.-C. C.; Francese, S.; Loadman, P. M.; Sutton, C. W.; Scriven, P.; Claude, E.; Snel, M. F.; Franck, J.; Salzet, M.; Clench, M. R. *Proteomics* **2009**, *9*, 2750–2763.
- (37) Morth, J. P.; Poulsen, H.; Toustrup-Jensen, M. S.; Schack, V. R.; Egebjerg, J.; Andersen, J. P.; Vilsen, B.; Nissen, P. *Philos. Trans. R. Soc., B: Biol. Sci.* **2009**, *364*, 217–227.
- (38) Poulsen, H.; Khandelia, H.; Morth, J. P.; Bublitz, M.; Mouritsen, O. G.; Egebjerg, J.; Nissen, P. *Nature* **2010**, *467*, 99–102.
- (39) Markesberry, W. R. *Free Radical Biol. Med.* **1997**, *23*, 134–147.
- (40) Zlokovic, B. V. *Nat. Rev. Neurosci.* **2011**, *12*, 723–738.