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Quantitative peptidomics of mice lacking peptide-processing enzymes

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Summary

Peptidomics is defined as the analysis of peptides present in a tissue extract, usually using mass spectrometry-based approaches. Unlike radioimmunoassay-based detection techniques, peptidomics measures the precise form of each peptide, including post-translational modifications, and can readily distinguish between longer and shorter forms of the same peptide. Also, peptidomics is not limited to known peptides and can detect hundreds of peptides in a single experiment. Quantitative peptidomics enables comparisons between two or more groups of samples, and is perfect for studies examining the effect of gene knock-outs on tissue levels of peptides. We describe the method for quantitative peptidomics using isotopic labels based on trimethylammonium butyrate, which can be synthesized in five different isotopic forms; this permits multivariate analysis of five different groups of tissue extracts in a single liquid chromatography/mass spectrometry run.

Keywords

Prohormone convertase; proprotein convertase; carboxypeptidase; peptidomics; proteomics; peptidase; protease

1. Introduction

Prohormone convertases PC1/3 and PC2 function as endopeptidases in the neuroendocrine system, contributing to the processing of peptide hormones and neuropeptides (1,2). In addition to the endopeptidase step, most bioactive peptides require an additional step mediated by carboxypeptidase E and/or D; these enzymes remove C-terminal basic residues from the prohormone convertase reaction products (3). For some peptides, additional modifications are required such as C-terminal amidation (4). To study the peptide processing enzymes, it is useful to have a method of measuring the precise form of each peptide present in a tissue. Mass spectrometry-based quantitative peptidomic techniques can be used to distinguish peptide processing profiles in various model biological systems (5). This technique involves extraction and purification of peptides from mouse brain regions followed by labeling with isotopic tags and analysis using liquid chromatography and electrospray ionization mass spectrometry. The analysis provides a detailed view of the

precise molecular forms of each peptide, including any post-translational modifications. By examining how the form and the relative amount of a peptide vary between wild-type mice and mice lacking a peptide-processing enzyme, one can understand the contribution of that enzyme in a given tissue (6,7,8,9,10,11). Peptides which increase in null mice relative to wild-type are putative substrates of the knocked-out peptidase, whereas peptides that decrease in null mice relative to wild-type mice are putative products of the enzyme. Peptides that do not change between null mice and wild-type mice are presumably processed redundantly by multiple peptidases, such that deletion of any one enzyme does not have an effect on peptide levels. By comparing the amino acid sequences of the peptides that do change with those that do not change in the absence of an enzyme, one can better understand the specificity of each enzyme. The chemical character of the amino acids surrounding the cleavage site, such as presence of hydrophobic, charged, or bulky side chains, in turn renders information about the physical aspects of the catalytic site. By comparing quantitative peptidomics data from several different brain regions in multiple groups of null mice, a global view of peptidase processing of neuroendocrine peptides is attained.

Most of the previous *in vivo* studies of PC1/3 and PC2 null mice have used radioimmunoassays to quantify changes in peptide processing (12). If antibodies are available that recognize a specific form of a peptide, this method provides useful information. However, this technique is limited by the requirement for specific antisera to known peptides. Despite the sensitivity of these antibody-based experiments, detection can be unspecific, as antibodies will often recognize several different forms of a peptide, including N- or C-terminally extended peptides or peptides with post-translational modifications such as amidation or acetylation. This makes determining the precise molecular form of the peptide difficult.

Using a mass spectrometry-based quantitative peptidomics approach, peptide levels in a variety of tissues from both prohormone convertase knockout mice and their wild-type counterparts can be analyzed in a systematic manner (7,8,11,10). This method allows for the relative quantification of both known peptides previously unknown peptides; these can often be identified from tandem mass spectrometry analysis. The following protocol describes the basic method of peptide extraction, isotopic labeling, and mass spectrometry analysis.

2. Materials

2.1 Extraction of Peptides

Distilled water Milli-Q system (Millipore, Bedford, MS, USA).

0.1M Hydrochloric Acid (6 N, sequanal grade, constant boiling) Pierce (Rockford, IL, USA)

0.4 M NaH₂PO₄ Sigma-Aldrich, Inc. (St Louis, MO, USA).

Ultrasonic processor (W-380, Ultrasonic Inc., Farmingdale, NY, USA).

Low retention microcentrifuge tubes, Eppendorf

2.2 Peptide Labeling

Several isotopic tags can be used for quantitative peptidomics, some of which are commercially available (such as succinic anhydride with 4 hydrogens or deuteriums). Reagents that react with primary amines are usually used for peptidomics because a free amine is frequently found on peptides either on the N-terminus and/or on Lys side chains (13). However, some peptides do not contain a free amine due to N-terminal acetylation, pyroglutamylation, or another modification, and the absence of Lys residues; these peptides will not be quantifiable using the following approach. In our experience, the optimal isotopic tags for labeling amines are based on 3-(2,5-dioxopyrrolidin-1-yl)oxycarbonylpropyl trimethylammonium chloride (14), which is the N-hydroxysuccinimide (NHS) ester of trimethylammoniumbutyrate (TMAB). One advantage of these reagents over succinic anhydride is that the TMAB reagent contains a positive charge (a quaternary amine) and so the positive charge(s) of the peptide's N-terminus and any Lys residues is/are maintained after labeling with TMAB-NHS. In contrast, peptides labeled with succinic anhydride will have the positive charges of the free amines converted into negative charges, and unless there is another positive charge on the peptide it will not be observed in mass spectrometry performed in positive ion mode (which is the most common procedure). Furthermore, succinic anhydride-labeled peptides tend to show weaker signals than TMAB-labeled peptides (15). In addition, peptides labeled with TMAB reagents containing 9 deuteriums co-elute from HPLC with peptides labeled with the 9 hydrogen form of the reagent; this is important for quantification. In contrast, peptides labeled with succinic acid containing 4 deuteriums do not precisely co-elute with peptides labeled with the hydrogen form of succinic acid (15). Finally, the TMAB reagents can be produced in five different isotopic forms containing either all hydrogen (referred to as D0-TMAB-NHS), three deuteriums (D3-TMAB-NHS), six deuteriums (D6-TMAB-NHS), nine deuteriums (D9-TMAB-NHS), and nine deuteriums and three atoms of ^{13}C (referred to as D12-TMAB-NHS because the mass difference is 12 Da greater than the D0 form) (16). Structures of the reagents are shown in Figure 1 along with the masses added to the peptide by the addition of the TMAB group to an amine and loss of one hydrogen atom. The advantage of multiple isotopic forms of a label is that it permits multivariate analysis of different tissue extracts, either from completely different groups of mice, or from replicates of the same genotype, as shown in Figure 2.

TMAB-NHS compounds. The synthesis of these compounds has been previously described (16).

1.0 M NaOH Sigma-Aldrich, Inc. (St Louis, MO, USA).

Dimethylsulfoxide Sigma-Aldrich, Inc. (St Louis, MO, USA).

NH_2OH HCl Sigma-Aldrich, Inc. (St Louis, MO, USA).

Glycine Sigma-Aldrich, Inc. (St Louis, MO, USA).

Hydriion pH Papers (8.0-9.5) Micro Essential Laboratory (Brooklyn NY, USA)

2.3 Peptide Purification

Amicon Ultra 4 mL Ultracel 10,000 Molecular weight cutoff Centrifugal Filter Devices (Millipore, Bedford, MS, USA).

PepClean™ C-18 spin column Pierce (Rockford, IL, USA)

Acetonitrile HPLC grade Fisher Scientific (Fair Lawn, NJ)

Trifluoroacetic acid Pierce (Rockford, IL, USA)

3. Methods

3.1 Peptide Extraction

1. Sacrifice and Microwaving—Mice are sacrificed by cervical dislocation. Mice are decapitated and heads are immediately placed in a conventional microwave oven at full power for 8 seconds, until the internal temperature of the brain reaches 80°C (17). Alternatively, mice can be sacrificed by microwave irradiation (18). Because the strength of different microwave devices vary considerably, it is essential to calibrate the device to ensure that the brain is heated to a temperature sufficient to inactivate proteases without damaging the brain structure by excessive heating.

2. Dissection—Brain is allowed to cool, then removed and cut into coronal sections using a razor blade for further dissection. Prefrontal cortex is removed by cutting at Bregma 1.94; all measurement references taken from Paxinos and Franklin 2001(19). Additional coronal cuts are made on either side of the hypothalamus at Bregma 0.00 and –3.00. The striatum is dissected from the 1.94 to 0.00 section by removing the cortex. This “striatum” sample includes the caudate putamen, nucleus accumbens, septum, and ventral palladium. The coronal section of Bregma 0.00 to –3.00 is dissected into the hippocampus, thalamus, amygdala, and hypothalamus. Cortex can be dissected from this section as well. Cerebellum is dissected from the remaining brain by removing the forebrain and brainstem sections from the cerebellum.

3. Tissue Storage—At this point, tissues from multiple animals can be pooled into single tubes, if multiple animals are to be used for each group. It is imperative that low retention tubes are used for this, as loss of peptides can occur if normal tubes are used. Washing the tubes with double distilled water beforehand is recommended. Tissues should be stored at –70°C until extraction.

4. Sonication—Dissected tissue pools are sonicated two times for 20 sec at 1 pulse/sec at duty cycle 3, 50% output, in an appropriate volume of ice-cold water using an ultrasonic processor. Tissues are sonicated in low retention 2 mL volume microfuge tubes, using 5 µL of water per µg of tissue, with a minimum of 200 µL water to prevent splatter. A lower additional volume of water is used to rinse the tip of the sonicator into the tube containing the brain extracts, ensuring all peptides are collected. The sonicator should be rinsed between tissue extractions.

5. Extraction of Peptides—The homogenates are incubated in a 70°C water bath for 20 min and then cooled on ice for 15 min. Extracts are acidified with ice-cold 0.1 M HCl to a final concentration of 10 mM HCl followed by vortex mixing. It is important to be sure extracts are ice-cold before adding acid to prevent acid-labile bonds from breaking. After incubation of extracts with acid for 15 minutes at 4°C, homogenates are centrifuged at 13,000 g for 40 min at 4°C and the supernatant is transferred to a new low-retention tube. These extracts can be frozen and concentrated to a lower volume in a vacuum centrifuge. This may be necessary if large volumes are to be combined during the filtration step (it is optimal if the total volume of all labeled extracts is under 4.0 mL to fit in filters used in a subsequent step).

6. Buffering—The pH of the peptide extracts is then adjusted to 9.5 by the addition of 0.4 M phosphate buffer (pH 9.5). These extracts can be stored at –70°C until labeling.

3.2 Labeling

The number of labels used and the number of replicates depends on the experimental questions being addressed. If comparing two conditions, such as wild-type (WT) and knock-out (KO) mouse brain extracts, only two isotopic tags need to be used. However, it is essential to perform replicates, and at least one of the replicates should have the tags switched so that WT and KO are labeled with the opposite tags used for the other groups, as shown for the example indicated in Run 2 as compared to Runs 1 and 3 (Figure 2, top). Because the resulting analysis provides relative values, and not absolute values, in order to perform statistical analysis of the data it is important to also compare the relative level of peptides in WT vs WT groups (Figure 2, middle). This allows ratios from the KO/WT experiments to be directly compared to the ratios from WT/WT experiments and statistical testing to be performed. An alternative approach is to use additional isotopic tags, such as the five forms of the TMAB-NHS labels; this allows for replicates to be included in a single run, and with 2 LC/MS runs it is possible to compare five KO and five WT groups (Figure 2, bottom).

1. Labels—Depending on how many samples are to be compared, two to five labels may be used to label sample extracts. The TMAB-NHS labeling reagents are dissolved in DMSO at 350 µg/µl and used to label each sample. Typically, a total of 5 mg of TMAB-NHS reagent is used for labeling of each mouse brain region (i.e. 5 mg for each hypothalamus, striatum, etc present in the tube).

2. Label Volume Calculation—To determine the volume of labeling solution added per round of labeling, divide the total volume of label/DMSO solution by seven, as seven rounds of labeling will be carried out.

3. Labeling—Each round of labeling consists of adding one seventh of the label volume to the appropriate sample individually. Samples are allowed to incubate at room temperature for 10 minutes before the pH is adjusted to 9.5 using 1.0 M NaOH, with pH paper used to test the pH of each sample by blotting <1 µL of sample onto the paper. Samples incubate another ten minutes at room temperature before the next round of label is added. This

process is repeated seven times to ensure all peptides are properly labeled. After the final addition of TMAB-NHS reagent, the samples should be incubated at room temperature for another 10-30 minutes. The entire labeling procedure requires approximately four hours.

4. Quenching—After the labeling and prior to combining the samples for subsequent steps, it is essential to quench any unreacted TMAB-NHS reagent; this is accomplished by the addition of 2.5 M glycine in water. For this, 10 μ l of the glycine solution is added per 5 mg of TMAB-NHS reagent and the mixture is incubated at room temperature for 40 minutes.

3.3 Peptide Purification

1. Pool and Filter—Labeled samples that are to be compared are pooled together; typical schemes are shown in Figure 2. After pooling samples are applied to Amicon Ultra 4 mL Ultracel filters to remove proteins >10 kDa. Before using the filters, they should be washed with 2 mL water to remove any glycerol that remains on the filter from the manufacturer. Once rinsed, labeled samples are pooled and filtered according to the filter instructions. The flow through is retained, this is the filtrate which will be analyzed.

2. Hydroxylamine Step—In order to ensure that all labeled peptides are labeled only on free amino termini and lysines, and not on tyrosines, the filtrate must be treated with hydroxylamine (NH_2OH). First the pH of the filtrate is adjusted to 9.0 using 1.0 M NaOH. Then, 2.0 M hydroxylamine in DMSO is added to the filtrate at a ratio of 3 μ l of hydroxylamine solution for every 10 mg of TMAB label in the filtrate (if 5 mg of D0- and 5 mg of D9-TMAB-NHS are used, the total amount after pooling is 10 mg; more TMAB-NHS reagent will be present if additional isotopic tags are used, and so additional hydroxylamine should also be used). The reaction is carried out in three rounds, with one third of the 2.0 M hydroxylamine added per round. After the first aliquot of hydroxylamine is added, the filtrate is incubated at room temperature for 10 minutes. The pH of the filtrate is adjusted back to 9.0 using 1.0 M NaOH. The addition of hydroxylamine followed by incubation and pH adjustment is then carried out twice more, for a total of three rounds. These filtrates can now be stored at -70°C until desalting.

3. Desalting—Desalting is carried out with a PepClean™ C-18 spin column (Pierce). There are many peptides in these filtered brain extracts, so for each filtered pool of samples, the resin from two C-18 columns are combined into one column by pouring the resin from one column to the other. Other than this step, desalting of the sample is carried out according to the manufacturers' instructions using solutions made with acetonitrile and trifluoroacetic acid. Peptides are eluted with 80 μ l of 70% acetonitrile and 0.1% trifluoroacetic acid in water. These eluents are frozen, and concentrated to 10-20 μ l in a vacuum centrifuge. Aliquots of the peptide samples are stored at -70°C until mass spectrometry (MS) analysis.

3.4. Liquid chromatography and mass spectrometry (LC/MS)

In order to obtain quantitative information on the relative levels of peptides, it is necessary to analyze the samples on MS. In our experience, best results are obtained by chromatography on a reverse phase column with direct electrospray ionization mass

spectrometry on a quadrupole time-of-flight (q-TOF) instrument. Other types of instruments, such as ion traps, provide less information, and some (such as the LCQ ion trap) did not yield any data for test samples. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) instruments are also less effective than q-TOF instruments because the MALDI-TOF laser causes decomposition of the TMAB tag and the loss of the isotopic label (i.e. the loss of trimethylamine). The extent of the decomposition depends on the intensity of the laser in the MALDI instrument, and can vary from a small percentage to the majority of the signal. The loss of the trimethylamine from the TMAB removes the isotopic-tag, and therefore the resulting signal cannot be quantified. The q-TOF instruments also show loss of the trimethylamine label, but only after collision-induced dissociation, which is actually an advantage; the MS mode has the trimethyl group intact and provides quantification, while the MS/MS mode shows nearly complete dissociation of the trimethylamine tag which makes interpretation of the data easier (i.e. all of the 5 isotopic forms of the TMAB tags produce identical MS/MS spectra because they lose the trimethylamine moiety).

A variety of LC systems have been used with success. When limiting amounts of sample are available, such as when analyzing individual mouse hypothalami (or other brain regions), nanospray is optimal. If larger sample sizes are available, then nanospray is not critical. A typical protocol for LC/MS analysis on a quadrupole time-of-flight mass spectrometer (Ultima Q-TOF, Waters/Micromass, Manchester, UK) is described below:

1. Frozen samples are thawed and briefly centrifuged in a microfuge to remove particulates.
2. An aliquot (typically 2-5 μ l) is injected onto a Symmetry C18 trapping column (5 μ m particles, 180 μ m i.d. $\times$ 20 mm, Waters, USA).
3. The material is desalted online for 15 min.
4. The trapped peptides are separated by elution with a water/acetonitrile 0.1% formic acid gradient through a BEH 130 - C18 column (1.7 μ m particles, 100 μ m i.d. $\times$ 100 mm, Waters, USA), at a flow rate of 600 nl/min.
5. Data are acquired in data-dependent mode and selected peptides dissociated by collisions with argon, using standard procedures. Note that for optimal MS/MS analysis, it may be helpful to use higher collision energy values for the TMAB-labeled peptides than typically used for non-labeled peptides.

3.5 Data analysis

Relative levels of peptides in treatment groups compared to controls are determined by measuring peak intensities of mass spectra in the appropriate software program (for example, Masslynx is used for viewing data obtained on a Waters instrument). Spectra are scanned in this program and peaks sets are recognized by number and distance from one another. Peptides usually show mass to charge ratios in the 300-1400 range (Figure 3, top) and elute over a 20-30 minute period, with individual peptides eluting within 5-30 seconds in a typical experiment. A typical spectrum is shown in Figure 3, with the 300-1400 range shown in the top panel for a single elute time. In this spectrum, a number of peak pairs can be observed, most of which show comparable peak intensities for the light and heavy forms

of each peptide. An expanded view of the mass/charge range from 480 to 540 is shown in the middle panel, revealing a set of peaks that are not at equal intensities. The m/z difference between the two sets of peaks is ~ 4.5 (507.183 - 502.662). In this example, the charge state is 2; this is determined by comparing the ^{13}C -containing peaks to the monoisotopic peak (507.183 for the monoisotopic peak, 507.682 for the peak containing one atom of ^{13}C ; the difference is 0.5 or $1/2$, meaning the charge is 2). Therefore, the m/z difference of 4.5 for $z=2$ means that the mass difference is 9 Da, which indicates that a single isotopic tag has been incorporated. The mass of this peptide without isotopic tags or protons can be calculated from the following formula.

$$\text{mass of unmodified peptide} = (m/z \cdot z) - (c \cdot T) - (1.008 \cdot (z - T))$$

where m/z is the observed mass to charge value for the monoisotopic peak

z is the charge state

c is the mass of the TMAB tag (128.118 for the D0 TMAB, 137.170 for the D9 TMAB)

T is the number of tags incorporated

1.008 is the mass of a proton,

and $(z-T)$ is the calculation of the number of protons (i.e. the difference between the charge and the number of tags). This last part of the equation is essential because the TMAB tags add a positive charge due to the quaternary amine group and therefore the charge state is not equal to the number of protons.

Typically, the mass of the unmodified peptide is taken as the average of the values determined from all of the isotopic tags; in the example shown in Figure 3, this would be the average of the D0-TMAB and D9-TMAB group. For samples using the five TMAB tags, the average of all five monoisotopic peaks would be used for this calculation.

The value of determining the mass of the unmodified peptide is for comparison to databases; because the tags were added as a means of quantifying the peptide, the mass of this tag needs to be subtracted in order to represent the endogenous form of the peptide. If additional modifications are detected (such as phosphorylation or acetylation), the extra mass of the modification is not subtracted because these modifications represent the endogenous form in the sample.

Quantification of the results is performed by measuring peak intensity. This can be done using the mass spectrometry program, although it is often faster to use a ruler and simply calculate the relative peak height. Typically, the monoisotopic peak and the peak containing 1 atom of ^{13}C are averaged so that the peak intensity is based on multiple points and not a single peak (which can show spikes that are not reflective of the overall level). In the example shown (Figure 3, middle panel inset) the average peak intensity for the sample from the KO mouse striatum is 18% of the value from the WT mouse striatum, and the ratio is therefore 0.18.

If five labels are used, the analysis is generally similar except that the final value is not a simple ratio but the relative peak height of each of the five peaks. For an experiment set up as in Figure 2, bottom, the controls would be averaged and the ratio of each KO peak group to the average control peak would be determined. When using five labels, spectra often contain overlapping peaks in which the signals with multiple ^{13}C atoms extend into the range of the next TMAB tag. In these cases, the isotopic distribution of the peptide must be calculated and the contribution from the ^{13}C -containing peaks subtracted as described (16). This is not a problem when using just the D0- and D9-TMAB labels because the 9 Da mass difference of these two labels is relatively much larger than the natural isotopic abundance.

Statistical significance is calculated using Student's two-tailed T-test to compare the ratio of each peptide determined from treatment versus control LC/MS runs to the ratio of the peptide in the control versus control LC/MS runs. If using two labels, as per the scheme in Figure 2, top, then this requires additional runs of WT vs WT samples. If using the five labels, as in Figure 2 bottom panel, then the variation among replicates of WT samples can be determined and included in the statistical calculations.

3.6. Identification of peptides by MS/MS sequencing

Interpretation of MS/MS data is usually performed by computer-assisted searching of databases consisting of proteins or translated cDNA. A number of programs are available for database searching. For peptides labeled with the TMAB reagents we have found the optimal program to be Mascot. This program currently has four of the five TMAB labels included as options (the missing one is the D12-TMAB). Most importantly, the Mascot program considers the neutral loss of TMA from the peptides during collision-induced dissociation; this causes the loss of 59 Da from peptides labeled with one D0-TMAB tag, 62 Da from peptides labeled with one D3-TMAB tag, 65 Da from peptides labeled with one D6-TMAB tag, and 68 Da from peptides labeled with one D9-TMAB. For example, the proenkephalin heptapeptide (YGGMFRR) incorporates one TMAB tag and when the D9-TMAB form is fragmented by collision-induced dissociation, both the precursor ion (507.183, 2+) and a 68 Da smaller form (473.119, 2+) are observed (Figure 3). The fragments resulting from cleavage of amide bonds (the b and y ions) usually lack the TMA group, as observed for the heptapeptide (Figure 3).

Mascot searches need to be followed by manual interpretation to eliminate false positives. Several criteria are used to accept or decline the peptides identified by Mascot:

1. The isotopic form of TMAB matched by Mascot is the correct one based on analysis of the peak set. While this may seem obvious, Mascot does not consider the peak set and know which of the individual peaks correspond to the D0 form, the D9 form, or any other form used. Therefore, if using the five isotopic forms of TMAB, there is a 1 in 5 chance that a false positive labeled with one tag is correct. If two tags, there is a 1 in 25 chance that a false positive has the correct tags. Therefore, a correlation of the isotopic TMAB form in the observed peak set with the predicted Mascot match is a simple and necessary step.
2. The number of tags incorporated into the peptide matches the number of free amines (N-terminus and side chains of Lys). If multiple tags, all should be the same

isotopic form on a particular peptide (i.e. all D0-TMAB, or D9-TMAB, and not one D0-TMAB and one D9-TMAB on the same form of a peptide).

3. The Mascot score is either the top score of all potential peptides, or the other peptides with comparable scores can be excluded by the following criteria, leaving only one peptide that matches all criteria.
4. The majority (>80%) of the major MS/MS fragment ions match predicted *a*, *b*, or *y* ions, or precursor ions with loss of trimethylamine.
5. The mass accuracy of the fragment ions is within the accepted specification for the q-TOF instrument used for the analysis.
6. A minimum of 5 fragment ions match *b* or *y* ions. For small peptides, this can be a problem.
7. The charge state should be reasonable based on the peptide sequence.

4. Notes

While the quantitative peptidomics technique is able to identify hundreds of peptides and their specific processing forms from a single LCMS run, the method does not detect every peptide in the sample. For example, peptides lacking an N-terminal free amine (due to post translational modification such as acetylation or pyroglutamylation) that also lack an internal lysine residue are not labeled by the TMAB reagent. These peptides are therefore present as single peaks, and no information on relative levels can be obtained using this method. In other cases, intrinsic factors can cause low ionization efficiency of certain peptides during mass spectrometry. The mass/charge ratios used in these studies (300-1800 amu) excludes some very small or very large peptides from detection. Finally, the dynamic range of peptide levels in biological samples varies beyond the detection limit of the mass spectrometry equipment and low abundance peptides are not detectable above the background.

Avoiding contaminants is important. Small molecules and polymers can substantially interfere with the MS analysis. Clean water is essential. Some brands of microfuge tubes and filtration devices have polymeric contaminants that appear as polyethylene glycol-related compounds on MS; these contaminant signals completely overwhelm the signals from the tissue-derived peptides.

Using low-retention microfuge tubes and pipette tips is important, as peptides can bind regular tubes and tips, and many peptides can therein be lost during transfers and labeling steps. Whenever possible, rinse and dry tubes and tips with double deionized water before use.

Prepare labeling solutions as well as glycine and hydroxylamine solutions fresh for each set of labeling reactions. It is extremely important to prepare all solutions (HCl, NaOH, glycine, phosphate buffer and desalting solutions) with double-deionized water to avoid contamination from small organic molecules that can interfere with the mass spectrometry.

It is extremely important to clean Centricon filters before filtering peptides. These filters often contain glycerol which needs to be washed off before filtering to avoid sample

contamination which will substantially interfere with mass spectrometry. Make sure that filters are covered with double-deionized water, and then run the water through the filters at the designated centrifugal speed. Be sure to empty any remaining water before loading extracts for filtering.

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D0-TMAB-NHS (+ 129 Da)
D3-TMAB-NHS (+ 132 Da)
D6-TMAB-NHS (+ 135 Da)
D9-TMAB-NHS (+138 Da)
D12-TMAB-NHS (+141 Da)

Figure 1.

TMAB labels used for isotopic tagging. The form of peptide shown includes the N-hydroxysuccinimide (NHS) moiety, which is replaced by the amine (N-terminus or Lys side chain) in the reaction with the peptide. The indicated mass is the net additional mass after one tag is incorporated into the peptide, replacing a hydrogen on the amine. Note the quaternary amine group which provides a positive charge after labeling, maintaining the charge of the primary amine on the peptide. The TMAB-NHS labeling reagents are synthesized as the chloride salt (not shown).

Labeling scheme with 2 isotopic tags

Labeling scheme with 5 isotopic tags

Figure 2.

Scheme for isotopic labeling of peptides extracted from tissues. Top: If two experimental conditions are to be compared (such as WT and KO), only two isotopic tags are needed for each replicate. Peptides are extracted from the appropriate brain region or other tissue, labeled with isotopic tags, and pooled as indicated. Note that the samples shown for Run 2 are labeled with tags in the reverse scheme as those in Runs 1 and 3; this is done to control for possible artifacts that could result from differential reactivity of the isotopic tags (not previously found to be a problem, but if the synthesis or storage of one of the labels is not

optimal, this could result in stronger labeling with one of the reagents). To obtain data for statistical tests, it is necessary to repeat the analysis with comparable groups of WT animals, as shown for Runs 4-6. This provides a control for peptide variability among groups of animals and allows for statistical comparison of the results from Runs 1-3. Bottom: The use of five isotopic labels to compare two different groups of animals allows for replicates to be performed within each LC/MS run, as shown in the scheme. Note the reversal of the labels in the scheme for Run 2, as compared to Run 1.

Figure 3.

Representative MS and MS/MS data. Top: A spectrum showing MS over the mass/charge (m/z) range 300-1400 at 58.2 minutes from an experiment in which PC2 KO mouse striatal extract was labeled with D0-TMAB and WT striatal extract was labeled with D9-TMAB. Note the appearance of several peak pairs that appear roughly equal in intensity: these include 686.869 and 691.721; 704.216 and 710.783; 744.016 and 749.269; 774.553 and 781.770 (some of these are not labeled in the figure). The 704/710 ions were subsequently identified by MS/MS as Purkinje cell protein 4 with 8 TMAB tags and 3 protons ($11+$

charge state) while the 774/781 ions represent this protein with 8 TMAB tags and 2 protons (10+ charge state). The 686/691 and 774/749 ions represent an unidentified peptide with 7 TMAB tags and charge states of 13+ and 12+, respectively. Middle: The spectrum in the top panel was analyzed over the 480-540 mass/charge range, showing ions of 502.662 and 507.183. These ions co-elute (not shown), have a charge of 2+ (based on analysis of the difference between m/z of the monoisotopic and ^{13}C -containing peaks), and have a mass difference of 9.0 Da indicating that a single TMAB tag was incorporated. Quantification is performed by measuring peak intensity of the monoisotopic peak and the peak containing one atom of ^{13}C , taking an average of these two values, and then comparing the ratio of the experimental group (KO) to the control group (WT). In this example, the ratio of KO to WT is 0.18. Bottom: The 507.183 ion was selected by the mass spectrometer for collision-induced dissociation, resulting in the MS/MS spectrum shown. From this spectrum, and using computer search programs, it is possible to identify this peptide as the proenkephalin heptapeptide (sequence YGGFMRF). Fragment ions labeled “b” represent N-terminal pieces of the peptide while those labeled “y” represent C-terminal pieces. Note that the trimethylamine (TMA) moiety of the TMAB tags is removed from the labeled peptide upon the collision-induced dissociation, resulting in b ions that contain only the butyryl tag (which adds 69 Da to the mass of the peptide). Strong peaks for the precursor ion (507.183, 2+) and precursor ion after loss of TMA (473.119, 2+, and 945.106, 1+) are visible in the spectrum.