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An Automated Method for Scanning LC−MS Data Sets for Significant Peptides and Proteins, Including Quantitative Profiling and Interactive Confirmation

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Abstract: Differential quantification of proteins and peptides by LC−MS is a promising method to acquire knowledge about biological processes, and for finding drug targets and biomarkers. However, differential protein analysis using LC−MS has been held back by the lack of suitable software tools. Large amounts of experimental data are easily generated in protein and peptide profiling experiments, but data analysis is time-consuming and labor-intensive. Here, we present a fully automated method for scanning LC−MS/MS data for biologically significant peptides and proteins, including support for interactive confirmation and further profiling. By studying peptide mixtures of known composition, we demonstrate that peptides present in different amounts in different groups of samples can be automatically screened for using statistical tests. A linear response can be obtained over almost 3 orders of magnitude, facilitating further profiling of peptides and proteins of interest. Furthermore, we apply the method to study the changes of endogenous peptide levels in mouse brain striatum after administration of reserpine, a classical model drug for inducing Parkinson disease symptoms.

Keywords: LC−MS • quantitation • differential display • neuropeptides · DeCyder MS · label-free quantitation

Introduction

The measurement of protein abundances is fundamental to understanding biological processes. Creating protein profiles and comparing amounts of individual proteins in various sample types, between healthy and diseased tissue or body fluid samples, between samples representing different stages of disease, and under differing biological conditions, is key to a better understanding of the role proteins play in disease. The proteomic approaches have great potential to add more disease-specific drug targets to the discovery pipeline and to allow the identification of biomarkers with higher diagnostic and prognostic value. There are many methods for quantifying proteins, including well-established methods used in most laboratories such as separating proteins by SDS-PAGE and comparing the staining of the bands. This requires that the sample is not too complex, else other proteins will interfere with the quantification. 2D gels can be used when the sample is more complex.[1] If highly specific antibodies are available, protein amounts can be measured using either ELISA,[2] to measure a single protein, or a protein array,[3] to measure a set of known proteins. In cases where it is feasible to genetically tag the protein of interest, the amount of the protein can be measured with a labeled antibody against the tag. For example, this has been done for a majority of the proteins in *Saccharomyces cerevisiae*.[4]

Numerous new methods have been developed during the past few years for the quantification of proteins and peptides using mass spectrometry (MS). Proteins are typically digested prior to MS analysis because the resulting peptides are more amenable to MS analysis due to their inherent composition and can be measured with higher accuracy. Many of these methods include labeling the samples with different isotopes and subsequently mixing them to minimize the effect of variation in the sample handling and measurement.[5] In these methods, the peptides are usually separated by reversed-phase liquid chromatography (RPC) and analyzed by mass spectrometry. The quantification is done by comparing the MS peak areas for the same peptide labeled with the different isotopes and therefore originating from different samples. It is an advantage to introduce the isotopic labeling at an early stage of the sample handling, and most isotopic labeling strategies therefore incorporate labels designed to have very similar behavior during separation. Ideally, the isotopic label is introduced *in vivo* by metabolic labeling.[6−8] After the cells have been disrupted, the proteins can be labeled using isotope-coded

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affinity tags (ICAT),[9−11] quantifying the cysteine-containing peptides. During protein digestion, the C-termini of all proteolytic peptides can be labeled with ^{18}O.[12,13] After digestion, isobaric tags for relative and absolute quantification (iTRAQ)[14] can be used to label the N-termini of all proteolytic peptides. Synthetic peptides with isotopically labeled amino acids can also be added to quantify the corresponding peptides in the sample.[15,16]

Improvements in the stability of chromatography systems and mass spectrometers have allowed differential analysis of protein amounts without the use of isotopic labels.[17−29] The MS intensity of the same peptide in different LC−MS runs is then used for quantitative comparison.

In this paper, we describe an automated data analysis method for LC−MS data from unlabeled samples, and demonstrate how it can be used to detect statistically significant differences in the levels of peptides and proteins using samples of known composition. We also illustrate the utility of the method by studying the changes of endogenous peptide levels in mouse brain striatum after administration of reserpine. Reserpine, a classical tool to induce and study parkinsonism in animal models,[30−32] depletes dopamine stores in the central nervous system through a competitive inhibition of monoamine uptake into intracellular vesicles of dopamine neurons,[33] and the dopamine depletion in striatum induces increased proteolytic processing of secretory peptides.[34,35]

Materials and Methods

Synthetic Samples and Data Acquisition. A tryptic digest of eight proteins was used as a background in all the experiments on synthetic samples. The original background mixture contained 16.1 pmol/μL carbonic anhydrase, 36.0 pmol/μL α-lactalbumin, 16.2 pmol/μL glyceraldehyd-3-phosphate dehydrogenase, 17.7 pmol/μL ovalbumin, 26.1 pmol/μL bovine serum albumin, 8.8 pmol/μL transferrin, 80.8 pmol/μL lysozyme, and 37.7 pmol/μL cytochrome C and was diluted to 1 pmol/μL total concentration, Three peptides, bradykinin, angiotensin I, and neurotensin were added as internal standards at 1.2 pmol/μL each. Different aliquots of the eight-protein mixture including the internal standards were then spiked with 0.01, 0.05, 0.1, 0.5, 1, and 3 pmol/μL tryptic digest of equine apomyoglobin and with 0.01, 0.05, 0.1, 0.5, 1, 5, and 10 pmol/μL of the peptides angiotensin III, substance P, fibrinopeptide B, and ACTH 1−24.

One-microliter aliquots of the resulting peptide mixtures were analyzed by RPC LC−MS/MS using an Ettan MDLC (GE Healthcare, Uppsala, Sweden) coupled with a Finnigan LTQ linear ion trap mass spectrometer (Thermo Electron Corp., San Jose, CA) fitted with a nanospray interface. Full scan mass spectra were collected in profile mode and MS/MS spectra in centroid mode to minimize the data size, while optimizing the data for both quantification and identification. A profile mass spectrum is a readout of observed ion current versus mass/charge ratio, while a centroid mass spectrum only contains discrete peaks of zero width.

It is important to collect the full scan data in profile mode because quantification is performed by integrating under the peaks, and therefore, profile mode gives more accurate quantification. The tandem MS data is used for identification, and for this purpose, centroid data is sufficient, because the identification process is much less sensitive to the shape of the peaks.

Endogenous Peptide Samples and Data Acquisition Mice were administrated 10 mg/kg reserpine, a substance commonly used to induce and study parkinsonism in animal models[30−32] known to deplete dopamine stores in the central nervous system through a competitive inhibition of monoamine uptake into intracellular vesicles of dopamine neurons.[33] Groups of mice were sacrificed by focused microwave irradiation at 1 h ($n = 5$), 6 h ($n = 5$), and 12 h ($n = 6$) after injection.[20] One group received an injection of saline (controls, $n = 6$). Each group contained five to six animals. The striatum was dissected, extracted, and sonicated in 75% MeOH and 0.25% acetic acid, and cell debris was spun down at 20 000g for 45 min. The peptide fraction of the sample was isolated by centrifugation through a 10 kDa cutoff filter (Microcon YM-10, Millipore, Bedford, U.K.). The peptide filtrate was analyzed using an Ettan MDLC (GE Healthcare) coupled with a Q-Tof, (Waters) for quantification by LC−MS analysis and a Finnigan LTQ linear ion trap mass spectrometer (Thermo Electron Corp.) for peptide identification. The reproducibility of the experimental system has previously been shown to be between 5 and 17% (mean 12%).[19]

Identification. The X! Tandem[36] search engine was used to identify peptides in the spiked samples by matching tandem mass spectra against peptides derived from a protein sequence database. A parent mass error of 4 Da and a fragment mass error of 0.4 Da were used to search the tandem MS data from the ion trap. We considered peptides with an expectation value cutoff of $e < 10^{-3}$ as identified. The peptides in the spiked samples were identified by searching all mammalian sequences from NCBI's nonredundant protein sequence collection. Because when using the conventional search engines, it is more difficult to obtain significant identities of endogenous peptides , these were identified by matching the list of deconvoluted masses generated by DeCyder MS against SwePep.[37] The absolute mass difference between the theoretical and experimental mass was selected not to exceed 0.2 Da in order for a match to be valid. The search result from SwePep was verified by searching MS/MS data against UniProtKB version 51.0 taxonomy Rodent, using unspecific cleavage, with X! Tandem and Mascot.[38] The search was performed using a peptide mass tolerance of ±2 Da and a fragment mass tolerance of ±0.7 Da. For more information about the identification of the endogenous peptides, see Supporting Information.

Visualization. The LC−MS data from the different samples was displayed as two-dimensional signal intensity maps with retention time and m/z on the two axes and a gray scale representing the intensity of a peak at a certain retention time and m/z.[16,18,26,39,40] This type of visualization gives a quick overview of the data and can be utilized to optimize the chromatography and the MS data acquisition.[40,41]

Peptide Detection, Quantification, and Matching. The DeCyder MS Differential Analysis Software (GE Healthcare) was used to analyze the signal intensity maps. First, peptides were detected in individual LC−MS intensity maps. The detection was performed automatically by the software in a sequence of steps as follows: (i) modeling of the background intensity as a function of m/z and elution time, based on the intensity distribution; (ii) detection of local maxima in the intensity map, discarding points not sufficiently above background; (iii) detection of chromatographic peaks, characterized by m/z and an elution time interval, starting from the local maxima; (iv) charge assignment, where the charge of each peak was deduced from

the spacing between isotopes if the resolution was high enough, or from the presence of other charge states of the same peptide; (v) quantification, carried out as the sum over all m/z bins covered by the peptide followed by an integration over the time points (i.e., mass spectra). The modeled background intensity level was removed from all intensity measurements in the process.

Peptides within a mass and time tolerance window (0.5 Da and 2 min, respectively) were matched automatically across the different signal intensity maps. This window corresponds to a "two-dimensional resolution" of about 1000 Da/0.5 Da × 60 min/2 min = 60 000 if the assumption of evenly distributed peptides is made. This is, of course, not exactly the case. On the other hand, there were only about 700 peptides in each spot map at the most in the present study. Addressing much more complex samples would require a prefractionation step to reduce the complexity.

DeCyder MS allows quantitative comparison using the raw peak intensities, or the intensities can be normalized using two different methods: (i) if peptides of known amounts have been added to all samples and detected, these intensities can be used to normalize the peak intensities between different samples; (ii) assuming that a majority of the peaks correspond to peptides that are present at the same amount in all samples, the entire peptide intensity distributions can be used for normalization.

Results and Discussion

Scanning for Significantly Varying Tryptic Peptides. The signal intensity maps for the synthetic samples were used to scan for significantly varying peptides. Different experimental groups were created by spiking different amounts of peptides (digested myoglobin, angiotensin III, and fibrinopeptide B) into a background of a mixture of a tryptic digest of eight proteins (1 pmol/μL total). Two peptides (bradykinin and neurotensin), added in the same amount to all samples, were used as internal standards. Each experimental group was analyzed in six replicates. Peptides were detected and compared using DeCyder MS. About 700 peptides were automatically detected in each signal intensity map. Student's t test statistics were used to extract the peptides showing a significant variation between the two experimental groups. All peptides with $p < 10^{-3}$ were considered significant. This is a quite conservative level where one would expect one false positive out of 1000 peptides measured. Identity of the peptides was confirmed using the available MS/MS data.

Table 1 shows the significantly varying peptides between experimental groups with spiking amounts of 50 and 100 fmol/μL, respectively. The two spiked peptides and four myoglobin peptides showed the lowest p-values. The average fold change for the myoglobin peptides was 1.8, and the standard deviation was 0.3, which is consistent with the 2-fold difference in spike amount added to the samples.

Table 2 shows the significantly varying peptides between experimental groups with spiking amounts of 500 fmol/μL and 1 pmol/μL, respectively. At these higher spiking amounts, we observe many more significantly varying peptides. A majority of these can be identified as spiked peptides. The average fold change for the myoglobin peptides was 2.2, and the standard deviation was 0.3. The difference in signal levels was thus consistent with the 2-fold difference in spike amount also in this case.

Table 1. The Peptides with p-Values $<10^{-3}$ from Comparison between Samples Spiked with 50 and 100 fmol/μL, Showing Their Mass and Retention Time, the Standard Deviation of the Retention Time, the p-Value from the t Test, the Fold Change, and the Identity Including Information about Amino Acid Extent in Corresponding Protein for Proteolytic Peptides[a]

mass (Da)	rt(min)	t test (p)	fold change	identity
897.0	18.4(0.49)	2.9E-06	1.8	angiotensin III
940.9	22.0(0.58)	2.9E-05	1.7	myoglobin 146−153
1270.9	19.3(0.45)	1.9E-04	1.7	myoglobin 32−42
1378.2	31.2(0.50)	<1.0E-12	2.2	myoglobin 64−77
1501.8	19.3(0.48)	5.3E-04	1.5	myoglobin 119−133
1569.8	26.9(0.51)	3.2E-10	2.6	fibrinopeptide B

[a] The average fold change for the myoglobin peptides was 1.8, and the standard deviation was 0.3. This should be compared to the expected fold change of 2.0.

Table 2. The Peptides with p-Values $<10^{-3}$ from Comparison between Samples Spiked with 500 fmol/μL and 1 pmol/μL, Showing Their Mass and Retention Time, the p-Value from the t Test, the Fold Change, and the Identity Including Information about Amino Acid Extent in the Corresponding Protein for Proteolytic Peptides[a]

mass (Da)	rt (min)	t test (p)	fold change	identity
649.3	24.1	7.0E-04	1.9	myoglobin 148−153
747.5	25.9	3.4E-04	2.1	myoglobin 134−139
847.8	19.0	2.1E-04	1.9	myoglobin 32−42
897.1	18.2	4.5E-07	1.9	angiotensin III
900.1	12.6	2.7E-04	3.2	substance P
933.0	17.3	4.2E-04	1.8	
940.9	21.7	1.1E-05	2.1	myoglobin 146−153
1001.7	19.0	1.3E-05	1.9	myoglobin 119−133
1230.9	36.0	2.6E-04	1.8	
1270.8	19.0	2.4E-05	2.1	myoglobin 32−42
1293.2	18.8	1.9E-05	1.4	
1378.1	23.1	1.2E-04	0.4	
1378.5	30.8	8.0E-05	2.1	myoglobin 64−77
1400.0	30.8	1.8E-05	3.4	
1415.9	30.7	5.9E-04	1.8	
1501.9	19.0	2.5E-05	2.1	myoglobin 119−133
1506.0	27.4	2.2E-04	2.1	myoglobin 63−77
1569.9	26.5	8.0E-12	2.0	fibrinopeptide B
1581.8	11.9	4.9E-04	2.5	myoglobin78−96
1606.2	23.1	1.5E-05	2.2	myoglobin17−31
1661.4	22.3	9.8E-05	1.9	myoglobin 32−45
1815.4	40.9	1.2E-04	2.9	myoglobin 1−16
1853.2	12.6	2.5E-05	2.8	myoglobin 80−96
1884.8	41.7	1.4E-04	6.4	myoglobin 103−118
2931.8	18.7	3.6E-05	2.3	ACTH
2993.2	18.7	7.2E-05	2.1	
3002.6	19.9	2.2E-05	1.7	
5399.2	30.9	4.7E-04	1.6	
7181.5	18.6	3.1E-04	1.5	

[a] The average fold change for the myoglobin peptides was 2.2, and the standard deviation was 0.3. The myoglobin fragment (amino acids 103−118) at 1884.8 Da was considered an outlier and omitted from the calculations. This emphasizes the need for including several peptides in the quantitative calculations. The result should be compared to the expected fold change of 2.0.

However, a few individual peptides show an inconsistent fold change that may be caused by interference from other peptides with similar m/z and retention time. But when several tryptic peptides are observed originating from the same protein, these outliers can be removed in a fully automated analysis, optionally in combination with interactive confirmation based on raw data visualization in the quantification process.

Profiling Proteins of Interest. To investigate the potential for profiling selected peptides over several experimental groups

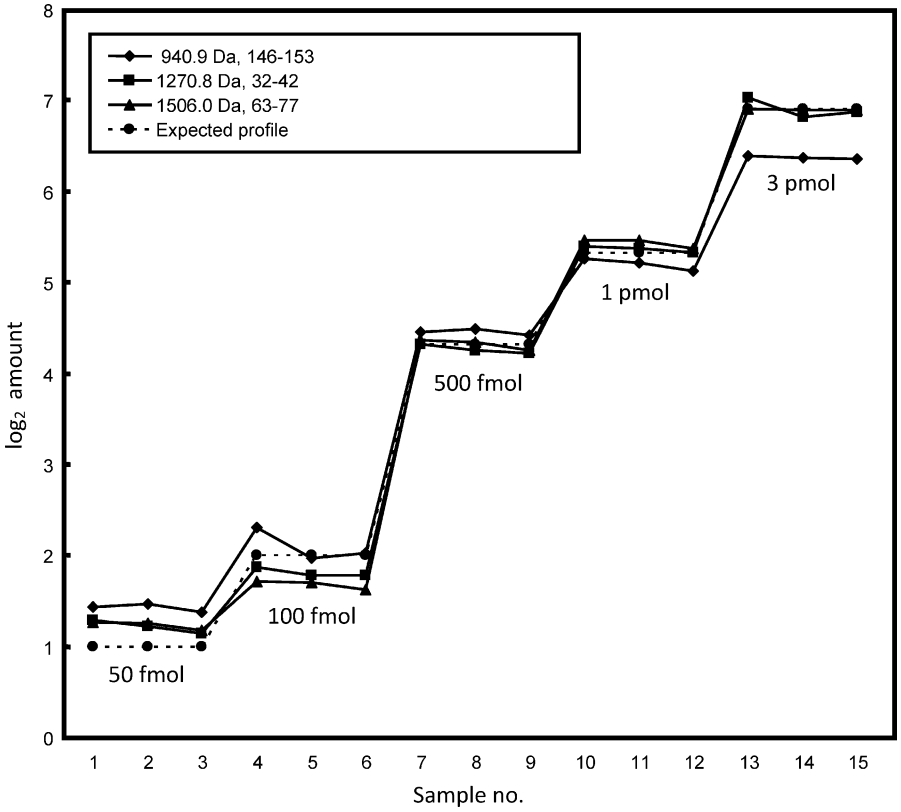

Figure 1. The expected profile based on the amount of myoglobin spiked into the sample is depicted (dashed line) together with the volumes of peaks for the three most prominent tryptic peptide ions from myoglobin. Each solid line corresponds to a different tryptic peptide. The profiles were normalized to the expected profile by subtracting each data point with the average $\log_2$ amount for the corresponding profile. The 50 fmol data points for the expected profile were set to 1.0. Absolute comparison is not possible because the mass spectrometric response is dependent on the amino acid composition. The peptide masses and corresponding amino acid extents in myoglobin are listed in the legend.

Table 3. Expected Log_2 Signal Levels and Corresponding Experimental Average Signal Levels for Each Spike Level in the Myoglobin Profile Depicted in Figure 1[a]

spike amount [fmol]	expected signal [Log_2]	experimental average SD [Log_2]
50	1.0	1.3 ± 0.1
100	2.0	1.9 ± 0.2
500	4.3	4.3 ± 0.1
1000	5.3	5.3 ± 0.1
3000	6.9	6.7 ± 0.3

[a] The expected signal is based on the amount of myoglobin spiked into the sample and set to 1.0 for the 50 fmol spike amount. The experimental data was normalized to the expected data so that the experimental average over all spike levels became equal to the expected average over all spike levels. Experimental data are in good agreement with expected data, except for a slight deviation for the 50 fmol spike level.

with large differences in amounts, a few tryptic peptides from myoglobin (50 fmol/μL to 3 pmol/μL) were profiled in a background of the digested eight-protein mixture (1.2 pmol/μL total). The results are shown in Figure 1 and summarized in Table 3 where average experimental signal levels are compared to expected signal levels. As seen in Table 3, experimental data are in good agreement with expected data, except for a slight deviation for the lowest spike level. The changes in the relative ion intensities are proportional to the amount of peptide in the sample over the whole range of peptide amounts (almost 2 orders of magnitude). In contrast, the absolute intensity of the different myoglobin peptide ions cannot be predicted from the peptide amount because the mass spectrometric response is dependent on the amino acid composition.

Linearity. Standard peptides were profiled over triplicates of samples to investigate the linearity of the quantification. The result is displayed in Figure 2, left panel, as spiked amount (shown on a log scale) versus $\log_2$ of the peak volume, which shows that a linear response over almost 3 orders of magnitude change in peptide amount was obtained by fully automatic analysis. The slight nonlinearity at lower concentrations is a result of low peak heights in relation to background signal. By visual inspection of the corresponding signal intensity maps, it was concluded that the angiotensin III peak at 10 fmol/μL was disturbed by tailing from a high-intensity peak at a nearby m/z and a slightly shorter retention time and is therefore too high (Figure 2, right panel). Though not shown with this data set, spike amounts above 10 pmol/μL show nonlinearity as well, which is probably the result of overloading the RPC column and/or saturation of the signal in the MS detector.

Internal Standards. Three peptides were added at the same amount to all samples to serve as internal standards (bradykinin, neurotensin, and angiotensin I). Because of the quality of the resulting spot pattern and interference from other peptides in the sample with similar m/z and retention time, angiotensin I was not suitable as an internal standard in this experiment, and it was therefore omitted from the normalization procedure. It is always recommended that a few different

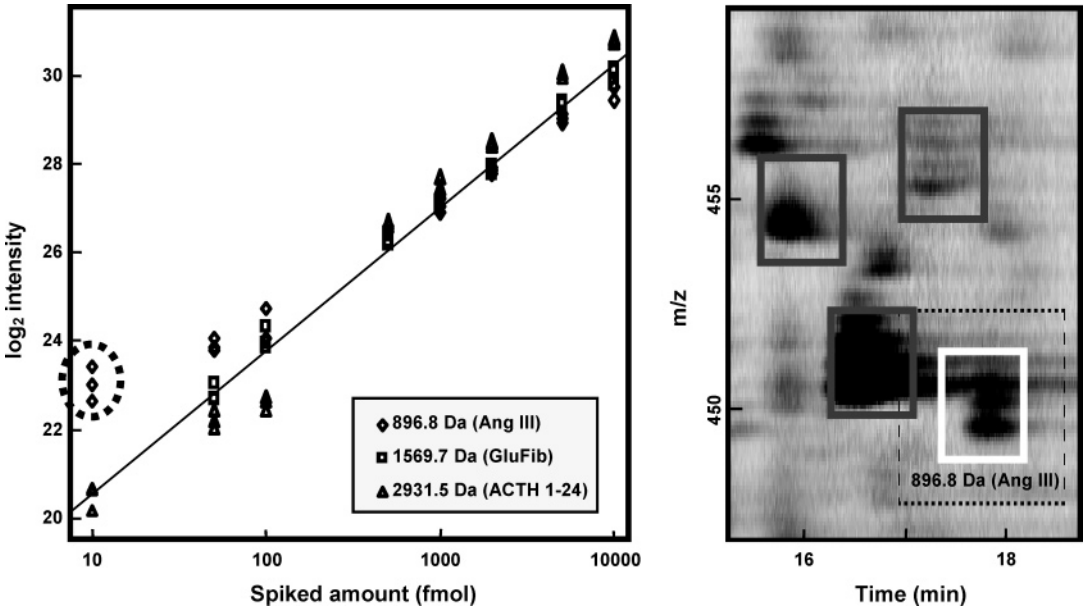

Figure 2. Peak volumes of technical replicates of the peptides angiotensin III, fibrinopeptide B, and ACTH (aa 1–24) are shown as a function of spiked amount in the left panel. Since both axes are logarithmic, one would expect a linear relation to result in a straight line. The quantification of angiotensin III is systematically distorted at the 10 fmol spike level. This may happen when different peptide ions have similar *m*/*z* and retention time, which also could be confirmed to be the case (see right panel).

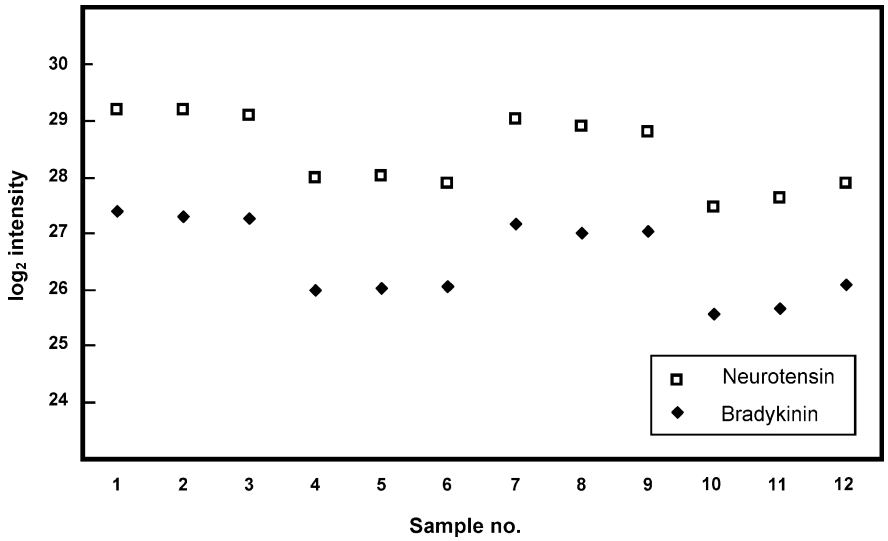

Figure 3. The signal intensities of two peptides (bradykinin and neurotensin) are shown for data from 12 different samples. The two peptides were added at the same amount to all the samples to serve as internal standards. The samples were run during two separate sessions with an interval of several days; samples 1, 2, 3, 7, 8, and 9 were run during the first session, and samples 4, 5, 6, 10, 11, and 12 were run during the second session. The two peptides show very similar signal intensity variation patterns among the samples. Since they were added to all samples at the same amount, the variation in the signal intensities does not reflect differences in peptide amounts, but it is caused by small differences in the experimental conditions during and between the two experimental sessions.

internal standards are used, because the probability of interference with sample peptides is not negligible. The variation of signal intensities for bradykinin and neurotensin are shown in Figure 3. The two peptides show almost identical signal intensity variation patterns among the samples. Since they were added to all samples at the same amount, the variation in the signal intensities is caused by small differences in the experimental conditions. The bradykinin and neurotensin internal standards were used to normalize the abundance data among the samples. Samples 1 to 6 represent the low-spike amount group, and samples 7 to 12 represent the high-spike amount group (Table 2). The samples were run at two separate occasions with an interval of several days, yielding different overall intensities, but the results could still be compared through the sample-to-sample normalization.

Endogenous Peptide Expression Changes Following Reserpine Administration. The brain area striatum was analyzed using nanoLC–MS and DeCyder MS for the detection of significantly altered endogenously processed peptides at different time points following reserpine administration. Approximately 300 peptides were automatically detected in each sample, and the level of 20 peptides changed significantly

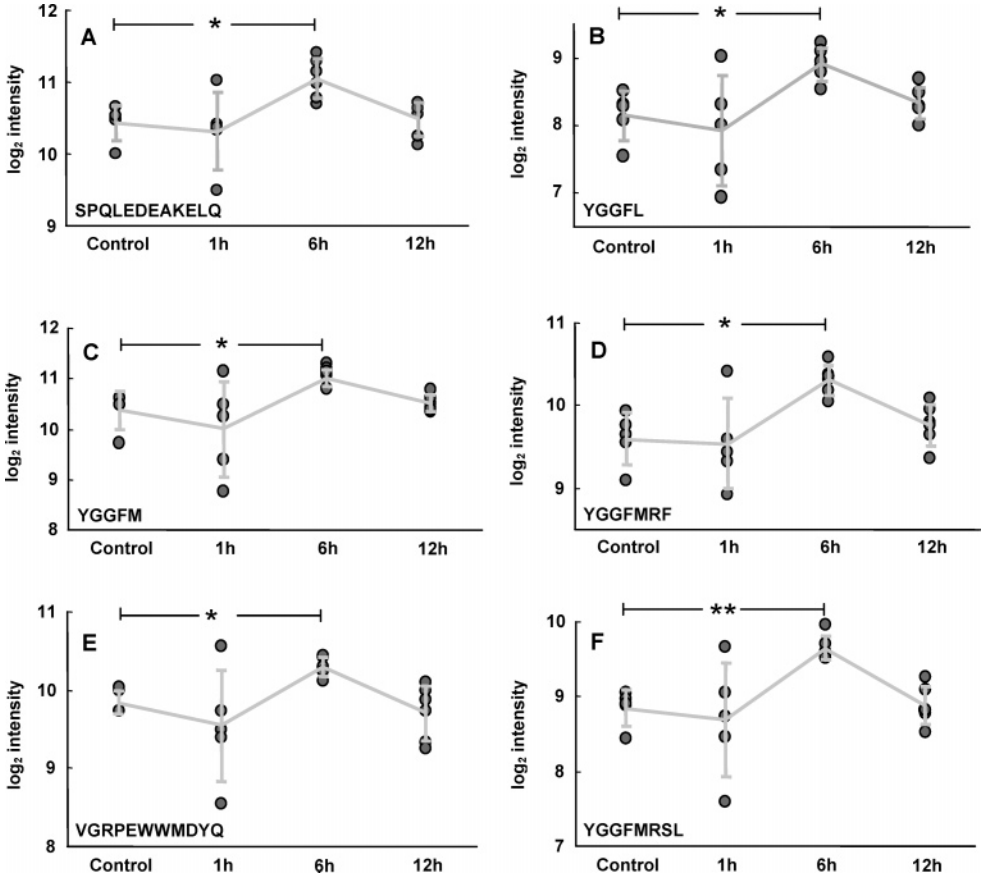

Figure 4. Levels of endogenous peptides at 1, 6, and 12 h post reserpine administration to mice. Six neuropeptides originating from the proenkephalin A precursor were significantly altered according to ANOVA ($p < 0.05$) (panels A−F). The individual measurements are shown as filled circles, mean values are connected by solid lines, and standard deviations for the different time points are represented by bars in the graphs. Student's *t* tests were performed to compare the different time points to the control samples. The significant change was observed when comparing 6 h to control (* $p < 0.01$, ** $p < 0.001$). The fold changes, 6 h to control, for the peptides in panels A−F were 1.5, 1.7, 1.5, 1.6, 1.4, and 1.7, respectively.

according to ANOVA analysis ($p < 0.05$) using a filtering criteria of at least five observations in each group. Out of these 20 peptides, 8 were identified. Student's *t* tests were performed to compare the different time points after reserpine administration against control samples. At a significance level of $p < 0.01$, 1 and 11 peptides were found to be regulated at 1 and 6 h, respectively. Using a significance level of $p < 0.001$, only 2 peptides were regulated at 6 h. No significant regulation was detected at 12 h after reserpine administration.

Six of the significantly altered peptides originated from the proenkephalin A precursor (P22005). All these peptides display a similar expression profile throughout the time course (Figure 4). One hour post reserpine administration, a diverse response to the drug was observed. Six hours post administration, all the peptides originating from proenkephalin A displayed a significant up-regulation compared to controls, and at 12 h, their levels had returned to levels close to control levels.

The effect of reserpine on the levels of endogeneous peptides can be mediated by increased processing[34,35] and increased expression of secretory proteins.[42−44] Reserpine has been shown to increase the processing of a number of secretory proteins including proenkephalin[34] in cell cultures. Processing of the secretory peptides is most likely carried out by the prohormone convertases PC1/3 and PC2. PC1/3 has been shown to be inhibited by catecholamines *in vitro*. Therefore, the increase in processing has then been suggested to be triggered by the depletion of dopamine caused by reserpine administration.[35] Dopamine depletion also produces other neurochemical changes within the striatum, including increased expression of preproenkephalin mRNA and decreased expression of prodynorphin and preprotachykinin mRNA.[45−51] Acute reserpine administration has been shown to induce increased expression of preproenkephalin mRNA in rat striatum after 24−120 h.[42−44] The present study confirms the previous findings of increased processing of proenkephalin derived peptides.[34]

In conclusion, we have shown that DeCyder MS Differential Analysis Software provides useful tools for: (i) automatic scanning for peptides with significant variation between groups of samples for large data sets; (ii) visual confirmation of detection results against original raw data; (iii) differential data analysis over a wide dynamic range with and without using internal standards. In addition, we have applied this method to detect, quantify. and compare endogenous peptide patterns in the mouse brain (striatum) following reserpine administration.

Supporting Information Available: Additional information about the identification of the endogenous peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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