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Mass Spectral Analysis of Neuropeptide Expression and Distribution in the Nervous System of the Lobster *Homarus americanus*

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Abstract

The lobster *Homarus americanus* has long served as an important animal model for electrophysiological and behavioral studies. Using this model, we performed a comprehensive investigation of the neuropeptide expression and their localization in the nervous system, which provides useful insights for further understanding of their biological functions. Using nanoLC ESI Q-TOF MS/MS and three types of MALDI instruments, we analyzed the neuropeptide complements in a major neuroendocrine structure, pericardial organ. 57 putative neuropeptides were identified and 18 of them were *de novo* sequenced. Using direct tissue/extract analysis and bioinformatics software SpecPlot, we charted the global distribution of neuropeptides throughout the nervous system in *H. americanus*. Furthermore, we also mapped the localization of several neuropeptide families in the brain by high mass resolution and high mass accuracy mass spectrometric imaging (MSI) using a MALDI LTQ Orbitrap mass spectrometer. We have also compared the utility and instrument performance of multiple mass spectrometers for neuropeptide analysis in terms of peptidome coverage, sensitivity, mass spectral resolution and capability for *de novo* sequencing.

Keywords

Homarus americanus; MALDI FTICR MS; MALDI TOF/TOF; nanoLC ESI QTOF; MALDI LTQ Orbitrap; pericardial organ; neuropeptide; bioinformatics; mass spectrometric imaging (MSI)

Introduction

The American lobster *Homarus americanus* is used as an important model organism in many areas of physiology. The neural circuits contained within the *H. americanus* cardiac nervous system and the stomatogastric nervous system (STNS) provide an important platform for the study of neuromodulatory control of rhythmic behavior, such as generation of cardiac activity and the swallowing, chewing and filtering of food items.¹⁻³ In addition, this model organism has long served as a model for studies of hormonal control of aggression⁴⁻⁶ and environmental stress related responses.⁷⁻¹¹ Furthermore, *H. americanus* is also widely used to study the roles of neuromodulators in maturation of neural circuits.¹²⁻¹⁵

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Given the biological and economical importance of *H. americanus*, considerable effort has been devoted to characterize the signaling molecules it uses to generate and modulate behaviors, with greater emphasis placed on its diverse neuropeptide complement. To date, close to 100 neuropeptides have been identified from this species, using various biochemical methods including state-of-the-art mass spectrometric techniques.¹⁶⁻¹⁹ A recent study by our group characterized 83 neuropeptides, including 28 novel ones via *de novo* sequencing,¹⁷ using a combination of online LC-MS/MS and MALDI MS methodology. Despite a large number of neuropeptides identified in the central nervous system (CNS) and the STNS of the lobster, only a few neuropeptides have been identified from the pericardial organ (PO), one of the most important neuroendocrine organs in crustacean model organisms. In order to better understand the hormonal regulation of crustacean and how it affects the activities of neuronal circuits, such as STNS, it is crucial to perform a comprehensive characterization of neuropeptide contents of this organ. A complete knowledge of the neuromodulators/hormones present in the lobster *H. americanus* and their distribution patterns is an important first step towards a better understanding of the complex interplay of these signaling molecules in a functional network.

Here, we analyzed the neuropeptide content in the pericardial organ using a combination of nanoscale liquid chromatography coupled to electrospray ionization quadrupole time-of-flight tandem mass spectrometry (nanoLC ESI Q-TOF MS/MS) and three types of matrix-assisted laser desorption/ionization (MALDI) instruments. Distinct types of neuropeptides were observed in different regions of PO, suggesting its possible multiplex functions as reflected by heterogeneity and complexity of peptidomic profiles. In total, 57 neuropeptides were identified from the lobster POs, including 18 novel ones reported here for the first time. This study represents the first comprehensive characterization of the peptidome in this important neuroendocrine organ of *H. americanus*. Furthermore, we systematically examined the neuropeptide complements in eight different neuronal tissues by direct tissue/extract profiling. Using in-house developed software SpecPlot we charted the global distribution of neuropeptides throughout the nervous system in *H. americanus*. Because the brain is an extremely complex structure that contains numerous neuropils and neuronal clusters, we also mapped the localization of several neuropeptide families in the brain using mass spectrometric imaging (MSI). Our report represents the first demonstration of crustacean neuropeptide imaging using the high mass accuracy and high mass resolution offered by the MALDI LTQ OrbitrapTM instrument. The high resolving power and wide dynamic range of the Orbitrap analyzer offer distinct advantages for MALDI imaging as compared to other more commonly employed MALDI TOF/(TOF)-based instruments. The direct tissue analysis and MALDI imaging showing localization of neuropeptides within the nervous system of *H. americanus* displays useful information about the large diversity of neuropeptides and the complexity of their expression patterns, thus providing an important link to their potential biological functions.

Methods

Animals and Tissue Collection

American lobsters, *H. americanus*, were purchased from Simply Lobster Company (Lewiston, ME) and were maintained in flow-through natural seawater aquaria at ambient seawater temperature (approximately 12-13 °C) before use. All animals were anesthetized by packing them in ice for 30-60 minutes, after which the dorsal carapace was removed from each individual and its supraoesophageal ganglia (brain) and thoracic ganglion (TG), subesophageal ganglion (SEG), commissural ganglion (CoG), stomatogastric ganglion (STG), sinus gland (SG) in the eyestalk and pericardial organ (PO) from the pericardial chamber were dissected free from surrounding muscle and connective tissues in chilled (approximately 10 °C) physiological saline (composition in mM: 479.12 NaCl, 12.74 KCl, 13.67 CaCl₂, 20.00

MgSO₄, 3.91 Na₂SO₄, 5.00 HEPES [pH 7.4]). Following dissection, tissue samples were directly analyzed with direct tissue method or immediately placed in acidified methanol (90% methanol: 9% glacial acetic acid: 1% deionized water) and stored at -80 °C until utilized for peptide extraction or direct tissue mass spectral analysis.

Direct Tissue Analysis

Direct tissue analysis was performed as described previously.²⁰ Briefly, the tissue was rinsed in a droplet of acidified methanol, desalted in a droplet of dilute MALDI matrix (10 mg/ml 2, 5-dihydroxybenzoic acid (DHB), aqueous), and placed on the MALDI plate. DHB (100 mg/ml in 50% methanol, v/v) matrix was deposited on top of the tissue to adhere it to the MALDI target and allowed to crystallize at room temperature. Direct tissue mass spectrometric analysis was performed using the MALDI TOF/TOF mass spectrometer.

MALDI Imaging Sample Preparation

The freshly dissected brain was rinsed briefly by dipping in deionized water for two seconds to eliminate the salt content, and then embedded in gelatin (100 mg/ml, aqueous) and snap-frozen. Sectioning into 12 µm slices was performed at -20 °C on a cryostat (Microtom HM505E, Waldorf, Germany), and the slices were thaw-mounted onto a MALDI TOF/TOF plate or a microscope glass slide (for MALDI LTQ Orbitrap) that had been coated with a thin layer of DHB. Before imaging acquisition, five coats of DHB (150 mg/ml in 50% methanol, v/v) were applied on the tissue surface using an airbrush (Paasche airbrush company, Chicago, IL) with 30 second intervals between each cycle.

Tissue Extraction and HPLC Separation

Tissues were pooled, homogenized, and extracted with acidified methanol. Extracts were dried in a Thermo Scientific Savant SC 110 SpeedVac concentrator (Thermo Fisher Scientific, Waltham, MA) and re-suspended in approximately 100 µl (depending on the starting sample size) of 0.1% formic acid. The re-suspended extracts were then vortexed and briefly centrifuged. The resulting solution was either analyzed directly after desalting with C₁₈ ziptip or separated using HPLC. Two pooled pericardial organ tissue extract samples were analyzed in this study.

Rainin Dynamax HPLC system (Rainin Instrument Inc., Woburn, MA) equipped with a Dynamax UV-D II absorbance detector was used for offline separation. The mobile phases were: deionized water containing 0.1% formic acid (Solution A), and acetonitrile (HPLC grade, Fisher Scientific) containing 0.1% formic acid (Solution B). For each separation run, 20 µl of extract was injected onto a Macrosphere C₁₈ column (2.1 mm i.d. × 250 mm length, 5 µm particle size; Alltech Assoc. Inc., Deerfield, IL). The separation consisted of a 120 minute gradient of 5%-95% Solution B with fractions automatically collected every three minutes using a Rainin Dynamax FC-4 fraction collector. The fractions were dried with SpeedVac and resuspended in 5 µl of 0.1% formic acid before use.

Formaldehyde Derivatization

An aliquot of 3 µL tissue extract was labeled in solution by adding 0.7 µL borane pyridine (C₅H₈BN, 120 mM in 10% methanol), and then mixed with formaldehyde (FH₂, 15% in H₂O, 0.5 µL). The samples were then placed in a 37 °C water bath for 20 minutes for the labeling reaction to complete.

MALDI FTICR MS

MALDI FTICR MS experiments were performed on a Varian ProMALDI Fourier transform ion cyclotron resonance (FTICR) mass spectrometer (Lake Forest, CA) equipped with a 7.0

Tesla actively-shielded superconducting magnet. This FTICR MS instrument contains a high pressure MALDI source coupled with 355nm Nd:YAG laser where the ions from multiple laser shots can be accumulated in the external hexapole storage trap before the ions are transferred to the ICR cell via a quadrupole ion guide. The ions were excited prior to detection with an rf sweep beginning at 7050 ms with a width of 4 ms and amplitude of 150 V base to peak. The filament and quadrupole trapping plates were initialized to 15 V, and both were ramped to 1V from 6500 to 7000 ms to reduce baseline distortion of peaks. Detection was performed in broadband mode from m/z 108.00 to 4500.00. For sample spotting, equal volume of 0.4 μ l sample solution and matrix solution (100 mg/ml DHB in 50% methanol) were mixed and allowed to dry.

MALDI TOF/TOF

A model 4800 MALDI TOF/TOF analyzer (Applied Biosystems, Framingham, MA) equipped with a 200 Hz, 355 nm Nd:YAG laser (spot diameter $\sim$ 75 μ m) was used for direct tissue/extract analysis, and MALDI imaging of brain sections. Acquisitions were performed in positive ion reflectron mode. Instrument parameters were set using the 4000 Series Explorer software (Applied Biosystems). Mass spectra were obtained by averaging 900 laser shots covering mass range m/z 500-4000. MS/MS was achieved by 1 kV collision induced activation (CID) using air as collisional gas. For sample spotting, equal volume of 0.4 μ l sample solution and α -cyano-4-hydroxy-cinnamic acid (CHCA) matrix solution in 60% acetonitrile were mixed and allowed to dry.

Imaging acquisition was performed using the 4800 Imaging application software (Novartis, Basel, Switzerland) available through the MALDI MSI website (www.maldi-msi.org). To generate images, spectra were collected at 100 μ m intervals in both the x and y dimensions across the surface of the sample. Each mass spectrum was generated by averaging 200 laser shots over the mass range m/z 600-1800. Individual spectra were acquired using 1.0 ns binning to yield 27,812 data points per spectrum. Image files were processed and extracted ion images were created using the TissueView software package (Applied Biosystems, Framingham, MA, USA).

MALDI LTQ Orbitrap

The same LTQ Orbitrap XL instrument from electrospray applications is used for MALDI, with minor modifications to accommodate the MALDI source.²¹ It uses a commercial 60 Hz N₂ laser at 337 nm (LTB Lasertechnik Berlin GmbH, Berlin, Germany) in direct beam configuration. Two microscans of averaging and $\sim$ 80 laser shots (for a total of 160 laser shots/pixel) were used for both MS and MS/MS experiments with Orbitrap detection. All experiments were performed with automatic gain control (AGC) turned on. AGC determines (in a prescan event) the number of laser shots to fill the Orbitrap to a predetermined target value. The default target values, a dimensionless number that is related to the number of charges allowed at one time, were used for all experiments (10^5 - 10^6 ions for MS detection). Both MS and MS/MS experiments were acquired in centroid mode. A 2 Da mass window was used for MS/MS precursor selection. For Higher Energy Collisional Dissociation (HCD), 35% normalized collision energy (NCE) was applied and 32% NCE was used for Pulse-Q Dissociation (PQD) experiments. Qualitative data were obtained using standard Xcalibur™ software and images were extracted using ImageQuest™ 1.0.1 software. Mass spectral images were normalized using the 'MassRange/TIC' plot type in ImageQuest. The Orbitrap analyzer was fully tuned and calibrated with the aid of two calibration peptide mixtures (MSCal4, Sigma Aldrich, St. Louis, MO) for optimization in two mass ranges: 150-2000 and 200-4000. The mass calibration of the Orbitrap analyzer alone was checked once a day with external calibrant to ensure operation within the <3 ppm instrument specifications.

Capillary LC ESI Q-TOF MS/MS

Nanoscale LC ESI Q-TOF MS/MS was performed using a Waters NanoAcquity Ultra Performance capillary LC system coupled to a Q-TOF Micro mass spectrometer (Waters Corp., Milford, MA). Chromatographic separations were performed on a C₁₈ reversed phase capillary column (75 μ m internal diameter $\times$ 150 mm length, 3 μ m particle size; Micro-Tech Scientific Inc., Vista, CA). The mobile phases used were: deionized H₂O with 5% acetonitrile and 0.1% formic acid (A); acetonitrile with 5% deionized H₂O and 0.1% formic acid (B); deionized H₂O with 0.1% formic acid (C). An aliquot of 6.0 μ l of an HPLC fraction (see 2.3) was injected and loaded onto the trap column (PepMapTM C₁₈; 300 μ m column internal diameter $\times$ 1 mm, 5 μ m particle size; LC Packings, Sunnyvale, CA, USA) using mobile phase C at a flow rate of 30 μ l/min for 3 minutes. Following this, the stream select module was switched to a position at which the trap column became in line with the analytical capillary column, and a linear gradient of mobile phases A and B was initiated. A splitter was added between the mobile phase mixer and the stream select module to reduce the flow rate from 15 μ l/min to 200 nl/min.

The nanoflow ESI source conditions were set as follows: capillary voltage 3200 V, sample cone voltage 35 V, extraction cone voltage 1 V, source temperature 120°C, cone gas (N₂) 10 l/hr. A data dependent acquisition was employed for the MS survey scan and the selection of precursor ions and subsequent MS/MS of the selected parent ions. The MS scan range was from m/z 300-2000 and the MS/MS scan was from m/z 50-1800. The MS/MS *de novo* sequencing was performed with a combination of manual sequencing and automatic sequencing by PepSeq software (Waters Corp.).

Data Analysis and SpecPlot

An in-house developed bioinformatics software SpecPlot was used to process and organize the neuropeptide profiling data from eight different regions in the lobster. The algorithm of this software was described elsewhere.²² Briefly, the peak list of a mass spectrum from MALDI TOF/TOF was exported from the Data Explorer software, and pasted into Microsoft Excel. The peak lists were compared to the in-house database of crustacean neuropeptides, and filtered manually for known neuropeptide peaks. The excel files were then converted to DTA files to import into SpecPlot. To transform the peak lists into feature lists, individual bins were created across spectra and the presence or absence of a peak was determined in each bin from each spectrum using 30 ppm as mass error tolerance.

Results

Detection and Sequencing of Neuropeptides on Different Types of Mass Spectral Instruments

We compared three commonly employed MALDI mass spectrometers for neuropeptide analysis, including MALDI TOF/TOF, MALDI FTICR and MALDI LTQ Orbitrap mass spectrometers. As shown in Figure 1, numerous peaks are detected in an HPLC fraction of PO extract by each type of instruments. Some peptide peaks are commonly observed using all three instruments, whereas others are only uniquely identified by one or two of the instruments, highlighting the preferential detection of certain peptide ions with different mass analyzers. For example, MALDI TOF/TOF is more sensitive for ions with lower masses. As seen, peptide peaks at m/z 965.54 and m/z 995.55 are detected as two highly abundant ions on the TOF/TOF, but they are detected as low level peaks on the other two instruments. However the FTICR MS and LTQ Orbitrap instruments exhibit favorable detection for peptide ions in the mass range of m/z 1000-1500. Furthermore, TOF mass analyzer offers modest resolving power as compared to Orbitrap and FTICR MS analyzers. Broader peaks are observed in the MALDI TOF/TOF spectra. Furthermore, as shown in the inset of Figure 1b, two peaks around m/z

1213.5 are detected on MALDI LTQ Orbitrap, showing the presence of both SSEDMDRLGFG and FDAFTTGFGHN peptide peaks in the *H. americanus* PO. However, these two peaks are not resolved on MALDI TOF/TOF. Although the FTICR mass analyzer offers comparable resolving power as the LTQ Orbitrap mass spectrometer, the latter instrument offers better dynamic range and higher ion transmission efficiency that enables a more comprehensive coverage of peptide detection. As shown in Figure 1d, for the same PO HPLC fraction, 139 peaks are detected on the MALDI LTQ Orbitrap instrument, and 85 are observed on MALDI TOF/TOF, but only 50 are seen on MALDI FTICR MS. Neuropeptides detected using different instruments are listed in Table 1.

MALDI LTQ Orbitrap provides several useful ion fragmentation reactions for peptide sequencing, including collision induced dissociation (CID), higher energy collisional dissociation (HCD),²³ and pulsed-Q dissociation (PQD).²⁴ As shown in Figure 2, we have studied and compared these three fragmentation reactions for *de novo* sequencing of an unknown peptide at m/z 1084.6 from the *H. americanus* brain. This peptide is detected in multiple neural structures of the lobster, and it is also observed with differential expression level in the brain from lobsters at different developmental stages, showing its possible function related to animal development (data unpublished). As shown in Figure 2a, CID in the LTQ suffers from the 1/3 cutoff rule and only fragments with higher masses are detected. PQD enables the detection of low-mass fragments in MS/MS mode including immonium-type fragment ions in the linear ion trap (Figure 2b), however the overall signal intensities are relatively low, suggesting less than optimal fragmentation efficiency and detection, especially for higher mass fragment ions. Much improved fragmentation patterns are observed for HCD, which populates the low mass range (down to m/z 50). The fragment ions are detected in the Orbitrap, thus providing high mass accuracy (Figure 2c). High energy CID on a TOF/TOF instrument provides MS/MS spectra with noisier background (Figure 2d) compared to HCD, however it can induce more complete fragmentation compared to other types of fragmentation methods. Combining the information generated by multiple complementary fragmentation reactions, we assigned the sequence of this novel peptide as HI/LASLYKPR. The biological activities of this peptide will be further investigated by electrophysiological testing on the STNS of crustacean model organisms. The fragmentation information obtained on MALDI instruments can also provide valuable complementary information to the LC ESI MS/MS. As shown in Figure 3b, y ions are more favorably detected on ESI QTOF for APSKNFLRFa (m/z 1078.5) and most other FMRFamide-related peptides (FaRPs) because of the presence of highly basic arginine residue at the C-terminus, and the formation of b ions are suppressed. However, in the HCD on the MALDI LTQ Orbitrap instrument (Figure 4a) and CID on the MALDI TOF/TOF (Figure 4b), the formation of b ions is more favorable or comparable with the production of y ions. Almost complete series of b ions are observed using both fragmentation methods, with multiple a-type fragment ions (28 Da mass differences from the corresponding b ions) being produced to help confirm the identities of b ions with *de novo* sequencing.

Identification of Novel Neuropeptides from the Lobster Pericardial Organs

Pericardial organ is one of the major neuroendocrine sites in the crustacean nervous system, which can release peptide hormones into the circulating fluid -- hemolymph and modulate the functions of distant organs.²⁵ To obtain a complete description of neuropeptides present in this organ, we combined online nanoLC ESI QTOF and offline HPLC coupled with three different MALDI MS instruments for *de novo* sequencing and peptide matching by accurate mass measurements. Two pooled pericardial tissue extract samples have been analyzed. As shown in Table 1, 57 putative neuropeptides were detected in the POs from 9 families, including A-type, B-type, and C-type allatostatins, FaRP, orckinin and many others. The nanoLC ESI QTOF usually generates multiply charged ions, which often exhibit higher efficiency for MS/

MS fragmentation thus facilitating *de novo* sequencing of unknown peptide peaks. Using this technology and MS/MS information obtained from various MALDI types of instruments, 18 neuropeptides were *de novo* sequenced for the first time in this study.

Allatostatins—Allatostatins (AST) are pleiotropic peptides for which one function in insects is the inhibition of juvenile hormone synthesis.²⁶ A-type AST is a peptide family possessing –YXFGLamide C-termini motif (where X is a variable amino acid), and so far more than 100 different isoforms from this family have been characterized.²⁶ In this study, several novel A-type ASTs are identified, such as PRNYAFGLamide (Figure 3a), which further expands this family. The CID fragmentation on ESI Q-TOF mass spectrometer provides sufficient information for *de novo* sequencing. The predicted amino acid sequence is searched with BLAST against crustacean protein database, and the same sequence is encoded in an allatostatin precursor protein (BAF64528) from a closely related spiny lobster *Panulirus interruptus*, further supporting the derived peptide sequence. In addition, two B-type ASTs TNWNKFQGSWamide (m/z 1266.6) and STNWSSLRSAWa (m/z 1293.6) are observed in this study. Furthermore, C-type AST peptide pQIRYHQCYFNPISCF (m/z 1899. 9) is also detected in the PO. The C-type ASTs were first discovered from insects, and very recently, two C-type ASTs were detected and sequenced from two crustacean species, including pQIRYHQCYFNPISCF (m/z 1899. 9) from the POs of the crab *C. borealis* and another C-type AST from the brain of the lobster *H. americanus*.²⁷ Both C-type AST peptides contain characteristic disulfide bond in the peptide structure. In this study, the identification of pQIRYHQCYFNPISCF (m/z 1899. 9) was based on accurate mass matching, and it was further confirmed by DTT reduction, where $\Delta 2$ Da mass shift was observed in the resulting spectrum due to breakage of disulfide bond in the putative C-type AST peptide. This result represents the first evidence about the presence of C-type AST peptide in the lobster PO, suggesting the conservation of this peptide in similar organs between different crustacean species.

FMRFamide-Related Peptides (FaRPs)—FaRP family is another large and diverse group of peptides found in both invertebrates and vertebrates.²⁸ Several subfamilies have been identified in arthropods, including the sulfakinin, the myosuppressin and the short neuropeptide F.^{29–31} In this study, a large number of FaRPs were identified from PO tissue extract including 10 new peptides *de novo* sequenced (Table 1). Most of the *de novo* sequenced RFamides share similar C terminal motif as –NFLRFamide. As an example, APSKNFLRFamide (m/z 1078.6, Figure 3b) was fragmented on the ESI QTOF using CID fragmentation, and almost complete series of y ions were observed. Furthermore, the sequence was confirmed by dimethylation reaction with a mass shift of 56 Da upon derivatization, suggesting the presence of lysine in this peptide. In addition, several peptides possessing –NYLRFamide C termini were also sequenced, such as DRNYLRFamide (m/z 982.5) and YSDRNYLRFamide (m/z 1232.6). Compared to our previous study,¹⁷ two peptides, with sequences of SMPSLRLRFa and pQDLHDHVFLRFa, were not detected in the PO. This discrepancy could be due to differences in dissecting the PO tissues (as it is a highly heterogeneous structure) or false positive detection caused by sample contamination in the previous study. It is interesting to note that a molecular mass similar to the peptide pQDLHDHVFLRFa (1271.68 Da) was detected in the PO and *de novo* sequencing analysis revealed a novel amino acid sequence of GYPSRNYLRFamide (1271.66 Da). The former peptide along with its non-pGlu modified counterpart is widely distributed in the brain and STNS, whereas the latter form is uniquely present in the PO. Some of these FaRPs may be processing or degradation products originated from the same prohormones, such as RNFLRFa/DRNFLRFa, SKNFLRFa/APSKNFLRFa among some others. Further studies using analysis of neuronal release, electrophysiological test using standards, and molecular analysis of prohormones are required to definitively determine their biological origins and functional roles. Overall, the identification of this large array of closely

related FaRPs in conjunction with the well-characterized crustacean nervous system provides an excellent opportunity to further investigate the functional consequences of peptide diversity.

Crustacean Hyperglycemic Hormone—Crustacean hyperglycemic hormone (CHH) is a class of well studied peptide hormones that regulate the blood sugar levels and glycogen metabolism in crustacean, which have high impact on the control of reproduction and stress response.^{32, 33} CHH precursor-related peptides (CPRPs) are produced during the proteolytic processing of CHH preprohormones. In this study, we detected multiple CPRP truncated peptides, indicating the presence of CHH in this neural tissue. As shown in Table 1, multiple truncated CPRP peptides were observed, both from the N terminus (i.e. RSVEGASRMEKLL) and C terminus (i.e. PLGFLSQDHS) of CPRPs. In addition, we observed truncated CPRP peptide fragments from two different types of CHH prohormones encoded by the *H. americanus* genome. For example, peptides possessing RSVEGA- were from A-type CHH prohormone, whereas peptides with sequence motif of RSVEGV- were from B-type CHH prohormone, suggesting the presence of both types of CHH peptides in this tissue. However only one native CPRP was detected from the direct tissue/extract profiling of PO, and neither of the intact CHH peptides was detected. In our previous study, two CHHs were detected in the sinus gland.¹⁷ This is possibly due to their relatively low-level of expression in the PO compared to the commonly studied X-organ/sinus gland. In addition, the large size of the CHH peptides (>70 amino acids) generally makes them more difficult to be detected by MS. These truncated peptides are very likely to be present in the tissue *in vivo*, because these peaks were also observed by direct tissue analysis, in which freshly obtained tissue samples were rapidly dipped in acidified methanol to prevent protein degradation. The detection of these truncated peptides provides evidence for the presence of large CHH peptides; however whether they have any biological activities requires further investigation.

Direct Tissue Analysis of Different Regions in PO

Pericardial organ in the lobster has more complex structure as compared to other crustacean species.^{15, 34} Cell bodies in the ventral nerve cord project to the neurosecretory regions of the PO, which appear as bluish-white enlargement of the nerve stretching across the opening of the brachiocardiac veins. In our direct tissue PO profiling experiments, another type of tissue with more transparent appearance was observed in the same region, stretching along the surrounding muscle and projecting to the heart. As shown in Figure 5, very different neuropeptide profiles were observed from these two types of PO tissues. The bluish-white enlargement shows rich content of RFamides, proctolin, and multiple CPRP truncated peptides (Figure 5a). In contrast, as shown in Figure 5b, orckinins were observed as the most abundant neuropeptide family in the long-fiber along the muscle, and a low level of crustacean cardioactive peptide (CCAP) was also detected in this tissue. The differential direct tissue profiles from these two types of tissues demonstrate the complexity of the PO structure in the lobster and potentially different regulatory functions exerted by different regions of the POs.

Distribution of Neuropeptides throughout the Nervous System of *H. americanus*

To understand the function of neuropeptides, it is crucial to study their distribution in the nervous system because the localization of neuropeptides is often linked to their potential biological functions. Extensive immunocytochemical studies have been conducted to localize and map neuropeptide distributions in neuronal tissues from *H. americanus*.³⁵⁻³⁹ However, this approach suffers from the difficulty to distinguish multiple peptides with similar structures and relatively low throughput due to longer time required for antibody development and staining. Here we describe a mass spectrometry-based method to combine direct tissue/extract profiling and the use of bioinformatics software SpecPlot for a large scale mapping for neuropeptide distribution throughout the nervous system. Figure 6 shows the direct tissue/extract profiles of eight major regions in the lobster nervous system obtained on MALDI TOF/

TOF, including three regions from the central nervous system (CNS): brain, subesophageal ganglion (SEG), thoracic ganglion (TG) along the ventral nerve cord; three major organs in the STNS including commissural ganglion (CoG), stomatogastric ganglion (STG), esophageal ganglion (OG); and two major neuroendocrine organs, the PO surrounding the heart and the sinus gland (SG) in the eyestalk. Two neuroendocrine organs PO and SG exhibited very different neuropeptide compositions compared to the other regions of nervous system. We performed more than 10 replicates of direct tissue/extract experiments on each region, and the data was combined to generate a composite neuropeptide list and was imported into the SpecPlot software (Figure 6i). The software compares these peptide lists against the calculated peptide mass list using a pre-determined mass tolerance of 30 ppm. The presence of a specific neuropeptide is indicated with a blue square and the absence is shown as a black square for each region of interest. Overall 51 neuropeptides were examined for their presence in each type of tissue (Table S1). As a result, we find that some neuropeptides are distributed extensively in different regions of nervous system, while some are only localized in certain regions.

MALDI Mass Spectrometric Imaging of Neuropeptides in the *H. americanus* Brain

The functional roles that various compounds play in the complex tissue or organism are highly related to their locations. The brain of the lobster is a complex structure consisting of numerous neuropils and neuronal clusters (Figure 7a). Direct tissue analysis is usually compatible with small tissues; however, to obtain detailed and accurate description of neuropeptide distribution in a heterogeneous structure as complex as brain, another method with higher spatial resolution is required. Here we demonstrate the first use of a MALDI LTQ Orbitrap instrument for mapping of several neuropeptide families in the lobster brain. As shown in Figure 7, three major neuropeptide families, including CabTRPs, SIFamides and Orcokinins are mapped. The CabTRP 1a (m/z 934.5) and Val-SIFamide (m/z 1423.8) are localized in olfactory lobe (OL) and accessory lobe (AL). This observation is in agreement with previous immunocytochemical report of SIFamide in the crayfish brain that strong positive staining was seen in the olfactory lobe cells and fiber plexuses in the olfactory and accessory lobe neurophils.⁴⁰ OL and AL are two major neuropils in the olfactory system of lobster. Crustaceans detect odors through the dendrites of bipolar olfactory receptor neurons on their antennae, from which axons project to olfactory lobes within the midbrain. The accessory lobes receive secondary signals from olfactory lobes, and both of them can send output signals via projection neurons. This system plays important roles in chemosensory reception. However the presence of neuropeptides and their distribution patterns are not well documented. Our MALDI MS imaging experiments revealed that both CabTRP 1a and Val-SIFamide were localized in the olfactory system with relatively high signal intensities, suggesting their potential functional roles in olfactory regulation. Modest level of orcokinin NFDEIDRSGFGFN (m/z 1517.7) was also observed in the OL and AL, however it was more concentrated in the area of antenna II neuropil (AnN) and lateral antennular neuropil (LAN), which showed similar pattern as that reported previously in brain sections of crab *C. borealis*.⁴¹ The detection of orcokinin in this region suggested possible functional link to tactile sensory.

Lobster brain sections were also imaged on a MALDI TOF/TOF mass spectrometer (Figures 7 b and c), which showed similar distribution pattern for both VYRKPPFNGSIFamide (m/z 1423.8) and NFDEIDRSGFGFN (m/z 1517.7), as compared to the images acquired on the MALDI LTQ Orbitrap instrument (Figures 7d and f). For higher intensity peaks, such as VYRKPPFNGSIFamide (m/z 1423.8), a clear image of the candidate peptide can be obtained. However, for NFDEIDRSGFGFN (m/z 1517.7) greater interference from noise peaks was seen in the TOF/TOF imaging experiments. The signals from embedding material gelatin outside the edge of the tissue can also interfere with generating the ion images using a mass analyzer with lower resolution and mass accuracy. Although a faster imaging tool due to shorter acquisition times inherent of TOF mass analyzers, MALDI TOF/TOF produced mass spectra

with lower resolution (resolution ~ 10,000 to 15,000) and higher background of noise as compared to the MALDI LTQ Orbitrap system (resolution ~ 60,000 to 100,000). Because of the highly resolved spectra generated by the Orbitrap analyzer, single isotopes can be extracted to create images. Those in Figure 7 were extracted at ± 0.0025 amu. The selected reaction monitoring (SRM) MS/MS experiment in the LTQ ion trap monitors a specific reaction pathway by following the generation of a product ion from a specific parent ion, as shown in Figure 7i. This experimental scheme is useful to reduce interfering ions from the spectrum and therefore can be used successfully with complex tissue samples.

Discussion

Comparison of Multiple Mass Spectrometers for Neuropeptide Analysis

Characterization of neuropeptides is inherently challenging due to the extreme chemical complexity of neuronal tissues. Mass accuracy, mass spectral resolution, detection sensitivity and dynamic range of the instruments are all essential parameters for acquiring high quality mass spectra. In this study, four different types of mass spectrometers were employed, including nanoLC ESI QTOF, MALDI TOF/TOF, MALDI FTICR, and MALDI LTQ Orbitrap mass spectrometers. The MALDI LTQ Orbitrap mass spectrometer combines the benefits of high tolerance for complex samples and little sample consumption provided by the MALDI ion source, with the high mass accuracy and resolution of the Orbitrap mass analyzer, offering a powerful analytical tool for neuropeptide study. As for *de novo* sequencing, higher mass accuracy and mass spectral resolution provided by HCD on LTQ Orbitrap makes it easier to interpret the data and assign the fragment peaks with greater confidence. Furthermore, the accurate mass measurements offered by an orbitrap instrument can also help distinguish lysine (K) from glutamine (Q) in the sequence. On the other hand, high energy CID on MALDI TOF/TOF provides MS/MS spectra with relatively noisier background. However more complete fragmentation was observed. In addition, the abundant low mass ions detected by TOF/TOF analyzer are particularly valuable for *de novo* sequencing. For example, in Figure 2d, the formation of immonium ion m/z 136.1 confirms the presence of tyrosine in the sequence and the detection of immonium ion m/z 110.1 suggests the presence of histidine in the peptide sequence. Furthermore, the w ion formed in the high energy CID on the TOF/TOF mass spectrometer facilitates differentiation between leucine (L) and isoleucine (I) in this peptide.

MALDI MS instruments are not typically considered as the best tool for *de novo* sequencing because primarily singly charged ions are produced by MALDI MS, yielding limited fragmentation efficiency. However in our study, we found that the sequence-specific fragmentation obtained by MALDI tandem MS provides valuable complementary information to the LC ESI MS/MS data, thus providing more complete information for enhanced *de novo* sequencing of peptides from this model organism.

Region-Specific Localization of Neuropeptides in the Nervous System of *H. americanus*

Some neuropeptides are detected with very broad distribution in the nervous system. Orcokinin is one of the largest families that are detected in all of the eight regions examined in the nervous system of the lobster, including CNS, STNS and two neuroendocrine organs. Electrophysiological studies have shown their modulation activity on the STNS of *H. americanus*.¹⁸ Nonetheless, very little is known about the physiological functions of various orckinin isoforms. In this study, abundant orckinins are seen in every region examined, especially in the SG, where multiple orckinins are detected with high intensity (Figure 6h), suggesting multiple functions of this peptide family. FMRFamide related peptides (FaRPs) with C terminal motif of NFLRFamide and YLRFamide are detected with high intensity in the lobster PO (Figure 6g), suggesting their hormonal roles. Some of the smaller FaRPs, such as NFLRFa (m/z 695.4), are not detected in the CNS and STNS, which may be due to their

relatively lower abundance. The three -RLRFamide containing peptides are only detected in the CNS and the STNS, including GPPSLRLRFamide (m/z 1041.6), SMPSLRLRFamide (m/z 1105.6), and DTSTPALRLRFamide (m/z 1275.7). These peptides exhibit -RXRFamide C-termini (where X represents a variable residue), which places them into the short neuropeptide F (sNPF) subfamily. sNPF peptide has been found in both insects and crustaceans, and it is believed that these peptides can regulate multiple behaviors, such as feeding, reproduction among several other functions.⁴² The distinct locations of these sNPF peptides in the lobster as compared to other FaRPs suggest possible different functional roles of this subfamily, despite the common C-terminal motif of RFamide in the peptide sequences.

Allatostatins were first discovered in insects and named based on their function of inhibiting juvenile hormone synthesis.²⁶ The three different families of allatostatins have been reported to have similar effects on STNS motor pattern generations.²⁷ The A-type allatostatins are widely distributed throughout the CNS and STNS as well as neurohemal structures,^{35, 43} suggesting they have both neuronal and hormonal functions. In order to further investigate co-localization patterns and potential different functions of recently reported B- and C- type ASTs, it would be useful to map the distribution of these peptides throughout the nervous system and neurohemal organs. In this study, both of these neuropeptide families were observed to be broadly distributed in the lobster CNS and STNS, whereas they were found to be absent from the SG. In addition, among the two C-type AST peptides, only one C-type AST peptide pQIRYHQCYFNPISCF (m/z 1899. 9) and its non-pGlu isoform (m/z 1916.9) were observed in the PO, whereas the other C-AST peptide SYWKQCAFNAVSCFamide (m/z 1650.8) was observed in the CNS and the STG and CoG, suggesting possible different roles play by these two C-type ASTs. The B-type AST peptide TNWNKFQGSWa (m/z 1266.6) was detected in all analyzed regions except the SG. Overall, similar distribution patterns were seen for three different families of ASTs.

Some neuropeptides are localized in specific regions, for example both red pigment concentrating hormone (RPCH, m/z 930.5) and one of the pigment dispersing hormones (PDHs, m/z 1927.0) were only detected in the SG and the other PDH (m/z 1973.0) was also seen in the brain. Both of these peptide families are hormones that regulate pigment movements in integumental chromatophores and in the eyes of crustacean, which mediate the dispersion of mainly pigment granules for light/dark adaptation.^{44, 45} Previous immunocytochemical studies showed anti-PDH immunoreactivities were observed in eyestalk tissue/sinus gland and the neuropils in brain of lobster,⁴⁶ which was in agreement with the result in this study. The results in this study demonstrate that both of these families are present in the SG, which may be correlated to their physiological functions of pigment regulation and the circadian rhythm generation. Crustacean cardioactive peptide (CCAP) was only seen in the PO, consistent with the previously published immunocytochemical data.⁴⁷ In our previous study¹⁷, CCAP (956.38 Da) was reported to be present in the brain; however in this study we found that this peak was in fact the sodium adduct of CabTRP 1a (956.48 Da), whose mass was about 100 ppm off the theoretical mass of CCAP based on internal calibration. CPRP and CHH were primarily seen in endocrine organs, suggesting that they function as hormones rather than neurotransmitters. Furthermore, proctolin was detected in the PO with relatively high signal intensity. It was also observed in the direct tissue analysis of STG with lower signal intensity. This result is in agreement with previous immunocytochemical studies of proctolin in lobster that this peptide was observed in the PO with much higher signal intensity compared to other tissues.⁴⁸

Some neuropeptides are only present in the CNS and STNS, such as *Cancer borealis* tachykinin related peptides (CabTRPs). As shown in Figure 6i, three different CabTRPs were detected, including CabTRP Ia APSGFLGMRamide (m/z 934.5), which was observed as the highest intensity peak in majority of the tissues surveyed; the methionine oxidized form of this peptide was also seen (m/z 950.5); CabTRP II TPSGFLGMRamide (m/z 964.5) was detected to be

colocalized with APSGFLGMRamide. However none of these peptides were detected in SG and PO. Previous study shows CabTRPs present in the hemolymph of *Cancer borealis*⁴⁹, and it is very likely these circulating CabTRPs were secreted from midgut epithelial cells instead of PO and SG.^{50, 51} Val-SIFamide is another neuropeptide that is only present in the CNS and STNS. The Val-SIFamide was detected with high signal intensity in several neuronal tissues, especially brain and STG. This result is in agreement with the electrophysiological study that SIFamide can modulate the pyloric motor patterns generated by isolated lobster STNS.¹⁶

The tissue distribution patterns of most peptide families revealed by our MS-based study show good correlations with those revealed by immunocytochemical studies reported in the literature (shown in Supplemental Information, Table S1). However several discrepancies are noted. For example, RCH has been reported to be present in the brain and STNS by immunocytochemistry,⁵² but it was only detected in the SG in our study. This is possibly due to its low abundance and relatively low ionization efficiency for MS detection.⁴⁵ On the other hand, this study enables a large amount of information to be extracted from neuronal tissues as peptide identification and their localization can be achieved simultaneously in high throughput manner using the described MS based technology, which lays the foundation for future physiological studies toward understanding their biological functions.

Although our MS-based peptidomic mapping and characterization provide more comprehensive characterization of neuropeptides in *H. americanus* including many novel peptides reported for the first time, several differences from a previous study is noted.¹⁷ For example, CHH was reported to be present in the STG, CabTRP 1a was seen in the SG from a previous study, but these peptides are not observed in these respective tissues in the current analysis. The absence of detection of these peptides here could be a combination of the low-level of expression of a given peptide, limited MS detection sensitivity due to different amount of starting materials, and the population- and/or individual-specific variants.

MALDI Mass Spectrometric Imaging of Neuropeptides in the *H. americanus* Brain

The high resolving power, the sub-ppm mass accuracy and the wide dynamic range of the MALDI LTQ Orbitrap platform make it a superior alternative for MALDI-based tissue imaging. The high mass accuracy and high mass spectral resolution of the MALDI LTQ Orbitrap instrument allows unambiguous assignment of ion signals for each peptide, and the wide dynamic range and reduction of MALDI matrix adducts provide improved signal-to-noise for peptide detection.²¹ MALDI FTICR MS has recently been reported for high-mass-accuracy and high-mass-resolution MALDI imaging; however the data acquisition speed on the MALDI FTICR instrument is on the order of 15 sec/pixel, as shown in preliminary experiments.⁵³ The MALDI Orbitrap data shown in Figure 7 was collected at rate of ~6.6 sec/pixel, however depending on the concentration level of analytes present in the tissue, MALDI Orbitrap spectrometer can also operate at even higher speed (i.e. 1.2-1.8 sec/pixel in a recent study).⁵⁴ In addition, the imaging data size of the same tissue section obtained on FTICR MS is usually 100 times larger compared to those from the LTQ Orbitrap spectrometer, which could cause difficulties for data analysis and storage.

Compared to traditional immunocytochemistry, MALDI-based tissue imaging provides lower resolution (~75-100 μ m) due to instrument limitations, such as laser beam size and certain degree of analyte diffusion during matrix application and sample preparation. However, MALDI MS imaging is capable of distinguishing isoforms in the same peptide family and also it can be performed in a much higher throughput manner to enable a large scale mapping and documentation of neuropeptide localization. Furthermore, using the overlay function of the imaging software, it is convenient to use MALDI imaging to study the colocalization pattern of numerous neuropeptides simultaneously.

Conclusion

In this study, multiple MS instruments were employed and compared for the characterization of neuropeptides in a major neuroendocrine structure from the lobster *H. americanus*. In total, 57 neuropeptides were detected, including 18 novel ones that are *de novo* sequenced. Direct tissue analysis was combined with bioinformatics software SepcPlot to study the region-specific expression and distribution of neuropeptides throughout the nervous system of the lobster. Furthermore, a MALDI mass spectrometric imaging technique using the MALDI LTQ Orbitrap platform, with high mass accuracy and high mass spectral resolution, was employed to map the distribution of multiple neuropeptide families in the lobster brain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Analysis of an HPLC fraction from the lobster PO tissue extract on three different MALDI instruments, including (a) MALDI TOF/TOF, (b) MALDI LTQ Orbitrap and (c) MALDI FTICR MS. (d) Venn diagram showing the number of peaks detected using each type of instrument. Each spot was analyzed three times on each instrument.

Figure 2.

Comparison of multiple fragmentation reactions on the MALDI TOF/TOF and MALDI LTQ Orbitrap mass spectrometers for *de novo* sequencing of an unknown peptide peak at m/z 1084.6. (a) Collision induced dissociation (CID) on the LTQ Orbitrap instrument, (b) pulsed-Q dissociation (PQD) on the linear ion trap of the LTQ Orbitrap instrument, (c) high energy collisional dissociation (HCD) on the LTQ Orbitrap instrument, and (d) Collision induced dissociation on the TOF/TOF mass spectrometer. The predicted sequence is HI/LASLYKRP, and the presence of b and y ions is indicated by horizontal lines above (y ions) or below (b ions) the corresponding amino acid residues in the peptide sequence.

Figure 3.

Collision-induced dissociation spectra of *de novo* sequenced neuropeptides from the pericardial organ of the lobster on nanoLC ESI QTOF instrument. (a) A-type allatostatin: PRNYAFGLamide; (b) FMRamide-related peptide (FaRP): APSKNFLRFamide. Both precursor ions are doubly charged. The sequence-specific b- and y-types fragment ions and immonium ions are labeled. The presence of lysine in peptide (b) was confirmed by dimethylation of peptide via formaldehyde labeling of the crude sample extract.

Figure 4.

MS/MS spectra of peptide APSKNFLRFamide acquired on two MALDI tandem MS instruments. (a) High energy collisional induced dissociation on the LTQ Orbitrap instrument, and (b) Collision induced dissociation on the TOF/TOF mass spectrometer. The presence of b and y ions is indicated by horizontal lines above (y ions) or below (b ions) the corresponding amino acid residues in the peptide sequence in each spectrum. The presence of lysine in the sequence was confirmed by formaldehyde labeling (data not shown).

Figure 5.

Direct tissue profiling of two areas of the lobster pericardial organ using MALDI TOF/TOF: (a) white enlargement; (b) long fiber that projects along the muscle and into the heart. The sequences of detected peptide peaks are indicated in the spectra, and peptides from different families are distinguished by different colors.

Figure 6. Mapping the distribution of neuropeptides throughout the nervous system in *H. americanus* by a combination of direct tissue analysis performed on MALDI TOF/TOF and bioinformatics. Direct tissue/extract analysis was performed on eight neuronal organs: (a) brain, (b) commissural ganglion (CoG), (c) thoracic ganglion (TG), (d) subesophageal ganglion (SEG), (e) stomatogastric ganglion (STG), (f) esophageal ganglion (OG); and two major endocrine organs (g) pericardial organ (PO), and (h) sinus gland (SG). (i) The presence of multiple neuropeptide families and isoforms were compared and organized using in-house developed bioinformatics software SpecPlot. The molecular mass of each neuropeptide was listed on the top row organized in the order of families, and the first column indicates the tissue source for each row. Blue squares indicate the presence of certain neuropeptides in the specific organs, and black squares indicate absence of peptide signals.

Figure 7. MALDI imaging of neuropeptide localization in the *H. americanus* brain using both MALDI TOF/TOF and MALDI LTQ Orbitrap mass spectrometers. (a) Schematic drawing of the lobster brain, which contains multiple neuropils, including anterior (AMPN) and posterior medial protocerebral neuropils (PMPN), olfactory lobe (ON), accessory lobe (AL), antenna I neuropil (AnN) and lateral II antenna neuropil (LAN). Ion images of (b) VYRKPPFNGSIFamide (m/z 1423.8) and (c) NFDEIDRSGFGFN (m/z 1517.7) were obtained using MALDI TOF/TOF. Ion images of multiple known neuropeptides were acquired by MALDI LTQ Orbitrap, including: (d) VYRKPPFNGSIFamide (m/z 1423.8); (e) APSGFLGMRamide (m/z 934.5); (f) NFDEIDRSGFGFN (m/z 1517.7); and (g) overlay of the above three neuropeptides. A novel peptide HI/LASLYKPR (m/z 1084.6) in the lobster brain was also mapped using MALDI LTQ Orbitrap instrument by (h) precursor ion scanning of m/z 1084.6 and (i) selected reaction monitoring of transition between m/z 1084.6 and sequence-specific fragment ion m/z 685.4 (b_6).

Table 1
Neuropeptides Detected in the Lobster *H. americanus* Pericardial Organs

Neuropeptide families	[M+H] ⁺	Sequence	TOF/TOF	QTOF	FTICR MS	LTQ Orbitrap
<i>Proctolin</i>	649.37	RYLPT	x		x	
<i>AST-A</i>	808.45	VPRYAFGa		x		
	824.43	ASPYAFGLa			x	x
	853.47	RQYAFGLa	x	x	x	x
	923.47	pERAYSFGLa	x		x	x
<i>CCAP</i>	936.51	PRNYAFGLa	x	x	x	x
	937.49	PRDYAFGLa	x	x	x	x
	956.37	PECNAFTGCa	x	x	x	x
	695.40	NFLRFa	x		x	
<i>FaRP_s</i>	851.50	RNFLRFa	x	x	x	x
	910.53	SKNFLRFa	x	x		x
	965.54	GGRNFLRFa	x	x	x	x
	966.53	DRNFLRFa		x		x
	982.52	DRNYLRFa		x		
	995.55	SGRNFLRFa	x	x	x	x
	1005.57	GPRNFLRFa	x	x	x	x
	1007.58	SPKNFLRFa	x	x		
	1022.56	GNRNFLRFa	x	x	x	x
	1023.55	GDRNFLRFa	x	x	x	x
	1069.55	SDRNYLRFa		x		x
	1076.61	QPRNFLRFa		x		x
	1078.62	AFPSKNFLRFa	x	x	x	x
	1103.59	HDRNFLRFa	x	x	x	x
	1104.61	GAHKNYLRFa	x		x	x
	1190.64	pQLDRNFLRFa	x	x	x	x
	1208.63	DQNRNFLRFa	x		x	x
	1232.62	YSDRNYLRFa		x		
	1271.66	GYPSRNYLRFa	x	x	x	x
	1289.64	GYSDRNYLRFa	x	x	x	x

Neuropeptide families	[M+H] ⁺	Sequence	TOF/TOF	QTOF	FTICR MS	LTQ Orbitrap
AST-B	1337.67	FSHDRNFLRFa	x	x	x	x
	1293.63	STNWSSLRSAW ^a	x			x
	1266.60	TNWNKFQGSW ^a		x	x	
Orcokinin related peptide	1186.52	FD ^a FTTGF ^b GH ^c S			x	x
	1213.53	FD ^a FTTGF ^b GH ^c N	x		x	x
	1213.52	SS ^a EDMDRLGFG				x
	1474.63	SS ^a EDMDRLGFGFN	x	x		
	1490.62	SS ^a EDM(O)DRLGFGFN		x	x	x
	1280.66	VYGP ^a RIANLY	x	x	x	x
Corazonin	1198.55	NF ^a DEIDRS ^b GF ^c a	x			x
	1256.55	NF ^a DEIDRS ^b GFG	x		x	x
	1403.62	NF ^a DEIDRS ^b GFGF	x	x	x	x
	1502.69	NF ^a DEIDRS ^b GFGFV	x	x	x	x
	1517.67	NF ^a DEIDRS ^b GFGFN	x	x	x	x
	1540.68	NF ^a DEIDRS ^b GFGFH	x	x	x	x
	1369.63	pQTFQY ^a SRGW ^b TNa		x		
	1899.85	pQIRYHQC ^a YFNPIS ^b CF	x			x
	1100.54	PLGFLSQDHS		x		x
	1149.57	RSVEGV ^a SRME	x		x	x
CPRP	1199.61	PLGFLSQDHSV		x	x	
	1475.80	RSVEGASRMEKLL		x		
	1503.83	RSVEGVSRMEKLL		x		
	1562.89	RSVEGASRMEKLLS	x	x	x	x
	1576.85	RSVEGASRMEKLLT	x			
	1590.86	RSVEGVSRMEKLLS		x		x
	1736.90	RSVEGASRMEKLLSSS		x		
	1850.94	RSVEGASRMEKLLSSSN		x	x	
	3604.766	CPRP I	x		x	

AST, allatostatin; CCAP, crustacean cardioactive peptide; CPRP, crustacean hyperglycemic hormone precursor related peptide; FaRP, FMRFamide related peptide. Previously reported peptides are shown in normal font; novel peptides are shown in bold. Disulfide-bonded cysteine residues are indicated with underline.