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C-terminal methylation of truncated neuropeptides: An enzyme-assisted extraction artifact involving methanol☆

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ABSTRACT

Neuropeptides are the largest class of signaling molecules used by nervous systems. Today, neuropeptide discovery commonly involves chemical extraction from a tissue source followed by mass spectrometric characterization. Ideally, the extraction procedure accurately preserves the sequence and any inherent modifications of the native peptides. Here, we present data showing that this is not always true. Specifically, we present evidence showing that, in the lobster *Homarus americanus*, the orcokinin family members, NFDEIDRSGFG-OMe and SSEDMDRLGFG-OMe, are non-native peptides generated from full-length orcokinin precursors as the result of a highly selective peptide modification (peptide truncation with C-terminal methylation) that occurs during extraction. These peptides were observed by MALDI-FTMS and LC–Q-TOFMS analyses when eyestalk ganglia were extracted in a methanolic solvent, but not when tissues were dissected, co-crystallized with matrix, and analyzed directly with methanol excluded from the sample preparation. The identity of NFDEIDRSGFG-OMe was established using MALDI-FTMS/SORI-CID, LC–Q-TOFMS/MS, and comparison with a peptide standard. Extraction substituting deuterated methanol for methanol confirmed that the latter is the source of the C-terminal methyl group, and MS/MS confirmed the C-terminal localization of the added CD₃. Surprisingly, NFDEIDRSGFG-OMe is not produced via a chemical acid-catalyzed esterification. Instead, the methylated peptide appears to result from proteolytic truncation in the presence of methanol, as evidenced by a reduction in conversion with the addition of a protease-inhibitor cocktail; heat effectively eliminated the conversion. This unusual and highly specific extraction-derived peptide conversion exemplifies the need to consider both chemical and biochemical processes that may modify the structure of endogenous neuropeptides.

1. Introduction

Peptides are the largest class of signaling molecules used by nervous systems to modulate physiology and behavior. Members of this class of signaling agents can function as locally released neuromodulators and/or as circulating hormones. Peptides are initially synthesized as larger prepro-hormones, which undergo at least one cleavage, and often extensive post-translational modification, prior to assuming their final bioactive conformations [7].

Crustaceans, particularly members of the Decapoda, have a long history in peptide research [7]. In these animals, mass spectrometry (MS) has played a major role in peptide discovery [20,28]. The MS-based identification of neuropeptides from crustaceans has frequently relied upon matrix assisted laser desorption/ionization (MALDI)-based analysis of small tissue samples removed from an individual animal by microdissection techniques (direct tissue analysis). Alternatively, peptides can be extracted from single tissues or tissues pooled from many individuals prior to MALDI or

electrospray ionization (ESI). Regardless of method, the identification of novel neuropeptides relies upon the assumption that the tissue isolation/preparation and/or extraction procedures used accurately preserve the sequence and any inherent modifications of the native peptides.

One group of crustacean peptides that has been the subject of extensive MS investigations is the orcokinin family, members of which are typified by an overall length of 13 amino acids and the structure NFDEIDR*XXX*GF*X*, where *X* represents a variable residue [7]. First described from the crayfish *Orconectes limosus* [41], members of this peptide family have subsequently been identified from many crustacean species (summarized in [7]), with many members identified by MS-based analysis. In most crustaceans, multiple orcokinins are present, all derived from a common prepro-hormone, which is also the source of a variety of peptides in addition to the orcokinins. For example, in the American lobster *Homarus americanus*, the orcokinin precursor protein contains the orcokinins NFDEIDRSGFGFN (3 copies), NFDEIDRSGFGFH (2 copies) and NFDEIDRSGFGFV (2 copies), as well as one copy each of FDAFTTGFGHN (commonly referred to as ocomyotropin), SSEDMDRLGFGFN (an orcokinin-like peptide), GPIKVRFLSAIFIPIAAPARSSPQQDAAAGYTDGAPV, GDYDVYPE, VYG-PRDIANLY and SAE [10].

In recent MS-based analyses of *H. americanus* neural tissues, each of the full-length orcokinins was detected, as were FDAFTTGFGHN, SSEDMDRLGFGFN, GDYDVYPE, and VYGPRDIANLY [10]. In addition, a number of truncated orcokinin and orcokinin-like peptides were characterized in this study [10] and in studies by other researchers [4,6,10,27,40]. Surprisingly, in studies conducted by Li and colleagues, several truncated orcokinins seemingly not derived from the known full-length peptides, i.e. NFDEIDRS-GFA and SSEDMDRLGFA, were characterized [30]; NFDEIDRSGFA has also been sequenced via MS-analysis from the crab *Cancer borealis* [21], where it too does not correspond to any of the known full-length orcokinin isoforms. Given this discrepancy, we became interested in determining the origin of NFDEIDRSGFA and SSEDMDRLGFA in the lobster. In the data that follow, we present evidence showing that extraction with acidified methanol yields the C-terminally methylated peptides NFDEIDRSGFG-OMe and SSEDMDRLGFG-OMe, and find no evidence to support the detection of NFDEIDRSGFA and SSEDMDRLGFA in *H. americanus*, suggesting that these peptides have been mis-identified. Using high resolution MALDI-Fourier transform mass spectrometry measurements, sustained off-resonance irradiation collision-induced dissociation (SORI-CID), high performance liquid chromatography-Chip nano-electrospray ionization quadrupole time-of-flight mass spectrometry (HPLC Chip–nanoESI Q-TOF MS), and isotopic labeling, we also show that methylation at the C-terminus of these truncated peptides arises as the result of a highly selective peptide modification during neuropeptide extraction from certain crustacean tissue samples in the presence of methanol, a solvent commonly used for peptide extraction. Furthermore, we show that this modification is not a simple chemical artifact, but rather is likely an enzymatically mediated process involving methanol. Taken collectively, the data presented in this study demonstrate the need to consider that unexpected neuropeptide modifications may occur in the process of neuropeptide extraction from tissue samples, giving rise to artifactual isoforms that can be misconstrued as naturally occurring, native isoforms.

2. Materials and methods

2.1. Animals and tissue collection

American lobsters, *H. americanus*, were purchased from local suppliers (Brunswick and Harpswell, ME, USA) and maintained in
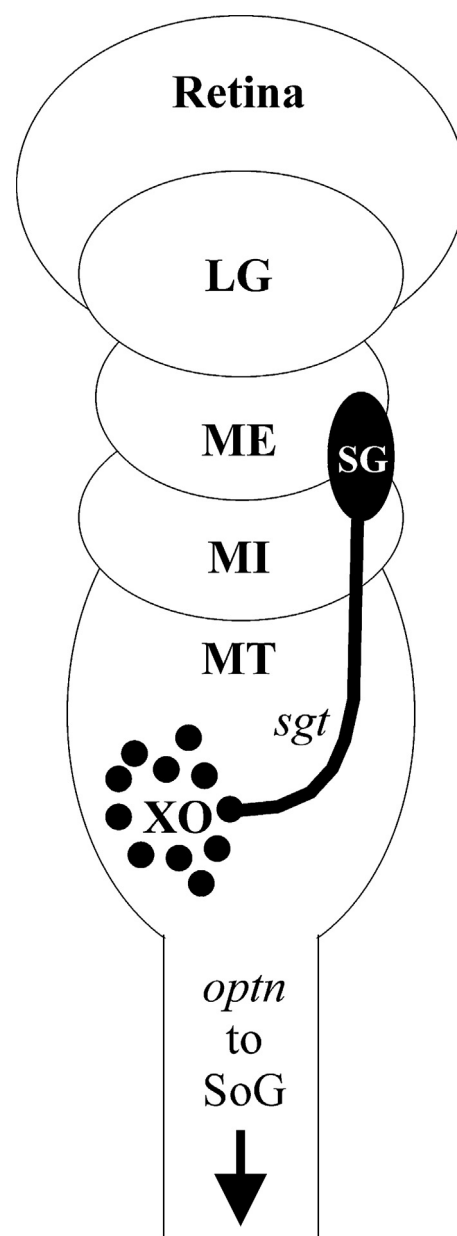
Fig. 1. Schematic diagram of the *H. americanus* crustacean optic ganglia. Distinct regions include the retina, the lamina ganglionaris (LG), the medulla externa (ME), the medulla interna (MI), the medulla terminalis (MT), the X-organ (XO), and the sinus gland (SG). The XO is a collection of neurosecretory cells located within the MT, which produce hormones that are released to the SG. The XO and SG are linked by the sinus gland tract (*sgt*). The optic nerve (*optn*) connects the eyestalk to the supraesophageal ganglion (SoG).

aerated seawater tanks at 8–10 °C. Prior to dissection, animals were anaesthetized by packing in ice for approximately 30 min. After icing, the eyestalk ganglia were isolated via manual microdissection in chilled (8–10 °C) physiological saline. For some experiments, eyestalk ganglia were divided in a regional specific manner to allow for analysis of the individual regions of this system, i.e. the medulla terminalis (MT), which includes the X-organ (XO), the medulla interna (MI), the medulla externa (ME), the lamina ganglionaris (LG), and the sinus gland (SG), a neuroendocrine organ derived from the XO somata (Fig. 1). Techniques used for the isolation of entire stomatogastric ganglion (STG) and commissural ganglion (CoG), or small pieces of the supraesophageal ganglion (brain), or pericardial organ (PO) have been described [10,11].

2.2. Sample preparation for direct tissue MALDI-FTMS analysis

For direct tissue MALDI-FTMS, dissected tissues were rinsed sequentially in two 12 μL droplets of 0.75 M D-fructose solution (reagent grade; Fisher Scientific, Pittsburgh, PA, USA) and placed on a face of a ten-faceted probe tip, minimizing co-transfers of solution. The tissue was then sliced 10–20 times with a 0.2 mm minuten pin, gathered together, and covered with a 0.5 μL droplet of 1.0 M 2,5-dihydroxybenzoic acid [DHB; Sigma–Aldrich (sublimed prior to use)] prepared in 1:1 acetonitrile:water containing 2% phosphoric acid.

2.3. Tissue extraction

2.3.1. Extraction with methanol:water:acetic acid

For most extractions in acidified methanol, the extraction solvent was 30% deionized water, 65% methanol (CH_3OH; HPLC-grade; Fisherbrand), and 5% glacial acetic acid (CH_3CO_2H; reagent grade; Sigma–Aldrich, ≥99%), as a %[v/v] mixture. A single eyestalk ganglion was rinsed sequentially in two 12 μL droplets of 0.75 M D-fructose solution, placed in a 0.6 mL low retention centrifuge tube (Fisherbrand) with 50 μL of extraction solvent (smaller volumes were used when smaller tissues were analyzed), and then homogenized by one of the following methods. In early work, the tissue was repeatedly sliced with spring scissors; in most of the work reported in this study, tissues were ground by inserting a longer, smaller diameter polypropylene tube (0.25 mL; Fisherbrand) into the 0.60 mL tube and repeatedly twisting the tube for homogenization. In some experiments, deuterated methanol (CD_3OD; 99.8% deuterated; Cambridge Isotope Laboratories, Andover, MA, USA) was substituted for the standard CH_3OH in the extraction buffer (the same solvent composition was used). After tissue homogenization, the sample was sonicated for 2–5 min and centrifuged at 15k rpm for 5–15 min. The supernatant was removed from the sample and placed in another 0.6 mL tube. In early experiments, samples were delipidated prior to analysis. For delipidation, 25 μL of nanopure water was added to the supernatant along with 25 μL chloroform (NMR-grade $^{13}CDCl_3$; Cambridge Isotope Laboratories) in order to remove lipids from the aqueous solution. The two layers were sonicated for 2 min and centrifuged for 10 min. The bottom organic layer was removed. Chloroform was added and the extraction was repeated two more times, but with a 5-min centrifugation.

The aqueous layer was either stored at −20 °C or concentrated to dryness in a SpeedVac vacuum concentrator (UVS400 Universal Vacuum System, Thermo Electron Corporation) at 36 °C. Once dried, the extract was reconstituted to a total volume of 50 μL in 1:1 ACN:H_2O in preparation for analysis by MALDI-FTMS or HPLC Chip–nanoESI Q-TOF MS. For some samples analyzed by MALDI-FTMS, the extracts, reconstituted in 0.1% TFA water, were desalted using C18 ZipTip pipette tips (Millipore, Billerica, MA, USA). For MALDI-FTMS analysis of extracts, 0.5 μL of the extract was mixed with 0.5 μL of DHB matrix on one face of the MALDI probe and the extract–matrix mixture was allowed to co-crystallize.

2.3.2. Extraction with acetone:water:hydrochloric acid or saturated DHB

For extractions in acidified acetone (85% acetone [Sigma–Aldrich, ≥99%], 13% deionized water, and 2% HCl [reagent grade; Fisherbrand], as a %[v/v] mixture, a single eyestalk ganglion was rinsed sequentially in two 12 μL droplets of 0.75 M D-fructose solution and placed in a 0.6 mL low retention centrifuge tube (Fisherbrand) with 50 μL of extraction solvent. The tissue was ground by inserting a longer, smaller diameter polypropylene tube (0.25 mL; Fisherbrand) into the 0.60 mL tube and repeatedly twisting the tube for homogenization. After tissue homogenization, the

sample was sonicated for 2–5 min and centrifuged at 15k rpm for 5–15 min. The supernatant was removed from the sample and dried prior to being reconstituted in 1:1 ACN:H_2O in preparation for analysis by MALDI-FTMS.

For extraction in saturated DHB, the extraction protocol described above was followed, using 50 μL of a freshly prepared, saturated solution of DHB in deionized water as the extraction solvent.

2.3.3. Extractions with protease inhibitors

Paired eyestalk ganglia were dissected from individual lobsters, with the ganglion from one eyestalk used as a control and the ganglion from the second used as a test to determine if a protease inhibitor cocktail, included in the extraction protocol, reduces or eliminates the C-terminal methylation reaction. The protease inhibitor cocktail was prepared by dissolving one tablet (complete, Mini; Roche Applied Science, Indianapolis, IN, USA) in 1.5 mL deionized water to prepare a stock solution, which was further diluted 1:7 with deionized water. In initial experiments, the control eyestalk tissue was homogenized in normal extraction solvent (65:30:5, methanol:water:acetic acid), while the test eyestalk tissue was homogenized in extraction solvent in which water had been replaced with protease inhibitor cocktail solution. After homogenization, the tissues were sonicated for 5 min, centrifuged for 15 min, and the supernatant was removed from the tissue pellet. In later experiments, the control tissue was first homogenized and sonicated in 30 μL of nanograde water; the test tissue was homogenized and sonicated in 30 μL protease inhibitor cocktail solution. Then, 65 μL of methanol and 5 μL of glacial acetic acid were added to each tube. The samples were resonicated and centrifuged; the supernatant was then removed from the tissue pellet. Most samples were dried and subjected to ZipTip purification prior to analysis.

2.3.4. Extractions with heat

Paired eyestalk ganglia were dissected from individual lobsters, with the ganglion from one eyestalk used as a control and the ganglion from the second used to test the effect of submerging the tissue in boiling water prior to homogenization. Each tissue was placed in 50 μL of normal extraction solvent. The control tissue sample sat at room temperature for 5 min; the test tissue sample was placed in a boiling water bath for 5 min. The two samples were then homogenized, sonicated, centrifuged and the supernatant was removed from the tissue pellet.

2.3.5. Liquid nitrogen freezing of tissues

Prior to the standard tissue extraction procedure detailed above, the ganglion from one eyestalk was immediately placed in a beaker of liquid nitrogen with forceps for 15 s in order to freeze the tissue. The tissue was then placed in a 0.6 mL microcentrifuge tube and homogenized by grinding with a smaller centrifuge tube. Then 30 μL of extraction solvent was added to each tissue sample and the tissues were then extracted as described above.

2.4. Standards with extraction solvent

To determine whether methyl esterification occurs with synthetic standards, 2 μL of 10^{-3} M [Asn^{13}]-orcokinin or Orc[1-11] (NFDEIDRSGFGFN or NFDEIDRSGFG, respectively; GenScript Corporation, Scotch Plain, NJ, USA) was mixed with 30 μL of either CH_3OH:water:acetic acid (65:30:5), CD_3OD:water:acetic acid (65:30:5), or nanopure water. The solutions sat at room temperature for 23 h before they were dried, reconstituted with 25 μL 1:1 ACN:water, and analyzed by MALDI-FTMS.

2.5. [Ala13]-orcokinin standard added to tissue and extraction solvent

To determine whether an exogenous orcokinin peptide undergoes truncation and C-terminal methylation, 1 nmol of a synthetic [Ala13]-orcokinin standard (NFDEIDRSGFGFA, a gift from Drs. L. Li [University of Wisconsin-Madison] and E. Marder [Brandeis University]) was added to two 0.6 mL microcentrifuge tubes, each containing 50 μL of extraction solvent [CH$_3$OH:water:acetic acid (65:30:5)]. The first tube contained only the solvent and standard; to the second tube, one eyestalk ganglion was added and homogenized. Both samples were sonicated for 5 min and centrifuged for 15 min; the solvent fraction was then analyzed by MALDI-FTMS. The solvent in the tissue-containing samples was analyzed without separating the supernatant from the tissue pellet. An additional 1 nmol of the [Ala13]-orcokinin standard was added to the tissue/extraction solvent mixture, and the sample was resonicated and centrifuged and analyzed before and after sitting at room temperature overnight.

2.6. Tryptic digestion of NFDEIDRAAFGFA in methanolic buffer

To determine whether C-terminal methylation can compete with hydrolysis by trypsin, 1 nmol of NFDEIDRAAFGFA was mixed with 0.09 nmol of bovine trypsin (Sigma–Aldrich) in 25 mM, pH = 4.0, citrate buffer prepared with water or 25% methanol. The digestion proceeded at room temperature for 1–4 days with analysis by HPLC Chip–nanoESI Q-TOF MS.

2.7. Instrumentation

2.7.1. MALDI-FTMS

Most samples were analyzed using a HiResMALDI Fourier transform mass spectrometer (Varian, Lake Forest, CA, USA) with a Cryomagnetics (Oak Ridge, TN, USA) 4.7 Tesla actively shielded superconducting magnet. Ions were generated using a pulsed nitrogen laser (337 nm) and were transported from the external ion source to the closed cylindrical ICR cell using a quadrupole ion guide. The ion guide radio frequency potential and trapping delay time were optimized to transmit and trap ions of a selected mass range (optimized for m/z 1500 for the results presented here). A pulse of argon was introduced to the vacuum system during trapping to elevate the system pressure transiently for collisional cooling. All spectra were measured using ion accumulation techniques, where ions from 7 to 30 successive laser shots were accumulated in the cell. A delay of 5–10 s preceded ion detection, which occurred with analyzer pressures of $1–2 \times 10^{-10}$ Torr. Transients (4 or 10 s) from direct tissue spectra were apodized using a Blackman function and zero-filled prior to fast Fourier transformation; SORI-CID spectra (see below) were processed without apodization.

Mass spectra were calibrated using the m/z values for two previously identified peptides [2]: APSGFLGMRamide (C. borealis tachykinin-related peptide I [CabTRP I]; m/z = 934.4927) and VYRKPPFNGSIFamide (Val1-SIFamide; m/z = 1423.7845). Peaks for these two peptides were present in the spectra and served as internal calibrants.

For SORI-CID experiments, argon was used as the collision gas, the frequency offset was set to −1.8% of the reduced cyclotron frequency and the voltage amplitude was in the range of 6–8.5 V$_{bp}$. SORI-CID spectra were calibrated externally, with a one-point adjustment based upon a [MH−NH$_3$]$^+$ fragment mass. The standards NFDEIDRSGFGA and NFDEIDRSGFG-OMe were characterized by SORI-CID.

2.7.2. HPLC Chip–nanoESI-Q-TOF MS

Mass spectrometric analysis was performed using a 6530 quadrupole time-of-flight (Q-TOF) mass analyzer (Agilent Technologies, Santa Clara, CA). Mass spectra (MS and MS/MS) were collected in positive ion mode; the ionization voltage ranged from 1850 to 1950 V and the ion source temperature was held at 350 °C. Spectra were internally calibrated using methyl stearate (C$_{17}$H$_{35}$CO$_2$CH$_3$) and hexakis(1H, 1H, 4H-hexafluorobutyloxy)phosphazine (HP-1221; C$_{24}$H$_{18}$O$_6$N$_3$P$_3$F$_{36}$), continuously infused and detected as [M+H]$^+$. CID-MS/MS experiments were executed with precursor ions subjected to CID using nitrogen as the target gas with the collision energy set to 26 V. Chromatographic separation and nano-electrospray ionization (ESI) were performed using a 1260 Chip Cube System (Agilent Technologies) using a ProtID-chip with a 40 nL enrichment column and a 43 or 150 mm × 75 μm analytical column (Agilent Technologies). The enrichment and analytical columns were packed with 300 Å, 5 μm particles with C18 stationary phase. The mobile phases were 0.1% formic acid/H$_2$O (A) and 0.1% formic acid/acetonitrile (B). Samples (0.2–2 μL) were loaded on the enrichment column using 98:2 (A:B) at 4 μL/min. Tryptic digest samples were analyzed with the 43 mm analytical column using a gradient of 98:2 (A:B) to 20:80 (A:B) over a period of 12 min at 0.6 μL/min. Eyestalk extracts and peptide standards were analyzed with the 150 mm analytical column using a linear gradient of 98:2 (A:B) to 65:35 (A:B) over a period of 5 min, to 40:60 (A:B) at 25 min and 2:98 (A:B) at 35 min using a flow rate of 0.3 μL/min.

2.8. Data analysis and figure production

Calibrated mass spectral peak lists were generated using the Omega 8.0 software (IonSpec, Lake Forest, CA, USA). MALDI-FTMS figures were generated using the Boston University Data Analysis software (B.U.D.A.; provided by Dr. Peter O'Connor, University of Warwick, Department of Chemistry, Coventry, England) to produce graphics that were imported into CorelDRAW X4 for final figure production. HPLC Chip–nanoESI Q-TOF MS figures were generated by exporting Mass Hunter (Agilent) chromatograms or mass spectra as metafiles and importing these graphics into CorelDRAW X4 for final figure production.

3. Results

The paired eyestalks of the lobster, H. americanus, and other crustaceans are composed of several distinct regions (shown schematically in Fig. 1) that contain unique complements of neuropeptides [18]. Only the SG of H. americanus has been characterized by mass spectrometry [6,8,10,15,30], and this tissue has been found to be a rich source of crustacean neuropeptides. This study was originally initiated in an attempt to more fully characterize the complement of neuropeptides present in different regions of H. americanus optic (eyestalk) ganglia, using MALDI-FTMS and the direct analysis of tissue samples, complemented by the analysis of tissue extracts. For this study, the analysis of tissue extracts was needed to characterize tissues that were too large to be fully characterized directly. We also planned to extract and analyze entire optic ganglia to obtain a full measure of the neuropeptide components. For the extraction of neuropeptides from tissue samples, we used an approach applied in previous studies in our lab, which involved microdissection of the desired tissue, tissue homogenization in a methanolic solvent mixture (65:30:5, methanol:water:glacial acetic acid for the work reported here), followed by sonication and centrifugation prior to MALDI-FTMS analysis. In early experiments, samples were delipidated with chloroform. This step, which
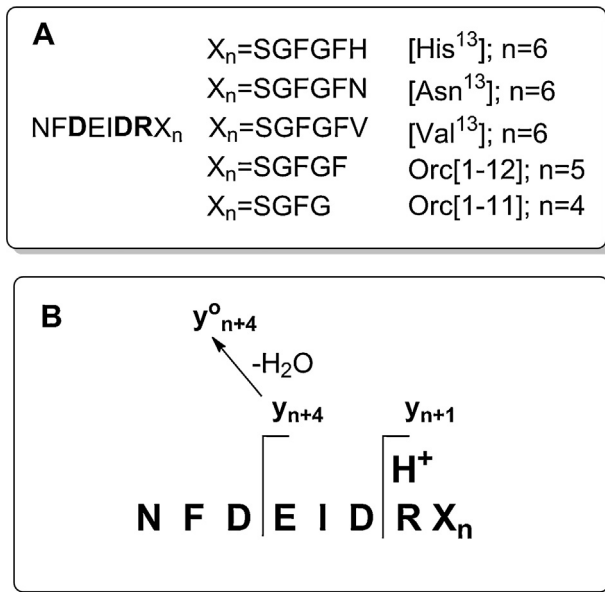
Fig. 2. (A) A summary of *H. americanus* full-length ([Asn13], [His13], and [Val13]) and truncated (Orc[1-12] and Orc[1-11]) orcokinin family peptides relevant to this study. (B) A summary of ions produced from orcokinin family peptides via metastable decomposition upon analysis by vacuum UV-MALDI-FTMS or by SORI-CID.

did not impact our results, was eliminated for most work reported here.

In previous studies, we have used MALDI-FTMS to analyze individual *H. americanus* SGs directly, or following single tissue extraction of a single gland [10]. A comparative analysis of neuropeptide profiles for each mode of sample preparation showed good agreement in terms of the neuropeptides detected and their relative abundance. Our work [10,43] and reports by other researchers [6,15,30] have consistently shown that orcokinin family peptides are abundant neuropeptides present in this tissue. A summary of the full-length orcokinin family peptides ([Asn13]-, [His13]-, and [Val13]-orcokinin) predicted by molecular cloning [10] and observed in our previous work with *H. americanus* appears in Fig. 2A. Two additional peptides encoded by the orcokinin gene and detected by mass spectrometry are the orcomyotropin peptide FDAFTTGFGHN (*m/z* 1213.53) and the orcokinin-related peptide SSEDMDRLGFGFN (*m/z* 1474.63). Additional truncated orcokinins, including Orc[1-12] and Orc[1-11] (Fig. 2A) have been observed mass spectrometrically in our lab [10,43] and by other researchers [6,15,30,40].

An important aspect of the work described below is an appreciation of the nature of the neuropeptide signals produced on our MALDI-FTMS instrument, which is particularly relevant to the identification of orcokinin family peptides. As described in previous publications [10,43], orcokinin family peptides produce a unique mass spectral signature, characterized by the fragment ions summarized in Fig. 2B, when analyzed by our vacuum UV-MALDI-FTMS instrument. The mass spectral signature is a consequence of the orcokinin amino acid sequence, which incorporates a basic, proton sequestering, arginine (R) residue and two acidic aspartate (D) residues. When a peptide sequence containing this amino acid combination is coupled with the energy imparted by the vacuum UV-MALDI ionization and the long trapping times required for FTMS analysis (>10 s), singly protonated orcokinin family peptides undergo so-called "Asp-Xxx cleavages" [47], which result in the production of characteristic C-terminal (y-type) fragments (see Fig. 2B). Our identification of orcokinin family peptides by MALDI-FTMS relies on the detection of both the [M+H]+ ion and the
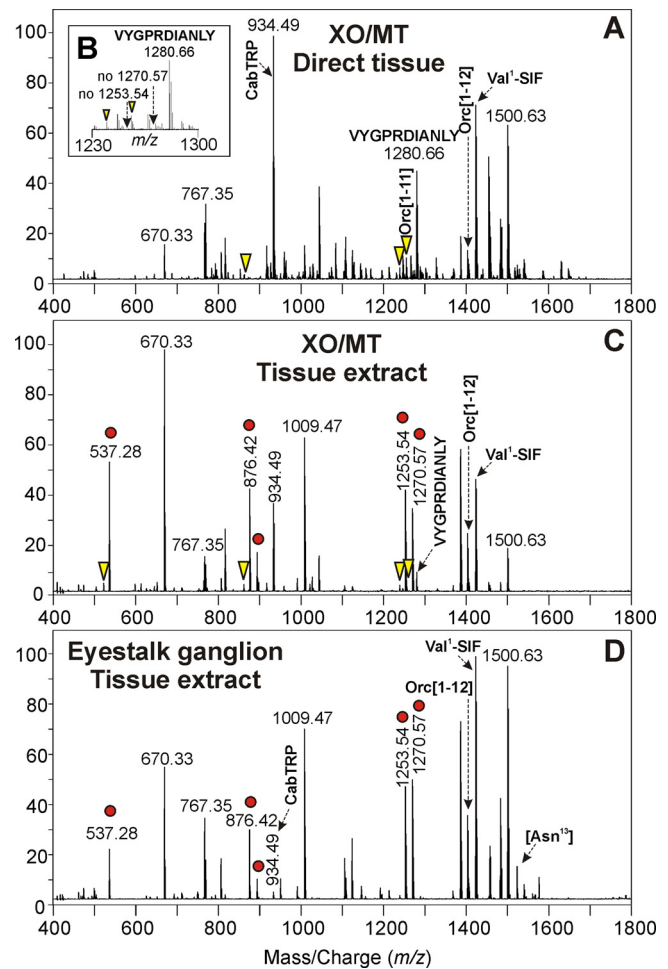

Fig. 3. MALDI-FT mass spectra of *H. americanus* eyestalk tissue and tissue extracts. (A) Spectrum from a small piece of freshly dissected tissue from the XO/MT region of the eyestalk. (B) An expansion of spectrum (A) showing the region where the peaks at *m/z* 1270.56 and 1253.54 ([M+H]+ and [MH−NH₃]+ from an orcokinin family peptide) would appear, if detected. (C) Spectrum of XO/MT tissue extracted with methanol:water:acetic acid (65:30:5). (D) Entire eyestalk ganglion extracted with methanol:water:acetic acid (65:30:5). All spectra were measured using DHB as the matrix and conditions optimized for *m/z* 1500. Spectra were calibrated using known peptide peaks [42], including APSGFLGMRamide (CabTRP) and VYRKPPFNGSIFamide (Val1-SIFamide) at *m/z* 934.4927 and *m/z* 1423.7845, respectively. The peaks at *m/z* 1270.57, 1253.54, 894.43, 876.42, and 537.28 (labeled with filled circles) in (C) and (D) were attributed to an orcokinin family peptide that was not detected in directly analyzed tissues, such as (A); peaks for Orc[1-11] at *m/z* 1256.55, 1239.53, 862.41, and 523.26 are labeled with filled triangles.

observation of characteristic y-type ions resulting from Asp-Xxx cleavages.

3.1. MALDI-FTMS analysis of eyestalk tissue extracts shows an orcokinin family peptide not predicted by genomics

When we analyzed small pieces of eyestalk ganglion tissues directly by MALDI-FTMS, we detected neuropeptide peak profiles that reflected differential distributions of neuropeptides in localized regions of the eyestalk ganglia. For example, the peptides CabTRP I (APSGFLGMRamide at *m/z* 934.49) and Val1-SIF (VYRKPPFNGSIFamide at *m/z* 1423.78) were detected in tissues from the LG, XO/MT, MI, and ME but not in the SG. Orcokinin family peptides were detected in many tissues, including the XO/MT, MI, ME, and SG. A representative spectrum from a small piece of XO/MT tissue is shown in Fig. 3A.

In contrast with previous studies [10], where we found good agreement between single tissues analyzed directly and by single tissue extraction, our analysis of extracts of tissues from the aforementioned regions of the eyestalk ganglion revealed the presence of a new peptide that had not been detected by direct tissue MALDI-FTMS. For example, Fig. 3C shows the spectrum observed when a small piece of XO/MT tissue was removed by microdissection techniques, placed in extraction solvent, homogenized, sonicated, and centrifuged. While we continue to detect peaks for CabTRP I and Val[1]-SIF, an abundant signal at m/z 1270.57 was observed, which was detected in combination with additional peaks showing the characteristic orcokinin family pattern. The full collection of peaks appeared at m/z 1270.57, 1253.54, 894.43, 876.42, and 537.28; these peaks were assigned to [M+H]+, [MH−NH3]+, y_{n+4}, y^o_{n+4}, and y_{n+1}. Unexpectedly, these masses did not correspond to any orcokinin family members predicted from genomic information for *H. americanus* [10], nor did they correspond to masses expected from conventional post-translational modifications, including the truncation of full-length orcokinin family peptides. Instead, exact mass measurements (m/z 1270.5692, measured) were consistent with the orcokinin sequence, NFDEIDRSGFA (Orc[Ala[11]]; m/z 1270.5699, predicted). This peptide has been detected in other studies [4,16,19,30,31] with the first characterization, from the crab, *C. borealis*, reported in a study by Huybrechts et al. [21]; later reports include the detection of Orc[Ala[11]] in nervous system tissues and neuroendocrine organs from *H. americanus* [30]. While C-terminally truncated versions of full-length *H. americanus* orcokinin-family peptides, including Orc[1-12] and Orc[1-11] (Fig. 2A), detected in XO/MT extract (Fig. 3C) and direct tissue (Fig. 3A and B) spectrum, have been also been reported by our laboratory [10] and by other researchers [4,6,10,27,40], the alanine-containing peptide sequence is unusual because an alanine residue at this position is not known for any full-length orcokinins detected mass spectrometrically or predicted from genomic information.

When we analyzed the extract from an entire eyestalk ganglion, we again detected peaks for the m/z 1270.57, putative Orc[Ala[11]], peptide (see Fig. 3D). To ensure that the detection of this peptide was not the result of a mutation specific to the individual animal analyzed, localized tissue samples and entire eyestalk ganglia from additional individuals ($n > 30$) were extracted. Although the abundance of the m/z 1270.57 and other putative Orc[Ala[11]]-derived peaks varied relative to that of other detected peptides, signals for this peptide were consistently observed, except in extracted sinus gland samples, where these signals were weak or missing.

3.2. MALDI-FTMS with SORI-CID supports the identification of NFDEIDRSGFG-OMe, not NFDEIDRSGFA

To further characterize the amino acid sequence of the peptide appearing at m/z 1270.57, we subjected the peak to analysis by SORI-CID, the form of MS/MS used on our FTMS instrument. Isolation of the [M+H]+ at m/z 1270.57 from an eyestalk ganglion extract followed by SORI-CID yielded a spectrum showing an abundant peak at m/z 1253.54 (loss of NH3) and the production of y-type sequence ions, including the Asp-Xxx cleavage products at y_8, y^o_8, and y_5 (m/z 894.43, 876.42, and 537.28, respectively; see Fig. 4A). This experiment provided support for our assignment of the m/z 1270.57 peak as an ionized orcokinin family peptide and, furthermore, supported our attribution of the m/z 1253.54, 894.43, 876.42, and 537.28 peaks in the MALDI-FT mass spectrum of tissue extracts to this gas-phase precursor. However, the SORI-CID mass spectrum did not provide sufficient information to establish the full amino acid sequence.

In previous studies [43], we have shown that the variable C-terminal sequence of orcokinin-family peptides can be established by using the mass spectrometric isolation and dissociation of the
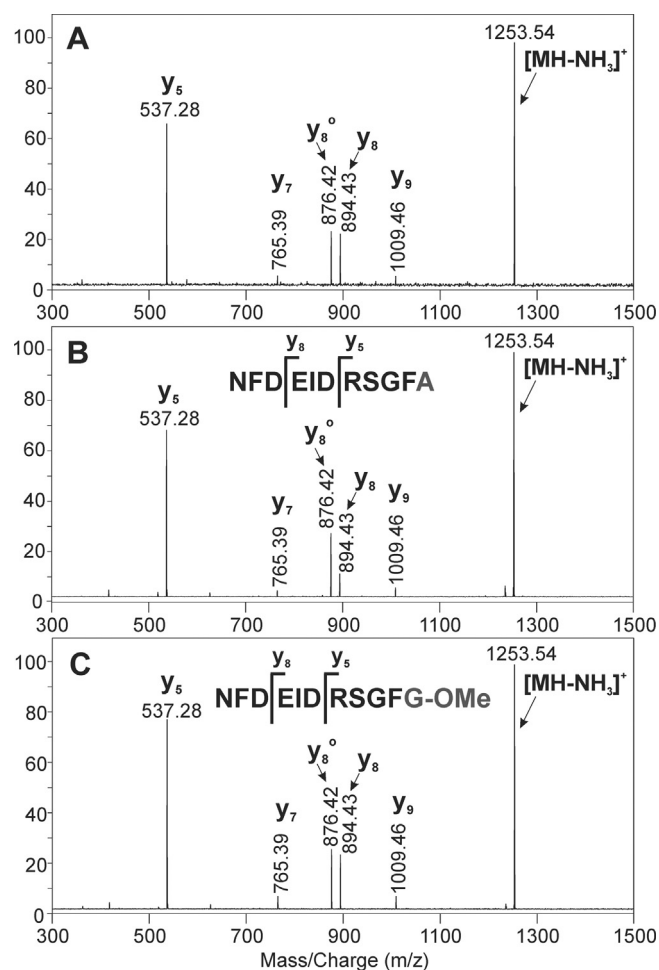

Fig. 4. SORI-CID of the [M+H]+ ion at m/z 1270.57 detected in a *H. americanus* eyestalk extract, compared with spectra derived with peptide standards, shows better agreement with that derived from the NFDEIDRSGFG-OMe standard; however, the spectrum for NFDEIDRSGFA is also very similar. (A) The SORI-CID mass spectrum of the m/z 1270.57 peak originating from the MALDI-FTMS analysis of a *H. americanus* eyestalk ganglion extract. (B) The SORI-CID mass spectrum of the [M+H]+, m/z 1270.57 peak produced from an Orc[Ala[11]] standard. (C) The SORI-CID mass spectrum of the [M+H]+, m/z 1270.57 peak produced from an Orc[1-11]-OMe standard. Each m/z 1270.57 peak was dissociated following ion isolation. Argon was used as the collision gas with an excitation amplitude of 6.5 V_{bp}. The y-type fragment ions are identified using the nomenclature established by Roepstorff [36].

y_{n+1} fragment by SORI-CID. The y_{n+1} fragment, produced via Asp-Xxx cleavage, contains the arginine (R) residue at the N-terminus and yields b-type sequence ions, which retain the N-terminal, arginine-containing, end of the peptide sequence. When the y_5 peak at m/z 537 was isolated and subjected to SORI-CID interrogation, we measured peaks, including b_1, b_2-NH3, b_3, and b_4 at m/z 157.11, 227.11, 301.16, and 448.23, respectively, that are consistent with the sequence RSGF (Fig. 5A). Other peaks in the spectrum (m/z 472.23, 489.26, 502.24) resulted from combinations of small neutral molecule losses (NH3, H2O, and CH2O). Working from the mass of the b_4 ion, the remaining mass (89.047 Da) is accounted for by an alanine (A) residue (71.037 Da) and water (18.011 Da), consistent with the sequence for putative Orc[Ala[11]]; however, we were surprised that the mass spectrum did not show a [b4+H2O]+ product ion at m/z 466.24. The [bn+H2O]+ ion is a C-terminal fragment that we have detected at >30% abundance in the SORI-CID spectra of y_{n+1} ions derived from many orcokinin family peptides, including Orc[1-12] [43], [Val[13]] [43], and [Ala[13]] (data not shown). The absence of this characteristic peak led us to question the sequence assigned to putative Orc[Ala[11]].
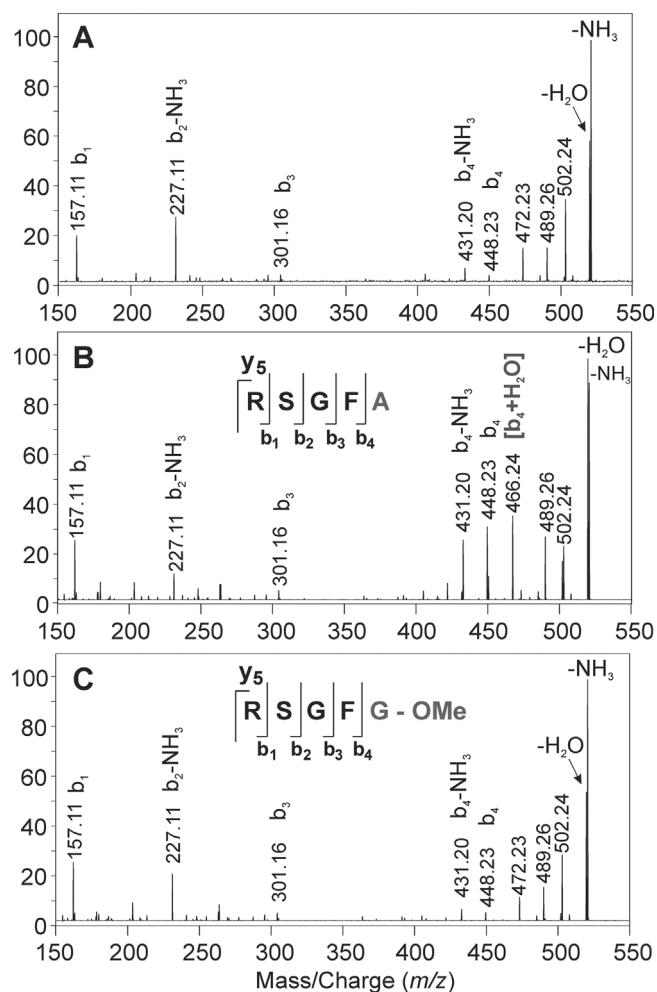
Fig. 5. SORI-CID of the y_5 ion at m/z 537.28 detected in *H. americanus* eyestalk extracts, and the comparison with spectra derived from peptide standards, demonstrates that the tissue-derived peptide sequence is NFDEIDRSGFG-OMe, not NFDEIDRSGFA. (A) The SORI-CID mass spectrum of the m/z 537.28 peak originating from the MALDI-FTMS analysis of a *H. americanus* eyestalk ganglion extract, showing no evidence for a $[b_4+H_2O]^+$ ion at m/z 466.24. (B) The SORI-CID mass spectrum of the y_5 peak at m/z 537.28, which was produced from an Orc[Ala[11]] standard. The spectrum shows the $[b_4+H_2O]^+$ ion at m/z 466.24, which is generated by fragmentation of peptides with a free acid at the C-terminus. (C) The SORI-CID mass spectrum of the $[M+H]^+$, m/z 537.28, y_5 peak produced from an Orc[1-11]-OMe standard. The $[b_4+H_2O]^+$ ion at m/z 466.24 is absent because methylation of the C-terminus blocks the rearrangement [45] required for ion formation. Each m/z 537.28 peak was dissociated following ion isolation. Argon was used as the collision gas with an excitation amplitude of 6.5 V_{bp}. The y- and b-type fragment ions are identified using the nomenclature established by Roepstorff [36].

To more conclusively establish if the m/z 1270.57 peptide is, in fact, Orc[Ala[11]], we measured SORI-CID mass spectra for a synthetic form of the peptide. As shown in Fig. 4B, SORI-CID of the m/z 1270.57, $[M+H]^+$, peak yields a spectrum that closely resembles that of the eyestalk extract-derived peptide; however, we note that the intensity of the y_8 peak (m/z 894.43) for the standard (Fig. 4B) is consistently lower than that observed for the putative Orc[Ala[11]] peptide (Fig. 4A). While this mass spectral difference was reproducible, the fact that the mass spectrum is dominated by Asp-Xxx cleavage ions (y_8, y_8^o, and y_5) limited our ability to carry out a more detailed comparison of structural features. In contrast, SORI-CID of the y_5 peak at m/z 537.28 proved to be more revealing. While similar ions and ion intensities were detected in the lower m/z range of the spectrum, more significant differences in ion intensities and ion identity were observed at higher m/z values (Fig. 5B). Most notably, the SORI-CID spectrum of the Orc[Ala[11]]-derived y_5 peak

(Fig. 5B) shows an abundant $[b_4+H_2O]^+$ product ion at m/z 466.24, which was not detected in the spectrum of the eyestalk extract-derived peptide (Fig. 5A). Production of the $[b_4+H_2O]^+$ ion from the Orc[Ala[11]]-derived y_5 ion is congruent with predicted fragmentation behavior, based upon studies of other orcokinin peptides (described above). The fact that this peak is not detected in the spectrum of putative Orc[Ala[11]] provides defining evidence that our eyestalk extract-derived peptide is not Orc[Ala[11]].

Furthermore, when structural elements that would block formation of the $[b_4+H_2O]^+$ ion are considered, we are able to propose a sequence for the eyestalk-derived m/z 1270.56 orcokinin peptide. Specifically, the mechanism responsible for the production of $[b_{n-1}+H_2O]^+$ product ions has been investigated, and it is known that ion formation involves a rearrangement at the C-terminus that requires a free C-terminal carboxyl group [45]. This rearrangement is prevented when the C-terminus is blocked by amidation or esterification. Based upon this information, we hypothesized that the m/z 1270.57, eyestalk extract-derived peptide was not Orc[Ala[11]], but was, instead, NFDEIDRSGFG-OMe (also m/z 1270.57), a C-terminally truncated orcokinin that has been specifically methylated at the C-terminus. To test this hypothesis, we carried out the SORI-CID characterization of synthetic Orc[1-11]-OMe (Figs. 4C and 5C). The SORI-CID mass spectra for both the m/z 1270.57 (Fig. 4C) and 537.28 (Fig. 5C) peaks derived from synthetic Orc[1-11]-OMe showed excellent agreement with spectra from the eyestalk extract-derived peptide (Figs. 4A and 5A), particularly with respect to the absence of the diagnostic $[b_4+H_2O]^+$ ion, which provided strong support for our hypothesis regarding the identity of this peptide.

3.3. LC/MS analysis of tissue extracts provides additional support for the identification of Orc[1-11]-OMe, not Orc[Ala[11]], based upon chromatographic retention times

To provide further evidence in support of our identification of Orc[1-11]-OMe, we analyzed the extract from a single *H. americanus* eyestalk tissue by HPLC Chip–nanoESI Q-TOF MS (Fig. 6A and B). Under the same chromatographic conditions, we analyzed standards of Orc[Ala[11]] (Fig. 6C) and Orc[1-11]-OMe (Fig. 6D). The analysis of the eyestalk extract revealed the presence of a single peak at 16.5 min in the extracted ion chromatogram (EIC) for the m/z 635.789, $[M+2H]^{2+}$, ion for the isobaric Orc[1-11]-OMe or Orc[Ala[11]] (Fig. 6B). A comparison with the retention times for the Orc[Ala[11]] (Fig. 6C) and Orc[1-11]-OMe (Fig. 6D) standards showed that Orc[1-11]-OMe elutes at the same time as the eyestalk extract peptide (16.5 min), while Orc[Ala[11]] elutes at an earlier time (15.5 min). The enhanced retention for Orc[1-11]-OMe relative to Orc[Ala[11]] is expected given the higher hydrophobicity of the C-terminal ester group compared with that of the free acid. Additional confirmation of our identification of putative Orc[1-11]-OMe was provided by enriching the eyestalk sample with Orc[1-11]-OMe standard and observing signal enhancement at the retention time for the eyestalk peak at 16.5 min (data not shown). These experiments provided additional support for our identification of Orc[1-11]-OMe in eyestalk tissue extracts.

3.4. Q-TOF-CID analysis yields MS/MS spectra that are not useful for distinguishing Orc[1-11]-OMe and Orc[Ala[11]]

While LC retention times proved to be diagnostic for distinguishing Orc[1-11]-OMe and Orc[Ala[11]], CID experiments carried out by HPLC Chip–nanoESI Q-TOF MS yielded MS/MS spectra for the two standard and the eyestalk-extract peptide that were virtually indistinguishable (see Fig. 7A, E, and G). In contrast with SORI-CID mass spectra of these compounds, where dissociation of the m/z 1270.56, $[M+H]^+$ precursor ions (Fig. 4A–C) provided very limited
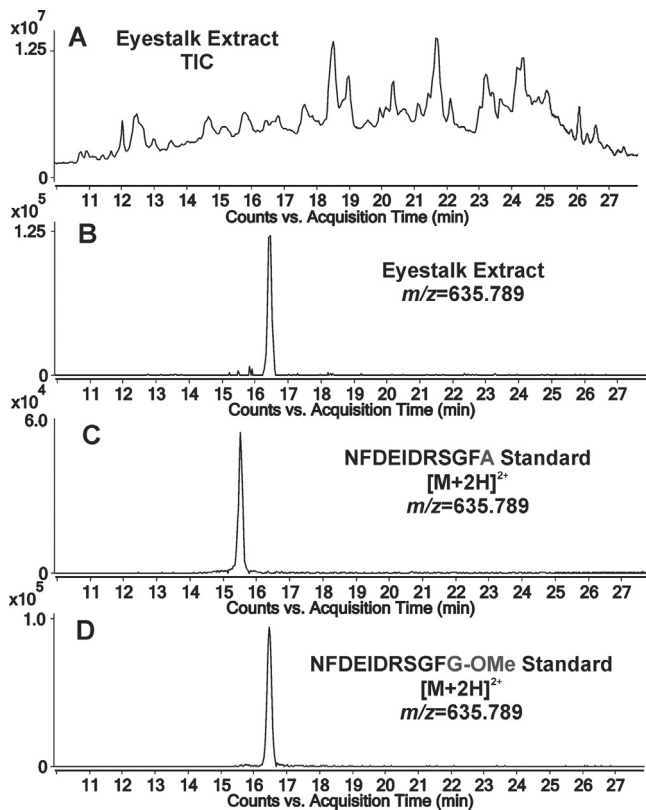
Fig. 6. Chromatograms from HPLC Chip–nanoESI Q-TOF MS analyses, which show the *H. americanus* eyestalk ganglion-derived peptide eluting at the same time as NFDEIDRSGFG-OMe, not NFDEIDRSGFA. (A) Total ionization chromatogram (TIC) for a single eyestalk ganglion extracted with methanol:water:acetic acid (65:30:5). (B–D) Extracted ion chromatograms (EICs) for the $m/z = 635.789$, $[M+2H]^{2+}$, ion characteristic of isobaric Orc[1-11]-OMe and Orc[Ala^{11}]. (B) EIC of the extract from a single *H. americanus* eyestalk ganglion showing a single peak that elutes with the same retention time as Orc[1-11]-OMe. (C) EIC for an Orc[Ala^{11}] standard, which elute with a shorter retention time than Orc[1-11]-OMe. (D) EIC for an Orc[1-11]-OMe standard. EICs were generated using a 15 ppm mass window.

sequence information as a consequence of proton localization by the charge-sequestering arginine residue, dissociation of the m/z 635.79, $[M+2H]^{2+}$, precursor ions on the Q-TOF instrument yielded MS/MS spectra that provided excellent sequence coverage through the formation of y- and b-type ions (see Fig. 7A, E, and G); however, the MS/MS spectra still precluded structural differentiation because product ion masses were identical (Ala and G-OMe are isobaric) and the structurally similar residues did not influence relative ion intensities in ways that were useful for distinguishing the two peptide sequences.

3.5. Methanol in the extraction solvent is the source of added methyl group

Because our extraction solvent contains 65% methanol, the methanolic solvent was hypothesized to play a role in the production of Orc[1-11]-OMe. To determine if this was the case, we carried out tissue extractions using CD_3OD, instead of CH_3OH, in the extraction solvent. To eliminate variations that may result when comparing tissues extracted from different individuals, the paired eyestalk tissues were removed from one lobster. One eyestalk ganglion was placed in extraction solvent containing CH_3OH and the other in extraction solvent containing CD_3OD. The samples were homogenized, sonicated, and centrifuged, and the supernatant was separated from the tissue pellet. The samples were dried and reconstituted for analysis by MALDI-FTMS. The MALDI-FT
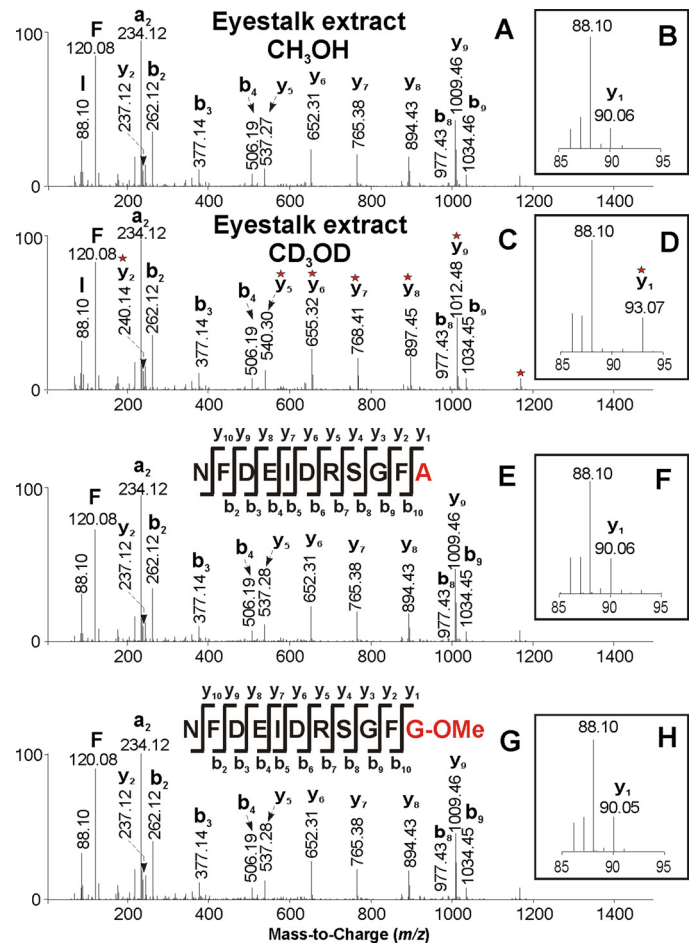

Fig. 7. HPLC Chip–nanoESI Q-TOF MS/MS spectra showing that Q-TOF MS/MS spectra are not useful for distinguishing the isobaric peptide sequences NFDEIDRSGFG-OMe and NFDEIDRSGFA, but do provide support for the sequence and C-terminal localization of the added methyl group when the peptide NFDEIDRSGFG-OCD$_3$ is produced by extraction in CD$_3$OD-containing solvent. (A) CID-MS/MS spectra for the $m/z = 635.789$, $[M+2H]^{2+}$, ion characteristic of isobaric Orc[1-11]-OMe and Orc[Ala^{11}] for the *H. americanus* eyestalk ganglion-derived peptide eluting at 16.5 min; single eyestalk ganglion extracted with CH$_3$OH:water:acetic acid (65:30:5). (B) Expansion of (A) showing m/z 85–95. (C) CID-MS/MS spectra for the $m/z = 637.298$, $[M+2H]^{2+}$, ion characteristic of Orc[1-11]-OCD$_3$ for the *H. americanus* eyestalk ganglion-derived peptide eluting at 16.5 min; single eyestalk ganglion extracted with CD$_3$OD:water:acetic acid (65:30:5). Filled stars show peaks that have shifted by 3.02 Da as a result of the incorporation of CD$_3$. (D) Expansion of (C) showing m/z 85–95. The filled star indicates that the y$_1$ sequence ion has shifted by 3.02 Da, demonstrating that the CD$_3$ group has been incorporated at the C-terminus. (E) CID-MS/MS spectrum for the $m/z = 635.789$, $[M+2H]^{2+}$, ion from the Orc[Ala^{11}] standard eluting at 15.5 min. (F) Expansion of (E) showing m/z 85–95. (G) CID-MS/MS spectrum for the $m/z = 635.789$, $[M+2H]^{2+}$, ion from the Orc[1-11]-OMe standard eluting at 16.5 min. (H) Expansion of (G) showing m/z 85–95. All CID-MS/MS spectra were acquired using a collision energy of 26 V, nitrogen as the collision gas, and a 4 Da window for ion isolation. The CID-MS/MS spectrum for the eyestalk-derived peptide, extracted using CH$_3$OH (A and B) is indistinguishable from the spectra for Orc[Ala^{11}] (E and F) and Orc[1-11]-OMe (G and H); however, the eyestalk-derived peptide, extracted using CD$_3$OD (C and D) shows y-series ions, including y$_1$ (see (D)), that are all shifted by 3.02 Da, demonstrating methylation at the C-terminus and providing confirmation of the Orc[1-11]-OMe sequence. The y- and b-type fragment ions are identified using the nomenclature established by Roepstorff [36].

mass spectra for the two eyestalk tissue extracts show that the Orc[1-11]-OMe-derived peaks at m/z 1270.57, 1253.54, 894.43, 876.42, and 537.28 for the eyestalk extracted with CH_3OH (Fig. 8A and B) have all shifted by 3 Da to m/z 1273.59, 1256.56, 897.45, 879.44, and 540.30, for the eyestalk extracted with CD_3OD (Fig. 8C and D).

MALDI-FT mass spectra and CID experiments carried out on the Q-TOF also support our localization of the added methyl group at
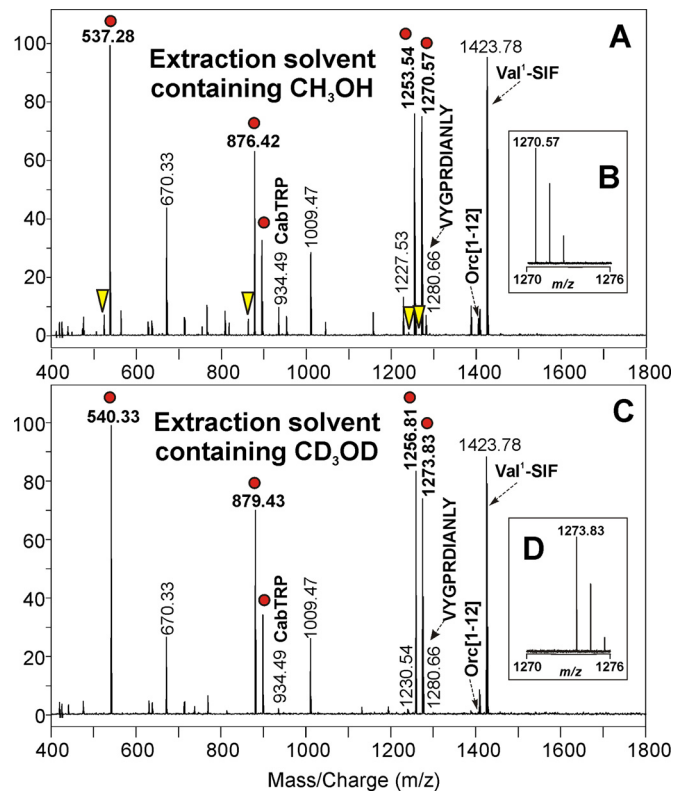
Fig. 8. Methanol in the extraction solvent is the methyl group source for Orc[1-11]-OCH₃. MALDI-FT mass spectra for *H. americanus* eyestalk ganglia extracted using (A) methanol extraction solvent (65:30:5; $CH_3OH:H_2O:CH_3CO_2H$) or (B) deuterated methanol extraction solvent (65:30:5; $CD_3OH:H_2O:CH_3CO_2H$). All spectra were measured using DHB as the matrix; conditions were optimized for *m/z* 1500. Spectra were calibrated using known peptide peaks [42], including CabTRP and Val¹-SIF at *m/z* 934.4927 and *m/z* 1423.7845, respectively. The peaks at *m/z* 1270.57, 1253.54, 894.43, 876.42, and 537.28 from Orc[1-11]-OMe (A) shift by 3 Da for the spectrum generated using CD₃OD-containing solvent (B), demonstrating that the added methyl group originates from the methanolic solvent. Orc[1-11]-OMe peaks labeled with filled circles; peaks for Orc[1-11] at *m/z* 1256.55, 1239.53, 862.41, and 523.26 are labeled with filled triangles.

the C-terminus of the peptide. This localization is supported by the 3 Da mass shifts for the y_8, y_8^0, and y_5 ion in the MALDI-FT mass spectra of eyestalks extracted with CD₃OD (Fig. 8), which localizes the added methyl group to the C-terminal sequence, and by the 3 Da mass shift for y-type (but not b-type) ions produced in the Q-TOF MS/MS analysis (Fig. 7C and D). The most diagnostic fragment is the y_1 peak, which undergoes a 3 Da mass shift (from *m/z* = 90.06 to 93.07) for the CD₃-labeled peptide (Fig. 7D). This fragment ion definitively localized the methyl addition to the C-terminus. These results document the incorporation of one CD₃ group for Orc[1-11]-OMe at the C-terminus and demonstrate that the methanolic extraction solvent is the source of the added methyl group.

3.6. Methylation is a biochemical, not chemical, extraction artifact

Acid-catalyzed esterifications have been recognized as the source of exogenous protein methylations that occur when methanol and acids are used, for example, in destaining SDS-PAGE gels [5,17,24,48]. In an early study by Haebel et al. [17], five test peptides were incubated in methanolic trichloroacetic acid (TCA) solutions (12.5:50:37.5, methanol:TCA:water) for 1–24 h to determine the propensity for methylation at different amino acid residues. The authors concluded that glutamate (E) undergoes the most rapid acid-catalyzed esterification, the C-terminus reacts with a rate that is lower by a factor of 2–6, and other groups (D, Q) are

less reactive by at least a factor of 10 [17]. When acetic acid replaced TCA, methylation was not observed [17].

A direct chemical modification appeared to be unlikely as an explanation for the production of Orc[1-11]-OMe under our conditions, based upon the following observations and considering the work by Haebel et al. [17]. First, our data clearly show that methylation occurs, with 100% specificity, at the C-terminus. It seems unlikely that an acid-catalyzed chemical conversion could account for this highly specific methylation, which leaves the remaining two aspartate and the single glutamate residues unmodified. Second, we find no evidence for methylation of any full-length orcokinin family peptides. Third, we find that significantly lower levels of Orc[1-11]-OMe are found in extracts from the SG (a neuropeptide storage site) compared with extracts of whole eyestalk ganglia or small pieces of eyestalk tissue, such as the XO/MT, where enzymes important for the synthesis and processing of neuropeptide prohormones are expected to be co-localized. Based upon these observations, we hypothesized that methylation must involve processing components endogenous to the eyestalk tissues.

To first establish that orcokinin family peptides are not methylated in vitro by exposure to our extraction solvent, we added 30 μL of extraction solvent (CH₃OH and CD₃OD versions) or 30 μL of water (used as a control) to [Asn¹³] and Orc[1-11] standards (2 nmol). [Asn¹³]-orcokinin was tested because this peptide is an abundant orcokinin family peptide in *H. americanus*. Orc[1-11] was included as the unmethylated form of Orc[1-11]-OMe, to determine if the sequence of this peptide, including the C-terminal glycine residue, makes it particularly susceptible to acid-catalyzed C-terminal methylation. The solutions sat at room temperature for 24 h, at which time each sample was dried, reconstituted, and subjected to MALDI-FTMS analysis. The spectra for both [Asn¹³]-orcokinin and Orc[1-11] showed no evidence for peptide methylation (data not shown), indicating that the extraction solvent, alone, is not responsible for the observed peptide modification. In addition, we found no evidence for peptide degradation (truncation or other modifications).

To test the hypothesis that components endogenous to the eyestalk tissues play a role in the C-terminal methylation, we carried out an experiment in which we started with two microcentrifuge tubes, each containing 1 nmol of a standard of [Ala¹³]-orcokinin, a full-length orcokinin that is not present in *H. americanus*. To one tube we added extraction solvent; to the other we added extraction solvent and a freshly dissected eyestalk ganglion. The tissue sample was homogenized and both samples were sonicated and centrifuged. As expected, the [Ala¹³]-orcokinin standard alone gave a strong MALDI-FTMS signal with characteristic orcokinin family fragments (see Fig. 9A) and showed no evidence for methylation. In contrast, the MALDI-FT mass spectrum for the tissue-containing sample showed abundant signals for Orc[1-11]-OMe (Fig. 9B) that were more intense than Orc[1-11]-OMe signals observed for other eyestalk tissue extracts. No signals for [Ala¹³]-orcokinin were observed. The fact that no [Ala¹³]-orcokinin signals were observed, coupled with the elevated Orc[1-11]-OMe signals, suggests that [Ala¹³]-orcokinin was converted to Orc[1-11]-OMe in the sample. To provide more evidence to support this conclusion, we added an additional 1 nmol of the [Ala¹³]-orcokinin standard to the tissue with extraction solvent mixture, resonicated and centrifuged the sample and immediately analyzed the supernatant by MALDI-FTMS. With the second addition of [Ala¹³]-orcokinin, we were now able to detect peaks for this peptide (see Fig. 9C). When the mixture was allowed to remain at room temperature overnight and was reanalyzed, we found that signals for [Ala¹³]-orcokinin had decreased, while those for Orc[1-11]-OMe had increased (see Fig. 9D), providing additional support for the conversion of the full-length [Ala¹³]-orcokinin to Orc[1-11]-OMe when the methanolic solvent was present with a tissue sample. These results are also
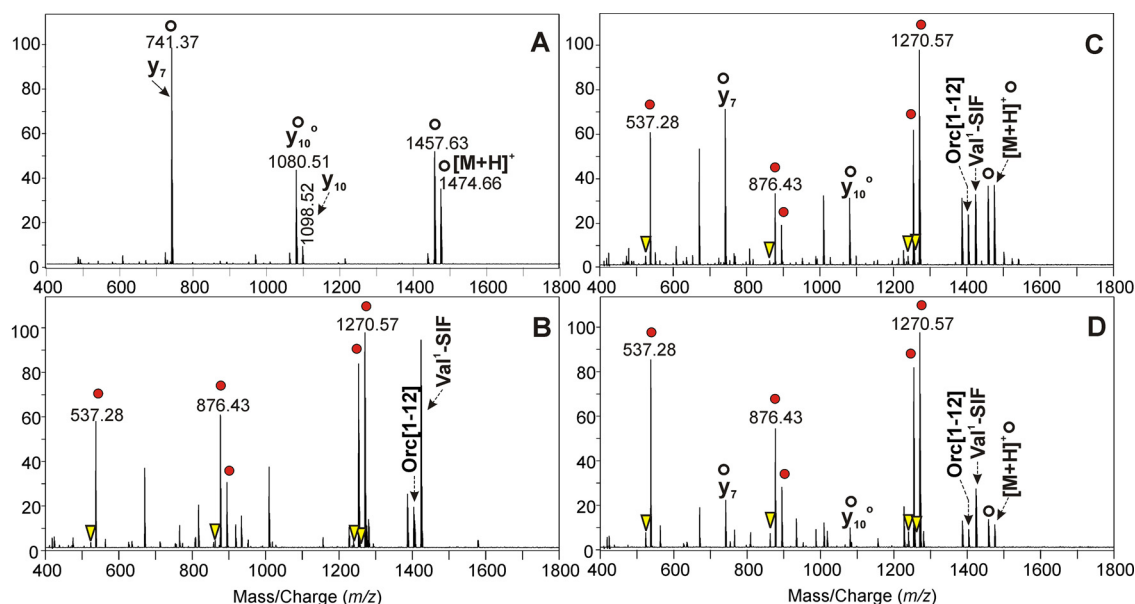
Fig. 9. An exogenous orcokinin peptide, NFDEIDRSGFGFA ([Ala[13]]-orcokinin), is converted to Orc[1-11]-OMe when added to a tissue extract, but not when exposed to the extraction solvent alone. MALDI-FT mass spectra for (A) NFDEIDRSGFGFA ([Ala[13]]-orcokinin standard (1 nmol) in 30 μL of extraction solvent. (B) [Ala[13]]-orcokinin standard (1 nmol), homogenized in 30 μL of extraction solvent with the ganglia from one *H. americanus* eyestalk prior to analysis. (C) [Ala[13]] standard (1 nmol) added to the solution from (B) and re-homogenized prior to analysis, (D) Solution from (C) after sitting at room temperature for 12 h. All spectra were measured using DHB as the matrix with conditions optimized for *m/z* 1500. [Ala[13]]-orcokinin standard peaks labeled with open circles; Orc[1-11]-OMe peaks labeled with filled circles. Spectra were calibrated using known peptide peaks [42], including CabTRP and Val[1]-SIF at *m/z* 934.4927 and *m/z* 1423.7845, respectively. Peaks for Orc[1-11] at *m/z* 1256.55, 1239.53, 862.41, and 523.26 are labeled with filled triangles.

consistent with our observation that stronger signals for Orc[1-11]-OMe are correlated with reduced intensities for full-length orcokinin peptides, an observation that is explained by the conversion of the full-length peptides to the truncated, C-terminally methylated form. Taken collectively, these results demonstrate that the methanolic extraction solvent alone is not responsible for the formation of C-terminally methylated Orc[1-11], and point to components, possibly enzymes, present in the tissue samples that facilitate the formation of Orc[1-11]-OMe from full-length orcokinin precursors.

3.7. Enzyme inhibitors reduce Orc[1-11]-OMe formation

To determine if enzymes play a role in promoting the formation of Orc[1-11]-OMe during extraction of eyestalk tissues, we attempted to reduce methylation by inhibiting enzymatic activity using a commercial protease inhibitor cocktail that contains a mixture of inhibitors designed to protect against a broad range of proteases. To include the protease inhibitor in our extraction protocol, we used two different approaches. The first approach involved including the aqueous inhibitor solution in place of water in our extraction solution. The second approach involved homogenizing the tissue in either the aqueous inhibitor solution or water (as a control), followed by the addition of acidified methanol to bring the solvent to the percentages that have been used in previous experiments. Experiments were carried out with paired eyestalk ganglia to directly compare the efficacy of the inhibitor treatment.

Our results show that the inclusion of protease inhibitor cocktails reduced the detected levels of Orc[1-11]-OMe compared with control measurements. For example, Fig. 10A shows the signals from Orc[1-11]-OMe for a control eyestalk ganglion, which was treated by homogenization in water before the addition of acidified methanol; Fig. 10B shows the result when homogenization took place in the protease inhibitor cocktail. Both samples, following sonication and centrifugation, were dried and

purified using C18 ZipTips to remove salts that interfered with our ability to produce good quality MALDI-FT mass spectra. As shown in Fig. 10B and C, only weak Orc[1-11]-OMe signals were detected in the sample with protease inhibitor present, while signals from this peptide were abundant when the sample was prepared without the inhibitor cocktail. We also noticed that signals for Orc[1-11] were also reduced with inclusion of the inhibitor cocktail. Upon carrying out multiple trials making use of the inhibitor cocktail, we consistently found reduced levels of both Orc[1-11]-OMe and Orc[1-11] when the inhibitor was present; however, inhibition was never complete. Regardless, these results provide evidence to support the hypothesis that an enzyme participates in production of the Orc[1-11]-OMe product.

3.8. Heat treatment significantly reduces Orc[1-11]-OMe formation; treatment with liquid N_2 does not

Heat has been used an effective means to reduce proteolytic degradation of proteins when processing vertebrate tissue samples [9,12,13,44]. Working from the hypothesis that an enzyme plays a role in promoting the formation of Orc[1-11]-OMe during extraction of eyestalk tissues, we attempted to deactivate enzymatic components with heat. To test this approach we removed the paired eyestalk ganglia from one lobster. The ganglion from a single eyestalk was placed in a microcentrifuge tube with 50 μL of extraction solvent and the tightly capped tube was placed in a boiling water bath for 5 min. Concurrently, the ganglia from the second eyestalk of the same lobster were placed in extraction solvent and left at room temperature for 5 min. Both eyestalk tissue samples were then homogenized, sonicated, and centrifuged prior to MALDI-FTMS analysis.

While the control eyestalk extract showed the Orc[1-11]-OMe-derived peaks at *m/z* 1270.57, 1253.54, 894.43, 876.42, and 537.28 (see Fig. 11A), no evidence for these peaks was found for the tissue/extraction solvent mixture that was placed in the boiling water
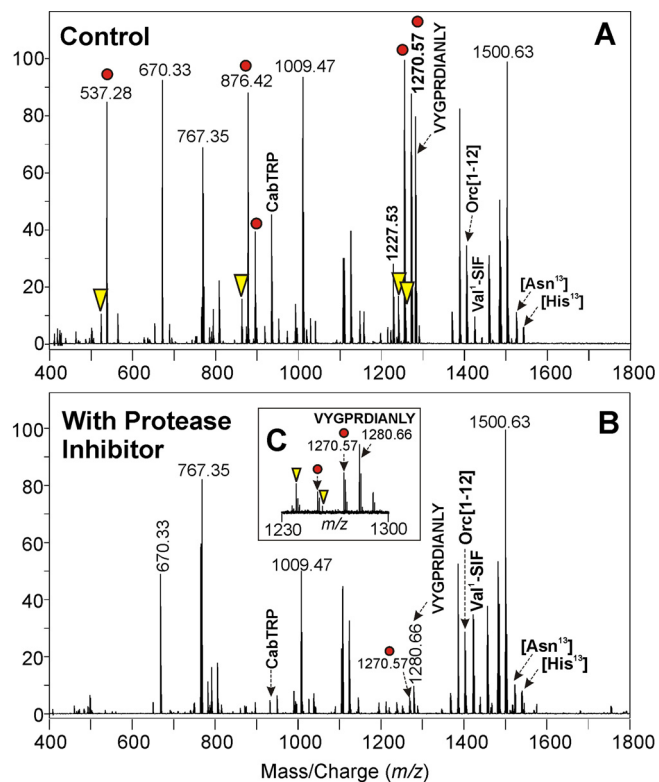
Fig. 10. MALDI-FT mass spectra of extracts from a matched pair of *H. americanus* eyestalk ganglia showing that extraction with protease inhibitors reduces the intensity of Orc[1-11]-OMe and Orc[1-11]-derived peaks, suggesting that enzymatic activity plays a role in production of this methylated peptide. (A) Control ganglion. The ganglion was homogenized in 30 μL of nanopure water, after which 65 μL of methanol and 5 μL of acetic acid were added and the sample was sonicated and centrifuged prior to analysis. (B) Test ganglion, with protease activity inhibited. The ganglion was homogenized in 30 μL of a protease inhibitor cocktail, after which 65 μL of methanol and 5 μL of acetic acid were added and the sample was sonicated and centrifuged prior to analysis. Only low intensity peaks for Orc[1-11]-OMe are detected for the extraction was carried out with the protease inhibitor cocktail. All spectra were measured using DHB as the matrix and conditions optimized for *m/z* 1500. Orc[1-11]-OMe peaks labeled with filled circles; Orc[1-11] peaks are labeled with filled triangles. Spectra were calibrated using known peptide peaks [42], including CabTRP and Val[1]-SIF at *m/z* 934.4927 and *m/z* 1423.7845, respectively. Peaks for Orc[1-11] at *m/z* 1256.55, 1239.53, 862.41, and 523.26 are labeled with filled triangles.

Fig. 11. MALDI-FT mass spectra of extracts from a matched pair of *H. americanus* eyestalk ganglia, showing that heating the tissue in extraction solvent eliminates Orc[1-11]-OMe-derived peaks, suggesting that heating effectively deactivates proteases. (A) Control ganglion. The ganglion was placed in 50 μL of extraction solvent (65:30:5; CH₃OH, H₂O, CH₃CO₂H), which sat at room temperature for 5 min, after which the sample was homogenized, sonicated, and centrifuged prior to analysis. (B) Test ganglion; with heat treatment. The ganglion was placed in 50 μL of extraction solvent (65:30:5; CH₃OH, H₂O, CH₃CO₂H). The capped microcentrifuge tube was placed in a boiling water bath for 5 min, after which the sample was homogenized, sonicated, and centrifuged prior to analysis. No peaks for Orc[1-11]-OMe, isobaric Orc[Ala[11]], or Orc[1-11] are detected for the extraction was carried out after boiling water heat treatment. All spectra were measured using DHB as the matrix and conditions optimized for *m/z* 1500. Orc[1-11]-OMe peaks labeled with filled circles. Spectra were calibrated using known peptide peaks [42], including CabTRP and Val[1]-SIF at *m/z* 934.4927 and *m/z* 1423.7845, respectively.

bath for 5 min (Fig. 11B and C). We also did not detect the truncated peptide, Orc[1-11]. When this approach was replicated (*n* > 6), the treatment consistently eliminated the production of Orc[1-11]-OMe and Orc[1-11].

We also tried freezing eyestalk ganglion tissues in liquid nitrogen before homogenizing and adding extraction solvent, but found that this treatment did not measurably reduce production of Orc[1-11]-OMe.

3.9. Lower water content in the extraction solvent reduces Orc[1-11]-OMe formation; acetic acid promotes formation of Orc[1-11]-OMe over Orc[1-11]

Many enzymes are known to function in aqueous-methanolic solvent mixtures [2,38]; however, enzymatic activity is generally reduced or eliminated when the water content is reduced [22,25]. We hypothesized that, if an enzyme plays a role in the production of Orc[1-11]-OMe, production of the peptide would be reduced if the extraction solvent contained a lower percentage of water. To determine if the percentage of water in the extraction solvent influenced the extent of methylation, we extracted eyestalk ganglia in solvents containing 1–30% water. The
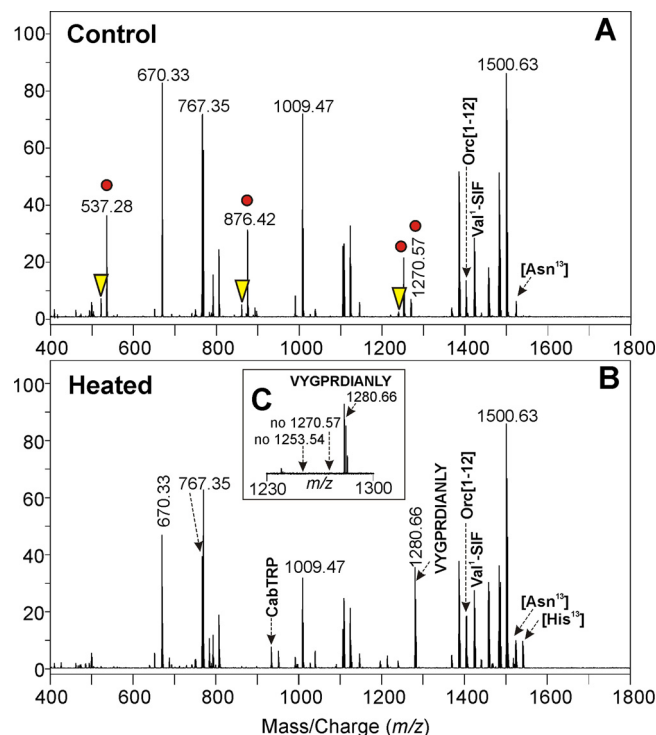
mixtures tested included our normal extraction solvent (65:30:5; methanol:water:glacial acetic acid), a solvent mixture (90:5:5; methanol:water:glacial acetic acid) containing a lower percentage of water, and the solvent mixture (90:1:9; methanol:water:glacial acetic acid) routinely used by the Li group [30] and used by Huybrechts and co-workers [21]. Experiments were again carried out with paired eyestalk ganglia to determine directly how the change in solvent composition impacted the methylation of tissues from a single animal. For these and additional experiments in which the percentage of water was held below 1%, we found detectable, but lower, yields of the Orc[1-11]-OMe product (data not shown).

Solution pH is an important determinant of enzymatic activity. Acidified aqueous and organic solvents are commonly used to extract neuropeptides from tissue samples [20]; however, a reduction in solution pH has also been found to promote methanolysis over hydrolysis reactions for enzymes that can function in methanolic solutions [3]. To determine if the addition of acid to the extraction solvent plays a role in the formation of Orc[1-11]-OMe, we carried out eyestalk ganglion extractions in the absence of acetic acid, using 65:35, methanol:water. When the eyestalk ganglion extract was analyzed by MALDI-FTMS, the peptide Orc[1-11]-OMe was not detected. Instead, we observed elevated signals for the truncated orcokinin, Orc[1-11] (see Fig. 12), which would be produced by the hydrolysis, not methanolysis, of a full-length
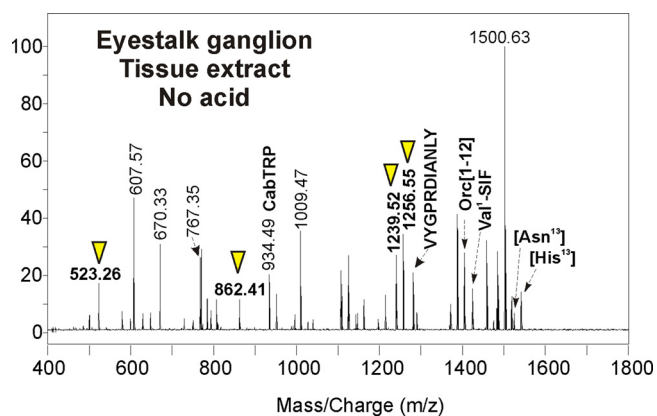
Fig. 12. MALDI-FT mass spectrum of an extract from an *H. americanus* eyestalk ganglion, showing that eliminating acetic acid from the extraction solvent promotes hydrolysis (formation of Orc[1-11]) over methanolysis (formation of Orc[1-11]-OMe), suggesting that pH plays a role in methanolysis. The ganglion was placed in 50 μL of extraction solvent (65:35; CH_3OH, H_2O) and was homogenized, sonicated, and centrifuged prior to analysis. No peaks for Orc[1-11]-OMe are detected for the extraction was carried without acetic acid in the extraction solvent. All spectra were measured using DHB as the matrix and conditions optimized for *m/z* 1500. Orc[1-11] peaks are labeled with filled triangles. Spectra were calibrated using known peptide peaks [42], including CabTRP and Val^1-SIF at *m/z* 934.4927 and *m/z* 1423.7845, respectively. Peaks for Orc[1-11] are labeled with filled triangles.

orcokinin. Collectively, these experiments provide evidence that the percentage of water, as well as solution pH, play a role in the putative enzymatic conversion of orcokinin family peptides to hydrolyzed or methylated, truncated forms.

3.10. Trypsin-catalyzed C-terminal methylation at low pH is demonstrated for NFDEIDRAAFGFA

To further test the hypothesis that an enzyme is responsible for the formation of Orc[1-11]-OMe, and that methanolysis can compete with proteolysis at acidic pH values that are not optimal for enzymatic activity [3], we carried out the proteolysis of NFDEIDRAAFGFA, a synthetic peptide, with and without 25% methanol in a pH = 4, 25 mM citric acid buffer. We used trypsin as a representative serine protease that should permit methanol to act as a competing nucleophile in the proteolysis reaction [2,38]. Following reaction, the products were analyzed by Chip–nanoESI Q-TOF MS. In the control experiment (no methanol present), trypsin cleaved the peptide C-terminal to the arginine residue to produce two hydrolysis products, NFDEIDR and AAFGFA (see Fig. 13A). In the presence of methanol, C-terminal methylation was observed through the formation of NFDEIDR-OMe (see Fig. 13B). No other methylated products were observed.

3.11. An additional truncated, methylated peptide, SSEDMDRLGFG-OMe, is identified

To determine if additional methylated peptides were present in eyestalk extracts, we compared spectra of eyestalks extracted with CH_3OH with those extracted with CD_3OD and searched for peptides that showed the predicted shift of 3 Da. This analysis results in the identification of the peptide SSEDMDRLGFG-OMe, which showed peaks at *m/z* 1227.53, 809.40, and 563.33 ([M+H]$^+$, y_7, and y_5, respectively). Exact mass measurements (1227.5298, measured; 1227.5310, predicted) supported this assignment. This peptide is presumably derived from the full-length precursor SSEDMDRLGFGFN.
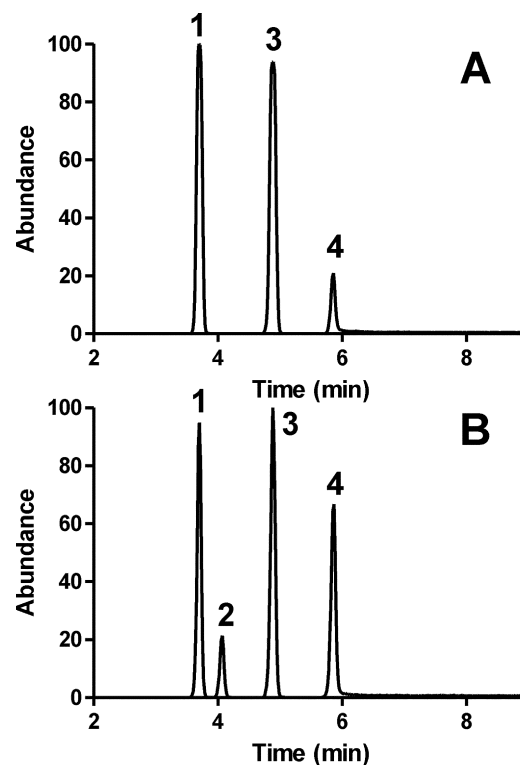

Fig. 13. Chromatograms from the HPLC Chip–nanoESI Q-TOF MS analysis of a tryptic digestion of synthetic peptide NFDEIDRAAFGFA in (A) pH = 4 citrate buffer and (B) 25% CH_3OH, pH = 4 citrate buffer, demonstrating that, under appropriate conditions, a serine protease can accommodate methanol as a competing nucleophile and generate a product methylated at the C-terminus. Peaks 1 (NFDEIDR) and 3 (AAFGFA) result from tryptic cleavage C-terminal to the arginine residue. Peak 4 is the undigested peptide. Peak 2 results from C-terminal methylation when methanol was present in the solvent, forming NFDEIDR-OCH$_3$. Samples were digested for 3 days at room temperature; starting peptide concentration was 2×10^{-6} M.

3.12. Eyestalk ganglia extractions using non-methanolic solvents fail to provide evidence for Orc[Ala^{11}]

Our analysis of *H. americanus* eyestalk tissue extracts provided structural characterization of Orc[1-11]-OMe and demonstrated that this peptide is not endogenous to *H. americanus* eyestalk tissues. The isobaric peptide Orc[Ala^{11}] has been localized to *H. americanus* eyestalk ganglion and sinus glands by Li and co-workers [30]. The fact that Orc[1-11]-OMe is isobaric with previously reported Orc[Ala^{11}] lead us to wonder whether Orc[Ala^{11}] was misidentified in previous studies or if Orc[Ala^{11}] is a neuropeptide endogenous to the lobster eyestalk ganglia. Misidentification is a possibility, especially when considering the fact that most MS/MS measurements would not reveal a structural difference between the two peptides (described above). To address these concerns, we attempted to detect Orc[Ala^{11}] using eyestalk extracts prepared using two non-methanolic extraction techniques, namely, extraction using HCl-acidified acetone [49] and extraction with aqueous, saturated DHB [37]. These solvent systems should preclude the formation of Orc[1-11]-OMe and reveal any Orc[Ala^{11}] that may have been overshadowed by Orc[1-11]-OMe, particularly if that peptide was present at higher abundance. These approaches should then provide two additional measures, complementing our data on heat-deactivated methanolic extractions where no evidence for Orc[Ala^{11}]/Orc[1-11]-OMe was found (see Fig. 11B). In measurements with acidified acetone (see Fig. 14) and saturated DHB (data not shown), where we extracted single eyestalk ganglia from a minimum of three individuals, no peaks characteristic of Orc[Ala^{11}] were detected by MALDI-FTMS.
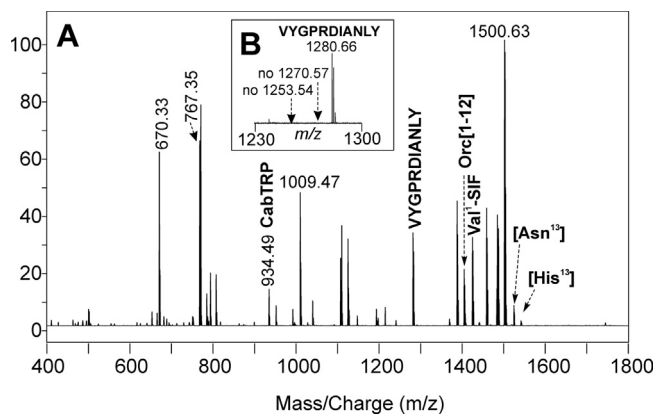
Fig. 14. MALDI-FT mass spectrum of an extract from an *H. americanus* eyestalk ganglion, showing that extracting the tissue in a non-methanolic solvent eliminates Orc[1-11]-OMe-derived peaks and fails to reveal peaks from Orc[Ala^{11}]. The ganglion was placed in 50 μL of extraction solvent (85:13:2; acetone:H$_2$O:HCl) and was homogenized, sonicated, and centrifuged prior to analysis. No peaks for Orc[1-11]-OMe or isobaric Orc[Ala^{11}] are detected for the extraction was carried out after acetone extraction. All spectra were measured using DHB as the matrix and conditions optimized for *m/z* 1500. Spectra were calibrated using known peptide peaks [42], including CabTRP and Val^1-SIF at *m/z* 934.4927 and *m/z* 1423.7845, respectively. Peaks for Orc[1-11] at *m/z* 1256.55, 1239.53, 862.41, and 523.26 are labeled with filled triangles.

3.13. Sinus gland and CoG extractions with full methyl esterification fail to provide evidence for Orc[Ala^{11}]

Because Orc[Ala^{11}] was previously detected in the SG and stomatogastric nervous system of *H. americanus* [30], we carried out a detailed reexamination of MALDI-FTMS spectra generated using extracts from single sinus glands and paired commissural ganglia (CoGs), which were reported in a previous study from our laboratory [10]. In this study, sinus glands and CoGs were analyzed by MALDI-FTMS using direct tissue analysis, analysis of methanolic tissue extracts, and analysis of methyl-esterified tissue extracts. The last method of sample preparation, in particular, allows the differentiation of Orc[1-11]-OMe and Orc[Ala^{11}]. Specifically, while Orc[1-11]-OMe undergoes a mass shift to *m/z* 1312.62 following acid-catalyzed methyl esterification of the two aspartate and single glutamate residues, any Orc[Ala^{11}], with a free C-terminal carboxylic acid, would undergo a mass shift to *m/z* 1326.63 resulting from the esterification of four, not three, acidic residues. A careful reexamination of these previously acquired data [10] showed no peaks characteristic of Orc[1-11]-OMe/Orc[Ala^{11}] for direct tissue analysis, low abundance peaks in some, but not all, sinus gland and CoG extracts, low abundance peaks *m/z* = 1312.62 in some, but not all, sinus gland and CoG methyl esterified, and no peaks characteristic of methyl esterified Orc[Ala^{11}] at *m/z* = 1326.63 in the methyl esterified extracts. Subsequent extraction of a single sinus gland using 65% CD$_3$OD:30% H$_2$O:5% acetic acid showed that the peaks detected for Orc[1-11]-OMe/Orc[Ala^{11}] were shifted in mass by 3 Da, indicating that the low abundance peptide is Orc[1-11]-OMe (data not shown). These results again failed to reveal any endogenous Orc[Ala^{11}] in the sample.

3.14. LC/MS analyses of heat-deactivated, pooled, eyestalk tissue extracts fail to show evidence of Orc[Ala^{11}]

To determine if our analysis of single, not pooled, eyestalk ganglion extracts was limiting our ability to detect signals from low abundance, endogenous Orc[Ala^{11}], we analyzed pooled extracts of 11 and 35 heat-treated, *H. americanus* eyestalk ganglia that were extracted with the solvent composition (90:1:9; methanol:water:glacial acetic acid) used in previous studies [21,30]

where Orc[Ala^{11}] was detected. To further increase the dynamic range for the detection of Orc[Ala^{11}], we analyzed the extracts by HPLC Chip–nanoESI Q-TOF MS. When we analyzed data for the pooled extracts and generated EICs for the *m/z* 635.789, [M+2H]$^{2+}$, ion for the isobaric Orc[1-11]-OMe or Orc[Ala^{11}], a single peak, eluting at the retention time characteristic of Orc[1-11]-OMe, was observed (data not shown). We found no evidence for a peak at the retention time for Orc[Ala^{11}].

3.15. Direct-tissue analysis by MALDI-FTMS reveals no peaks for Orc[1-11]-OMe/Orc[Ala^{11}] in H. americanus neuroendocrine organs and tissues

When we initially embarked upon our study of localized regions of *H. americanus* eyestalk tissues, we detected peaks attributed to Orc[1-11]-OMe in extracted tissue samples, but not in any eyestalk tissues analyzed directly by MALDI-FTMS. Because methanol is not used as a tissue washing solvent or as a matrix solvent in our protocol for the preparation of direct tissue samples, we felt confident that Orc[1-11]-OMe formation would be prevented during direct tissue analyses. To further explore the possibility that Orc[Ala^{11}] is an endogenous neuropeptide in the *H. americanus* eyestalk ganglion, we analyzed additional localized SG, LG, XO/MT, MI and ME eyestalk tissue samples dissected from a minimum of three individuals using direct tissue MALDI-FTMS to determine if sampling variability or differences between individuals could be responsible for our inability to detect putative Orc[Ala^{11}]. Furthermore, we collected between three and ten spectra from different regions of each MALDI sample to account for heterogeneity within each sample. In the case of SGs, a source of putative Orc[Ala^{11}] in a previous study, we have collected direct tissue spectra from more than 30 individuals. A representative MALDI-FT mass spectrum from a *H. americanus* SG gland is shown in Fig. 15A; an expansion of the mass range where Orc[Ala^{11}] would appear (Fig. 15B) reveals no signals characteristic of Orc[Ala^{11}], although other orcokinin family peptides are abundant in the full MALDI-FT mass spectrum. We detected peaks for Orc[1-11] in some, but not all, spectra. In the replicated direct tissue MALDI-FTMS characterizations of localized pieces of eyestalk ganglion tissues from multiple individuals, we failed to detect signals characteristic of Orc[Ala^{11}] in any spectra.

Because Orc[Ala^{11}] has been reported in other *H. americanus* neuroendocrine organs and tissues, including the supraesophageal ganglion (SoG/brain) [4,30], pericardial organ (PO) [6], and the adult and embryonic stomatogastric ganglion (STG) [4,23], we evaluated the direct tissue MALDI-FT mass spectra of these organs and tissues, as well as *H. americanus* commissural ganglia (CoG). We again characterized tissues derived from a minimum of three individuals to determine if sampling variability or differences between individuals could be responsible for our inability to detect putative Orc[Ala^{11}]. Furthermore, we collected between three and ten spectra from different regions of each MALDI sample to account for heterogeneity within each sample. For the brain and POs, we also analyzed multiple samples of tissue that were dissected from different locations from the larger sample. CoGs were analyzed in their entirety or split into two pieces prior to analysis, while the entire STG was co-crystallized with matrix. We also characterized the brain from a juvenile lobster. Representative spectra from the tissues analyzed in our laboratory are shown in Fig. 15.

In previous studies, abundant signals for putative Orc[Ala^{11}] and Orc[1-11] were detected by direct tissue analysis of small pieces of tissue dissected from the *H. americanus* PO [6]. Orc[Ala^{11}] and Orc[1-11] were found with other orcokinin family peptides in a long fiber that projects along the crustacean muscle and into the heart. In this study, MALDI samples were prepared by washing the tissues in acidified methanol followed by co-crystallization
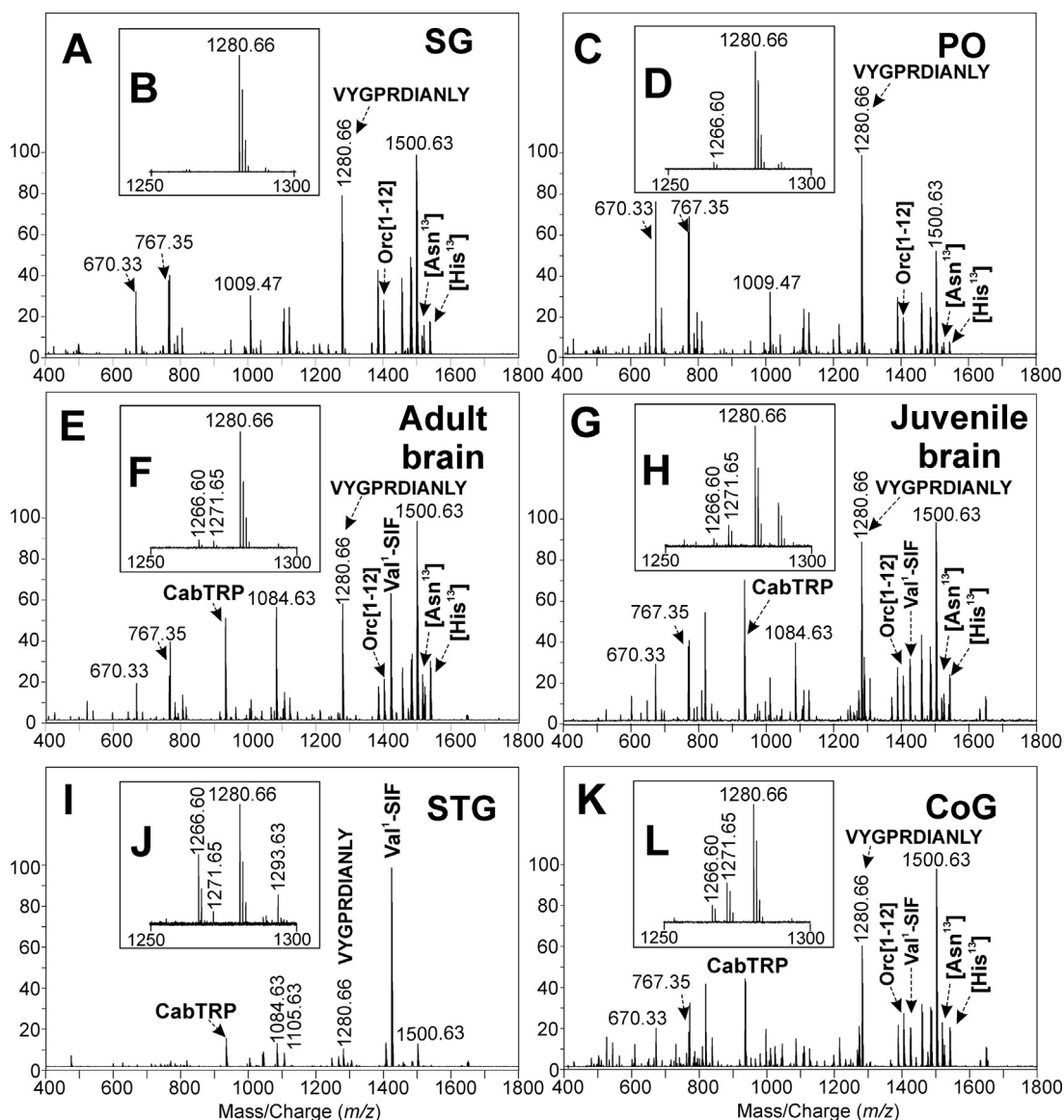
Fig. 15. Direct tissue MALDI-FT mass spectra of *H. americanus* neuroendocrine organs and tissue. Each spectrum contains an expansion showing the region where *m/z* 1270.56 and 1253.54 ([M+H]+ and [MH−NH3]+ from putative Orc[Ala11]) would appear, if detected. (A) Spectrum from a SG freshly dissected from the eyestalk ganglion. (B) An expansion of spectrum (A) showing a peak for VYGPRDIANLY at *m/z* 1280.66, but no detectable signals for Orc[Ala11]. (C) Spectrum from a small piece of freshly dissected PO from a fiber projecting from the muscle to the heart. (D) An expansion of spectrum (C) showing peaks for VYGPRDIANLY at *m/z* 1280.66 and TNWNKFQGSWamide (*m/z* 1266.60), but no detectable signals for Orc[Ala11]. (E) Spectrum from a small piece of freshly dissected brain tissue from an adult lobster. (F) An expansion of spectrum (E) showing peaks for VYGPRDIANLY at *m/z* 1280.66, TNWNKFQGSWamide (*m/z* 1266.60), and pQDLDHVFLRFamide (*m/z* 1271.65), but no detectable signals for Orc[Ala11]. (G) Spectrum from a small piece of freshly dissected brain tissue from a juvenile lobster. (H) An expansion of spectrum (G) showing peaks for VYGPRDIANLY at *m/z* 1280.66, TNWNKFQGSWamide (*m/z* 1266.60), and pQDLDHVFLRFamide (*m/z* 1271.65), but no detectable signals for Orc[Ala11]. (I) Spectrum from a freshly dissected STG. (J) An expansion of spectrum (I) showing peaks for VYGPRDIANLY at *m/z* 1280.66, TNWNKFQGSWamide (*m/z* 1266.60), pQDLDHVFLRFamide (*m/z* 1271.65), and STNWSSLRSAWamide (*m/z* 1293.63), but no detectable signals for Orc[Ala11]. (K) Spectrum from a freshly dissected STG. (L) An expansion of spectrum (K) showing peaks for VYGPRDIANLY at *m/z* 1280.66, TNWNKFQGSWamide (*m/z* 1266.60), and pQDLDHVFLRFamide (*m/z* 1271.65), but no detectable signals for Orc[Ala11]. All samples were prepared using a fructose tissue wash and co-crystallization using DHB in acetonitrile as the matrix; conditions were optimized for *m/z* 1500. Spectra were calibrated using known peptide peaks [42], including APSGFLGMRamide (CabTRP) and VYRKPPFNGSIFamide (Val1-SIFamide) at *m/z* 934.4927 and *m/z* 1423.7845, respectively.

with DHB in 50% methanol [6]. In our investigations, we excluded methanol from the sample preparation, washed tissues in fructose, and co-crystallized with DHB in acetonitrile prior to MALDI-FTMS interrogation. A representative PO spectrum from our analysis of samples along the long fibrous projection between the muscle and heart (Fig. 15C and D) shows strong signals from orcokinin family peptides. In agreement with the mass spectrum published by Li and co-workers [6], which was dominated by signals from orcokinin family peptides, we consistently detected peaks for the orcokinin family peptides [Asn13], [His13], [Val13], Orc[1-12], SSED-MDRLGFGFN, FDAFTTGFGHN, and VYGPRDIANLY, all with mass

measurement errors of less than 5 ppm. Furthermore, we detected Orc[1-11] in some, but not all, spectra; however, we failed to detect signals for Orc[Ala11] in spectra for any of the PO tissues we examined.

Signals for putative Orc[Ala11] and Orc[1-11] were also detected in *H. americanus* brain tissues through the analysis of tissue extracts [30] and using direct tissue analyses [4,30], where either saturated DHB in water [30] or acidified methanol [4] were used to wash tissue samples and tissue samples were co-crystallization with DHB in 50% methanol. We have carried out the extraction of *H. americanus* brain tissues using CD3OD in our extraction solvent and found only

evidence for Orc[1-11]-OMe (trideuterated; data not shown). In our direct tissue investigations, we excluded methanol from the sample preparation, and analyzed small samples of brain tissues from adult ($n > 4$) and juvenile ($n = 2$) lobsters. Representative spectra from an adult *H. americanus* brain (Fig. 15E and F) and from a juvenile brain (Fig. 15G and H) show complements of peptides similar to those detected by the Li group [4,30], including abundant signals from Val[1]-SIF and the orcokinin family peptides [Asn[13]], [His[13]], [Val[13]], Orc[1-12], SSEDMDRLGFGFN, FDAFTTGFGHN, and VYGPRDIANLY, all with mass measurement errors of less than 5 ppm. A careful examination of the mass range where putative Orc[Ala[11]] should appear (Fig. 15F and H) shows two peptides, TNWNKFQGSWamide (*m/z* 1266.60) and pQDLDHVFLRamide (*m/z* 1271.65), which had been detected in the previous work [4,30]. While we did detect weak signals for Orc[1-11] in a few spectra, we did not detect signals for Orc[Ala[11]] in spectra for any of the brain tissues we examined.

We also examined direct tissue spectra for the STG and CoG, two additional nervous system ganglia. Signals for putative Orc[Ala[11]] and Orc[1-11] were previously reported in *H. americanus* STG tissues using direct tissue analyses [4,23], where acidified methanol was used to wash tissue samples and the tissue samples were co-crystallization with DHB in 50% methanol. In our direct tissue investigations of these ganglia, we once again excluded methanol from the sample preparation. A representative spectrum from an STG (Fig. 15I and J) shows complements of peptides similar to those detected by the Li group [4,23], including an abundant signal from Val[1]-SIF. An examination of the mass range where putative Orc[Ala[11]] should appear (Fig. 15I and J) shows three peptides, TNWNKFQGSWamide (*m/z* 1266.60), pQDLDHVFLRamide (*m/z* 1271.65), and STNWSSLRSAWamide (*m/z* 1293.63), which have been detected in the previous work [4,23]; however, we did not detect signals for Orc[Ala[11]] in spectra of any STG tissues we examined. We also failed to detect signals for Orc[Ala[11]] in the MALDI-FTMS spectra for any CoGs (Fig. 15K and L), where we have examined samples from over 20 individuals.

4. Discussion

For any study aspiring to characterize the endogenous components of a biological system, an underlying assumption is that the sampling and analysis approach will leave the sample in an unaltered state. Our results document a highly specific neuropeptide structural alteration, namely the combined truncation and C-terminal methylation of orcokinin family peptides, that occurs only when biological components of a crustacean tissue sample are present in an acidified methanolic extraction solvent.

4.1. The peptide NFDEIDRSGFG-OMe is produced from full-length orcokinin peptides via methanolysis in acidified, methanolic extraction solvents

We used SORI-CID (product ion mass spectra from [M+H]+ and y5 ions) to identify an *m/z* 1270.57 peptide detected in *H. americanus* eyestalk tissue extracts as NFDEIDRSGFG-OMe, not isobaric, previously identified NFDEIDRSGFA, based upon comparisons with standard-derived SORI-CID reference spectra. Furthermore, we used LC/MS to show that the extraction-derived peptide eluted with the same retention time as an Orc[1-11]-OMe, not Orc[Ala[11]], standard. We used extraction with CD3OD to show that methanol from the solvent is the source of the single methyl group found in the *m/z* 1270.57 peptide detected in *H. americanus* eyestalk tissue extracts, and applied Q-TOF-CID to show that the added methyl group is found only at the C-terminus of the peptide, Orc[1-11]-OMe. A second truncated, C-terminally methylated peptide, SSEDMDRLGFG-OMe, was also

identified based upon mass measurements and labeling experiments with CD3OD.

Our work provides evidence to support the role of a tissue component, presumably an enzyme, in the production of the Orc[1-11]-OMe product. Our evidence supporting enzymatic participation in the formation of Orc[1-11]-OMe includes the following. First, the specificity associated with the observed single methylation at the peptide C-terminus is at odds with the outcome expected if a chemical acid-catalyzed esterification were responsible for methylation. The peptide sequence contains three additional targets for methylation (one glutamate and two aspartate residues) and, of the four methylation sites on the peptide, the glutamate residue is expected to be the favored target for acid-catalyzed methylation [17]. Second, we found no evidence for any products of acid-catalyzed esterification when full-length (NFDEIDRSGFGFN) and truncated (NFDEIDRSGFG) peptide standards were incubated in the acidic methanolic extraction solvent at room temperature for 24 h. Third, we found evidence to support the conversion of NFDEIDRSGFGFA, a full-length, non-native orcokinin family peptide, to Orc[1-11]-OMe when eyestalk tissues were mixed with the peptide and extraction solvent. This conversion involved peptide truncation (net loss of FA), with C-terminal methylation. This experiment provided evidence showing that tissue components play a critical role in Orc[1-11]-OMe formation and documented the conversion of a full-length orcokinin family peptides to the truncated, methylated Orc[1-11]-OMe product. Fourth, we observed significant, though not complete, inhibition of Orc[1-11]-OMe and Orc[1-11] production when an enzyme inhibitor cocktail was incorporated in the extraction protocol and, more definitively, found that insertion of the tissue/extraction solvent mixture into a boiling water bath provided the most effective method for preventing Orc[1-11]-OMe and Orc[1-11] formation. These two enzyme-deactivation methods provided the most specific evidence that an enzymatic, not chemical, reaction is responsible for the observed peptide conversion. Additionally, when we reduced the percentage of water in the extraction solvent, we observed that the formation of Orc[1-11]-OMe was reduced, but not eliminated. The reduced production of Orc[1-11]-OMe may reflect an inhibition of enzymatic activity if the higher percentage of methanol in the solvent denatured the enzyme or otherwise yielded conditions that impeded enzymatic activity. Fifth, we found that eliminating acetic acid from the extraction solvent resulted in enhanced levels of the Orc[1-11] peptide, while Orc[1-11]-OMe was no longer detected. This supported work showing that enzymatic methanolysis is favored over hydrolysis for enzymes functioning under more acidic pH conditions [3]. Finally, we also demonstrated that, under conditions where the pH is reduced, methanol can act as a competing nucleophile to yield a C-terminally methylated product using the serine protease, trypsin.

4.2. Orc[Ala[11]] is not detected in H. americanus tissues using direct tissue analyses or when eyestalk tissues are analyzed using three independent extraction-based approaches

Because previously reported Orc[Ala[11]] is isobaric with the extraction artifact, Orc[1-11]-OMe, we attempted to determine if low abundance levels of Orc[Ala[11]] were obscured and undetected in our analyses with methanol. To address these concerns, we carried out three independent extraction-based analyses of eyestalk tissues, namely, (1) MALDI-FTMS analyses of eyestalk ganglion extracts using non-methanolic solvent systems (acidified acetone and saturated DHB), (2) HPLC Chip–nanoESI Q-TOF MS analyses of pooled eyestalk extracts, all heat-treated to deactivate enzymes and extracted using a solvent composition that was used in previous studies, and (3) MALDI-FTMS of sinus gland tissues extracted with full methyl esterification, which provides an additional way

to distinguish Orc[Ala11] and Orc[1-11]-OMe. These three independent approaches failed to show any evidence to support the presence of Orc[Ala^{11}] as a peptide endogenous to the lobster.

To determine if we were able to detect Orc[Ala^{11}] by direct tissue analyses, we analyzed additional eyestalk tissues and other *H. americanus* neuronal glands and tissues (PO, brain, STG, and CoG) by direct tissue MALDI-FTMS, where methanol is not used in any steps of our tissue preparation protocol. All of our measurements, which often included multiple sub-samples dissected from larger tissues (PO and brain), and which represented measurement from a minimum of three individuals and a maximum of greater than 20 individuals for SG and CoG samples, failed to show any evidence of peaks characteristic of Orc[Ala^{11}] in any spectra.

While it is impossible to prove that a peptide is not present in an organism, our best efforts, using direct tissue analyses and three independent extraction-based approaches, failed to show signals supporting prior work identifying Orc[Ala^{11}] as a peptide endogenous to the lobster.

4.3. The observed truncation with C-terminal methylation calls into question some prior neuropeptide sequence identifications where acidic methanolic solutions are used for extraction or direct tissue preparations

Acidified methanol has been used as the extraction solvent of choice in many previous investigations of invertebrate neuropeptides [1,10,14,21,35,39]. For the extraction of crustacean tissues, research groups have commonly used methanolic solvent systems composed of 90% methanol, 1% water [10,14,21,39] or 9% water [46], and acetic acid. Furthermore, for many MALDI-based analyses of crustacean tissues, acidified methanol has been commonly used as a tissue washing protocol and methanol is a component of the DHB matrix solutions [16,26,29,30].

Orcokinin family peptides have been characterized in many crustacean species. Of the many characterized, full-length orcokinins [7], glycine, not alanine, is found exclusively at the 11th position in the sequence. Although genomic data for crustaceans is sparse, the available information documents no genes encoding full-length orcokinins with Ala^{11}; furthermore, no genes have been found for any truncated orcokinin variants. This sequence analysis, coupled with our demonstration that isobaric Orc[1-11]-OMe is an extraction artifact, has led us to question the identity of previously published truncated orcokinin family peptides with an alanine, not glycine, at the 11th position. This concern has been supported by our analysis of *H. americanus* tissues using approaches that either exclude methanol or permit differentiation of Orc[1-11]-OMe/Orc[Ala^{11}], where we failed to find any evidence of putative Orc[Ala^{11}].

The truncated orcokinin, Orc[Ala^{11}], was first reported by Huybrechts et al. as a novel peptide de novo sequenced from the Jonah crab, *C. borealis* [21]. For that study, brain and thoracic ganglion tissues from 50 animals were extracted in methanol:water:acetic acid (90:9:1) and peptides were sequenced using ESI-Q-TOF MS/MS. As was the case for *H. americanus*, this peptide sequence, with an alanine appearing as the 11th residue, is at odds with the sequences of full-length *C. borealis* orcokinin peptides, which have been established by many MS studies [21,32]. We suggest that the peptide reported by Huybrechts et al. is, in fact, Orc[1-11]-OMe; this assertion is supported by work carried out in our laboratory, where we have evidence for the detection of Orc[1-11]-OMe in *C. borealis* brain tissue extracts (data not shown), but not for any directly analyzed *C. borealis* tissues (CoG, SG, PO, brain) (data not shown). Because alanine (A) is isobaric with methylation at a C-terminal glycine residue (G-OMe), this distinction would not have been revealed from mass measurements. Furthermore, the MS/MS technique used for de novo peptide sequencing in the Huybrechts et al.

study would not have provided any obvious flags to distinguish the C-terminal alanine from G-OMe.

The orcokinins NFDEIDRSGFA, SSEDMDRLGFA, and NFDEIDRSSFA, all with an alanine as the 11th residue and all detected in tissues that had been analyzed following extraction with acidified methanol, have been reported in other publications [15,16,21,30–32]. In summary, our work calls into question the identification of these truncated peptides, which may have Gly-OMe, not Ala, at the C-terminus.

4.4. The C-terminal Gly-OMe motif may be misidentified as Ala-OH

A unique issue for the in vitro modification detected in this study is the localized methylation at the C-terminus. Because the Gly-OMe modification is isobaric with Ala-OH, this structural change is not detectable via mass measurements. In this study, where the y_5 fragment ion was isolated and dissociated, the arginine-containing, singly protonated y_5 precursors (protonated RSGFGFA or RSGFG-OMe, respectively) yielded MS/MS spectra sensitive to the methyl-esterified C-terminus. Specifically, we observed a $[b_4+H_2O]^+$ product ion when the C-terminus had a free carboxyl group (for Orc[Ala^{11}]), and that diagnostic ion was missing when the C-terminus was methyl esterified (for Orc[1-11]-OMe). In contrast, the MS/MS spectra generated on our Q-TOF instrument were insensitive to the structural difference, and this approach could not be used for distinguishing the two peptide sequences. Because MS/MS spectra may not provide the specific, diagnostic information needed to distinguish the peptide sequences, and because standards are not always available, other measures, such as running extraction solvent controls with isotopically labeled solvents, may be needed to distinguish this extraction artifact.

4.5. Hypotheses regarding the formation of Orc[1-11]-OMe

Protease-catalyzed reactions have been exploited by chemists to carry out a variety of transformations in nonaqueous solvents [2], including C-terminal peptide esterifications [3,22,33,34]. Most enzymes exploited for this purpose are serine or cysteine proteases, which form reactive acyl-enzyme intermediates that can be attacked by a competing nucleophile, such as methanol.

In considering mechanisms that may be responsible for the production of Orc[1-11]-OMe and SSEDMDRLGFG-OMe, we note that the longer precursors to these modified orcokinin family peptides are not amidated at the C-terminus. Most bioactive neuropeptides are C-terminally amidated to prevent proteolytic degradation; therefore, the orcokinin peptides would be expected to be more susceptible to both enzymatic degradation and enzyme-mediated methylation. Additionally, while other C-terminally truncated orcokinins (predominantly Orc[1-12] and Orc[1-11]), have been detected in our investigations [10] and by other researchers [4,6,27,40], the C-terminal methylations detected for Orc[1-11]-OMe and SSEDMDRLGFG-OMe have only been associated with Gly^{11}. This implies that there is something unique about this amino acid (G) or the amino acid sequence proximate to this location that, in some way, enhances selectivity toward methanolysis. Finally, the glycine-phenylalanine (GF) motif at positions 11 and 12 are highly conserved elements of crustacean orcokinin sequences, which also may signify that this motif is important to neuropeptide function or processing.

Based on this information, we speculate that methanol could participate in either exo- or endopeptidase-mediated pathways leading to the production of Orc[1-11]-OMe, as well as SSEDMDRLGFG-OMe, from full-length orcokinin family peptides. An important element of this mechanism is the acidity of the solvent system, which can promote enzymatic methanolysis over
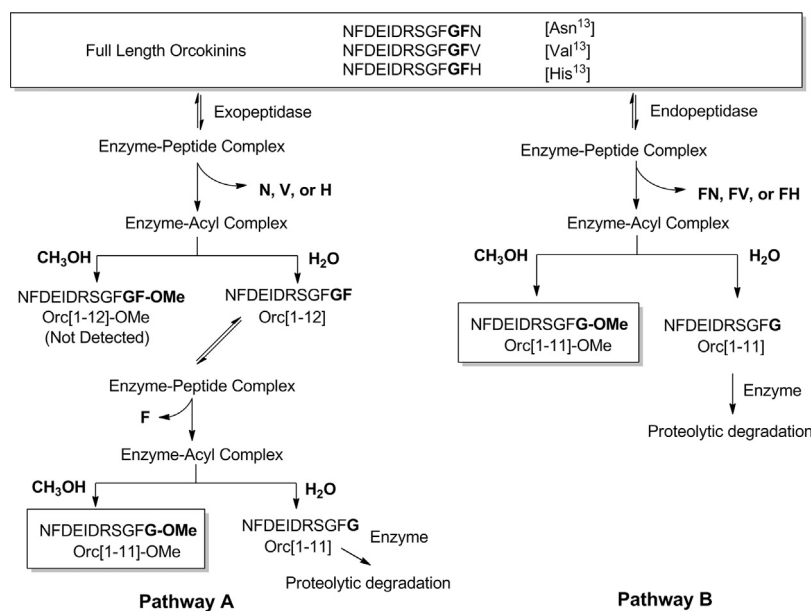
Fig. 16. Pathways that may result in C-terminal methanolysis to form Orc[1-11]-OMe. Pathways A and B include methanol as a competing nucleophile that attacks the acyl-enzyme intermediate formed by most serine endo- and exoproteases. Pathway A depicts possible reactions of an exopeptidase; pathway B depicts the pathway for an endopeptidase. The high abundance of Orc[1-11]-OMe may result from the action of a specific dipeptidase or an elevated reaction rate associate with methanolysis at the glycine residue. Solution pH may play a role in partitioning between hydrolysis (producing Orc[1-11]) and methanolysis (producing Orc[1-11]-OMe), with lower pH values favoring methanolysis.

hydrolysis [3]. One hypothesis, pathway A in Fig. 16, would involve C-terminal proteolysis of full-length orcokinin family peptides by an exopeptidase. Carboxypeptidases play an important role in the conversion of neuropeptide preprohormones into active neuropeptides, and these enzymes may be responsible for the production of truncated orcokinin peptides. For pathway A, selective detection of Orc[1-11]-OMe, but not Orc[1-12]-OMe, would require a kinetic effect favoring water addition (hydrolysis) to form Orc[1-12], with methanol able to compete effectively following loss of the phenylalanine (F) residue. A second hypothesis, pathway B in Fig. 16, invokes an endopeptidase with specificity toward cleavage between the Gly-Phe peptide bond. Again, to rationalize production of Orc[1-11]-OMe, methylation would occur in conjunction with the enzymatic cleavage of the peptide bond. Finally, we note that an amidated orcokinin, NFDEIDRSGFamide (Orc[1-10]-NH$_2$), has been reported in the literature for *H. americanus* [30] and detected in our lab (data not shown). In this peptide, the C-terminal Gly^{11} residue (methylated in Orc[1-11]-OMe) is the residue targeted by the peptidylglycine enzymes responsible for converting Gly^{11} into an amide group. The specificity associated with methylation of the Gly^{11} residue may be related to formation of an activated intermediate that is formed in the possible conversion of Gly^{11} to the amidated, Orc[1-10]-NH$_2$ product. Further experimentation is clearly needed to determine if any of these speculations about the highly specific conversion observed in this study have merit.

5. Conclusions

The truncated and C-terminally modified orcokinins, NFDEIDRSGFG-OMe (Orc[1-11]-OMe) and SSEDMDRLGFG-OMe were identified in eyestalk tissue extracts and the conditions responsible for production were explored using mass spectrometry. We found that the truncation with C-terminal methyl esterification occurs as a result of the extraction procedure, but the reaction is not a simple chemical acid-catalyzed esterification. Experiments with enzyme inhibitors and the use of heat for enzyme deactivation supported an enzymatically mediated conversion of full-length orcokinins to the truncated, methylated

NFDEIDRSGFG-OMe (Orc[1-11]-OMe) and SSEDMDRLGFG-OMe product. These products were not detected when tissues were analyzed directly. This study should heighten awareness regarding unexpected structural perturbations that may occur when neuropeptides are extracted from biological tissues.

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