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In situ identification and mapping of neuropeptides from the stomatogastric nervous system of *Cancer borealis*

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Abstract

RATIONAL—The crustacean stomatogastric nervous system (STNS) is a classic experimental model to derive basic knowledge about neuron functions and how they coordinate with each other to generate neural circuits. To investigate the components of the neuromodulators and how they are distributed in such system is essential to understand the underlying mechanism. In this study, in situ mass spectrometry based techniques were employed to fulfill this goal.

METHODS—Offline high performance liquid chromatography (HPLC) separation was coupled with matrix-assisted laser desorption/ionization time of flight/ time of flight (MALDI-TOF/TOF) to analyze the neuropeptides in the stomatogastric ganglion (STG) tissue extract from the Jonah crab *Cancer borealis*. Direct tissue analysis was also employed to investigate the neuropeptides presented in the STNS. MALDI imaging was also applied to map the localization of multiple neuropeptide families in the STG and the upstream nerve.

RESULTS—57 neuropeptides were detected from a single desheathed STG using direct tissue analysis, and they were from 11 different neuropeptide families, including FaRP, AST-A, AST-B, etc. Differential neuropeptide profiles from three different types of ganglia and two types of nerve fiber tissues from the STNS were documented. The direct tissue analysis was shown better for studying neuropeptides from small neural organs like the STG as compared to the large scale HPLC-MALDI analysis. MALDI images were also acquired to study the distribution of neuropeptides in the STG.

CONCLUSIONS—In this study, the components and distribution of neuropeptides have been analyzed in the STNS from *C. borealis* using direct tissue profiling and MALDI imaging. The results show that the direct tissue analysis of desheathed neural tissues can provide higher sensitivity for neuropeptide study compared to large scale HPLC-MALDI analysis of pooled tissues. The results are valuable for understanding the functions of neuropeptides in neural network generation.

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Keywords

Cancer borealis; direct tissue analysis; MALDI-TOF/TOF; mass spectrometric imaging (MSI); neuropeptide; stomatogastric nervous system (STNS)

INTRODUCTION

The Jonah crab *Cancer borealis* is a model organism long served for many areas of physiology, including the study of endocrinology and neurobiology [1–2]. Over the past several decades, the *C. borealis* stomatogastric nervous system (STNS) has become a premier system for the study of the generation, maintenance and modulation of rhythmic behavior at the cellular and network levels [3–4]. STNS consists of several linked motor pattern generating centers that work in concert to produce the complex coordinated motor output that controls ingestive and masticatory movements of the foregut [2]. The small number of easily identifiable neurons allows the establishment of connectivity diagrams among the neurons of the stomatogastric ganglion (STG). A central pattern generating (CPG) circuit within the STNS produces the gastric rhythm, which operates the gastric teeth, and the pyloric rhythm, which separates out food particles for further digestion. A number of studies have demonstrated that the functional output of these well-defined neural circuits is extensively modulated by various amines and neuropeptides [5–7]. Neuropeptides play a variety of important roles in reconfiguring the neural circuits that control behaviors. Some peptides act on their targets via release from neurons with well-defined projections, where they interact with receptors and modulate ion channels to transduce neuronal signals [8]. Many neuropeptides can also be transported via the hemolymph circulation [9–10]. For example, members from multiple neuropeptide families, i.e. RFamide, allatostatin, orckinin, etc, were detected from hemolymph in our previous study [10]. Investigation of the components and localization of those peptides in the STNS will help us to better understand the connections between different neural ganglia and provide valuable insights of their functions.

The application of mass spectrometry to the field of neuroscience has led to the discovery and characterization of many neuropeptides [11–13]. This is usually achieved by tandem mass spectrometry analysis of peptides fractionated using chromatography from crude nerve tissue extract. Alternatively, direct tissue methods, in which the tissue is coated with matrix and probed via matrix-assisted laser desorption/ionization (MALDI) MS analysis, enable sensitive detection of neuropeptides in single organs and even single cells [14–16]. More recently, the use of mass spectrometric imaging (MSI) to map the distribution of neuropeptides has gained increasing attention [17–21]. In contrast to the traditional biochemical techniques such as immunohistochemistry, MSI does not require *a priori* knowledge of the target analytes, and enables simultaneous mapping of different peptides even with significant sequence homology. These *in situ* mass spectrometric techniques can provide highly efficient detection of neuropeptides using limited amount of experimental material.

Although a large number of neuropeptides have been discovered in the neural tissues from various crustacean models using mass spectrometry [11–13], their presence in the STNS has not been studied in detail. In this study, we employed several mass spectrometry based techniques to investigate the neuropeptides in the STNS from the Jonah crab *C. borealis*. The sensitivity of in situ mass spectrometric methods including direct tissue analysis and MALDI imaging were improved by removing the sheath around the ganglia. The result obtained by direct tissue analysis on a single STG ganglion was compared with offline HPLC-MALDI-TOF/TOF analysis using 100 organs. Furthermore, MALDI imaging was employed to map the localization of multiple neuropeptide families in the STG. The presence and distribution of multiple neuropeptide families in the STNS were documented.

EXPERIMENTAL

Chemicals and Materials

Methanol, acetonitrile, formic acid, acetic acid and EDTA disodium salt were purchased from Fisher Scientific (Pittsburgh, PA). 2, 5-dihydroxybenzoic acid (DHB) was obtained from MP Biomedicals, Inc. (Solon, OH). Acidified methanol (90% methanol, 9% glacial acetic acid, and 1% water) was used for neuropeptide extraction. Composition of the physiological saline is 440 mM NaCl; 11 mM KCl; 13 mM CaCl₂; 26 mM MgCl₂; 10 mM HEPES acid; pH 7.4–7.5.

Animals and Dissection

Jonah crabs *C. borealis* were purchased from The Fresh Lobster Company (Gloucester, Massachusetts, USA) and maintained without food in an artificial seawater tank at 12–13 °C before use. Crabs were cold anesthetized by packing on ice for 15 minutes before use. Dissection was performed in chilled (approximately 10 °C) physiological saline. For cresyl violet staining, STG was first fixed with 10% formalin (3.7% formaldehyde) for 3 hours right after dissection and tissue harvest. After fixation, tissue was rinsed in PBS buffer for 2–3 times. Then cresyl violet acetate working solution (Electron Microscopy Sciences, Hatfield, PA) was poured into the petri dish for staining. During staining, the sheath on STG was gradually peeled off. After 90 min of staining, tissue was differentiated in ethyl alcohol rapidly for one to two times. Before taking pictures on microscope, the tissue was re-immersed in artificial crab saline.

Tissue Extraction and HPLC Separation

100 STG tissues were pooled and neuropeptides were extracted by homogenizing the tissues in cold acidified methanol. The extract was first dried in SpeedVac (Thermo Electron Corporation, West Palm Beach, FL) and then resuspended in 50 µL 0.1% formic acid. The resulting supernatants were subsequently fractionated via HPLC.

Peptide separation was performed on a Rainin Dynamax HPLC system equipped with a Dynamax UV-D II absorbance detector (Rainin Instrument Inc., Woburn, MA). 50 µL of tissue 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 following solvents were used for peptide separation: deionized water containing 0.1% formic acid (mobile phases A) and

acetonitrile containing 0.1% formic acid (mobile phase B). The peptides were eluted from the column using a 120 minutes gradient of 5%–95% mobile phase B. Fractions were automatically collected every two minutes using a Rainin Dynamax FC-4 fraction collector (Rainin Instrument Inc., Woburn, MA). The fractions were dried in the SpeedVac and resuspended in 20 μ L of 0.1% formic acid solution prior to mass spectrometric analysis.

Direct Tissue Analysis

Intact STNS was removed from the animal's stomach and pinned to a clear dish. The sheath around STG and associated nerves was then removed using a tungsten needle attached to a pin holder (Fine Scientific Tools, Foster City, CA). The tissue was rinsed quickly in a droplet of acidified methanol, and desalted in a droplet of 10 mg/mL aqueous DHB solution on the MALDI plate. Then a droplet of 0.3 μ L DHB matrix solution (50 mg/mL, 50% methanol) was deposited on top of the tissue and allowed to crystallize at room temperature. The sample spots were then analyzed using MALDI-TOF/TOF.

MALDI-TOF/TOF and Mass Spectral Imaging

A model 4800 MALDI TOF/TOF analyzer (Applied Biosystems, Framingham, MA) equipped with a 200 Hz, 355 nm Nd:YAG laser (spot diameter of 75 μ m) was used for all mass spectral analyses. Acquisitions were performed in positive ion reflectron mode. Instrument parameters were set using the 4000 Series Explorer Software (Applied Biosystems, Framingham, MA). Mass spectra were obtained by averaging 900 laser shots covering mass range m/z 500–4000. Tandem mass spectra (MS/MS) were achieved by 2 kV collision induced dissociation (CID) using air as the collision gas. 750 laser shots were averaged for each MS/MS spectrum, and the peptide sequences were interpreted manually.

MALDI mass spectrometric imaging was performed as previously described [18, 22]. Briefly, desheathed STG was placed on the MALDI plate, and 5 coats of DHB matrix was applied by airbrush. The tissue region to be imaged and the raster step size were controlled using the 4800 Imaging application (Novartis, Basel, Switzerland) available through the MALDI MSI Web site (<http://www.maldi-msi.org>). To generate images, spectra were collected at 50 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. Mass spectra were externally calibrated using peptide standards applied directly to the stainless steel MALDI plate.

Image files were processed, and extracted ion images were created using the Tissue View software package (Applied Biosystems, Framingham, MA). Tissue View was used to create the MSI images by reconstructing the x and y coordinates of the spectra in the acquired image file with their original locations on the tissue. Images can be extracted over an m/z window and visualized using an intensity-based color scale.

RESULTS AND DISCUSSION

Off-line HPLC-MALDI

HPLC separation is an extensively used sample preparation method to deal with complex biological tissue samples. In this study, we first obtained a tissue extract from pooled STG tissues and then fractionated the neuropeptides using C18 reverse phase HPLC. The fractions were then analyzed using MALDI-TOF/TOF. Mass spectra were analyzed and searched against an in-house *C. borealis* neuropeptide database established using multiple organs, including brain, pericardial organ (PO), etc [23]. CID fragmentation was also performed to further confirm their identities. As shown in Table 1, 30 neuropeptides were detected from this organ using offline HPLC-MALDI-TOF/TOF analysis. These peptides belonged to 10 different families, including FMRFamide related peptide (FaRP), a type allatostatin (AST-A), b type allatostatin (AST-B), RYamide, orcokinin, orcomyotropin, *Cancer borealis* tachykinin related peptide (CabTRP), pyrokinin, SIFamide and YRamide.

To minimize the introduction of artificial peptides caused by protein and peptide degradation during analysis, several measures have been taken to reduce the endogenous protease activities. First, the dissection was performed on ice to keep the tissue fresh. Second, the animals were dissected as fast as possible, and cell damage was avoided during dissection. Finally, once removed from the animal, the tissues were immediately immersed in acidified methanol, which denatured all the proteins in the tissue and deactivated the proteases. The fractionation of tissue extract using HPLC greatly simplified the sample and reduced ion suppression; more importantly, it removed the impurities, such as proteins, lipids and salts from the sample. Very few peaks were detected from the crude extract without any sample processing. However, the HPLC separation requires a large amount of materials, especially for small neural organs such as the STG. About 100 STG ganglia were used in this study. All of the detected neuropeptides have been previously observed in the STG from *C. borealis* [23]. Some of these neuropeptides have been investigated in the STNS using electrophysiological studies. For example, the three families of allatostatins are reported to reduce the pyloric network burst frequency in a state-dependent manner, while application of proctolin on STG shows robust excitatory actions [24–25]. However, the neurological effects of some peptides, such as RYamide and YRamide, have not been discovered. Further investigation should be conducted to fully understand the biological functions of these neuropeptides within STNS.

In Situ Identification of Neuropeptides from STG

The sensitivity of direct tissue MALDI profiling was significantly improved by removing the sheath around the ganglia and nerve fibers. As shown in Figure 1a, STG is wrapped in a tube-like sheath and after removing the sheath layer, the center neuropil and the surrounding neurons can be observed clearly under microscope after cresyl violet staining (Figure 1). As shown in Figure 2b, a large number of peptide peaks with decent signal intensities were observed from freshly dissected desheathed STG. In contrast, much fewer peptides were detected when the tissue was briefly smashed using forceps on the MALDI plate (Table 1). By removing the sheath surrounding the STG, neuropeptides inside the tissue can be extracted into the matrix solution more efficiently, facilitating better peptide detection. In

addition, it is possible that less lipid content was introduced into the sample-matrix mixture by removing the sheath, resulting in better matrix crystallization.

The observed peptides were identified based on mass matching using the in-house *C. borealis* neuropeptide database. About 60–80% of the observed peptide peaks in the MALDI spectra were positively identified. As shown in Table 1, 57 neuropeptides were detected from a single desheathed STG using direct tissue analysis, which are from 11 different families, including FaRP, AST-A, AST-B, RYamide, orcokinin, orcomyotropin, CabTRP, pyrokinin, SIFamide, YRamide and proctolin. In the previous study, 142 neuropeptides were detected from five different neural tissues, including brain, CoG, STG, PO and sinus gland (SG) [23]. 37 neuropeptides were detected from STG combining MALDI-FTMS analysis and MALDI-TOF/TOF analysis. All these 37 neuropeptides were observed in the direct tissue analysis of STG in this study, and additional 20 more neuropeptides were detected compared to the previous study. These newly detected neuropeptides were seen in other types of tissues from *C. borealis* before, but observed for the first time in the STG in this work. The number of the detected peptides was greatly increased compared to the other two sampling methods. 27 more peptides were detected compared to direct tissue analysis of intact STG, and 27 more compared to off line HPLC-MALDI. For example, only 3 AST-A and only 1 AST-B were detected using offline HPLC-MALDI. In comparison, 12 AST-A and 9 AST-B were detected directly from the desheathed STG. These peptides present in the STG with relatively lower intensities compared to the other peptide families, and the sample loss during chromatography separation could make them more difficult to be detected. The direct tissue analysis technique requires much less amount of starting material and minimum sample processing, thus shows unique advantages for detecting low abundant neuropeptides from small neural tissues.

Distribution of Neuropeptides in the STNS

STNS consists of a group of four linked ganglia, the paired commissural ganglia (CoGs), the unpaired esophageal ganglion (OG), and the STG (Figure 2f). Each of the CoGs contains approximately 400 neurons, and the OG contains approximately 18 neurons. And the center ganglia STG itself contains around 30 neurons. In this part of study, we examined the neuropeptides from multiple regions of STNS, including the four major ganglia and the nerves connecting them. As shown in Figures 2b and 2c, a large number of neuropeptides were observed from the STG and CoG. However, the overall peptide profiles were distinct between them. For example, CabTRP 1a (m/z 934.5) was detected with the highest peak intensity from CoG, while it was observed as a very small peak in the STG. Compared to the STG, neuropeptides profile from the median protocerebrum (MPC) region in the *C. borealis* brain showed greater degree of similarity to CoG [18]. This is consistent with the fact that CoGs are connected to the cell clusters in the MPC region of brain via *coc* nerve fiber [26]. Only one known neuropeptides CabTRP1a (m/z 934.5) was detected from OG with much lower signal intensity compared to the other tissues. This may be due to the fact that OG is a smaller organ containing much fewer neurons.

As shown in Figure 2, direct tissue MALDI analysis was also performed on several nerve tissues, including the stomatogastric nerve (*stn*) and the superior esophageal nerve (*son*).

Similar neuropeptide profiles were observed in these nerve fibers as compared to their adjacent ganglion STG, but with relatively lower signal intensities. No signal can be detected directly from intact nerves without desheathing, which further demonstrated that removing sheath can improve the detection sensitivity for neural tissues. Furthermore, tandem mass experiment can be performed directly on the tissue samples, which helps to confirm the identities of the observed peptides. Two examples were shown in Figure 3. The CID fragmentation spectra were analyzed manually, and the peptide sequences were assigned by comparing with the theoretical fragmentation pattern of known peptides.

MALDI imaging of STG

Using the oversampling method [27], MALDI imaging was performed to study the localization of specific neuropeptides in the STG using a 50 μm step size. STG was desheathed before transferred onto a MALDI plate. As shown in Figure 4a, the integrity of the organ was well preserved during dissection. As a result, multiple neuropeptides were imaged, and several examples were shown in Figure 4, including orcokinin (NFDEIDRSFGFA, m/z 1474.6), CabTRP (APSGFLGMRa, m/z 934.4), RFamide (NRNFLRFa, m/z 965.5), SIFamide (GYRKPPFNGSIFa, m/z 1381.6) and YRamide (HIGSLYRa, m/z 844.5). All these neuropeptides were detected in the direct tissue analysis. These peptides showed slightly different distribution patterns, indicating that these neuropeptides were likely to be expressed in different neuronal processes. This intriguing observation could implicate that the differentially localized neuropeptides may have distinct neurobiological functions in the neuronal circuits within STG. As shown in Figure 4, the lipids on the tissue were more evenly distributed across the tissue with slightly higher intensity in the stn nerve tissue compared to the STG neuropil, while the neuropeptides were more concentrate in the center region of the ganglion, where neuropil was formed by the processes from the projection neurons in the other regions in STNS. Similar phenomenon was observed in the STG from blue crab *Callinectes sapidus* in our previous study [21]. MALDI-TOF/TOF and MALDI-FTICR equipped with smartbeam™ (Bruker Daltonics, Billerica, MA) were used in the previous study, resulting in MALDI images with higher resolution. Laser beam with smaller diameter is preferable to perform mass spectral imaging for such small tissues.

CONCLUSIONS

In this study, in situ mass spectrometry based techniques including direct tissue analysis and MALDI imaging were employed to study the identities and distribution of neuropeptides in the *C. borealis* STNS. We improved the analysis sensitivity by removing the sheath around the tissue, enabling the detection of much larger number of neuropeptides compared to large scale offline HPLC-MALDI-TOF/TOF analysis. In addition, MALDI imaging was employed to map the localization of multiple neuropeptide families in the STG.

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Figure 1.

The isolated STG from *C. borealis*. (a) Intact STG with sheath; (b) the top layer of sheath surrounding the STG was removed, and neurons were exposed; (c) the optical image of STG tissue stained with cresyl violet.

Figure 2.

Direct tissue analysis of neuropeptides present in the STNS. Mass spectra profiles of peptides present in tissues from (a) nerve *son*, (b) ganglion STG, (c) ganglion CoG, (d) nerve *stn*, and (e) ganglion OG. (f) Schematic drawing of the STNS along with the brain. The STNS consists of four major ganglia and nerve fibers that connect them.

Figure 3.

Representative MS/MS sequencing of two neuropeptides obtained directly from the STG tissue acquired using MALDI-TOF/TOF. (a) MS/MS fragmentation of NRNFLRFa (m/z 965.57); (b) MS/MS fragmentation of HIGSLYRa (m/z 844.50). The presence of fragment ions is indicated by blue color (a and b ions) or red color (y ions).

Figure 4.

MALDI images of lipids and neuropeptides present in the STG. (a) Optical image of STG on the MALDI plate. (b) Optical image of STG covered by matrix during MALDI imaging. MALDI images of (c) lipid PC 34:1 (m/z 760.5), (d) FaRP NRNFLRFa (m/z 965.5), (e) orokinin NFDEIDRSGFGFA (m/z 1474.6), (f) SIFamide, GYRKPPFNGSIFa (m/z 1381.6), (g) CabTRP 1a APSGFLGMRa (m/z 934.5), and (h) YRamide HIGSLYRa (m/z 844.5). The scale bars were 500 μm.

Table 1List of neuropeptides detected in the STG from *C. borealis* using different analytical methods.

m/z	Sequence	HPLC-MALDI	DTA	DTA Desheathed
<i>FaRP</i>				
926.52	SKNYLRFa			x
965.54	NRNFLRFa	x	x	x
966.53	DRNFLRFa	x		
1019.59	APRNFLRFa			x
1022.56	GNRNFLRFa	x	x	x
1104.61	GAHKNYLRFa	x	x	x
1105.63	SMPSLRLRFa			x
1146.61	GYSKNYLRFa	x	x	x
1147.65	APQRNFLRFa	x	x	x
1172.63	AYNRSFLRFa	x		x
1174.64	FTSKNYLRFa	x		
1181.62	SENRNFLRFa	x	x	x
1271.65	pQDLDHVFLRFa		x	x
1288.68	QDLDHVFLRFa			x
1342.81	DVRTPALRLRFa			x
<i>AST-A</i>				
585.30	YSFGLa			x
770.38	GGAYSFGLa			x
795.40	EPYAFGLa			x
810.41	AGPYSFGLa			x
909.49	ARPYSFGLa			x
923.47	pQRAYSFGLa	x		x
925.49	SRPYSFGLa			x
937.48	PRDYAFGLa	x		x
940.43	QRAYSFGLa	x		x
962.51	APQPYAFGLa			x
984.51	SKSPYSFGLa			x
1266.55	HSPSSASYDFGLa			x
<i>AST-B</i>				
1107.51	QWSSMRGAWa		x	x
1165.59	NWNKFQGSWa			x
1182.57	TSWGKFQGSWa			x
1222.58	GNWNKFQGSWa			x
1252.59	NNWSKFQGSWa			x
1260.66	SGKWSNLRGAWa	x	x	x
1293.63	STNWSSLRSAWa			x
1366.63	NNNWSKFQGSWa			x
1470.70	VPNDWAHFRGSWa		x	x

m/z	Sequence	HPLC-MALDI	DTA	DTA Desheathed
<i>RYamide</i>				
784.41	FVGGSRYa			x
976.46	SGFYANRYa			x
1030.5	pEGFYQRFa	x	x	x
<i>Orcokinin</i>				
1098.52	EIDRSFGFa	x	x	x
1198.55	NFDEIDRSFGa	x	x	
1256.55	NFDEIDRSFG		x	x
1270.57	NFDEIDRSFGa	x	x	x
1271.55	DFDEIDRSFGa			x
1403.62	NFDEIDRSFGF	x	x	x
1474.66	NFDEIDRSFGFa	x	x	x
1502.69	NFDEIDRSFGFV	x	x	x
1547.68	NFDEIDRSSFGFN	x	x	x
1554.70	NFDEIDRTGFGFH		x	x
<i>Orcomytropin</i>				
1186.52	FDAFTTGFGHS	x	x	x
<i>Corazonin</i>				
1369.63	pQTFQYSRGWTNa		x	
<i>CabTRP</i>				
766.40	SGFLGMRa		x	x
934.49	APSGFLGMRa	x	x	x
950.49	APSGFLGM(O)a	x		
980.50	TPSGFLGM(O)a	x		
964.50	TPSGFLGMRa	x	x	x
<i>Pyrokinin</i>				
878.52	LYFAPRLa			x
1037.55	SGGFAFSPRLa	x	x	x
1051.57	TNFAFSPRLa	x	x	x
<i>SIFamide</i>				
1161.65	RKPPFNGSIFa		x	x
1381.74	GYRKPPFNGSIFa	x	x	x
<i>YRamide</i>				
844.48	HIGSLYRa	x	x	x
<i>Proctolin</i>				
649.37	RYLPT			x

DTA, direct tissue analysis; FaRP, FMRFamide related peptides; AST, allatostatin; CabTRP, *Cancer borealis* tachykinin related peptide. x indicated a specific neuropeptide was detected using the corresponding sampling method.