High Identification Rates of Endogenous Neuropeptides from Mouse Brain

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Supporting Information

ABSTRACT: Mass spectrometry-based neuropeptidomics is one of the most powerful approaches for identification of endogenous neuropeptides in the brain. Until now, however, the identification rate of neuropeptides in neuropeptidomics is relatively low and this severely restricts insights into their biological function. In the present study, we developed a high accuracy mass spectrometry-based approach to enhance the identification rates of neuropeptides from brain tissue. Our integrated approach used mixing on column for loading aqueous and organic extracts to reduce the loss of peptides during sample treatment and used charge state-directed tandem mass spectrometry to increase the number of peptides subjected to high mass accuracy fragmentation. This approach allowed 206 peptides on average to be identified from a single mouse brain sample that was prepared using 15 μL of solutions per 1 mg of tissue. In total, we identified more than 500 endogenous peptides from mouse hypothalamus and whole brain samples. Our identification rate is about two to four times higher compared to previously reported studies conducted on mice or other species. The hydrophobic peptides, such as neuropeptide Y and galanin, could be presented and detected with hydrophilic peptides in the same LC−MS run, allowing a high coverage of peptide characterization over an organism. This will advance our understanding of the roles of diverse peptides and their links in the brain functions.

KEYWORDS: neuropeptidomics, neuropeptide, identification rate, charge state-directed tandem MS

INTRODUCTION

Neuropeptides, including endogenous peptide neuromodulators and hormones, are involved in diverse physiological and behavioral processes, such as food intake, sleep and reward processing.1 Neuropeptides are produced from neuropeptide precursors under the selective action of endopeptidases, and some of them undergo further posttranslational modifications (PTMs), which can profoundly influence their bioactivity. Individual peptides in general have distinct biological functions, even though they are derived from the same prohormone precursor. It is thus important to identify with high confidence as many peptides as possible derived from particular prohormone precursors, to understand the biological functions.

Mass spectrometry (MS)-based neuropeptidomics, a sub-branch of proteomics, has now emerged as the method of choice for the sequencing of endogenous neuropeptides in biological samples.2−4 An explicit goal of neuropeptidomics is the complete characterization of the neuropeptides, including classical neuropeptides and potentially bioactive peptide fragments derived from prohormone precursors, in a targeted organism. Neuropeptides are endogenous peptides of various lengths and thus different hydrophobicities. This diversity leads to a generally low identification rate in neuropeptidomics. Until now, the identification rate is believed to only cover a small fraction of the peptides derived from neuropeptide precursors.5−7 This is evident because in any single study, many classical neuropeptides, which are known to be present in the brain, remain undetected. This low overall identification rate restricts possibilities for the discovery of neuropeptide functions in biological studies.5

A number of studies have addressed the discovery of as many neuropeptides as possible from brain samples by using various methods such as novel sample treatment methods,6,7 derivatization,4 two dimension separation,8 high resolution MS,9,10 and database construction.11 This work has substantially improved the neuropeptide identification rate and greatly enlarged our knowledge of neuropeptides in brain. Previous single studies have generally been able to report around 50−150 peptides, including but a few classical neuropeptides, regardless of whether the sample was prepared from a specific brain area or the whole brain. Current studies showed that in

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the brain, signaling molecules work in a concerted manner to maintain the brain functions. Therefore, a high identification rate of neuropeptides is of great interest in the field of basic neuroscience and pharmaceutical research, because it could provide a more comprehensive understanding of the functional roles of various neuropeptides in the brain.

Neuropeptidomics typically require several serial procedures, including tissue preparation, peptide extraction, tandem MS (MS/MS) data acquisition and data interpretation, to perform a comprehensive identification of peptides. The peptide identification rate thus depends critically on the success of each procedure. Leaving restrictions due to bioinformatics aside, we found that the low identification rate of neuropeptides could be caused mainly by two issues: (i) the pronounced loss of peptides during sample treatment and (ii) the loss of the tandem MS information of many peptides, in particular low abundance ones, during data acquisition. We reasoned that the reduction of such losses would enable the significant enhancement of the peptide identification rate.

In this study, we have developed a new approach to increase the identification rate of endogenous peptides from brain extracts. Our integrated approach used mixing on column (MOC) for sample treatment and used charge-state-directed (CSD) tandem MS to increase the number of peptides subjected for high mass accuracy fragmentation. Our approach used a separation-and-pool mode at both the sample treatment and data processing stages. It contains several key methods, which include mixing on column (MOC) sample treatment, charge state-directed (CSD) liquid chromatography (LC)-Fourier transform (FT)-MS/MS, and hybrid spectral analysis. Using our integrated approach, more than 500 endogenous peptides in total were identified from mouse hypothalamus and whole brain samples, demonstrating previously unreached identification power in neuropeptidomics.

### EXPERIMENTAL PROCEDURES

#### Materials

LC–MS grade acetonitrile and formic acid were purchased from Fisher Scientific (New Jersey, USA) and Fluka (Wisconsin, USA), respectively. Acetic acid was purchased from Fluka (Buchs, Switzerland). Pure water was prepared by GenPure system (TKA, Niederelbert, Germany). Siliconized microcentrifuge tubes (2 mL) were purchased from Eppendorf (Hamburg, Germany). Microcon centrifugal filter devices (Vivacoon 500) were purchased from Sartorius AG (Goettingen, Germany).

#### Animals

An inbred strain of black mice (C57BL/6, n = 8) were used in the experiments. Animals were housed in a standard facility with a 12 h light/dark cycle under constant temperature and humidity. Mice were fed with normal chow diet, and they were given free access to food and water. All procedures with live animals were conducted with protocols approved by the veterinary office of Fribourg, Switzerland.

#### Brain Tissue Preparation

The degradation of proteins or neuropeptides themselves, which occurs during the post-mortem period, greatly influences the qualitative results and also affects the reproducibility in quantitative analysis. In all the sample preparation experiments, each step was under temperature control to minimize the degeneration of the endogenous peptides and also to reduce the amount of interference peptides produced from fragmentation of proteins. Male mice of 8–10 weeks old were sacrificed, and then their brains were removed immediately and heat stabilized using Denator irradiation (Denator AB, Gothenburg, Sweden) as described elsewhere. Three mouse brains were pooled and homogenized as entire brain (EB) samples for method comparison. Three samples prepared using the MOC method were further subjected to LC-FT-MS/MS analysis for identification of peptides from EB. Another five mouse brains were used only for identification of peptides from the hypothalamus. The hypothalami of both hemispheres from each brain were dissected at −10 °C, pooled as one sample, and then submitted for extraction.

#### Extraction of Peptides

To acquire the maximum extraction efficiency for diverse peptides of different hydrophobicities, three different extraction methods, acetic acid solution (AAS), mixing in solution (MIS), and mixing on column (MOC), were examined. AAS sample treatment method was as follows: each piece of homogenized EB was extracted two times with 0.2% acetic acid aqueous solution. Each step used 7.5 μL of solution per 1 mg of tissue. The two-step extractions were mixed and submitted to filtration. This method has been shown to have high reproducibility in peptide quantitative analysis. MIS sample treatment method was as follows: each piece of homogenized EB was extracted three times using (i) water–methanol–acetic acid solution (89.8:10:0.2, v/v/v), (ii) water–methanol–acetic acid solution (69.8:30:0.2, v/v/v), and (iii) water–methanol–acetic acid solution (49.8:50:0.2, v/v/v), respectively. Each step used 5 μL of solution per 1 mg of tissue. Before filtration, all the three extractions were mixed directly. MOC sample treatment method was as follows: each piece of homogenized EB was extracted four times using (i) 0.2% acetic acid aqueous solution, (ii) 0.2% acetic acid aqueous solution, (iii) water–methanol–acetic acid solution (79.8:20:0.2, v/v/v), and (iv) water–methanol–acetic acid solution (49.8:50:0.2, v/v/v), respectively. Each step used 3.75 μL of solution per 1 mg of tissue. Before filtration, the first two step aqueous extractions and the last two step organic extractions were mixed, respectively. The last two steps used methanol to increase the capability in the extraction of hydrophobic peptides.

In each extraction step of all the three extraction methods, every piece of tissue was homogenized twice (each time 20 s with a pause of 20 s between the homogenizations) for 1 min by a Precellys 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). Then, the sample was centrifuged at 22000g for 60 min at 4 °C. All the supernatants obtained in the extraction methods were filtered on a 10 kDa cutoff filter (Vivacoon 500, Sartorius AG, Goettingen, Germany) by centrifuging for 90 min at 14000g at 4 °C.

#### Comparative Analysis of Three Sample Preparation Methods

Three pooled, homogenized entire brains (EB) were used for comparative analysis between the three sample treatment methods. The test of each method used three EB samples (30 mg/sample) with two replications of LC-FT-MS analysis. Each analysis was conducted on a nanoLC–MS system (see details in LC-FT-MS/MS Data Acquisition). The LC parameters were identical to those used for LC-FT-MS/MS data acquisition except for the use of a short elution gradient. The gradient profile was as follows: 0–3.5 min, 2% B; 3.5–8 min, 2–25% B;
The peptide extracts were analyzed using a LTQ-Orbitrap Discovery (Thermo Fisher Scientific, Bremen, Germany) coupled with a 2D NanoLC (Eksigent Technologies, California, USA). In each LC–MS run, 5 μL of sample was injected with 1D pump to a trap column (100 μm ID, 2 cm long), which was packed with a C18 AQ particles (5 μm, 100 Å, Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) in a PEEK column holder (Upchurch, Oak Harbor, WA, USA). For each sample prepared in MOC, the organic extract was loaded on the trap column 3 min prior to the injection of the aqueous extract. Prior to injections, the trap column was conditioned using 2% acetonitrile and 98% water containing 0.2% formic acid. The elution direction of trap column was reversed through a 10-port valve when it started to couple with the analytical column. The analytical column of 25 cm long used C18 AQ particles (3 μm, 100 Å) as medium, which was packed in a Picofrit capillary with an emitter tip of 10 μm (New Objectiv, Woburn, MA, USA). The mobile phase A and B in 2D pump (Chanel 2) were 0.2% FA, and 95% acetonitrile (0.2% FA), respectively.

The gradient profile was as follows: 0–6 min, 2% B; 6–12 min, 2–20% B; 12–80 min, 20–50% B; 80–85 min, 50–60% B; 85–90 min, 60–95% B; and 90–100 min, 95% B. After each LC–MS sample analysis, the LC system was cleaned by injection of 75% acetonitrile (0.2% FA) one or two times and conditioned for 10 min with 50% B.

Data dependent acquisition (DDA)-LC-FT-MS/MS: Data acquisition on the LTQ-FTMS instrument consisted of a full FTMS scan event at mass range of 350–2000 m/z and data-dependent collision-induced dissociation (CID) MS/MS scans (30 000 resolution) of the five most abundant peaks from the previous full FTMS scan. The mass resolution for each scan event was kept at 30 000. Mass spectrometric experimental parameters were selected to provide high quality fragmentation spectra of the majority from the possible compounds of interest. Minimum signal threshold for the first and fifth most intensive ions were selected to 100 000 counts, respectively; isolation width was selected to m/z = 2; maximum accumulation time was 300 ms, normalized collision energy, 30%; activation Q, 0.25; and activation time, 50 ms, Dynamic exclusion was set as a repeat count of 1, an exclusion duration of 30 s, and a repeat duration of 30 s. Dynamic exclusion used 25 ppm mass tolerance.

CSD-LC-FT-MS/MS: The selection of charge state 1, 2, 3, ≥4 was conducted by using the charge rejection function in the Xcalibur software. To select the charge state of interest, the options for the rejection of other charge states are enabled. In this manner, four CSD-LC-FT-MS/MS analyses were conducted for each sample by the instrument automatically selecting the peptides of directed charge state(s) for fragmentation. The Xcalibur software does not allow specifically selection of peptides with charge ≥4. To compensate, the inclusion list of peptides of charge 4 or 5 in the previous CSD-LC-FT-MS/MS analysis was prepared, respectively. Software Sieve (1.3 version, Thermo, CA, USA) was used to extract the features with charge states 4 or 5 from raw LC-FT-MS/MS data. The inclusion mass lists were then loaded to the FT-MS/MS scan for another two CSD-LC-FT-MS/MS runs for each sample.

**Hybrid Spectral Analysis**

All the raw LC-FTMS/MS data were subjected to Peaks Studio 5.3 (BSI, Canada) for spectral interpretation. Database search was conducted against the Swepep mouse neuropeptide precursors database. The Swepep precursor database was downloaded from www.swepep.org and loaded to Peaks studio 5.3 by using the UniprotKB/TrEMBL FASTA format and taxonomy options. Peaks Studio 5.3 was used for various functions such as Data Refinement, Auto De Novo, and Peaks Search (homology search). The Data Refinement program allows correcting of the precursor mass and charge states to provide the accurate monoisotopic mass of a peptide. Data processing, including peak centroiding, charge deconvolution, and deisotopization, was conducted for data refinement. The refined data were subjected to database search with the mass tolerance of precursor ions and product ions set at 10 ppm and 0.05 Da, respectively. No enzyme was specified for cleavage. Variable PTMs, including amidation (C-terminal), acetylation (N-terminal), phosphorylation, pyroglycaminylation from glutamic acid and glutamine (N-terminal), were selected in sequencing. Estimation of false positives was conducted by searching all spectra against decoy databases. The cutoff of PDR (peptide sequence) for peptide identification in Peaks search was <1%. The peptides of FDR between ≥1% but <10 logP > 20 were submitted to manual inspection to confirm their sequences. A sequence was considered correct only if it matched all the following criteria: (1) the mass of a peptide must have been calculated from the monoisotopic ions of a peptide, (2) all of the database search results were inspected with de novo sequencing results, (3) the peptide mass was within 10 ppm of the theoretical mass, (4) the major fragments observed in MS/MS had to match within 0.05 Da to predicted monoisotopic production ions, and (5) the fragmentation information must be enough to recognize the alignment of amino acids, in particular if they fall in the substitution positions across the adjacent species.

**RESULTS**

Enhanced Peptide Extract and Load Efficacy with Mixing-on-Column Sample Treatment

We first examined the influence of different sample treatment methods on peptide extraction efficacy. The intensities (peak areas) of each feature (peptide) were compared across the samples prepared with three methods, AAS, MIS, and MOC. The normalized intensities of the 2000 most intensive features were plotted against their corresponding retention time (Rt).
The three methods show remarkable difference in their intensity−Rt patterns. The AAS method had overall low extraction efficacy, while the MOC method in general allowed high extraction efficacy. In AAS samples, the number of features with relative intensity >33.3% decreased with the increase of retention time, and plenty of features were almost undetectable (relative intensity close to 0%). In MOC samples, the number of features with relative intensity >33.3% (1330) is larger than those in AAS samples (279) and MIS (1035) samples. Compared to MIS samples, MOC samples had much fewer features with relative intensities close to 0%. In contrast, their features had moderate to high relative intensities across the whole time course. The overall intensity−Rt pattern indicated that the MOC method allowed a large number of peptides to be extracted and loaded to LC−MS analysis with high coverage and high abundance.

The examination on individual peptides further verified the high extraction efficacy of the MOC methods (Figure 1B). Thymosin β4, a polar peptide hormone of high abundance, was used as a reference peptide in comparative analysis. The relative intensities of this peptide were similar across the three methods. In a sharp contrast, somatostatin-28 (AA1−12), met-enkephalin, neurotensin, and neuropeptide Y were unequally extracted and loaded for LC−MS analysis. Somatostatin-28 (AA1−12), a polar peptide, had much more lower abundance in MIS samples than in AAS and MOC samples, while neuropeptide Y was well extracted in MIS and MOC samples but not in AAS samples. Compared to the MOC method, the direct mixing of aqueous and organic extracts before filtration and loading also led to the abundance reduction of peptides somatostatin 28 (AA1−12) and neuropeptide Y. These observations indicated that the extraction efficacy of a peptide heavily depended on the used solutions and procedure. As the quality of the tandem MS spectra of a peptide depends on its abundance, the use of the MOC method could increase the peptide identification rate because of its high coverage of peptides of different hydrophobicities.
To evaluate the performance of our integrated approach, we compared the number of peptides identified from mouse entire brain (EB) using different LC-FT-MS/MS methods and different sample treatment methods. Overall, our new approach allowed 206 peptides on average to be identified from a single mouse brain sample, which was prepared using 15 μL of solutions per 1 mg of tissue. The cumulative number of peptides identified from three EB samples reached a value of 272. Figure 2A shows that the CSD LC-FT-MS/MS method allows more peptides, no matter counting in average and cumulative numbers, to be identified than the DDA LC-FT-MS/MS method. Further comparative analysis shows that our new approach allowed more peptides to be identified than DDA LC-FT-MS/MS analysis combined with the commonly used acetic acid solution (AAS) sample treatment method or mixing in solution (MIS) multistage extraction method. The overlap of identified peptides between different methods. The homogenized entire brain (EB) samples are analyzed using different LC-FT-MS/MS analysis methods (DDA or CSD) with different sample treatment methods (AAS, MIS, and MOC). Each sample is analyzed using six LC-FT-MS/MS runs, and each method is examined with three brain tissue samples. The bars indicate the average numbers (mean ± s.e.m) of identified peptides per EB sample (blue) and the sum of unique identified peptides from three EB samples (red). All the EB samples used for method comparison are prepared from the homogenized tissues of three pooled mouse brains. The peptide sequence FDR is less than 1%.

Figure 2. Comparison of different neuropeptidomic approaches. (A) The number of peptides identified with different methods. The overlap of identified peptides between different methods. The homogenized entire brain (EB) samples are analyzed using different LC-FT-MS/MS analysis methods (DDA or CSD) with different sample treatment methods (AAS, MIS, and MOC). Each sample is analyzed using six LC-FT-MS/MS runs, and each method is examined with three brain tissue samples. The bars indicate the average numbers (mean ± s.e.m) of identified peptides per EB sample (blue) and the sum of unique identified peptides from three EB samples (red). All the EB samples used for method comparison are prepared from the homogenized tissues of three pooled mouse brains. The peptide sequence FDR is less than 1%.

allows more peptides, no matter counting in average and cumulative numbers, to be identified than the DDA LC-FT-MS/MS method. Further comparative analysis shows that our new approach allowed more peptides to be identified than DDA LC-FT-MS/MS analysis combined with the commonly used acetic acid solution (AAS) sample treatment method or mixing in solution (MIS) multistage extraction method. The results showed that about 82.0% of the peptides identified using method AAS+DDA had sequences identical with those using MOC+CSD. The peptides identified using method MIS+DDA and MOC+DDA had 83.1 and 84.7% sequences identical with those using MOC+CSD, respectively (Figure 2B). The results demonstrated the peptide identification rate is method dependent, while our new approach allowed an overall high overlap of identified peptides with other approaches.

Prohormone-Derived Peptides Identified from Mouse Brain

Overall High Peptide Identification Rate from Mouse Whole Brain and Hypothalamus Samples. When we applied our integrated approach on samples taken from the mouse hypothalamus, a peptide-rich brain region, we were able to identify 367 peptides from neuropeptide precursors. In total, 457 unique peptides were identified from both the homogenized EB and hypothalamus of wild type mice at confidence level peptide sequence FDR (false discovery rate) <1%. An additional 46 peptides were identified by manual inspection on other peptides (FDR ≥1% but −10 logP > 20) using strict criteria (see the Experimental Procedures). Overall, 503 unique peptides were identified from mouse brain (see Supporting Information Table S1).

Identification of Long and Hydrophobic Peptides. The brain expresses various neuropeptides with long sequence and high hydrophobicity. We unambiguously identified a number of long neuropeptides, such as galanin, orexin-B, neuropeptide Y, CLIP, and oxyntomodulin. The use of FT-MS/MS showed that peptides can be identified in high confidence. Figure 3 represents the identification of glucagon-like peptide 1 (7–36) and neuropeptide Y. Additionally, our new approach allows a number of high hydrophobic peptides to be identified along with polar peptides. Figure 4 shows representative high abundance peptides identified from one mouse brain (EB) sample. The base peak LC–MS chromatography indicated that peptides of different hydrophobicity, including polar somatostatin 28 (AA1–12) and hydrophobic secretoneurin, could be extracted, loaded, and detected in a single LC–MS run.

Discovery of Novel Peptides. The high identification rate of our integrated approach allowed the discovery of a large number of novel peptides (n = 189) that have not been previously reported in mouse or other species (see Supporting Information Table S1). Figure 5 represents an example of spectral identification for a novel peptide, Q(−17.03)-LRAPGAMLQIEALQEVLKKLKS, derived from precursor cocaine-and-amphetamine-regulated transcript (CART). This peptide has classical dibasic cleavage sites and is thus a possible candidate for a bioactive neuropeptide. Further experimental work is required to gain insight into the biological functions of this and other newly discovered peptides.

Identification of Peptides with PTMs. Post translational modifications (PTMs) commonly occur in neuropeptides and have important biological functions. They can alter functional properties of neuropeptides, for example, increasing the binding affinity to receptors or make peptides more resistant to enzymatic degradation. In the present study, we characterized 69 peptides containing PTMs (see Supporting Information Table S1), which include 6 phosphorylations, 7 acetylations, 18 pyrrol glutamations, and 41 amidations. Some of the peptides exhibited more than one PTMs. For example, both pyroglutamylisation and amidation modifications were detected on vasoactive intestinal peptide (VIP), Q(−17.03)-MAVKKYLNSILN(−0.98). The PTM of C-terminal amidation, which is specific to endogenous peptides and is required for the functional activation of many neuropeptides, occurred most frequently in our data set.

DISCUSSION

The improvement in the identification rate of endogenous peptides is a major current goal of neuropeptidomics. Our study developed an integrated approach to increase the peptide identification rate. This approach used mixing on column (MOC) to increase the peptide loaded onto the column and used charge state-directed (CSD) tandem MS to increase the number of peptides subjected for high mass accuracy fragmentation. Using our integrated approach, more than 500 endogenous peptides in total were identified from mouse hypothalamus and whole brain samples, demonstrating previously unrivaled identification power in neuropeptidomics.
Sample quality critically influences the data output in peptidomics and proteomics. Neuropeptides are a large family of compounds with a wide range of hydrophobicities, including strongly hydrophobic and hydrophilic molecules. This diversity of hydrophobicities makes it difficult to extract and preserve diverse neuropeptides in a single solution, such as the commonly used aqueous buffers. The extraction procedure therefore has a crucial influence on the number and amount of peptides delivered to the LC−MS/MS analysis. Remarkable losses of peptides in sample treatment have been previously observed during preservation. Our study also showed that some peptides can be lost during direct mixing of different solutions in a multistage extraction (see Supporting Information Figure S1). We therefore used a MOC method to sequentially extract and load peptides with aqueous and organic solutions to increase the coverage of both hydrophilic and hydrophobic peptides. As the hydrophilic peptides have low retention times and thus can be easily washed out from trap column with high content of organic solvents used for samples, the load of an organic extract was conducted prior to the load of an aqueous extract. Using these procedures, both hydrophilic and hydrophobic peptides were well extracted and loaded for further LC−MS analysis.

Our study increased the peptide identification rate with fully using the advantage of high accuracy tandem MS. Many of the endogenous peptides have long sequences, for example, Figure 3. Annotated FT-MS/MS spectra of representative peptides neuropeptide Y (A) and glucagon-like peptide 1(7–36) (B). The high accuracy of the MS and MS/MS information allows these peptides to be identified with high confidence.

Figure 4. Representative prohormone-derived peptides identified from a mouse brain (EB) sample. These peptides include somatostatin-28–14 (1), nociceptin (2), cerebellin-1 (3), dynorphin B (4), neurokinin A (5), neuropeptide-glutamic acid-isoleucine (6), met-enkaphalin (7), neurotensin (8), somatostatin-28 (9), met-enkephalin-arg-ser-leu (10), galanin (11), leu-enkaphalin (12), vasoactive intestinal peptide (13), WE-14 (14), BAM18 (15), oxymontodulin (16), little SAAS (17), somatostatin-14 (18), manserin (19), neuropeptide Y (20), neokinin-B (21), GAV (22), PEN (23), and secretoneurin (24). These peptides are detected with high MS signals (intensity larger than 1E6). One small protein, ubiquitin, and two peptide hormones, thymosin β4 and thymosin β10, are used in practice as reference peptides to monitor the elution order of peptides.
Orbitrap MS, allowing the acquisition of a larger number of MS analysis thus compensates for the low scan speed of LTQ-precursor ions. The use of charge state directed LC-FT-MS/MS analysis can be used to select peptides with different charge states to guide the tandem MS analysis. Compared to LTQ-Orbitrap, significantly narrow the search space in databases, and serve as a powerful approach in peptide identification.

To obtain tandem MS information for as many peptides as possible, we acquired high accuracy tandem MS analysis, several peptides with different charge states coelute at the same time window. Our approach method allows peptides, including low abundance ones, to be selected by software (Xcalibur in our case) for fragmentation according to the charge states of their precursor ions. The use of charge state directed LC-FT-MS/MS analysis thus compensates for the low scan speed of LTQ-Orbitrap MS, allowing the acquisition of a larger number of high quality fragmentation spectra for the peptides present in samples.

It is worth noting that in the present study, we stabilized mouse brain tissue by using a novel method, which utilizes a combination of heat and pressure under vacuum to stop the degradation of brain tissue immediately after extracting brains from animals. A line of evidence has demonstrated that proteases and other protein-modifying enzymes can rapidly change the composition of the proteome following brain extraction, leading to extensive changes of the proteome.

Post-mortem changes of the brain cause major disturbance in the tissue homeostasis, which in turn causes rapid release of mediators of degradation, altering the levels and composition of proteins and post-translational modifications (PTMs). The application of rapid tissue stabilization has been shown to substantially reduce protein fragmentation interference, and importantly, to maintain the levels of neuropeptides in the brain tissue.

Our study allowed identifying a large number of unreported peptides, which included peptides with N- and C-terminal modifications and/or dibasic cleavage sites as well as peptide fragments such as truncated peptides. The identification of these novel peptides reflected the overall increased detection capability of our approach. Peptide fragments are commonly observed in various neuropeptidomics studies, even if strict sample preparation procedures are used. The formation of peptide fragments may come from in vivo peptide/protein processing, which are performed by intracellular proteolysis or by extracellular peptide decay after their release from neurons.

These processes are not fully understood yet. However, a line of evidence showed that many peptide fragments have distinct physiological functions, for example, activating particular receptors or inhibiting enzymes. The identification of novel peptides therefore provided possible candidates for bioactive neuropeptides.

Our study allowed a number of hydrophobic and large peptides, including neuropeptide Y and galanin, identified from mouse brain. Neuropeptide Y and galanin are of high interest in various biological studies due to their important roles such as food intake control and cognition. Although they are abundant in the brain, they have not been reported in previous neuropeptidomic studies with high confidence, probably because of their high hydrophobicity and long sequence.

The reliable identification of such long and hydrophobic neuropeptides using our approach assists an exploration of their roles in physiological and behavioral processes. Moreover, our approach allowed these peptides quantified, if necessary, along with other hydrophilic peptides because the high coverage of peptides in a LC−MS analysis.

Taken together, we have described an integrated mass spectrometric approach for acquiring high identification rates of endogenous peptides from the mouse brain. Using the developed approaches, hydrophobic peptides as well as hydrophilic peptides could be simultaneously extracted from brain tissue. The subsequent CSD-LC-FT-MS/MS fully used the advantage of high accuracy FT-MS/MS while compensating for its slow scan speed. Moreover, the database search conducted at the sample level rather than individual raw data level simplified data processing and made the analysis much less time-consuming. The application of this approach to the mouse brain allowed us to identify an unrivaled number of endogenous peptides from neuropeptide precursor proteins. Our study presents a straightforward and powerful approach for the discovery of peptides from complex biological samples in discovery neuropeptidomics.

## ASSOCIATED CONTENT

**Supporting Information**

Supplementary table and figures. 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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**Figure 5. Identification of a novel hydrophobic peptide CART (AA57−79).** This peptide, RR.Q(−17.03)LRAPGAMLQIEALQEVLKKLKS.KR, has dibasic cleavage sites and thus can be a candidate neuropeptide.
REFERENCES


