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Expanding the Crustacean Neuropeptidome using a Multi-Faceted Mass Spectrometric Approach

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Abstract

Jonah crab *Cancer borealis* is an excellent model organism long served for many areas of physiology, including the study of endocrinology and neurobiology. Characterizing the neuropeptides present in its nervous system provides the first critical step toward understanding the physiological roles of these complex molecules. Multiple mass spectral techniques were used to comprehensively characterize the neuropeptidome in *C. borealis*, including matrix assisted laser desorption/ionization Fourier transform mass spectrometry (MALDI FTMS), MALDI time of flight (TOF)/TOF MS and nanoflow liquid chromatography coupled to electrospray ionization quadrupole time of flight tandem mass spectrometry (nanoLC ESI Q TOF MS/MS). In order to enhance the detection signals and expand the dynamic range, direct tissue analysis, tissue extraction, capillary electrophoresis (CE) and off-line HPLC separation have also been employed. In total, 142 peptides were identified, including 85 previously known *C. borealis* peptides, 22 peptides characterized previously from other decapods, but new to this species, and 35 new peptides *de novo* sequenced for the first time in this study. Seventeen neuropeptide families were revealed including RFamide, allatostatin (A and B type), RYamide, orcokinin, orcomyotropin, proctolin, crustacean cardioactive peptide (CCAP), crustacean hyperglycemic hormone precursor-related peptide (CPRP), crustacean hyperglycemic hormone (CHH), corazonin, pigment-dispersing hormone (PDH), tachykinin, pyrokinin, SIFamide, red pigment concentrating hormone (RPCH) and HISGLYRamide. Collectively, our results greatly increase the number and expand the coverage of known *C. borealis* neuropeptides, and thus provide a stronger framework for future studies on the physiological roles played by these molecules in this important model organism.

Keywords

Cancer borealis; matrix assisted laser desorption/ionization Fourier transform mass spectrometry (MALDI FTMS); electrospray ionization quadrupole time of flight mass spectrometry (ESI-Q-TOF MS); neuropeptides; peptide sequencing; peptidomics; thoracic ganglia; stomatogastric ganglia; commissural ganglia; sinus gland; pericardial organ

Introduction

As the most diverse and complex group of signaling molecules, neuropeptides are present throughout the central nervous system (CNS) as well as in peripheral organs, which induce and regulate many important physiological processes.[1-3] Comprehensive profiling and characterization of the neuropeptides represents an important first step towards a better understanding of the structure and function relationship of these complex signaling molecules.

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However, such neuropeptidomic characterization is often challenging due to the large diversity of endogenous neuropeptides, wide dynamic range and difficulty of inferring its final products from neuropeptide genes or a complete lack of sequenced genomes for many organisms. Techniques such as Edman degradation and immunocytochemistry have been used for the analysis of neuropeptides in the nervous system.[4-6] However, limitations exist in these traditional techniques because of the requirement of extensive purifications from a large amount of starting materials and/or the need for specific antibodies and difficulties to simultaneously investigate multiple structurally related peptides. Recent advances in mass spectrometry have made it possible to identify and discover the neuropeptides in nervous systems, even in species without genomic sequence information.[7-11]

Jonah crab *Cancer borealis* is a model organism long served for many areas of physiology,[12, 13] including the study of endocrinology and neurobiology.[14-17] Over the past several decades, *C. borealis* stomatogastric nervous system (STNS) has become a premier system for the study of generation, maintenance and modulation of rhythmic behavior at the cellular and network levels.[17-22] Numerous studies have demonstrated that the functional output of these well defined neural circuits is extensively modulated by various neuropeptides.[2, 23, 24] Therefore, it is important and highly desirable to obtain a complete profile of the neuropeptides in this species.

Numerous studies have reported on the identification of specific neuropeptide families such as tachykinin, orcokinin and allatostatins in *C. borealis*.[23, 25, 26] In addition, several peptidomic analyses of neuroendocrine organs such as pericardial organ (PO) or sinus gland (SG) have been published.[9, 10, 25, 27] A neuropeptidomic study of *C. borealis* brain and thoracic ganglia was also reported.[28] Expanding upon these previous studies, here we describe a more comprehensive neuropeptidomic study in the *C. borealis* nervous system including brain, thoracic ganglia, PO, SG, commissural ganglia (CoG) and stomatogastric ganglion (STG) with the latter two located within the STNS. In order to obtain a more complete characterization of the full complement of neuropeptides expressed in *C. borealis* nervous system, different sample preparation methods and multiple complementary mass spectral techniques have been employed in this study (Figure 1). To begin determining the complement of peptides present in *C. borealis* nervous system, direct tissue analysis of fragments from various nervous tissues was performed using high resolution high mass accuracy matrix assisted laser desorption/ionization (MALDI) Fourier transform mass spectrometry (FTMS) analysis. Tissues were further extracted with acidified methanol and analyzed with nanoflow liquid chromatography electrospray ionization quadrupole time-of-flight (nano-LC-ESI-Q-TOF) for *de novo* sequencing. Various chemical derivatization techniques such as formaldehyde labeling and methyl esterification were employed to improve peptide fragmentation and resolve ambiguities in sequence assignments. Furthermore, to enhance the dynamic range and improve peptidome coverage, pooled tissue extracts were fractionated by reverse-phase HPLC followed by MALDI-TOF/TOF, MALDI-FTMS and nano-LC ESI QTOF MS/MS analyses. For several tissue extracts with lower concentrations of neuropeptides such as CoG and STG extracts or HPLC fractions, MALDI sample plate with parafilm coating was used for FTMS detection to enhance sensitivity. Capillary electrophoresis (CE) separation was also employed for micro-scale separation followed by MALDI-FTMS detections. Overall, the combined use of multiple complementary methodologies enabled the identification of most of the previously known peptides as well as the discovery of numerous novel peptides, including new members from the well-characterized peptide families, in this important model organism.

Materials and methods

Materials

Methanol, acetonitrile, formic acid and glacial acetic acid were purchased from Fisher Scientific (Pittsburgh, PA), $NaBH_3CN$, ammonia, acetic chloride and formaldehyde-H_2 (FH_2, 37% in H_2O) were purchased from Sigma-Aldrich (St. Louis, MO). 2, 5-dihydroxybenzoic acid (DHB) was obtained from ICN Biomedicals Inc.

Animal and Tissue Collection

Jonah crabs, *C. borealis*, were shipped from Marine Biological Laboratories (Woods Hole, MA) and maintained without food in an artificial seawater tank at 10-12°C. Animals were cold-anesthetized by packing in ice for 15-30 min prior to dissection. They were dissected by removing the stomach section, eyestalks, thoracic ganglia, and pericardial ridges located on either side of the heart. The pericardial organs (POs) were removed from the pericardial ridges and the sinus glands (SGs) were removed from the eyestalks. The brain, commissural ganglion (CoG) and the stomatogastric ganglion (STG) were removed from the stomach portion of the crab. All dissections were carried out in chilled physiological saline (composition in mM: NaCl, 440; KCl, 11; $MgCl_2$, 26; $CaCl_2$, 13; Trizma base, 11; maleic acid, 5; pH 7.45).

Tissue Extraction, Off-line HPLC Fractionation and CE Separation

Tissues were separately pooled, homogenized, and extracted with acidified methanol: 90% methanol (Fisher Scientific, Pittsburgh, PA), 9% glacial acetic acid (Fisher Scientific), and 1% deionized water. Extracts were dried in a speedvac concentrator (Thermo Electron) and re-suspended with minimum amount of 0.1% formic acid. The re-suspended extracts were then vortexed and briefly centrifuged. The resulting supernatants were subsequently fractionated via high performance liquid chromatography (HPLC).

HPLC separations were performed using a Rainin Dynamax HPLC system equipped with a Dynamax UV-D II absorbance detector (Rainin Instrument Inc., Woburn, MA). The mobile phases included: (Solution A) deionized water containing 0.1% formic acid, and (Solution B) acetonitrile (HPLC grade, Fisher Scientific) containing 0.1% formic acid. About 20-50 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 separations consisted of a 120 minute gradient of 5%-95% Solution B. Fractions were automatically collected every two minute using a Rainin Dynamax FC-4 fraction collector.

Off-line CE separation was performed on a home-built CE apparatus equipped with a capillary of 75 cm in length (50 μm i.d. × 360 μm o.d.) as described elsewhere.[29] The CE runs under -18kV using ammonium formate buffer (50 mM, 10% ACN, pH 3.5) at room temperature, 25 °C. The CE fractions were deposited, in every 30 s (or 60 s), onto the tiny matrix spots pre-deposited onto the hydrophobic Parafilm as described previously.[30]

Reductive Methylation, Acetylation and Methyl Esterification of Tissue Extracts or HPLC Fractions

For some experiments, peptides in extracts or HPLC fractions were derivatized with formaldehyde prior to mass spectral analysis. Ten microliters of crude extract was mixed with 10 μL of acetate buffer (1 M, pH 4.8), followed by the addition and mixing of 5 μL of formaldehyde (37% in H_2O vol/vol, Sigma Aldrich, St. Louis, MO), and subsequent addition of 2 μL of 2M $NaBH_3CN$ (Sigma Aldrich). The labeling reaction was allowed to take place for 1 hr at room temperature. Excess formaldehyde was quenched via the addition of 4 μL of ammonia (37% in H_2O vol/vol, Sigma-Aldrich). The resulting solution was stored at -20°C before LC MS/MS analysis.

For acetylation reaction, 0.3 μL of tissue extract or HPLC fraction was spotted on the MALDI plate, followed by the addition of 0.3 μL of 3:1 methanol-acetic anhydride. The solution was left at room temperature for 3 minutes, and then mixed with 0.3 μL of 50mM ammonia bicarbonate solution.

Esterification was performed at room temperature or 37 °C for 2 h by adding methanolic HCl to the tube with pre-dried sample. The reaction solution was then concentrated to dryness in a Speedvac. Esterified peptides were resuspended in 10 μL of 0.1% formic acid in 30% methanol.

MALDI-FTMS and Direct Tissue Analyses

Matrix assisted laser desorption/ionization Fourier transform mass spectrometry (MALDI-FTMS) experiments were performed on a Varian/IonSpec ProMALDI Fourier transform mass spectrometer (Lake Forest, CA) equipped with a 7.0 Tesla actively-shielded superconducting magnet. The FTMS instrument contains a high pressure MALDI source 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. A 355 nm Nd: YAG laser (Laser Science, Inc., Franklin, MA) was used to create ions in an external source. 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.

Peptide fragmentation was accomplished by sustained off resonance irradiation-collision induced dissociation (SORI-CID). An arbitrary waveform from 2000 ms to 2131 ms with a ±10 Da isolation window was introduced to isolate the ion of interest. Ions were excited with SORI Burst excitation (2.648V, 2500-3000 ms). A pulse of nitrogen gas was introduced through a pulse valve from 2500 to 2750 ms to introduce collision activation.

Off-line analysis of HPLC fractions was performed by spotting 0.3 μl of HPLC fraction of interest on the MALDI sample plate and adding 0.3 μl of the saturated DHB. The resulting mixture was allowed to crystallize at room temperature. The MALDI-FTMS analysis was then performed as described above.

For direct tissue analysis, tissue fragments were desalted by briefly rinsing in a solution of DHB prepared in deionized water (10 mg/ml). The tissue was then placed onto the MALDI sample plate followed by adding 0.3 μl of saturated DHB matrix on top of it and crystallizing at room temperature.

To further increase the sensitivity of analysis for HPLC fractions and tissue extracts, a Parafilm-coated MALDI sample plate was used with predeposited nanoliter volume DHB matrix spots for LC fraction collection followed by MALDI MS analysis. Detailed procedures were described in previous publications.[29, 30] Briefly, a narrow piece of Parafilm M was cut to the size of 2.5 cm (l) × 0.4 cm (w), stretched to 8.0 cm and directly placed onto the MALDI sample target. About 50 nL DHB matrix (150 mg/mL in 50:50/ methanol: water) was deposited by capillary onto the film. Upon drying small spots at about 400 μm were formed due to the solvent repellent property of the film.

MALDI-TOF/TOF

A model 4800 MALDI-TOF/TOF analyzer (Applied Biosystems, Framingham, MA) equipped with a 200 Hz, 355 nm Nd:YAG laser was used for direct peptide profiling in brain sample and HPLC fraction screening. 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 1000 laser shots covering mass range *m/z* 500-4000.

MS/MS was achieved by 1 kV collision induced dissociation (CID) using air. A saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA) in 70% acetonitrile was used as matrix. For sample spotting, 0.5 μl of sample was spotted on MALDI plate first and allowed to dry followed by the addition of 0.5 μl matrix.

Capillary LC-ESI-QTOF-MS/MS

Nanoscale LC-ESI-Q-TOF MS/MS was performed using a Waters capillary LC system coupled to a Q-TOF Micro mass spectrometer (Waters Corp., Milford, MA). Chromatographic separations were performed on a C18 reverse phase capillary column (75 μm internal diameter ×150 mm length, 3 μm particle size; Micro-Tech Scientific Inc., Vista, CA). The mobile phases used were: deionized water with 5% acetonitrile and 0.1% formic acid (A); acetonitrile with 5% deionized water and 0.1% formic acid (B); deionized water with 0.1% formic acid (C). An aliquot of 6.0 μl of an HPLC fraction was injected and loaded onto the trap column (PepMap™ C_{18}; 300 μm column internal diameter × 1 mm, 5 μm particle size; LC Packings, Sunnyvale, CA, USA) using mobile phase C at a flow rate of 30 μl/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 μl/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 (N_2) 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.).

Figure Production

MALDI-FTMS figures were produced by converting the initial spectra obtained using IonSpec version 7.0 software (IonSpec Corp.) to a bitmap image using Boston University Data Analysis (BUDA) software (version 1.4; Boston University, Boston, MA). The BUDA files were then pasted into Fireworks MX 2004 (Macromedia, Inc., San Francisco, CA) and resampled to improve the resolution. All MS/MS figures were produced using a combination of Fireworks MX 2004 and Microsoft Windows Paint tool (Microsoft Corporation, Redmond, WA).

Results and Discussion

Enhancing Neuropeptidome Coverage in *C. borealis* using a Combination of Microscale Separation Methods and Complementary Mass Spectral Techniques

The combined mass spectrometric approach involving nanoLC-ESI-Q-TOF, MALDI-FTMS and MALDI-TOF/TOF is used for comprehensive characterization of the neuropeptide complements expressed in *C. borealis* nervous system. With high sensitivity of MALDI-TOF/TOF, high resolution and high mass measurement accuracy of MALDI-FTMS and the *de novo* sequencing capability of ESI-Q-TOF, 142 neuropeptides were identified from this species including 85 previously known *C. borealis* peptides, 24 peptides identified in other species, but new to *C. borealis*, and 35 novel peptides *de novo* sequenced for the first time in this study. Herein we sorted the identified peptides and their tissue distribution along with techniques used for their identification in Table 1. As shown in Table 1, seventeen neuropeptide families were revealed in our peptidomic analysis with some of these peptides being uniquely expressed in one type of tissue. For example, neuropeptide PFCNAFTGCamide (CCAP) was only seen in the pericardial organ (PO), whereas others are commonly present in several tissues.

Multiple sample preparation methods including direct tissue analysis, extraction, CE and HPLC fractionation were used in order to get the most complete neuropeptide profile in *C. borealis* nervous system. Tissue samples were analyzed using MALDI-FTMS direct tissue analysis method developed in our laboratory to generate a quick snapshot of the peptide profile for each tissue sample.[31] The *in situ* direct tissue analysis technique is especially useful for neuropeptide profiling in individual tissue samples with very small size such as SG, STG and CoG when combined with high mass measurement accuracy provided by MALDI-FTMS. Figure 2 shows a direct tissue analysis of STG, CoG and SG with MALDI-FTMS. With high mass measurement accuracy of MALDI-FTMS, numerous neuropeptides from several peptide families were identified based on accurate mass measurements. Compared to direct tissue analysis, the extraction can provide a more complete profile of the peptides present in the whole tissue due to the heterogeneity of the tissue. As a complementary method, tissues were homogenized and extracted with acidified methanol for the analyses. It often requires more samples for the extraction method compared to the direct tissue analysis; however, it is more frequently used due to the compatibility with a downstream separation and MS coupling detection, such as LC-ESI-Q-TOF MS/MS analysis for *de novo* sequencing, which is essential in the discovery of the novel peptides (Figure 3). For the extracts from very complex structures of the nervous systems such as brain and PO, further separation is especially necessary to reduce the salt and lipid interference. Off-line reverse-phase HPLC and CE separations prior to MS analysis reduce the chemical complexity and thus offer expanded dynamic range in the MS detection. For example, many more peptides were detected in the PO and brain after reverse-phase HPLC separation followed by subsequent nanoLC MS/MS analysis in this study. A previous neuropeptide analysis of crude PO extract resulted in identification of 54 neuropeptides.[9] Here we report 85 neuropeptides from the same tissue extract with off-line RPLC fractionation prior to nanoflow HPLC MS/MS sequencing analysis, highlighting the advantages of prefractionation of a complex tissue extract to improve peptidome coverage. However, for tissues with very small size such as STG and CoG, a large amount of the samples are required for HPLC separation, which is laborious and time consuming to perform microdissection and tissue collection. In this case, a sample preparation method using minimum amount of the tissue is preferred. Off-line CE coupled to MALDI-FTMS analysis offers an attractive alternative. We previously developed such a method to analyze the samples prepared into minute volume without further treatment.[29] In this study CE fractionation was applied to CoG extract prior to MS analysis, which provided preconcentration, desalting, and separation to decrease the ionization suppression of peptides. Furthermore, for the low concentration samples, the pretreated Parafilm coating on MALDI plate significantly enhanced the detection signals in MALDI-FTMS. Figure 4 shows a comparison of MALDI-FTMS detection of a CoG HPLC fraction with and without Parafilm pre-coating. Ion signals were greatly enhanced from the plate with Parafilm pre-coating, which enabled fragmentation analysis by SORI-CID for sequence confirmation.

It is noted that the neuropeptides detected by different mass spectral techniques were complementary. Compared to MALDI-FTMS and MALD-TOF/TOF methods, more neuropeptides were identified by the nano-LC-ESI-Q-TOF MS/MS method, which is partially due to the second dimensional LC separation offered by nano-LC-ESI MS/MS. This additional separation further reduced the sample complexity and the multiple-charging effect inherent of ESI ionization mode provided more efficient fragmentation. In addition, MALDI and ESI may have different ionization efficiencies for peptide ions, with MALDI producing singly charged ions while ESI generating singly or multiply charged ions. The multiple-charging effect of ESI often leads to higher quality fragmentation, which is useful for peptide sequencing. Therefore, the two ionization techniques are inherently complementary and it has been reflected in the peptides identified by these two methods. For example, AST-A type neuropeptide family is mostly detected by ESI-Q-TOF. The lack of detection of this peptide family in MALDI is possibly due to the low ionization efficiency of this family with the absence of basic residue

in their sequence, leading to potential ion suppression in the complex extracts and thus cannot be detected. Because nanoLC interfaced with ESI-Q-TOF offers second dimensional LC separation, this peptide family is further separated from other ions that enabled detection. On the other hand, MALDI-FTMS and MALDI TOF/TOF analyses enabled the identification of several neuropeptides that were otherwise missed detection using ESI-Q-TOF MS/MS analysis. For example, the corazonin was detected in PO and CoG by MALDI-MS but not detected by ESI-Q-TOF MS approach. This observation might be due to the sample loss especially for low-abundance neuropeptides during LC separation coupled to ESI-Q-TOF MS analysis.

In the previous peptidomic study of *C. borealis* brain and thoracic ganglia by Huybrechts et al.,[28] 28 peptides from 4 families were identified, among which most of the FaRPs and orcokinins were identified in this study. However, for the AST-A type peptides, none of the peptides overlap with our study. In the previous study, all of the A-type AST peptides were detected by MALDI-TOF and the identifications were made by mass matching to those from *Carcinus maenas* and *Penaeus monodon.* In our study, most of the AST-A type peptides were unambiguously *de novo* sequenced by ESI-Q-TOF tandem MS. It is noted that most of the AST-A type peptides detected were singly charged in ESI-Q-TOF MS analysis. However, we normally exclude singly charged ions for the MS/MS fragmentation in the Q-TOF runs. In order to improve the detection of this particular peptide family, gas phase fractionation approach was used. Basically, one of the run was set to selectively fragment the singly charged precursors while the other set to fragment the doubly, triply and quadruply charged ions. In this way, it is not only possible to detect the singly charged ions but also simplify the spectrum to improve the detection. Overall, in our study, we identified 68 peptides from 11 different peptide families in *C. borealis* brain and thoracic ganglia, doubling the peptides identified in the *C. borealis* CNS.

In a previous peptidomic study of *C. borealis* POs, 54 peptides were identified by analyzing the crude extract of POs with ESI-Q-TOF.[9] Here, in combination of multiple sample preparation and multi-faceted mass spectrometric approach, the list of peptides identified in POs has been greatly expanded with 96 peptides being identified, including 28 novel peptides. Most of the novel peptides were detected in HPLC fractions, highlighting the advantages of prefractionation of a complex tissue extract to improve peptidome coverage.

It is also noted that only one CHH isoform was identified in this study. CHH is a big family includes CHHs, moult-inhibiting hormones (MIHs), gonad inhibiting hormones (GIHs), vitellogenesis-inhibiting hormones (VIHs) and mandibular organ-inhibiting hormones (MOIHs). Furthermore, CHH may exist in several isoforms in one or more neurosecretory organs such as the sinus glands and the pericardial organs. However, due to the big size and multiple posttranslational modifications such as disulfide-bond linkages and N-terminal pyroglutamylation, it is extremely challenge to *de novo* sequence the CHH family. The different dynamic range and abundances of these peptides in various tissues could further complicate detection. Our group recently developed a novel hybrid strategy that combines “top-down” and “bottom-up” approaches for large neuropeptide *de novo* sequencing.[32] In the future study, we will combine the separation techniques developed in this study with the hybrid *de novo* sequencing strategy, to search and identify additional CHH isoforms and other peptides in the CHH superfamily.

Derivatization Reaction Facilitated *De Novo* Sequencing

Challenges exist in the *de novo* sequencing due to the possible incompleteness of MS/MS fragmentation and the ambiguity occurring in a complex MS/MS spectrum. Therefore, a variety of derivatization reactions have been developed to overcome such difficulties and ambiguities in mass spectrometric sequencing. Derivatization techniques label the N- or C-terminus of a

peptide and thus increase the information content of a peptide fragmentation spectrum. In this study, various derivatization reactions including methyl esterification, reductive methylation, and acetylation were utilized to facilitate the *de novo* sequencing and resolve sequence ambiguities. Methyl esterification converts carboxylic acids on the side chains of aspartic and glutamic acids as well as the carboxyl terminus to methyl esters with a 14-Da mass increment. It also reacts with the amide group of asparagine, glutamine and amidated C-terminus with a 15-Da mass increment.[33] Here, the methyl esterification was utilized to confirm the number of free carboxylic acid and the amide group in a peptide. Reductive methylation and acetylation are designed to label the N-terminus and the ε-amino groups of lysine residue. These labeling methods not only allow the differentiation between b- and y-type fragment ions but also can be used to confirm the number of lysine residues in a peptide. We also used these derivatization methods to differentiate isobaric amino acid residues K and Q in this study. Furthermore, the reductive methylation is reported to be useful in the *de novo* sequencing of singly charged neuropeptides such as A-type AST by enhancing the a1 ion and simplifying the MS/MS fragmentation pattern.[9] Figure 5 shows an example of using reductive methylation to facilitate *de novo* sequencing of an AST-A type peptide AGLYSYGLamide. As shown in Figure 5(A), MS/MS spectrum of native peptide is very complex due to extensive internal fragmentations. Furthermore, it is difficult to resolve the ambiguity of AG/GA/Q/K due to their similar molecular mass at the N-terminus. In contrast, after the reductive methylation, a and b ion series are enhanced while internal fragmentations are suppressed, which yielded a much cleaner MS/MS spectrum (Figure 5(B)). In addition, enhanced a1 ion helps resolve the ambiguities of the N-terminal residue, suggesting that the N-terminal sequence is AG. Figure 5(C) shows another example of reductive methylation facilitated *de novo* sequencing. Upon the reaction, no mass shift is observed for the peptide *m/z* 967.51^{1+}, which indicates the pyroglutamylation at the N-terminus. So the peptide sequence is unambiguously resolved as pQRDYSFGLamide.

Diversity of FMRFamide-Related Peptides and the Novel Isoforms Identified in *C. borealis*

The FMRFamide family is a large and diverse group of peptides found in both invertebrates and vertebrates.[34] Several subfamilies have been identified in arthropods, including the sulfakinins, the myosuppressins and the neuropeptide Fs.[35-38] In our study, 41 FMRFamide-related peptides were identified from the nervous system of *C. borealis* including 18 novel ones. Of particular interest is the novel peptide DRNFVLRFamide (Figure 3(D)). Different from the previously identified *C. borealis* FMRFamide-related peptides, which have the C-terminal sequence FLRFamide, YLRFamide or RLRFamide, this novel peptide has a new C-terminal ending sequence of VLRFamide. The peptides possessing C-terminal consensus sequence of VLRFamide have been previously identified from *C. elegans* and *Ascaris suum* as FMRFamide-related peptides.[39,40] However, this is the first report of the presence of VLRFamide-peptide in any crustaceans. QDLDHVFLRFamide and pQDLDHVFLRFamide are two *de novo* sequenced peptides with the C-terminal motif —HVFLRFamide, which places them into the myosuppressin subfamily. Six peptides, PSLRLRFamide, PSMRLRFamide, PSM(O)RLRFamide, SMPSLRLRFamide, SM(O)PSLRLRFamide and DVRTPALRLRFamide, exhibit —RXRFamide C-termini (where X represents a variable residue), which places them into the short neuropeptide F (NPF) subfamily. This subfamily has been proposed to be the invertebrate homolog of the vertebrate neuropeptide Ys. It is noted that all of the six sNPFs were identified in the central nervous system but not present in the POs. Recent MS imaging study[41] shows that SMPSLRLRFamide and DVRTPALRLRFamide have different distribution patterns in the brain compared to the RFamide-related peptides with C-terminal sequence —FLRFamide, which indicates their possible different physiological roles. Of the remaining peptides, 27 possess C-terminal sequence —FLRFamide and 5 with —YLRFamide. RFamide-related peptides show a tremendous diversity in N-terminal sequence, and as a possible consequence, a broad array of the biological functions such as cardioexcitatory function, modulation of muscle contraction and regulation of feeding

behavior.[42-44] Several RFamide physiological studies even demonstrated that different isoforms might have opposite biological effects.[45] A recent study on several RFamide-related peptides in *C. borealis* showed differential degradation of several isoforms in the presence of extracellular peptidases.[46] Overall, the identification of this large array of closely related FaRPs in conjunction with the well-characterized STNS provides an excellent opportunity to further investigate the functional consequence of peptide diversity.

Expanding the List of A-type AST Neuropeptides in *C. borealis*

A-type AST is a peptide family possessing —YXFGLamide C-termini motif (where X is a variable amino acid). Numerous allatostatins have been reported to be present in many crustaceans such as from *C. maenas*, *Penaeus mododon*, and *C. borealis*.[9, 47, 48] Physiological studies showed their role as inhibitors of the pyloric motor pattern and stomatogastric neurotransmission.[21, 49] In this study, 37 A-type AST including 11 novel ones were identified, constituting the second largest neuropeptide family found in *C. borealis*. Most of the A-type AST peptides were *de novo* sequenced by ESI-Q-TOF MS. Formaldehyde labeling is especially useful to resolve the sequence ambiguities for this peptide family. The majority of the A-type AST peptides are singly charged in ESI-Q-TOF and the MS/MS spectra of this peptide family often exhibit complex fragmentation pattern due to extensive internal fragmentations, which makes *de novo* sequencing difficult. Formaldehyde labeling increases the signals of a/b ion series and reduces the complexity of the MS/MS fragmentation pattern of the singly charged A-type AST and thus facilitates *de novo* sequencing. In addition, the enhanced a1 ion after labeling facilitates resolving the N-terminal sequence ambiguity. Immunostaining results show that A-type AST is a brain-gut peptide in insects,[50] which indicates that this peptide family might be related to the feeding/food intake. In the future, we will focus on mapping the isoform distribution of this peptide family using imaging techniques and investigate the functional roles of different isoforms in feeding.

AST-B Neuropeptides

The B type allatostatins (AST-B) are a family of peptides possessing the C-terminal motif-WX_6Wamide (where X_6 is six variable amino acids). It was first discovered in cockroaches as a juvenile hormone biosynthesis inhibitor.[51] Recently, we characterized several B-type allatostatins from the pericardial organs of the crabs *C. productus* and *C. borealis*.[8, 25] In *C. borealis*, the peptide VPNDWAHFRGSWamide was identified and was shown to exhibit inhibitory action on the pyloric motor pattern, which drives the rhythmic filtering of food between the foregut and midgut.[25] In this study, we identified 10 AST-B type peptides, including three novel B-type AST peptides. Physiological studies of two novel AST-B peptides, SGKWSNLRGAWamide and QWSSMRGAWamide, demonstrated that these two peptides exhibit similar inhibitory actions on the pyloric motor pattern as the peptide CbAST-B1, VPNDWAHFRGSWamide (Data not shown, personal communications with Dr. Eve Marder). It would be interesting to test and compare physiological actions of these peptide isoforms on the gastric mill rhythm and neuromuscular junctions.

Neuropeptide Tissue Distribution and the Comparison with Previous Immunocytochemical Data

Many immunohistochemical studies had been conducted on neuronal tissues from *C. borealis*.[2, 21, 52-54] Compared to the previous immunohistochemical data, our study shows a good correlation for most of the peptide families. For example, orcokinin, pigment dispersing hormone (PDH), red pigment concentrating hormone (RPCH), AST and proctolin immunoreactivities were seen in the SG, while in our study at least one isoform in each family was identified. Immunohistochemical data showed that orcokinin, proctolin, AST, CCAP, FaRP, RPCH and cholecystokinin (CCK) were immunoreactive in *C. borealis* POs. All of these

peptide families have been detected in this study except RPCH and CCK. This discrepancy is likely due to the poor ionization efficiency of the RPCH and CCK. For STG, CCK, corazonin and RPCH immunoreactivities were seen, however, these peptides were not detected in our study. This difference in observation may be due to the very low concentration or poor ionization efficiency of these peptide families. In addition, MALDI was the only ionization technique used in the study of the STG, which could limit the peptides being detected. Table 1 shows the identified peptides via MS and their tissue distribution. Some peptides are distributed in all of tissues we investigated such as some orcokinins, orcomyotropin FDAFTTGFGHS, two RYamides: SGFYANRYamide and pEGFYSQRYamide, and the peptide HIGSLYRamide. However, some of the peptides are unique to a specific tissue. For example, PDHs are only present in the SG and brain while CCAP is only present in the PO, which is also consistent with the previously published immunocytochemical data.[52] Some isoforms of orcokinins, FaRPs, AST and proctolin identified in the PO or SG were also found in the STG, indicating their roles as circulating hormone as well as neuromodulators. Overall, the MS-based neuropeptide identification data agree with immunocytochemical data. However, MS-based approach enabled simultaneous mapping and characterizing multiple isoforms which is not available for immunocytochemical approaches.

Conclusion

In this study we combined multiple sample preparation methods and multifaceted mass spectral techniques to comprehensively characterize the neuropeptides present in the nervous system of the Jonah crab *C. borealis*. In total, 142 peptides were identified with 59 that are new to this species. These data greatly increase the number of known peptides present in this species and provide a strong foundation for future studies on the physiological roles played by these signaling molecules in a well defined neural network.

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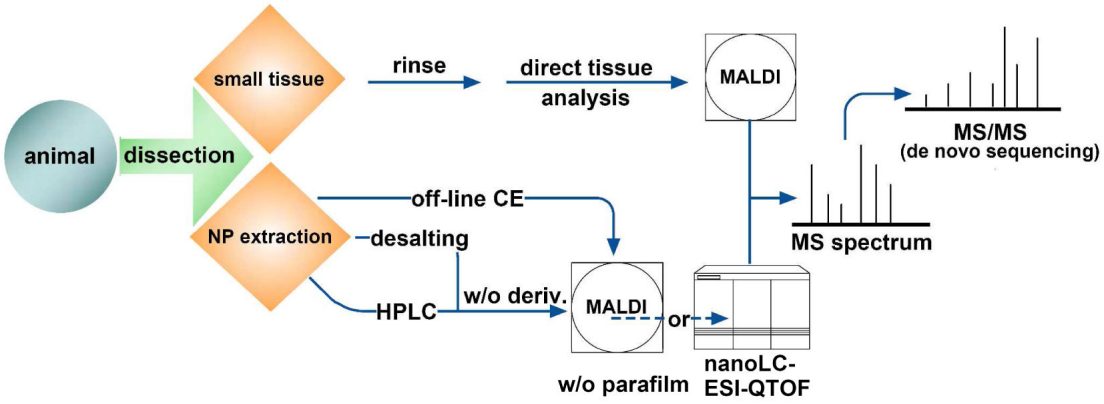

Figure 1.

Overview of a multi-faceted mass spectrometric approach for *C. borealis* neuropeptidomic study. First, the tissue is dissected free of the animal. A small piece of tissue can then be rinsed to reduce the salt content, and the direct tissue analysis is conducted on MALDI-FTMS or MALDI-TOF/TOF. Alternatively, the neuropeptide content can be extracted from the tissue sample. The extract can then be further processed by off-line CE separation, desalting with C_{18} Ziptip, off-line HPLC fractionation prior to the mass spectrometric study. Prior to MALDI and ESI-Q-TOF MS analyses, derivatization reaction is carried out for some of the fractions. For MALDI analysis, a parafilm coated MALDI plate is used for low concentration samples to enhance the MALDI MS detection. Once a mass spectrum is obtained, the neuropeptide sequence can be determined through tandem MS fragmentation followed by *de novo* sequencing.

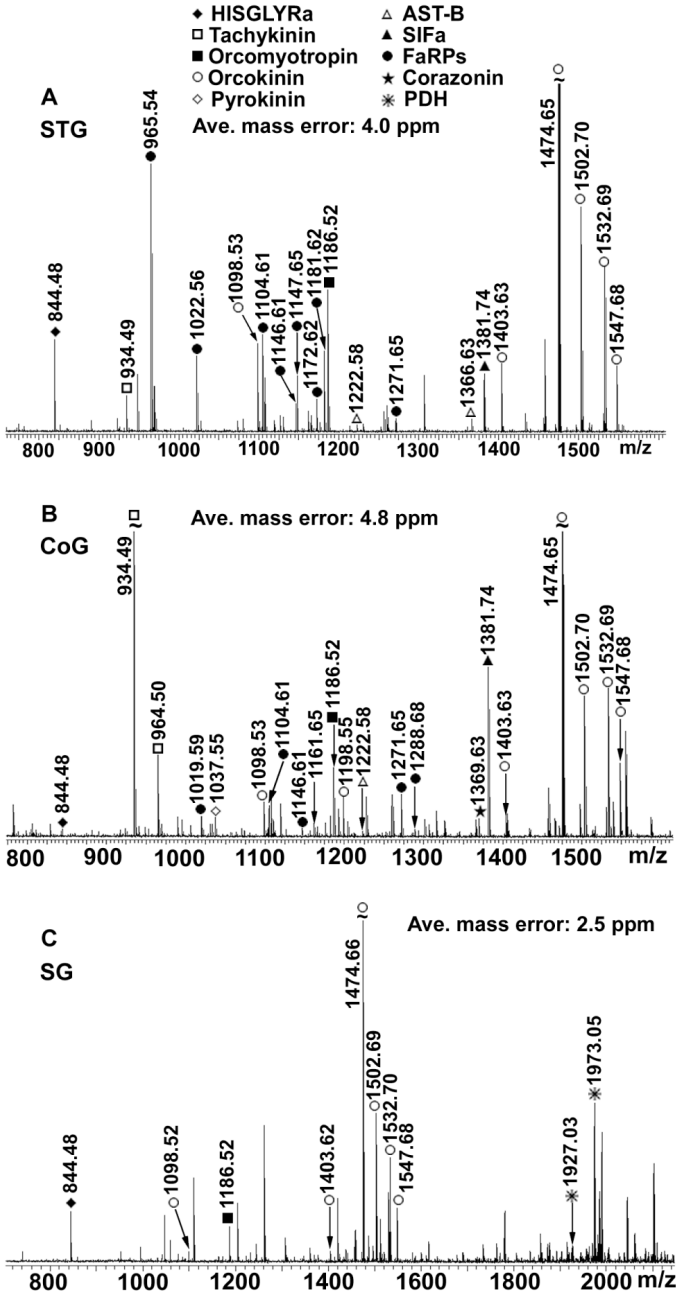

Figure 2.

Direct tissue peptide profiling of *C. borealis* STG (A), CoG (B), and SG (C) by MALDI-FTMS. Signals correspond to the protonated molecular ions, $[M+H]^+$, where M is the molecular weight of each peptide. The identified peptides are marked with the symbols indicating specific families to which they belong.

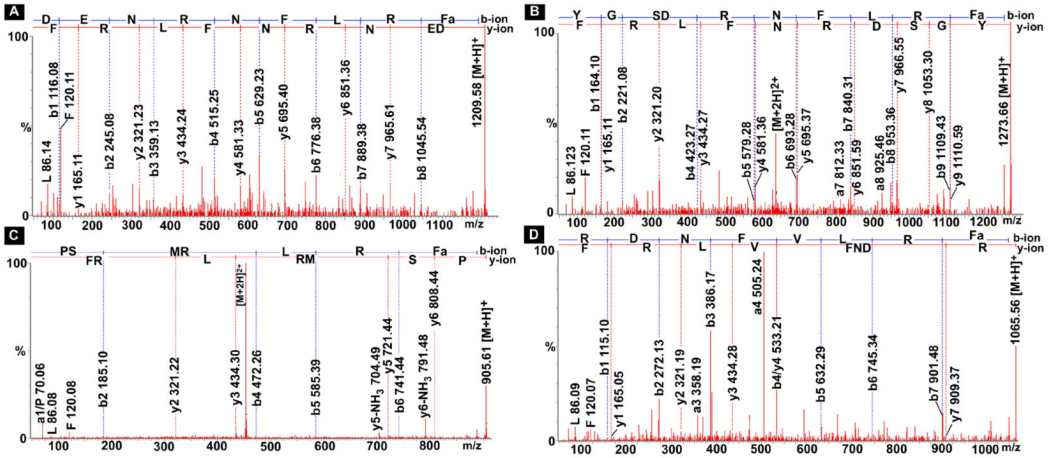

Figure 3.

Collision-induced dissociation spectra of four *de novo* sequenced peptides. ESI-Q-TOF MS/MS sequencing of four FaRPs: DENRNFLRFamide (605.43^{2+}; A), YGSDRNFLRFamide (637.26^{2+}; B), PSMRLRFamide (453.25^{2+}; C), and RDNFVLRFamide (533.30^{2+}; D).

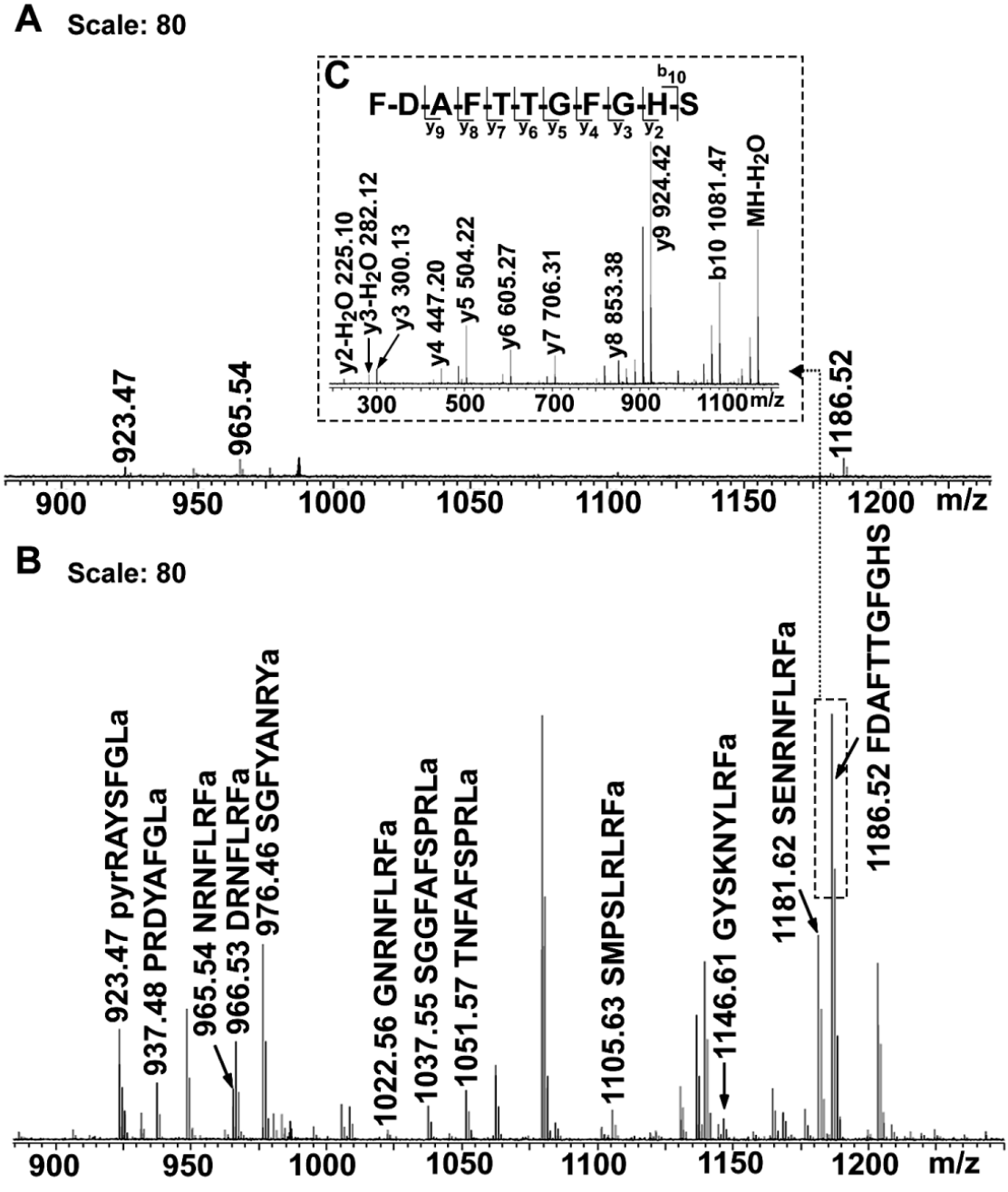

Figure 4.

MALDI-FT mass spectra of a HPLC fraction from *C. borealis* CoG extract. HPLC fraction detected without (A) and with (B) parafilm coated MALDI plate. Signals correspond to the protonated molecular ions, $[M+H]^+$, where M is the molecular weight of each peptide. The identified peptides are labeled with the amino acid sequence of the peptides. One scan was taken after the accumulation of the ions from 50 laser shots with the same laser power. (C) SORI-CID fragmentation spectrum of *m/z* 1186.52 with b- , y-ions and internal fragment ions labeled. The derived amino acid sequence is shown above the spectrum.

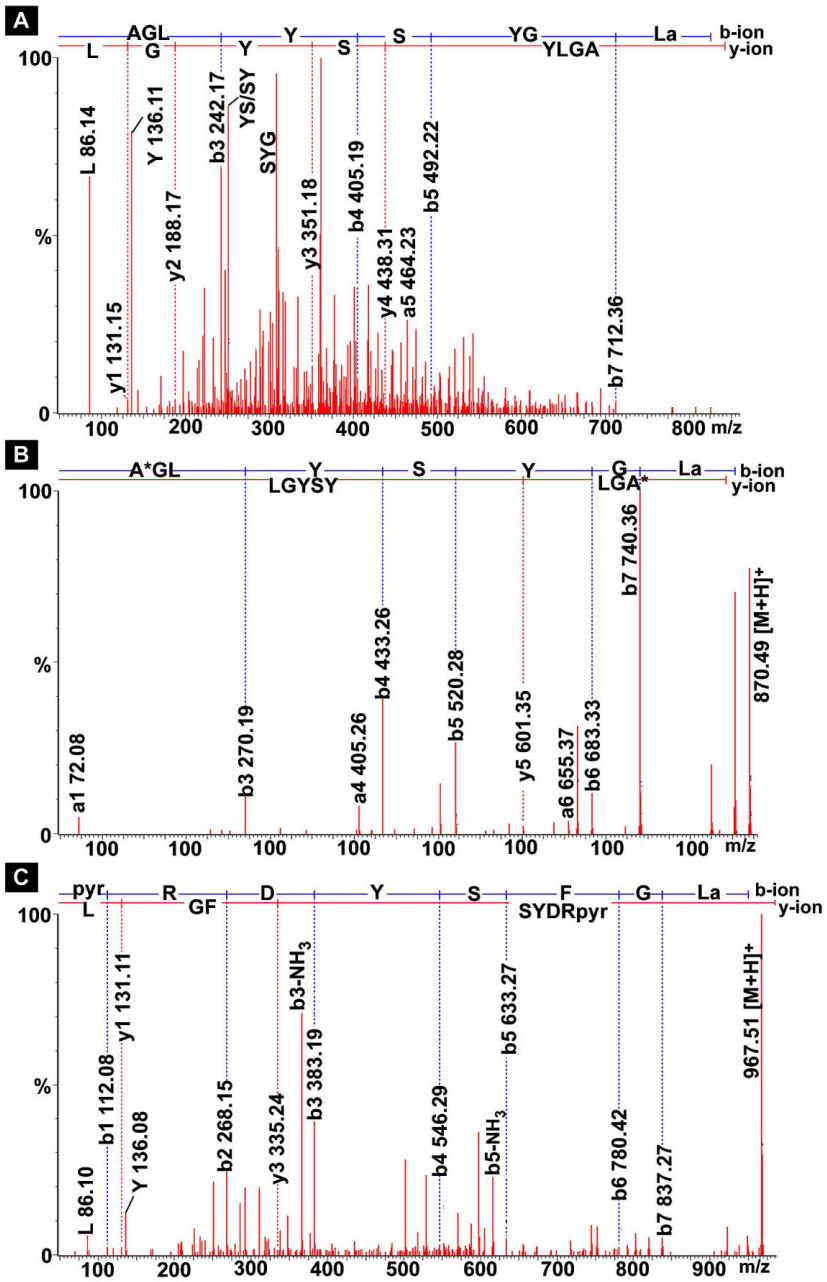

Figure 5.

ESI-Q-TOF MS/MS spectra of an A-type AST peptide AGLYSYGLamide (842.46^{1+}) before (A) and after (B) reductive methylation. In panel B, the a- , b- , and y-ion series are labeled according to the MS/MS of $(CH_3)_2$AGLYSYGLamide (870.46^{1+}). (C) ESI-Q-TOF MS/MS spectra of an A-type AST peptide after reductive methylation (967.51^{1+}). No mass shift is observed for this peptide after the reaction, which indicates the pyroglutamylation (pyr) at the N-terminus.

Table 1

Neuropeptides detected in multiple neuronal tissues from *C. borealis* by nano-LC-ESI-Q-TOF, MALDI-FTMS and MALDI-TOF/TOF.

/z	Sequence	QTOF					FTMS					TOF/TOF				
		B/T	PO	SG	STG	CoG	B/T	PO	SG	STG	CoG	B/T	PO	SG	STG	CoG
		e/f	e/f	e/f	e/f	e/f	e/d/f/CE	e/d/f/CE	e/d/f	e/d/f	e/d/f/CE	e/f	f	f	f	f
.40	*NFLRFamide*	f	f													
.43	**GPFLRFamide**		f													
.50	RNFLRFamide	f/e	f					e/f/CE								
.56	*PSLRLRFamide*	f/e														
.52	GRNFLRFamide		e									f/e				
.51	**SMRLRFamide**	e				e	f					f				f
.51	**PSM(O)RLRFamide**	f														
.52	SKNYLRFamide		f/e			e		f								
.53	NRSFLRFamide	e	f										f			
.53	pQRNFLRFamide		f					f/CE					f			
.54	NRNFLRFamide	f/e	f/e			e	f/e/d	f/e/d/CE		e/d	f/e/d/CE	f/e	f		f	f
.53	DRNFLRFamide	f/e	f/e				f/d	f/CE		f	CE				f	
.53	**NPSNFLRFamide**		f										f			
.51	**NPSDFLRFamide**		f													
7.58	**PKSNFLRFamide**		f													
9.59	APRNFLRFamide										d/CE	f				f
2.56	GNRNFLRFamide	f/e	f/e			e	f/e/d/CE	f/e/d/CE		d	d/CE	f/e	f		f	f
3.55	GDR NFLRFamide							CE								
9.56	**DHVPFLRFamide**		f													
1.59	**AHKNFLRFamide**		f													
5.58	**GHRNFLRFamide**		f					CE					f			
9.59	**AHRNFLRFamide**		f										f			
5.60	**RDNFVLRFamide**		e													
4.58	**ENRNFLRFamide**		f													
4.61	GAHKNYLRFamide		f/e				f/e/d/CE	f/e/d/CE		f/d	f/e/d/CE	f/e	f		f	f

/z	Sequence	QTOF					FTMS					TOF/TOF				
		B/T	PO	SG	STG	CoG	B/T	PO	SG	STG	CoG	B/T	PO	SG	STG	CoG
		e/f	e/f	e/f	e/f	e/f	e/d/f/CE	e/d/f/CE	e/d/f	e/d/f	e/d/f/CE	e/f	f	f	f	f
5.63	APNKNFLRFamide		f					f					f			
5.63	SMPSLRLRFamide	f/e				e	f/d/CE				f/d/CE	f/e				
1.62	*SM(O)PSLRLRFamide*	f/e														
2.63	*RDRNFLRFamide*		f					f				f/e				
6.61	GYSKNYLRFamide	e	f			e	f/e/d/CE	f/e/d/CE		f/e/d	f/e/d/CE	f/e	f		f	f
7.65	APQRNFLRFamide	f	f/e			e	f/d/CE	f/e/CE		d	d/CE	f/e	f		f	f
2.63	AYNRSFLRFamide	f/e	f				f/d	f/e/d/CE		d	CE	f/e	f		f	f
4.64	FTSKNYLRFamide		f					CE		d					f	
5.69	ARPRNFLRFamide		f										f			
1.62	SENRNFLRFamide	f/e	f/e			e	f/e/d/CE	f/e/d/CE		f/e/d	f/e/d/CE	f/e	f		f	f
9.61	DENRNFLRFamide		f										f			
8.63	SQPSKNYLRFamide		f										f			
1.68	pQDLDHVFLRFamide	e	f			e	f/d/CE			f/d	e/d/CE		f			
8.68	*QDLDHVFLRFamide*					e						f/e	f			f
8.64	YGSDRNFLRFamide		f										f			
2.81	*DVRTPALFLRFamide*	f/e				e	CE				CE	f/e				f
5.59	NWNKFQGSWamide	f	f/e				f	CE		d			f			
2.57	TSWGKFQGSWamide	e	f/e					f/CE					f			
2.58	GNWNKFQGSWamide		f/e			e	f/e	f/e/d/CE		d		f/e	f			
2.59	NNWSKFQGSWamide	f	f/e			e	f	f/e/d/CE		d		e	f			
7.51	QWSSMRGAWamide	f	e				f/d	f/d/CE		d	d	e	e			
0.66	SGKWSNLRGAWamide		f/e			e	CE	f/d/CE		d	e/d/CE		e			
2.66	LGNWSNLRGAWamide		f										f			
3.63	STNWSSLRSAWamide		f/e					f/e/d/CE		d		e	f			
6.63	NNNWSKFQGSWamide	f	f/e			e	f	f/e/d/CE		d			f			
0.70	VPNDWAHFRGSWamide							f/e/d			d	e	f			

/z	Sequence	QTOF					FTMS					TOF/TOF				
		B/T	PO	SG	STG	CoG	B/T	PO	SG	STG	CoG	B/T	PO	SG	STG	CoG
		e/f	e/f	e/f	e/f	e/f	e/d/f/CE	e/d/f/CE	e/d/f	e/d/f	e/d/f/CE	e/f	f	f	f	f
.31	YAFGLamide		f													
.30	YSFGLamide		f													
.34	SYAFGLamide		f													
.38	GPYSFGLamide		f													
.39	GQYAFGLamide		e										f			
.38	GGAYSFGLamide		f										f			
.39	DPYAFGLamide	f	f									e				
.40	EPYAFGLamide	f	f/e													
.40	NPYSFGLamide		f										f			
.41	AGPYSFGLamide		f													
.41	GDPYAFGLamide	f	f										f			
.42	AGGAYSFGLamide		f									e	f			
.44	AGLYSYGLamide		f													
.40	DGPYSFGLamide		f													
.43	PSMYAFGLamide		e													
.41	PDMYGFGLamide		f													
.44	GSGQYAFGLamide		f													
.49	ARPYSFGLamide		f													
.43	PDMYAFGLamide		e													
.41	PDM(O)YSFGLamide		f													
.40	SDMYSFGLamidef		f										f			
.47	pQRAYSFGLamide		f				f/CE	CE			f/CE	f/e	f	f		f
.49	*SRPYSFGLamide*		f													
.55	LVKYSFGLamide		f								CE					
.45	SSGQYAFGLamide		f													
.40	SDM(O)YSFGLamide		f													
.48	PRDYAFGLamide	e						f/CE		f	f					
.43	QRAYSFGLamide		f					f/CE			f/CE	f/e	f			

Proteome Res. Author manuscript; available in PMC 2010 May 1.

/z	Sequence	QTOF					FTMS					TOF/TOF				
		B/T	PO	SG	STG	CoG	B/T	PO	SG	STG	CoG	B/T	PO	SG	STG	CoG
		e/f	e/f	e/f	e/f	e/f	e/d/f/CE	e/d/f/CE	e/d/f	e/d/f	e/d/f/CE	e/f	f	f	f	f
.48	*pQRTYSFGLamide*		f													
.49	*LPMYNFGLamide*		f										f			
.48	DRPYSFGLamide		e													
.51	APQPYAFGLamide	f														
.46	**pQRDYSFGLamide**		f													
.51	**SKSPYSFGLamide**		f						CE				e	f		
3.51	PADLYEFGLamide		f										f			
6.55	PATDLYAFGLamide		f										f			
6.57	**HSPSSASYDFGLamide**		f						CE				f			
.41	FVGGSRYamide		f/e					f				e	f	f		
.41	FYANRYamide		f/e					f					f			
.42	FYSQRYamide							CE					f			
.46	SGFYANRYamide		f/e					f/e/d/CE	f	f	CE	f/e	f	f		f
4.58	SSRFVGGSRYamide		f					CE					f			
0.47	pEGFYSQRYamide		f/e				f/CE	f/e/d/CE	e/f/d	f	f/CE	f/e	f	f	f	f
3.52	*EIDRSGFGFA*	f/e					f/e/d/CE		f/d	f/d	f/e/d/CE	f/e		f		
3.55	*NFDEIDRSGFamide*			f		e	e/d				f/e/d/CE	f/e		f		f
9.53	*NFDEIDRSGF*	f														
6.55	*NFDEIDRSGFG*	e	f	f			f/d/CE		f/d	d	e/d/CE	f/e		f		
0.57	*NFDEIDRSGFA*	f/e		f		e	f/e/d/CE		f	d	f/d/CE	f/e		f		
1.55	**DFDEIDRSGFA**	f									f	f	f			
3.62	NFDEIDRSGFGF	f	f				f/d/CE		e/f/d	e/f/d	f/e/d	f/e	f	f		f
4.66	NFDEIDRSGFGFA	f/e		f		e	f/e/d/CE	f	e/f/d	f/d	f/e/d/CE	f/e	f	f	f	f
2.69	NFDEIDRSGFGFV		f				f/e/d/CE		e/f/d	e/f/d	f/e/d/CE	f/e	f	f		f
2.70	NFDEIDRSSFGFV		f				f/e/d/CE		e/f/d	e/f/d	f/e/d/CE	f/e	f	f		f
7.68	NFDEIDRSSFGFN	f		f			f/e/d/CE		e/f/d	f/d	f/e/d/CE	f/e		f		f

/z	Sequence	QTOF					FTMS					TOF/TOF				
		B/T	PO	SG	STG	CoG	B/T	PO	SG	STG	CoG	B/T	PO	SG	STG	CoG
		e/f	e/f	e/f	e/f	e/f	e/d/f/CE	e/d/f/CE	e/d/f	e/d/f	e/d/f/CE	e/f	f	f	f	f
4.70	NFDEIDRTGFGFH						f/d/CE	f	d	d	e/d	f/e	f	f		
6.52	FDAFTTGFGHS	f/e	f	e		e	f/d/CE		d	f/e/d	f/e/d	f/e		f	f	f
.37	RYLPT	f/e		e			f		f	f		e	f	f		f
.38	PFCNAFTGCamide		f					f/e/d				e	f			
9.63	pQTFQYSRGWTNamide						f/e				d/CE	f/e				
7.03	NSELINSLGLPKVMNDAamide			e					e					f		
3.05	NSELINSLGLPKVMNDAamide			e			f		e/d					f		
.40	SGFLGMRamide									d	d	e				
.49	*APSGFLGL(O)Ramide*	f/e		f		e	f				f/CE	f/e		f		f
.50	*TPSGFLGL(O)Ramide*	f				e	f				f/CE	f/e		f		f
.50	*TPSGFLGLRamide*					e	f/d/CE		f	d	f/e/d/CE	f/e				
.49	APSGFLGLRamide	e		f		e	f/d/CE		f	d	f/e/d/CE	f/e		f		f
.50	APSGFLGLRG					e								f		
.37	FSPRLamide	f														
.52	**LYFAPRLamide**	f										e				
1.57	TNFAFSPRLamide	f/e				e	f/CE				f/d/CE	f/e				f
7.55	SGGFAFSPRLamide	f/e				e	f/CE			f	f/e/d/CE	f/e				f
1.65	*RKPPFNGSIFamide*	f/e					f/d/CE			d	d/CE	f/e				
1.74	GYRKPPFNGSIFamide	f/e					f/d/CE		f	f/e/d	d/CE	f/e		f	f	f

J Proteome Res. Author manuscript; available in PMC 2010 May 1.

/z	Sequence	QTOF					FTMS					TOF/TOF				
		B/T	PO	SG	STG	CoG	B/T	PO	SG	STG	CoG	B/T	PO	SG	STG	CoG
		e/f	e/f	e/f	e/f	e/f	e/d/f/CE	e/d/f/CE	e/d/f	e/d/f	e/d/f/CE	e/f	f	f	f	f
1.73	*RSAQGLGKM(O)ERL*		f			f										
2.89	*RSAQGLGKM(O)ERLLAS*		f			e					f/e					
2.69	**RSAQGLGKMER**			e												
3.86	*RSAQGLGKMERLL*			e												
3.75	TPLGDLSGSLGHPVE			e												
9.89	RSAQGLGKMERLLA			e												
6.93	*RSAQGLGMERLLAS*			e										f		
0.95	*RSAQGLGMEHLLASY*			e										f		
6.05	**RSAQGLGKYLRLLASY**			e										f		
0.03	GALEPNTRLGDLSGSLGHPVE			e												
7.33	*RSAQGLGMERLLASYRGALEPN*			e												
3.05	CPRP I			f/e					f/e/d							
7.07	CPRPII			f/e					f/e/d							
3.02	CPRPIII			f/e												
1.08	CPRPIV			f/e												
	8545.80								f/e/d							
.45	pQLNFSPGWamide			e												
.48	HIGSLYRamide	f/e			f/e	e	f/d/CE		f/e/d	f/e/d	f/d/CE	f/e	f	f	f	f

ation methods include off-line CE separation method (CE), off-line HPLC separation method (f), direct tissue analyses (d) and crude extraction (e). The tissues analyzed include ganglia (B/T), pericardial organ (PO), sinus gland (SG), stomatogastric ganglion (STG) and commissural ganglion (CoG). Peptide families include FaRP (FMRFamide-related otostatin), CCAP (crustacean cardioactive peptide), CHH (crustacean hyperglycemic hormone), CPRP (crustacean hyperglycemic hormone precursor related peptide), PDH (pigment e), RPCH (red pigment concentrating hormone), and TRP (tachykinin-related peptide). pQ stands for N-terminal pyroglutamylation modification. Previously known *C. borealis* in black; peptides previously described from other decapods, but new to *C. borealis* are shown in blue; novel peptides are shown in red.