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COMBINING MICRODIALYSIS, NANOLC-MS, AND MALDI-TOF/TOF TO DETECT NEUROPEPTIDES SECRETED IN THE CRAB, *CANCER BOREALIS*

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Abstract

Microdialysis is a useful technique for sampling neuropeptides *in vivo* and decapod crustaceans are important model organisms for studying how these peptides regulate physiological processes. However, to date no microdialysis procedure has been reported for sampling neuropeptides from crustaceans. Here we report the first application of microdialysis to sample neuropeptides from the hemolymph of the crab, *Cancer borealis*. Microdialysis probes were implanted into the pericardial region of live crabs and the resulting dialysates were desalted, concentrated, and analyzed by LC-ESI-QTOF and MALDI-TOF/TOF mass spectrometry. Analysis of *in vitro* microdialysates of hemolymph revealed more neuropeptides and fewer protein fragments than hemolymph prepared by typical analysis methods. Mass spectra of *in vivo* dialysates displayed neuropeptides from ten peptide families, including the RFamide, allatostatin, and orcokinin families. In addition, GAHKNYLRFa, SDRNFLRFa, and TNRNFLRFa were sequenced from hemolymph dialysates. The detection of these neuropeptides in the hemolymph suggests that they are functioning as hormones as well as neuromodulators. *In vivo* microdialysis offers the capability to further study these and other neuropeptides in crustacean hemolymph, complementing current tissue-based studies and extending our knowledge of hormonal regulation of physiological states.

Keywords

Microdialysis; Neuropeptides; Mass spectrometry; LC-MS; MS/MS; LC-ESI-QTOF; MALDI-TOF/TOF; Crustacean; Hemolymph

Introduction

Neuropeptides are small signaling molecules, generally less than 50 amino acids long. They are present in a wide variety of animals, from the sponge all the way up to humans. Neuropeptides are known to be involved in the regulation of many physiological processes, including food intake, memory, pain, and stress^{1,2}.

Current neuropeptide analysis methods include tissue- and fluid-based methods. In tissue-based methods, the tissue of interest is dissected out of the animal and either directly sampled with matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) or the neuropeptides are extracted from the tissue before being analyzed by MS techniques³. These methods offer important information regarding peptide expression and processing. However,

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they have the disadvantage of nonspecific sampling of peptides. Because neuropeptides are synthesized from preprohormones that must undergo multiple steps of post-translational processing to become biologically active, sampling from the tissue yields both the non-active peptides residing in the cell as well as the biologically-active peptides that may have been secreted and it can be difficult to distinguish between them^{4, 5}.

In fluid-based methods, neuropeptides can be sampled locally from stimulated neuronal releasate⁶⁻⁸ or globally by sampling the blood or hemolymph using a needle attached to a syringe⁹. Although stimulated neuronal releasates sample biologically-active neuropeptides, the presence of extracellular peptidases in the surrounding fluid can quickly degrade the sampled peptides. For hemolymph collection, the stress the animal experiences during the hemolymph sampling process can be a source of artificial neuropeptide release and give misleading results. Further, most of these tissue- and fluid-based methods pick up a wide variety of molecules besides neuropeptides, such as lipids, albumins, clotting factors, and enzymes that degrade neuropeptides. This makes for a dirty sample requiring extensive clean-up to separate trace-level neuropeptides from more abundant molecules that will overwhelm their signal or degradation enzymes that will cleave neuropeptides into fragments too small to be identified.

For these reasons and others, many researchers have turned to *in vivo* sampling methods. *In vivo* sampling methods enable real-time monitoring of changes in the extracellular concentrations of molecules. The fluid output, or dialysate, can be collected into discrete fractions, each of which represents a sample of the molecules present at a different time point. Alternatively, the dialysate can be fed into a continuous online detection system, so molecules can be detected and monitored in real time. This allows the study of the transient expression of neuropeptides and how a neuropeptide profile changes over time.

Push-pull perfusion and microdialysis are the most common *in vivo* sampling methods. Push-pull perfusion utilizes two concentric tubes with different diameters where liquid is pumped through one tube (usually the inner one) and simultaneously withdrawn from the other tube. While push-pull perfusion enjoys a high recovery of the molecules present in the extracellular space, a disadvantage of this technique is that, traditionally, push-pull perfusion uses high flow rates (mL/min) which can often damage the tissue around the probe. Recently, Shippy and co-workers have modified push-pull perfusion to operate at very low flow rates (10-50 nL/min)¹⁰, yielding minimal tissue damage. However, these low flow rates require a long sampling period to collect enough material to detect and analyze. This is especially problematic for neuropeptides, which are estimated to be present at pM to μ M levels in the tissue¹¹.

Another *in vivo* sampling technique, microdialysis, works by pumping fluid through a probe that sits in the tissue of interest. This perfusion fluid usually has a composition similar to the extracellular fluid of the organism. On its way through the probe, the fluid passes by a semi-permeable membrane with a certain molecular weight cutoff (MWCO) and diffusion can occur between the fluid and the extracellular space. Molecules in the extracellular space, including neuropeptides, will diffuse into the fluid, according to the concentration gradient.

Since this technique relies on diffusion to collect desired molecules, there is minimal disturbance to the organism. Unlike traditional push-pull perfusion, the tissue damage caused by the probe is negligible. Also, because the molecules collected must first diffuse through a porous membrane, molecules larger than the pores will not be picked up. Typical membrane cutoffs are in the 5-50 kDa range, so albumins, degradation enzymes, and other large proteins will be filtered out by the membrane. This provides a cleaner sample with which to work, and abbreviates clean-up steps required for samples collected by tissue and fluid extraction methods or by push-pull perfusion.

LC-MS is well-suited to coupling with microdialysis for the detection of neuropeptides¹². LC-MS can exhibit high sensitivity without peptide derivatization, as shown by the detection of pM levels of neurotensin in different rat brain regions by Andren and Caprioli¹³. The microliter per minute flow rates of microdialysis are amenable to capillary LC, so microdialysis is capable of being coupled online to LC-MS. Further, since LC-MS detection is based on mass-to-charge ratios, it provides a relatively non-biased detection method for observing both known and unknown neuropeptides. Kennedy and co-workers demonstrated the ability of online microdialysis-LC-MS/MS to monitor several known neuropeptides at once and to discover previously unknown neuropeptides¹⁴.

Most of the published *in vivo* microdialysis studies have been performed using mammalian nervous systems. Decapod crustaceans are important model organisms for studying how neuropeptides modulate central pattern generating networks and how they affect the hormonal regulation of physiological processes^{15, 16}. Specifically, several motor circuits in the crustacean stomach (collectively called the stomatogastric nervous system) appear to be extensively modulated by peptide hormones secreted from major neurohemal organs such as the pericardial organs surrounding the heart and the sinus glands in the eyestalks. Numerous peptide analyses of neuronal tissues in various species of decapod crustaceans have been reported¹⁷⁻¹⁹. However, as these peptide hormones need to be released into circulating fluid to exert their effects, it is critical to develop effective analytical methodology to monitor peptide secretion. So far, only one study has reported the *in vivo* microdialysis of small molecule neurotransmitters in crayfish²⁰ and no report was found for neuropeptide studies.

To complement tissue-based neuropeptide analysis and provide a more complete picture of neurosecretion and peptidergic signaling in crustacean model systems, this study explores the first use of *in vivo* microdialysis sampling of neuropeptides from the crab, *Cancer borealis*. A concentric microdialysis probe was implanted into the pericardial region of the crab, in contact with the hemolymph above the heart. The crab was dialyzed for varying periods and the dialysates were concentrated and desalted before analysis by LC-electrospray ionization (ESI) MS/MS and MALDI MS/MS. These *in vivo* microdialysis experiments were compared with the typical approach for detecting neuropeptides in hemolymph. From these studies, nearly 40 neuropeptide masses were detected from ten peptide families, including the RFamides, allatostatins, and orcokinin. In addition, three known neuropeptides, TNRNFLRFa, SDRNFLRFa, and GAHKNYLRFa, were sequenced from microdialysates using MS/MS.

Experimental section

Animals

Jonah crabs, *Cancer borealis*, were shipped from The Fresh Lobster Company (Gloucester, MA) and maintained in an artificial seawater tank at 10-12°C with an 8 h/16 h light/dark cycle. The crabs were allowed to adjust to the tank for at least one week before performing microdialysis. Crabs were cold-anesthetized by packing in ice for 15-20 min before microdialysis probe implantation. To minimize tubing lengths, the crab was confined to a portion of the tank (approximately 11 in² footprint) with a perforated plexiglass sheet during microdialysis (Figure 1a). For *in vitro* microdialysis and typical hemolymph preparation, hemolymph was withdrawn from the pericardial sinus through the junction between the thorax and abdomen using a 22-gauge needle and a 3 mL plastic syringe. Hemolymph was immediately frozen and kept at -20°C until use.

Microdialysis supplies

Both homemade and commercial microdialysis probes were used in this study. Concentric-style homemade probes were constructed of Spectra/Por hollow fiber with a 13 kDa MWCO

and 3 mm active membrane length²¹. Purchased CMA/20 Elite probes had a 4 mm-long polyarylethersulfone membrane with a 20 kDa MWCO (CMA Microdialysis, North Chelmsford, MA). A tiny ball of hot glue was added 1 cm from the tip of commercial probes to provide a stopping point when inserting the probe into the crab; homemade probes were made with a glue ball at this location. A CMA/102 dual-syringe pump was used with 2.5 mL glass syringes. Tubing connections were made with fluorinated ethylene propylene (FEP) tubing (CMA) for commercial probes and lower density polyethylene tubing (Thomas Scientific, Swedesboro, NJ) for homemade probes.

***In vitro* microdialysis of hemolymph**

One milliliter of hemolymph was withdrawn and mixed with Arg vasopressin (AVP, CYFQNCPRGa with Cys₁-Cys₆ disulfide bridge, MW 1083.45) as an internal standard to a 10 μ M concentration. A CMA/20 probe was placed in the hemolymph solution on ice and perfused at 1 μ L/min with degassed, chilled physiological saline solution (composition in mM: NaCl, 440; KCl, 11; MgCl₂, 26; CaCl₂, 13; Trizma base, 11; maleic acid, 5; pH 7.45). Fractions were collected on ice every 15 min for 2 h total and stored at -20°C until quadrupole MS analysis.

Comparison of hemolymph preparation methods

Two milliliters of hemolymph were withdrawn from one crab and split into two samples, one for each method of analysis (Figure 2). For the typical preparation method, 1 mL of the hemolymph was combined with 1 mL of acidified methanol (90% methanol, 9% glacial acetic acid, 1% water), vortexed, and centrifuged at $2550 \times g$ (swing bucket rotor) for 10 min. The supernatant was filtered through a 10 kDa MWCO tube and the low-mass filtrate was kept. For the microdialysis method, a homemade microdialysis probe was placed in 1 mL of the hemolymph on ice and perfused with physiological saline at 0.5 μ L/min for 4 h total. The fraction was collected on ice and stored at -20°C until sample preparation was completed.

Both the low-mass filtrate and 100 μ L of the microdialysis fraction were dried down and resuspended in 0.1% formic acid (FA). They were desalted using ZipTips_{C18} (Millipore, Billerica, MA) and each eluted into 1 μ L of 50% acetonitrile (ACN), 0.1% FA. Both samples were spotted with MALDI matrix and run on MALDI-TOF/TOF on the same day.

Surgery and *in vivo* microdialysis

All surgery and *in vivo* microdialysis procedures were performed in a temperature-controlled room ($18\text{--}20^{\circ}\text{C}$). Plumber's epoxy (Pox Plus, Inc., Sussex, WI) was rolled into a ring (about 0.5 cm thick and 2.5 cm in diameter) and pressed around the region to be drilled. Then the crab was packed in ice except for the shell around the plumber's epoxy, which must stay dry. After 15–20 min, the crab was removed to a dish packed with ice for surgery. A 0.8 mm-diameter hole was made over the heart with an electric drill. A homemade or CMA probe was inserted about 1 cm deep into the hemolymph-filled pericardial sinus and then the plumber's epoxy ring was filled with super epoxy (Pox Plus, Inc.). The super epoxy was allowed to set for about 10 min, then the base of the probe shaft was coated with hot glue for stability. Once the hot glue dried, the crab was returned to the tank (Figures 1a, 1b, 3).

The outlet tubing of the microdialysis probe was placed in a 1.5 mL collection tube on ice. Chilled physiological saline solution containing 0.5% bovine serum albumin (BSA, w/v) and 100 nM somatostatin (AGCKNFFWKTFTSC with Cys₃-Cys₁₄ disulfide bridge, MW 1636.72) as an internal standard was then filtered and degassed for use as perfusate. All probes were perfused with the physiological saline perfusate for 15 min prior to beginning the experiment. The syringe pump was started at 10 μ L/min; once liquid could be seen flowing out of the outlet tubing, the flow rate was reduced to 0.5 μ L/min for the microdialysis experiment. A 30-min

equilibration period of the microdialysis probe was observed before microdialysis sampling was started in order to let the perfusion fluid equilibrate with the extracellular fluid. Samples were collected every 4–8 h during the day; one 16-h sample was collected overnight. Crabs were microdialyzed up to 3.5 days before being sacrificed.

Post-experiment, green food dye was perfused through the probe for a few minutes before cold-anesthetizing and sacrificing the crab. The location of the microdialysis probe in the pericardial region was verified by observing the dye's location in the connective tissue above the heart upon dissection of the crab (Figure 1c).

Sample preparation of *in vivo* dialysates

In vivo dialysates were collected on ice and immediately frozen (-20°C) after collection. The dialysates were spiked with AVP standard at 1 nM and then concentrated approximately 20-fold in a SpeedVac (Thermo Fisher Scientific, Waltham, MA). Twenty percent FA was added to the dialysates to a 0.1% final concentration. The supernatant was desalted and further concentrated to approximately 100-fold the initial concentration using either ZipTips or Vivapure C₁₈ Micro spin columns (Vivaproducts, Inc., Littleton, MA). For MALDI MS analysis, samples were eluted in 2 μL 50% ACN, 0.1% FA. For ESI MS analysis, samples were eluted in 5–10 μL 50% ACN, 0.1% FA, then diluted 1:2 with 5% ACN, 0.1% FA.

LC-ESI-Quadrupole Time-of-Flight (QTOF) MS/MS

NanoLC-MS/MS analysis was performed using a Waters capillary LC system coupled to a QTOF Micro mass spectrometer (Waters Corporation, Milford, MA). Chromatographic separations were performed on a reversed-phase analytical column (Microsil nano C18, 75 μm i.d. $\times$ 15 cm length, 3 μm particle size, Micro-Tech Scientific, Vista, CA). The mobile phases used were (A) deionized H₂O with 5% ACN and 0.1% FA, (B) ACN with 5% deionized H₂O and 0.1% FA, and (C) deionized H₂O with 0.1% FA. An aliquot of sample was injected and loaded onto the trap column (PepMap C₁₈, 300 μm column i.d. $\times$ 1 mm length, 5 μm particle size, LC Packings, Sunnyvale, CA) for desalting the sample. For dialysate samples, 1 to 3 μL of concentrated dialysate was injected for each analysis. The gradient for crab dialysates was from 10% B to 50% B in 52 min.

The nanoflow ESI source was set with a capillary voltage of 3200 V, a sample cone voltage of 36 V, and an extraction cone voltage of 1 V. The instrument was calibrated with phosphoric acid every 10–12 h to <20 ppm. A data-dependent acquisition was employed and the MS/MS threshold was set at 15 counts/s.

De novo sequencing was performed using a combination of the PepSeq peptide sequencer in MassLynx 4.0 (Waters) and manual sequencing. To verify the sequence assignments, raw MS/MS spectra were compared with the predicted fragmentation pattern (using the MS-Product software at <http://prospector.ucsf.edu>) of the proposed sequences.

More details about the stream select module, the ESI source, tandem MS, and *de novo* sequencing can be found in a previous publication²².

MALDI-TOF/TOF MS

A spot of 0.4 μL sample was placed on a stainless steel MALDI sample plate and allowed to dry before applying 0.4 μL of alpha-cyano-4-hydroxycinnamic acid (CHCA, saturated solution in 50/50 ACN/0.1% aqueous FA, v/v) matrix.

A model 4800 MALDI-TOF/TOF analyzer (Applied Biosystems, Framingham, MA) equipped with a 200 Hz, 355 nm Nd:YAG laser 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). Each mass spectrum was generated by averaging 1400 laser shots over the mass range m/z 500-4500. Mass spectra were externally calibrated using peptide standards applied directly to the MALDI sample plate.

Tandem MS were obtained using precursor ion selection windows of about 6 Da, and ion activation was achieved using 1 kV collision-induced dissociation with air as the collision gas. Fifteen hundred laser shots were averaged for each MS/MS spectrum, and sequence interpretation was performed manually.

Results and Discussion

Comparison of hemolymph preparation methods

Typically, hemolymph is analyzed for neuropeptides by first sampling the circulating fluid from the animal with a needle and attached syringe. The collected hemolymph is immediately mixed with cold acidified methanol to extract the peptides from the surrounding matrix, slow the activity of peptidases, and protonate the peptides in solution. This mixture is centrifuged and filtered to remove the larger molecules, such as peptidases and clotting factors, then desalted and dissolved in a solvent suitable to the detection method.

Since we are evaluating microdialysis as an alternative method to analyze hemolymph for neuropeptides, we began by comparing microdialysis with the typical hemolymph analysis method. Two equal-volume aliquots of hemolymph were processed, one using the typical hemolymph analysis method including acidified methanol extraction and one using *in vitro* microdialysis without acidified methanol (Figure 2). For the typical hemolymph analysis method we used a 10 kDa MWCO filter, which is commonly used since most neuropeptides are < 5 kDa. For the microdialysis method, we chose a probe with a 13 kDa MWCO membrane, which is the closest MWCO we could find. Although the filters have slightly different pore sizes, the steps following filtration were kept the same. Both filtrates were desalted with ZipTips, concentrated 100-fold, and analyzed by MALDI mass spectrometry on the same day.

The results of our comparison study are shown in Figure 4. We included representative spectra from each method of hemolymph analysis. There were several neuropeptide masses present in the two samples (Table 1, peptides found via *in vitro* microdialysis are indicated with single or double asterisks). The microdialysate contained three RYamides (m/z 784.5, 976.5, 1030.5), one RFamide (m/z 908.5), and one A-type allatostatin (m/z 949.5). The hemolymph prepared by typical methods contained one RFamide (m/z 1104.7). *Cancer borealis* pyrokinin I (CabPK-I, m/z 1051.6) was a prominent peak in both samples.

One difference between the two spectra is that the hemolymph prepared by typical methods contains larger masses than the microdialyzed hemolymph. Specifically, the mass spectrum of the hemolymph by typical preparation methods contains intense peaks at m/z 1395.8, 1508.9, 1769.9, and 2241.1, while the spectrum of microdialyzed hemolymph shows almost no signal above m/z 1300 (Figure 4). Since the hemolymph prepared by typical methods is centrifuged through the filter, while the microdialyzed hemolymph relies on diffusion to pass through the filter, the hemolymph prepared by typical methods is more likely to have higher masses than the microdialysate. This has implications for detection sensitivity, as larger proteins are usually present at concentrations that are several magnitudes higher than neuropeptide concentrations. Although some of these larger masses could be neuropeptides, most crustacean neuropeptides have masses less than 1500 Da, so it is more likely that these larger masses belong to protein fragments. Microdialysis, by better filtering out larger masses, produces samples with higher relative concentrations of neuropeptides compared to more abundant proteins, making it easier to detect low-level neuropeptides.

An investigation of the higher masses present in the hemolymph prepared by typical methods revealed no matches to known neuropeptides. However, two of the masses, m/z 1395.7 and 1508.8, appear to be fragments of cryptocyanin. Cryptocyanin is a molting protein found in crustacean hemolymph. It has been isolated and sequenced by molecular biology methods for a related crab, *Cancer magister*²³. In *Cancer borealis*, fragments of cryptocyanin have been sequenced from a tissue extract of the central nervous system²⁴. Here, an MS/MS spectrum of the peak at m/z 1508.8 is shown in Figure 5, confirming the peak as the cryptocyanin 1 fragment peptide, YKIFEPLRESNL. Furthermore, an MS/MS spectrum of m/z 1395.7 was also obtained, supporting the sequence derivation of YKIFEPLRESN, a related fragment of cryptocyanin 1 (data not shown).

In addition, several masses corresponding to other cryptocyanin fragments in hemolymph samples, including m/z 909.5 (YKIFEPL), m/z 1194.6 (YKIFEPLRE), and m/z 1373.7 (YKIFEPLREDNL), have also been detected. Some of these fragments have been observed at trace-levels in microdialysates, but most have been observed in hemolymph analyzed by typical preparation methods. These protein fragments suggest the presence of a degradation enzyme which is cleaving cryptocyanin into smaller peptides. Since there is always a brief delay between collecting the hemolymph and adding the acidified methanol, enzyme deactivation is not immediate and degradation enzymes could be cleaving abundant proteins during this time. Microdialysis, especially *in vivo* microdialysis, may better filter out these enzymes as well as clotting factors, making for cleaner samples from which neuropeptides can be detected more easily.

Although cryptocyanin fragments may not be the desired analytes, cryptocyanin is abundant in the hemolymph, particularly in the premolt stage, and cryptocyanin fragments could be incorrectly assigned as neuropeptides.

There are also many peaks in both spectra that cannot currently be assigned to any known neuropeptides or cryptocyanin fragments (Figure 4), leaving opportunities for further research into the peptide complement of hemolymph.

***In vitro* microdialysis of hemolymph**

The results of the comparison of microdialysis with typical hemolymph preparation methods were encouraging, so we repeated the *in vitro* microdialysis experiment, this time using a commercially-available probe with a larger MWCO (20 kDa vs. 13 kDa). The larger pore size should allow us to observe larger peptides and to collect greater amounts of smaller peptides. No discernable difference was observed in the MS of the resulting microdialysis fractions, so commercial probes were employed for all later experiments.

Several neuropeptide masses were observed in the resulting mass spectra. Two A-type allatostatins were observed at m/z 569.3 and 585.3, as well as one RYamide at m/z 784.4 and one RFamide at m/z 1104.6 (Table 1, *in vitro*). The peak at m/z 1104.6 could be assigned as one of two RFamides: GAHKNYLRFa or AQPSMRLRFa. To determine the correct sequence, we acquired a fragmentation spectrum of m/z 1104.6 (Figure 6a). This spectrum confirms the correct assignment of the m/z 1104.6 peak as GAHKNYLRFa.

GAHKNYLRFa was recently sequenced in several *Cancer* crabs as a novel member of the well-established RFamide peptide family; specifically, it was found in the pericardial organs, the commissural ganglia, and the stomatogastric ganglion²⁵. It was found that GAHKNYLRFa affected the bursting frequencies of the cardiac ganglion, pyloric rhythm (involved in filtering food), and gastric mill rhythm (involved in grinding food). This study presents the first evidence of GAHKNYLRFa in crab hemolymph. The fact that GAHKNYLRFa has been found in both the pericardial organs and the hemolymph suggests a hormonal role for this peptide.

GAHKNYLRFa may be secreted from the pericardial organs into the hemolymph to act on the crustacean heart and/or more distant stomatogastric motor neurons involved in the pyloric and gastric rhythms.

This experiment highlights the importance of analyzing the hemolymph, in addition to the tissue, in neuropeptide studies. To this end, microdialysis appears to offer distinct advantages to observing neuropeptides in hemolymph over typical hemolymph collection and analysis techniques. In particular, microdialysis of hemolymph provides cleaner samples that are free of protein fragments, clotting factors, and degradation enzymes. By using *in vivo* microdialysis, one can minimize the possibility of introducing artificial, stress-related neuropeptide release caused by sampling the hemolymph with a needle and syringe.

***In vivo* microdialysis**

To take full advantage of the benefits of microdialysis, we developed the first application of microdialysis sampling of neuropeptides from a live crab. We chose to dialyze from the pericardial sinus for a couple of reasons. First, this is a relatively large cavity in the crab, measuring about 2 cm³, so it was a good place to begin microdialysis studies. Second, this hemolymph-filled cavity is adjacent to the pericardial organs, which are known to be important neurosecretory structures that release many neuropeptides into the hemolymph, so it is likely that neuropeptides are present in relatively high concentrations in this region.

One of the biggest difficulties we encountered when developing our microdialysis procedure was dealing with the crab shell. Because there is no exposed tissue, we could not poke the probe into the organ of interest and rely on tissue elasticity and a bit of glue to seal the hole. The crab's shell is brittle and once drilled, hemolymph pumps out of the hole very quickly. The constant outflow of fluid makes it difficult for a glue to cure and we found that the presence of hemolymph itself prevents many otherwise good choices of glues from completely drying. Further, most glues that we applied to the probe would mix with the flowing hemolymph and run down the tail and away from the probe.

The best results were achieved by first creating a well of plumber's epoxy around the pericardial region. This had to be applied before the hole was drilled because the plumber's epoxy would not stick to a shell wet with hemolymph. When the super epoxy was applied inside this well, the plumber's epoxy served as a barrier to prevent the super epoxy from running down the crab's tail, allowing the super epoxy to stay in contact with the probe while it dried. Applying hot glue around the base of the probe shaft further stabilized the probe, which was important if the crab bumped the probe against the walls of the tank (Figures 1a, 3).

Another challenging aspect of crab microdialysis was dealing with the high salt content present in crab dialysates. Physiological saline contains about 500 mM of various salts, which is problematic for mass spectrometric detection. Initially, we attempted online desalting using the reverse-phase column on the LC-ESI-QTOF, but often this did not adequately desalt the dialysates. We found that it was important to first desalt the dialysates using ZipTips or micro spin columns before injecting them onto our LC-ESI-QTOF and MALDI-TOF/TOF instruments. These extra desalting steps had the added benefit of concentrating the dialysates, which proved useful as the neuropeptide signal was usually near the lower limit of detection for both instruments.

Both ESI-QTOF and MALDI-TOF/TOF mass spectrometers were employed for sample analysis. As ESI and MALDI have been shown to be complementary ionization techniques²⁶, use of both methods allowed a more thorough coverage of the neuropeptides present in hemolymph than if we had just used one ionization method alone. The internal standard, AVP, was observed in both ESI-QTOF and MALDI-TOF/TOF MS spectra. Based

on the intensity of AVP peaks in the MALDI-TOF/TOF mass spectra, we estimate that the neuropeptides identified in the *in vivo* dialysates were present at 1 nM to 500 nM concentrations. When fragmentation information of putative peptides was desired, the ESI-QTOF tended to give better MS/MS data than the MALDI-TOF/TOF, so MS/MS was performed on the ESI-QTOF instrument whenever possible.

One of the advantages of *in vivo* microdialysis is its ability to monitor analytes present in an organism over time. Figure S1 shows spectra from three dialysates collected during an *in vivo* microdialysis experiment. The first spectrum came from a dialysate collected during the first 24 h after probe implantation (Figure S1a). No neuropeptides are visible in this spectrum. The second and third spectra were taken from 30 min dialysates collected successively on the next day (Figures S1b, S1c). The first 30 min showed a small peak at m/z 781.4, which matches the mass of a known A-type allatostatin (DPYAFGLa). In the next 30 min fraction, the m/z 781.4 peak increased in intensity. While absolute quantification was not performed, the relative peak intensity changed from 1.6% to 11.3% of the tallest peak in the mass spectrum. This demonstrates the ability of microdialysis sampling and MS detection to monitor an endogenous peptide over time.

Table 1 lists all the neuropeptide masses that were found in the *in vivo* dialysates. Many neuropeptides were present from several families. Of the ten allatostatins that were observed *in vivo*, all except m/z 753.4 have been seen in *C. borealis* pericardial organ previously^{22, 27, 28} and the peptide at m/z 753.4 (SPYAFGLa) shares a similar sequence with the peptide at m/z 781.4 (DPYAFGLa). Interestingly, the peptides corresponding to m/z 569.3 (YAFLa), m/z 781.4 (DPYAFGLa), m/z 1519.8 (DPYAFGLGKRPADL), and m/z 2128.1 (DPYAFGLGKRPADLYEFLa) appear to be related. Several tachykinin-related peptides were detected, including *Cancer borealis* tachykinin-related peptide 1a (CabTRP 1a) at m/z 934.5, which has been previously observed in *C. productus* sinus gland by accurate mass measurement via Fourier transform MS analysis²⁷. All the orcokinins that we detected in our microdialysates have been identified previously in crustacean sinus gland or pericardial organ^{6, 18, 27, 29} and most of the RFamides have been reported as present in the pericardial organ of *C. productus* or *C. borealis*^{6, 22, 27, 28, 30}. In addition, tandem MS data was obtained for both SDRNFLRFa (m/z 1053.6, Figure 6b) and TNRNFLRFa (m/z 1066.6, Figure 6c), confirming the presence of these peptides in hemolymph.

The RYamide at m/z 784.4 and crustacean cardioactive peptide (CCAP, m/z 956.4) have both been observed before in *C. borealis* pericardial organ^{6, 22, 27, 28}. As CCAP has been shown to have excitatory actions on the isolated cardiac ganglion³¹, it is not unexpected that we found CCAP in the hemolymph bathing the heart. Red pigment concentrating hormone (RPCH, m/z 930.5) has been seen in both *C. borealis* pericardial organ and sinus gland previously^{6, 27, 29, 32}, so it is not surprising that we found it in hemolymph, as both of these organs are known to secrete peptide hormones into the hemolymph. A previous study reported *Cancer borealis* pyrokinin I (CabPK-I, m/z 1051.6) in the brain and stomatogastric nervous system, but it was not found in the pericardial organ³³. However, its detection in the hemolymph in the current study suggests that it may be present in the pericardial organ, sinus gland, or another neurosecretory structure of the stomatogastric nervous system.

In contrast to the multiple, high-abundance cryptocyanin fragments detected in the hemolymph collected with a syringe, only trace amounts of two cryptocyanin fragments were observed in the *in vivo* microdialysates (Table 1). This distinction highlights the advantages of the microdialysis probe membrane for separating the small neuropeptides from the large proteases in hemolymph that may produce such protein fragments.

Two families of neuropeptides that we did not observe are the crustacean hyperglycemic hormone (CHH) precursor-related peptides (CPRPs) and the pigment dispersing hormones (PDHs)³⁴. Their lack of detection in this study does not necessarily mean that they are not present in the hemolymph and that they could not potentially be collected by microdialysis of hemolymph. The CPRPs and PDHs could be present at lower concentrations in the hemolymph than the other neuropeptides that were detected and presented here. Alternatively, the large size of the peptides in both of these families makes them more difficult to detect by microdialysis (due to poor recovery) and LC-MS/MS than smaller peptides. Part of the reason is that microdialysis selects for smaller peptides, since smaller peptides can more easily diffuse through the pores and thus more of them will be recovered by the probe. Another reason is the inherent difficulty in detecting and sequencing larger peptides by MS/MS, due to the limited energy that can be imparted by collision-induced dissociation. Numerous highly-charged (3 or more protons), high mass (>1700 Da) ions were detected and fragmented but could not be assigned to known neuropeptides due to the relatively poor fragmentation that was observed. Some of these ions could be novel CPRPs, PDHs, or other larger neuropeptides.

There was a wealth of neuropeptides present in the hemolymph microdialysates. Many different peptide families were represented, including the A- and B-type allatostatins, tachykinin-related peptides, orcokinin, RFamides, and RYamides. Most of these neuropeptides have been observed previously in crab pericardial organs or sinus glands, supporting their roles as hormones that are secreted from these structures into the hemolymph to act on distant targets. The application of microdialysis to study the hemolymph in a live crab has made the detection of these neuropeptides possible.

The main purpose of this study was to demonstrate the microdialysis technique on a freely-moving crab for neuropeptide detection. Future studies will focus on the capability of microdialysis for real-time monitoring of these signaling molecules. Specifically, isotopic labeling could be combined with physiological experiments to observe how neuropeptide profiles change with different physiological states. Additionally, efforts should be made to improve the detection of neuropeptides from microdialysates³⁵, either by increasing recovery through the probe or decreasing sample loss during sample treatment.

Conclusions

In this paper we have demonstrated an improved method for detecting neuropeptides from crab hemolymph. Hemolymph microdialyzed *in vitro* showed more neuropeptides and less protein fragments than the same hemolymph prepared by the typical analysis method. Further, we developed the first microdialysis method to be used *in vivo* on a freely-moving crab. With this new technique we were able to observe a wide variety of neuropeptides in the hemolymph of the pericardial region, including members of the allatostatin, orcokinin, and RFamide neuropeptide families. This result suggests that the identified neuropeptides, including GAHKNYLRFa, SDRNFLRFa, and TNRNFLRFa, are not only acting as neuromodulators but also as hormones, probably secreted from the pericardial organs and traveling through the hemolymph to their target neuronal receptors. In summary, *in vivo* microdialysis offers an alternative method of detection for neuropeptides in the crab which, when combined with current tissue-based techniques, can provide a more complete picture of how neuropeptides function in a well-defined nervous system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Photographs of the *in vivo* microdialysis setup for the Jonah crab, *Cancer borealis*. (A) *In vivo* microdialysis of crab in tank. (B) Caudal view of probe implanted in pericardial region of crab. (C) Dorsal view of dissected pericardial region. A small amount of green dye on the heart indicates the probe's location above the heart during the experiment.

Figure 2.

Overview of experiment to compare hemolymph prepared by typical methods with microdialyzed hemolymph. Two milliliters of hemolymph were withdrawn from one crab with a syringe and needle and split into two equal-volume aliquots. One aliquot underwent typical preparation methods and the other aliquot was *in vitro* microdialyzed with a homemade probe. After desalting and concentrating 100-fold, both samples were analyzed by MALDI-TOF MS on the same day.

Figure 3.
Schematic representation of probe placement in pericardial region and the glues used to stabilize the probe.

Figure 4.

MALDI mass spectra of *C. borealis* hemolymph processed using (A) the typical hemolymph analysis method and (B) *in vitro* microdialysis. Two milliliters of hemolymph were withdrawn with a syringe and split into two equal-volume samples, one for each method of analysis. After processing, both samples were concentrated 100 times and 0.4 μ L aliquots were spotted for MS analysis on MALDI-TOF/TOF instrument. Peaks were assigned based on a mass measurement accuracy of within 50 ppm of the molecular weight of the corresponding neuropeptide.

Figure 5.

The MS/MS sequencing of m/z 1508.8 from *C. borealis* hemolymph prepared by the typical analysis method. The presence of b- and y-ions was determined in PepSeq using an intensity cutoff of 0.75% of the base peak and their detection is indicated by horizontal lines above (b-ions) or below (y-ions) the corresponding amino acid in the peptide sequence. Ions that met this cutoff but were not clearly visible in the spectrum are not labeled.

Figure 6.

The product ion spectra of three neuropeptides collected from *C. borealis* hemolymph microdialysates. (A) LC-ESI-QTOF MS sequencing of *m/z* 1104.6 from the *in vitro* microdialysis of hemolymph, yielding the peptide sequence GAHKNYLRFa. LC-ESI-QTOF MS/MS sequencing of (B) *m/z* 1053.5 and (C) *m/z* 1066.6 from the *in vivo* microdialysis of hemolymph in the pericardial region of the crab. The presence of b- and y-ions was determined in PepSeq using an intensity cutoff of 0.75% of the base peak and their detection is indicated by horizontal lines above (b-ions) or below (y-ions) the corresponding amino acid in the peptide sequence. Ions that met this cutoff but were not clearly visible in the spectrum are not labeled.

Table 1
Neuropeptides Identified in *Cancer borealis* Hemolymph via Mass Spectrometric Detection

Peptide Sequence	t _R (min)	Precursor ions		Monoisotopic mass		Instrument	
		m/z	q	Measured	Theoretical	QTOF	TOF/TOF
A-type Allatostatins							
YAFGLa ^{Vi}	36.6	569.29	1	569.29	569.31	x	
YSFGLa ^{Vi}	37.1	585.28	1	585.28	585.30	x	
SPYAFGLa		753.39	1	753.39	753.39		x
DPYAFGLa		781.39	1	781.39	781.39		x
PDMYAFGLa	35.5	912.44	1	912.44	912.43	x	
pERPYSFGLa ^{Vit}		949.47	1	949.47	949.49		x
B-type Allatostatins							
NWNKFQGSWa		1165.60	1	1165.60	1165.55		x
TSWKFQGSWa	39.4	1182.59	1	1182.59	1182.57	x	
NNNWSKFQGSWa		1366.57	1	1366.57	1366.63		x
Allatostatin Combos							
DPYAFGLGKRPADL		1519.77	1	1519.77	1519.79		x
DPYAFGLGKRPADLYEFLa	12.1	532.76	4	2128.02	2128.09	x	
Tachykinin-Related Peptides							
APSGFQa	36.6	605.31	1	605.31	605.30	x	
GFLGMRa	27.6	679.37	1	679.37	679.37	x	
APSGFLGMRa	12.3	467.75	2	934.49	934.49	x	x
APSGFLGM(O)Ra		950.51	1	950.51	950.49		x
Orcokinin							
NFDEIDRSFGF	38.5	1256.58	1	1256.58	1256.55	x	
NFDEIDRSFGGF	44.2	702.34	2	1403.67	1403.62	x	
NFDEIDRSFGGFV	46.0	751.85	2	1502.69	1502.69	x	
NFDEIDRSFGGFN	41.4	759.36	2	1517.71	1517.67	x	
NFDEIDRSSFGFV		1532.66	1	1532.66	1532.70		x
NFDEIDRSFGGFH		1540.68	1	1540.68	1540.68		x
RFamides							
RSFLRFa	54.0	824.48	1	824.48	824.49	x	
RNFLRFa	24.3	426.24	2	851.47	851.50	x	
GGRNFLRFa	44.3	483.29	2	965.57	965.54	x	
DRNFLRFa	22.5	483.76	2	966.51	966.53	x	
SGRNFLRFa		995.57	1	995.57	995.55		x
APRNFLRFa		1019.58	1	1019.58	1019.59		x
SDRNFLRFa	25.4	527.29	2	1053.57	1053.56	MS/MS	x
TNRNFLRFa	24.2	533.80	2	1066.59	1066.59	MS/MS	
DGGRNFLRFa	28.2	1080.55	1	1080.55	1080.57	x	
GAHKNYLRFa *	19.3	552.80	2	1104.60	1104.61	MS/MS	x
SENRNFLRFa	41.4	1181.58	1	1181.58	1181.62	x	
RYamides							
FVGGSRYa ^{Vi}	30.7	784.43	1	784.43	784.41	x	x
SGFYANRYa ^{Vit}		976.45	1	976.45	976.46		x
pEGFYSQRYa ^{Vit}		1030.51	1	1030.51	1030.51		x
Other neuropeptides							
pQLNFSPGWa (RPCH)	14.4	465.74	2	930.47	930.45	x	x
PFCNAFTGCa (CCAP)		956.36	1	956.36	956.38		x
TNFAFSPRLa (CabPK-I) *		1051.57	1	1051.57	1051.57		x
Cryptocyanin Fragments							
YKIFEPL		909.49	1	909.49	909.51		x
KIFEPLREDNL		1373.69	1	1373.69	1373.74		x
YKIFEPLRESN ^{Typ}	23.1	465.91	3	1395.71	1395.73	MS/MS	MS/MS
YKIFEPLRESNL ^{Typ}	42.1	503.59	3	1508.77	1508.81	MS/MS	x

An “x” indicates that the mass was present when analyzed with that instrument. A mass was considered to be present if it was within 50 ppm of the theoretical mass. A “MS/MS” label means that the sequence was confirmed with tandem MS.

“Typ” indicates that the peptide was present only in hemolymph prepared by typical methods. “Vit” indicates that the peptide was present only in *in vitro* hemolymph microdialysates. No mark indicates that the peptide was present only in *in vivo* hemolymph microdialysates. “Vi” indicates that the peptide was present in both *in vitro* and *in vivo* microdialysates.

RPCH, red-pigment concentrating hormone; CCAP, crustacean cardioactive peptide; CabPK-I, *Cancer borealis* pyrokinin I.

* An indicates that the peptide was present in hemolymph analyzed by all three methods of analysis (typical preparation, *in vitro* microdialysis, and *in vivo* microdialysis).