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Multifaceted Mass Spectrometric Investigation of Neuropeptide Changes in Atlantic Blue Crab, *Callinectes sapidus*, in Response to Low pH Stress

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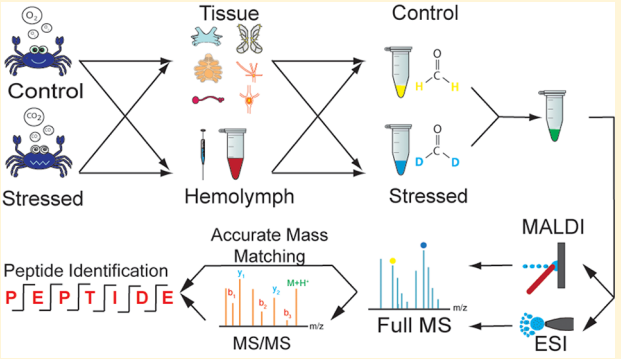
Supporting Information

ABSTRACT: The decrease of pH level in the water affects animals living in aquatic habitat, such as crustaceans. The molecular mechanisms enabling these animals to survive this environmental stress remain unknown. To understand the modulatory function of neuropeptides in crustaceans when encountering drops in pH level, we developed and implemented a multifaceted mass spectrometric platform to investigate the global neuropeptide changes in response to water acidification in the Atlantic blue crab, *Callinectes sapidus*. Neural tissues were collected at different incubation periods to monitor dynamic changes of neuropeptides under different stress conditions occurring in the animal. Neuropeptide families were found to exhibit distinct expression patterns in different tissues and even each isoform had its specific response to the stress. Circulating fluid in the crabs (hemolymph) was also analyzed after 2-h exposure to acidification, and together with results from tissue analysis, enabled the discovery of neuropeptides participating in the stress accommodation process as putative hormones. Two novel peptide sequences were detected in the hemolymph that appeared to be involved in the stress-related regulation in the crabs.



KEYWORDS: *Atlantic blue crab, Callinectes sapidus, neuropeptide, hemolymph, acidification, isotopic reductive dimethylation, mass spectrometry, LC−ESI−MS, MALDI−MS*

INTRODUCTION

Neuropeptides are the largest and most diverse group of endocrine signaling molecules in the nervous system. Recent studies have focused on characterization of neuropeptide signaling pathways[1] and their regulatory roles in physiological processes, such as rhythmic motor pattern,[2,3] food intake,[4−6] molting,[7] and accommodating stress.[8−10] With a relatively simple, well-characterized nervous system, invertebrates have become a popular model system for neuropeptide-based studies.[11−16] The decapod crustacean nervous system consists of a central nervous system (including the brain), neuroendocrine system (*e.g.*, pericardial organ and sinus glands), and stomatogastric nervous system (STNS), which contains 4 ganglia: paired commissural ganglia (CoG), unpaired stomatogastric ganglion (STG), and unpaired esophageal ganglion (OG).[17] Due to the crustacean open-circulatory system, all neuropeptide-rich tissues are bathed in fluid (i.e., hemolymph), functioning similarly as blood in mammals. Mature neuropeptides are secreted into the hemolymph,[17] after proteolytic processing from proneuropeptides, to achieve targeted modulation.[18] Thus, the study of neuropeptides in crustacean hemolymph may help to uncover their hormonal roles in regulating physiological processes, in addition to studying the tissue.[9,13,19]

As a species of marine animals, crustaceans are directly exposed to the water environment variation. Low pH stress is one of the most common stresses that crustaceans routinely experience in their habitats.[20] The global pH decreases in the seawater is mainly caused by the uptake of carbon dioxide (CO_2) in the atmosphere. Usually, water holds more CO_2 compared to oxygen (O_2) due to chemical reactions[21] which produce bicarbonate, carbonate, and protons,[22] consequently causing acidification. Human activities have been considered to be the most significant contributor to CO_2 release. As a principal sink of CO_2, oceans have been acidified from approximately 8.25 to 8.14[23] throughout this period, and their pH levels are now decreasing 50 times faster[24] compared to the preindustrial measurement.[25] It is predicted that by 2100, the pH levels of the oceans will decline by 0.15 to 0.31 units,[26] causing ecological and environmental problems.[27−31]

Studies of a diverse range of crustacean species showed that acidified environments induce a decline in locomotive activities,

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decrease in growth rate, and dysregulation in gene expression.[32−38] The Atlantic blue crab, *Callinectes sapidus*, is a remarkably mobile crustacean whose foraging, reproduction, and survival from predators and stress are highly dependent on frequent locomotion. The animal has been found to get tired in low O_2 (hypoxia) environments; however, the onset of fatigue was delayed when the animal was treated with additional CO_2 at the same time.[39] Notably, the pH of the crustacean circulatory fluid (hemolymph) showed no distinguishable change from the passive in vitro response in the initial 2−3 h after exposure.[40] The delay of CO_2-induced response may be brought about by an increase in hemolymph HCO_3^{-1} or a CO_2-specific effect on the blue crab's hemocyanin, which exhibited increasing affinity to oxygen at low pH values, supporting locomotive ability.[39,41] Unfortunately, there is still no sufficient explanation of the molecular mechanism of the underlying changes and the regulation of physiological response to pH stress.

In this study, we performed quantitative investigation of neuropeptide changes in different neural tissues in Atlantic blue crab, *Callinectes sapidus*, in response to low pH stress. Two complementary mass spectrometry (MS)-based methods, relying on either matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization (ESI) coupled with liquid chromatography (LC) were both adopted and evaluated by comparing the neuropeptide identifications and covered mass range. Crustacean neural tissues were collected at different time points (i.e., 2 h (hours) and 4 h) to monitor the dynamic changes of neuropeptides during development of the stress and the regulation of the adaptation process occurring in the nervous and neuroendocrine systems. In the circulating fluid, both known and newly discovered peptides were quantitatively measured to study their hormonal roles. This study provides a comprehensive analysis of crustacean neuropeptides in response to stress and will contribute to the future functional studies of neuropeptides in physiological regulation.

METHODS

Chemicals and Materials

Methanol (MeOH), acetonitrile (ACN), formic acid (FA), ammonium bicarbonate, and glacial acetic acid were purchased from Fisher Scientific (Pittsburgh, PA). Borane pyridine, formaldehyde, and deuterium formaldehyde were from Sigma-Aldrich (St. Louis, MO). 2,5-Dihydroxybenzoic acid (DHB) was obtained from Acros Organics (Morris Plains, NJ), and α-cyano-4-hydroxycinnamic acid (CHCA) was purchased from Sigma-Aldrich (St. Louis, MO). Acidified methanol was prepared as 90% methanol, 9% water, and 1% glacial acetic acid (v/v/v). All water used in this study was doubly distilled on a Millipore filtration system (Burlington, MA) or Fisher HPLC grade.

Animals and Acidification Experiments

Female Atlantic blue crabs, *Callinectes sapidus*, were purchased from local markets (e.g., Midway Asian Market (Madison, WI) or the Louisiana Crawfish Company (Natchitoches, LA)). All acquired crabs were maintained in recirculating artificial seawater, a salinity of 30 ppt, with a 12h:12h light/dark cycle, and without food for at least 1 week to minimize the stress-related effects due to the transportation. Inactive crabs or with missing limbs were not eligible for the experiment. Crabs used in one experiment (control and exposed) were selected from the same batch of purchased animals and were of similar body sizes, to ensure the baseline consistency in quantitative analysis (Figure S1 of the Supporting Information, SI). The selected crab was placed into a 10-gallon tank and was allowed to equilibrate at 18−20 °C for at least 3 days before experiments. Marine buffer purchased from Seachem Laboratories (Madison, GA) was added to avoid any deviation of initial pH level from 8.3, which is a normal pH for seawater. All housing parameters were the same between control and stressed groups. The acidified environment of the blue crabs' native habitat is quite complicated.[20] Here in this study, the model was simplified and simulated by introducing CO_2 gas. A pH sensor (American Marine Pinpoint pH Monitor) and a dissolved oxygen (D.O.) sensor (American Marine Pinpoint Oxygen Monitor) were placed at the corner of the tank farthest from the CO_2 gas tank tube. A moderate pH level (7.6−7.8) was achieved quickly by introducing CO_2 via gas tank into the housing tank. The air saturation was at 50%−60%, which was considered as moderate hypoxic condition. The pH level was allowed to stabilize before the stress experiment started. D.O. level and pH level were monitored throughout the experiment. The crabs were kept in the acidified environment for the desired durations (2 or 4 h). A tarp was placed on the water surface before the CO_2 sparge to prevent water−air exchange for the experimental duration. Control and stressed crabs were then cold-anesthetized on ice for 15−20 min followed by dissection, which usually took 45−50 min. Neuropeptide-rich organs, including brain, POs, SGs, TG, STG, and OG, were dissected in chilled physiological saline (Composition: 440 mM NaCl, 11 mM KCl, 13 mM $CaCl_2$, 26 mM $MgCl_2$, and 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) acid; pH 7.4, adjusted with NaOH). The dissection was carried out as described previously.[42] Hemolymph was collected by inserting a 25-gauge needle connected to a 1 mL plastic syringe through the base of one of the legs. An aliquot of 0.75 mL was withdrawn from the same crab before and after the low pH stress.

Tissue Extraction

Three animals' tissues, either control or stressed, were pooled into one sample to minimize individual variability and manually homogenized using chilled acidified methanol. Undissolved large proteins were removed by centrifugation at 16 100*g* for 10 min. The pellet was washed twice with acidified methanol. Supernatants were combined, dried down, and resuspended in 150 μL of 0.1% FA in water, followed by desalting using OMIX C_{18} Pipette Tips (Agilent Technologies, Santa Clara, CA). 150 μL 0.1% FA in 50% acetonitrile (v/v) was used to elute the peptides, and the elution was dried down and subject to labeling steps.

Hemolymph Sample Preparation

0.75 mL of hemolymph was added to an equal volume of acidified methanol to extract peptides and precipitate large proteins. The samples were centrifuged at 16 100*g* for 10 min. The pellet was washed twice by acidified methanol and the supernatants were combined, followed by ultrafiltration through a 10 kDa MWCO filter (rinsed by 0.1 M NaOH and 50/50 MeOH/H_2O, v/v) being centrifuged at 14 000*g* for 10 min. Filtrate was concentrated to dryness using a SpeedVac concentrator (Thermo Fisher Scientific, Waltham, MA, U.S.A.) and was resuspended in 150 μL of 0.1% FA in water. Samples were then desalted by OMIX C18 pipet tips (Agilent Technologies, Santa Clara, CA) and eluted to 150 μL 0.1% FA in 50% acetonitrile (v/v). Elution was dried down and subject to labeling steps.

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Reductive Dimethylation Labeling

Purified samples were dissolved into 10 μL 0.1% FA in water and labeled with formaldehyde-H_2 (1% v/v) and formaldehyde-D_2 (1% v/v), for control and stressed sample, respectively. Borane pyridine (30 mM) was added to the samples, which were then incubated for 15 min in a 37 °C water bath. Ammonium bicarbonate (100 mM) was added to quench the reaction. For each biological replicate, one heavy labeled (stressed, formaldehyde-D_2) sample and one light labeled (control, formaldehyde-H_2) sample were mixed in equal volume, then dried down and redissolved in 15 μL 0.1% FA in water. A 10 μL aliquot was subjected to further desalting process using C_{18} Ziptip Pipette Tips (Merck Millipore Ltd., Tullagreen, Carrightwohill, Co. Cork, IRL) before being loaded to LC−ESI−MS, and the remaining undesalted 5 μL was subjected to MALDI−MS analysis.

Mass Spectrometry Analysis

For ESI−MS analysis, the desalted sample in 10 μL 0.1% FA was subjected to online separation with a Waters nano-Acquity Ultra Performance LC system equipped with a self-packed column (150 mm length of 1.7 μm C_{18} with a 3 μm C_{18} cap) connected to a Q-Exactive quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). A 120 min gradient was used at flow rate of 0.3 μL/min, starting from 100% A (0.1% formic acid in water) and 0% B (0.1% formic acid in ACN), increasing to 10% B at 1 min, 35% B at 90 min, 95% B at 92 min (and remaining for 10 min) and then dropping back to 0% at 105 min. Typical mass spectrometry conditions were as listed: spray voltage, 2.1 kV; no sheath and auxiliary gas flow; heated capillary temperature, 275 °C. Data were collected in data-dependent mode, with top 15 abundant precursor ions selected for HCD fragmentation with the following settings: Full-MS, resolution, 70 000; AGC, $1e^6$; maximum injection time, 250 ms; scan range, m/z 200−2000; dd-MS^2, resolution, 17 500; AGC, $2e^5$; maximum injection time, 120 ms; loop count, 15; isolation window, m/z 2.0; fixed first mass, m/z 100.0; and normalized collision energy, 30.

For MALDI−MS, samples were separated into two fractions and spotted on MALDI target plate with DHB (2,5-dihydroxybenzoic acid, 150 mg/mL, 50% MeOH/50% H_2O/0.1% FA) and CHCA (α-cyano-4-hydroxycinnamic acid, 10 mg/mL, 84% acetonitrile/13% ethanol/0.003% trifluoroacetic acid) before analysis by MALDI−LTQ−Orbitrap XL mass spectrometer (Thermo Scientific Bremen, Germany) in the mass range of m/z 200−2000.

Data Processing

LC−ESI−MS raw data were analyzed by PEAKS Studio 7 (Bioinformatics Solution Inc., Waterloo, ON, Canada). Variable post-translational modifications (PTMs) were selected as C-terminal amidation, methionine oxidation, pyroglutamation, dehydration, dimethylation-H_2, and dimethylation-D_2. Parent mass error tolerance was 50 ppm, and fragment mass error tolerance was 0.02 Da. No enzyme cleavage was specified in de novo sequencing and database search. An in-house crustacean neuropeptide database was used. Quantitative analysis was conducted with a mass tolerance of 0.2 Da and retention time range of 1.0 min. Peptide spectrum matches (PSMs) with a −10logP value cutoff of 20 in PEAKS were considered for data filtration and validation.

MALDI−MS raw data were imported to a self-coded Java program to filter peak pairs that had mass increments of 28.0313 and 32.0564 Da from exact masses of NPs in database with mass error tolerance at ±5 ppm. Peak intensities of heavy (stressed) and light (control) labeled samples were calculated for fold changes in neuropeptide expression.

In both instrument methods, 5 biological replicates were analyzed for the brain, PO, SG, TG, STG, and OG at 2 h stress, as well as the brain and SG at 4 h stress. Four biological replicates were analyzed for the PO, TG, STG at 4 h stress. The hemolymph also had 4 biological replicates. Each biological replicate consisted of 3 technical replicates. Student's t-test was applied (unpaired t-test for tissue analysis, paired t-test for hemolymph analysis), and a p-value smaller than 0.05 was considered to be a statistically significant change.

RESULTS AND DISCUSSION

Neuropeptidome Analysis by MALDI−MS vs ESI−MS

As an essential and indispensable analysis method for complex samples, mass spectrometry (MS) has been widely used in

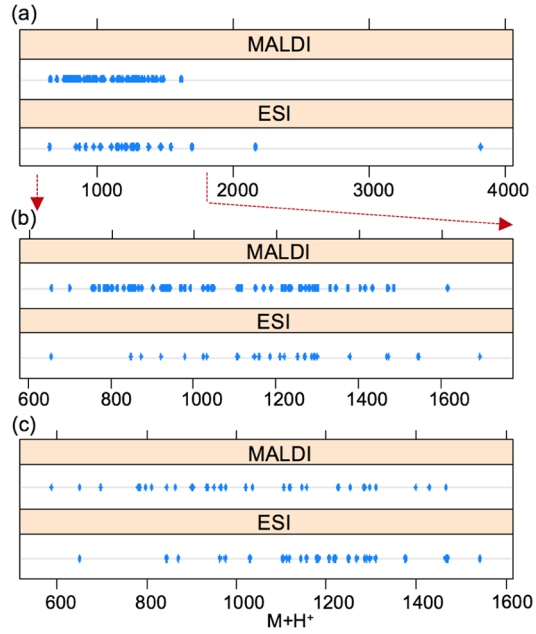

Figure 1. Mass distribution of neuropeptides detected in response to 2 h acidification stimulus in the PO (a) and the TG (c). Low mass range of neuropeptides in the PO was enlarged (b).

proteomics,[43−45] peptidomics,[46−48] and lipidomics[49−51] studies across species. Especially in the last several decades, the advent of ESI and MALDI have revolutionized the analysis of large biomolecules. MALDI is featured with relatively high tolerance to contaminants and salts, which allows for simple sample preparation, leading to less sample loss. It has been successfully applied to crustacean neuropeptide profiling[52,53] and stress-induced response study.[9,11] However, MALDI mostly produces singly charged ions, which often have limited fragmentation efficiency to generate sufficient sequence information. The simplified sample preparation of MALDI also leads to analyte suppression issues, which is detrimental to the detection of low level of endogenous neuropeptides in the crustacean.[54] ESI coupled with LC offers better separation followed by more efficient fragmentation due to the production of multiply charged ions. In this study, we adopted both methods in order to facilitate a more comprehensive neuropeptidome analysis. Furthermore, results from MALDI−MS were collected using

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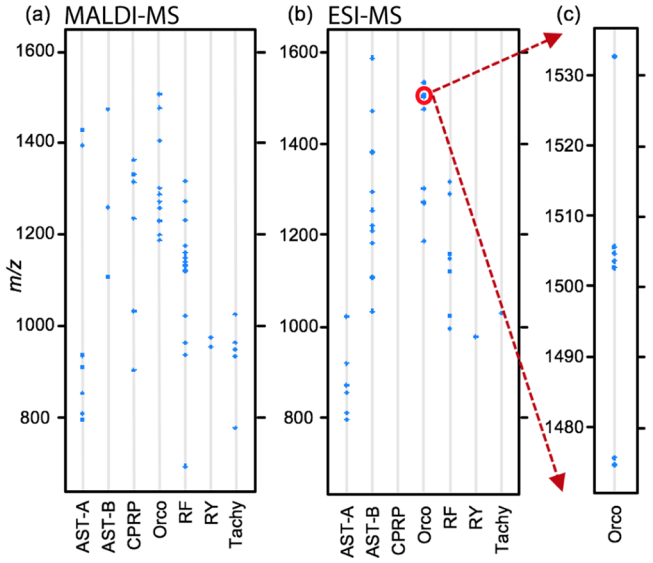

Figure 2. Neuropeptide families observed in the brain in response to 2 h low pH stress using (a) MALDI−MS and (b) ESI−MS. Mass range between 1460 and 1540 in the orcokinin family detected by ESI−MS was zoomed in (c). AST-A, A-type allatostatins; AST-B, B-type allatostatins; Orco, Orcokinin; RF, RFamide; RY, RYamide; and Tachy, Tachykinin.

both CHCA and DHB as matrices. These two matrices yielded complementary identifications of neuropeptides, which allowed better coverage of the neuropeptidome. Also, the expression level changes of neuropeptides detected in both matrices exhibited high consistency (data not shown), supporting the reproducibility and reliability of the results.

To compare the coverage contributed by each ionization method, MALDI−MS and ESI−MS were both applied to analyze brain, PO, SG, TG, STG, and OG at 2 h (SI Tables S1−S6) and 4 h time points (Supporting Information, SI Tables S7−S11). MALDI usually performed better in the detection of neuropeptides at the low mass range, whereas ESI facilitated detection at a higher mass range. In the POs treated with 2 h stress (Figure 1a,b), the mass of neuropeptides detected by ESI−MS could be up to 3.8 kDa, whereas MALDI−MS covered mass range up to 2 kDa. Differences in mass distribution could be observed throughout the experiments. As an example, after 2 h stress, the molecular weights of neuropeptides in the TG (Figure 1c) detected by MALDI−MS showed predominant occupancy in low mass while ESI−MS contributed to the detection of a greater number of larger peptides.

The differences in distribution between MALDI− and ESI−MS were also found among neuropeptide families. After 2 h incubation in low pH environment, in the brain tissue, more neuropeptides from allatostatin B-type (AST-B) and tachykinin families were detected by ESI−MS (Figure 2b) than that by MALDI−MS (Figure 2a). The responses of several peptides from the crustacean hyperglycemic hormone precursor-related peptide (CPRP) family to the stress were found in the brain using MALDI−MS; nevertheless, none of them was detected in the brain using ESI−MS. Interestingly, more orcokinin isoforms at a higher mass range within their family were detected by ESI−MS than by MALDI−MS (Figure 2c). The reason for the differences in family distribution could be attributed to the fact that some peptides ionized better with the assistance of MALDI

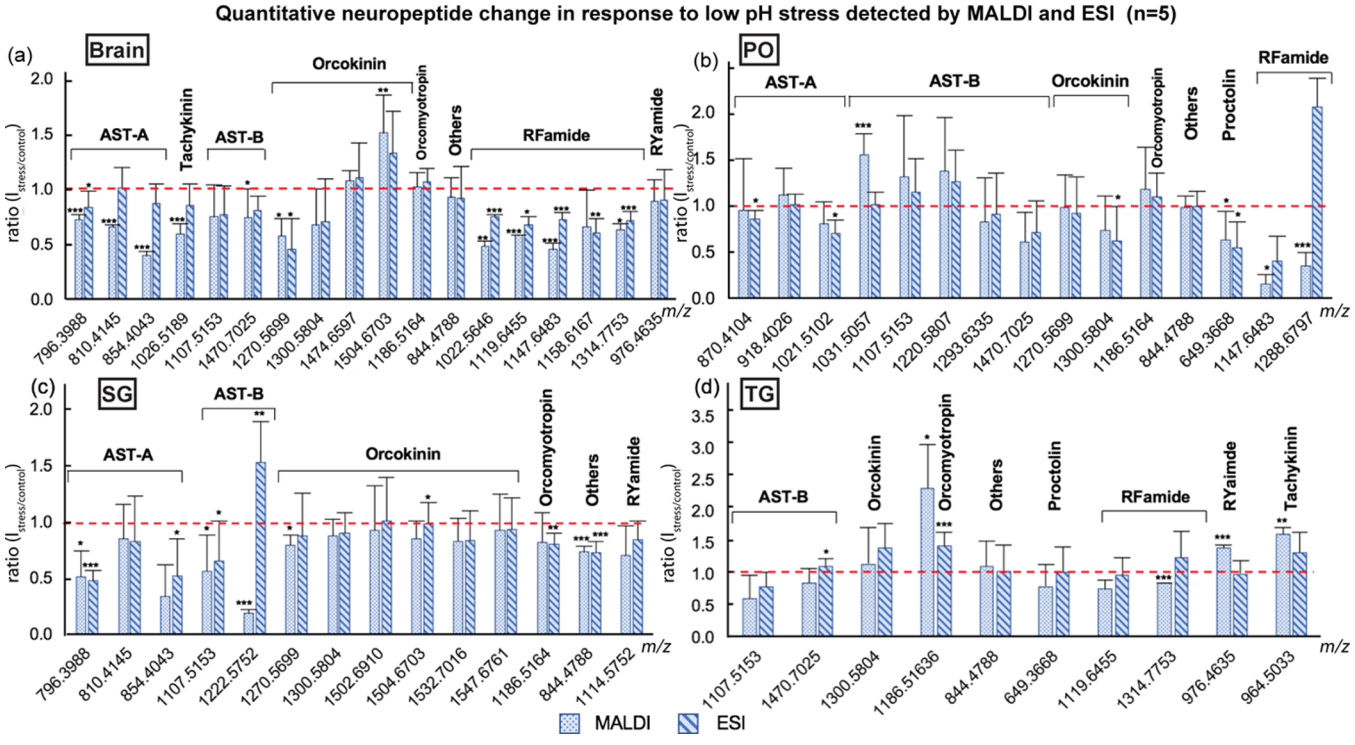

Figure 3. Neuropeptide changes in the (a) brain, (b) pericardial organ (PO), (c) sinus gland (SG), and (d) thoracic ganglia (TG) upon 2 h acidified incubation ($n = 5$), acquired by MALDI−MS and ESI−MS. Ratios were calculated by dividing the heavy-labeled groups (stressed, pH 7.6−7.8) by the light-labeled (control, pH 8.3). *X-axis*, exact mass of neuropeptides ($M + H^+$); *Y-axis*, peak intensity ratio of stress over control; *Error bar*, standard deviation; *Red dash line* indicated a ratio equal to one or no change in peptide levels. Student's *t*-test was applied to evaluate the significance of neuropeptide level changes upon stress compared to control groups. $*p < 0.05$; $**p < 0.005$; $***p < 0.001$.

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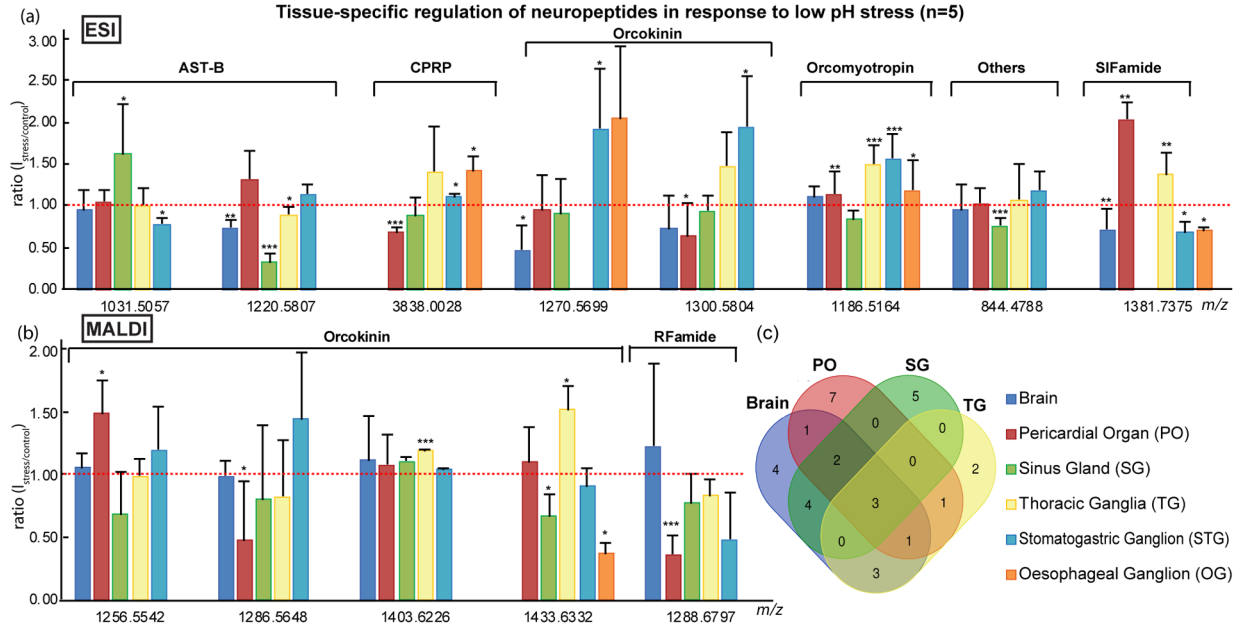

Figure 4. Expression level changes of single neuropeptide isoforms across various tissues under 2 h low pH stress ($n = 5$) analyzed by ESI−MS (a) and MALDI−MS (b). Venn diagram (c) for the numbers of neuropeptides detected after 2 h low pH stress in four major neural tissues (Brain, PO, SG, and TG). Only neuropeptides identified by both MALDI− and ESI−MS were included in this graph. *X-axis*, exact mass of neuropeptides ($M + H^+$); *Y-axis*, peak intensity ratio of stress over control; *Error bar*, standard deviation; *Red dash line* (a ratio of one) indicated no change in peptide levels. Student's *t*-test was applied to evaluate the significance of neuropeptide level changes upon stress compared to control groups. $*p < 0.05$; $**p < 0.005$; $***p < 0.001$.

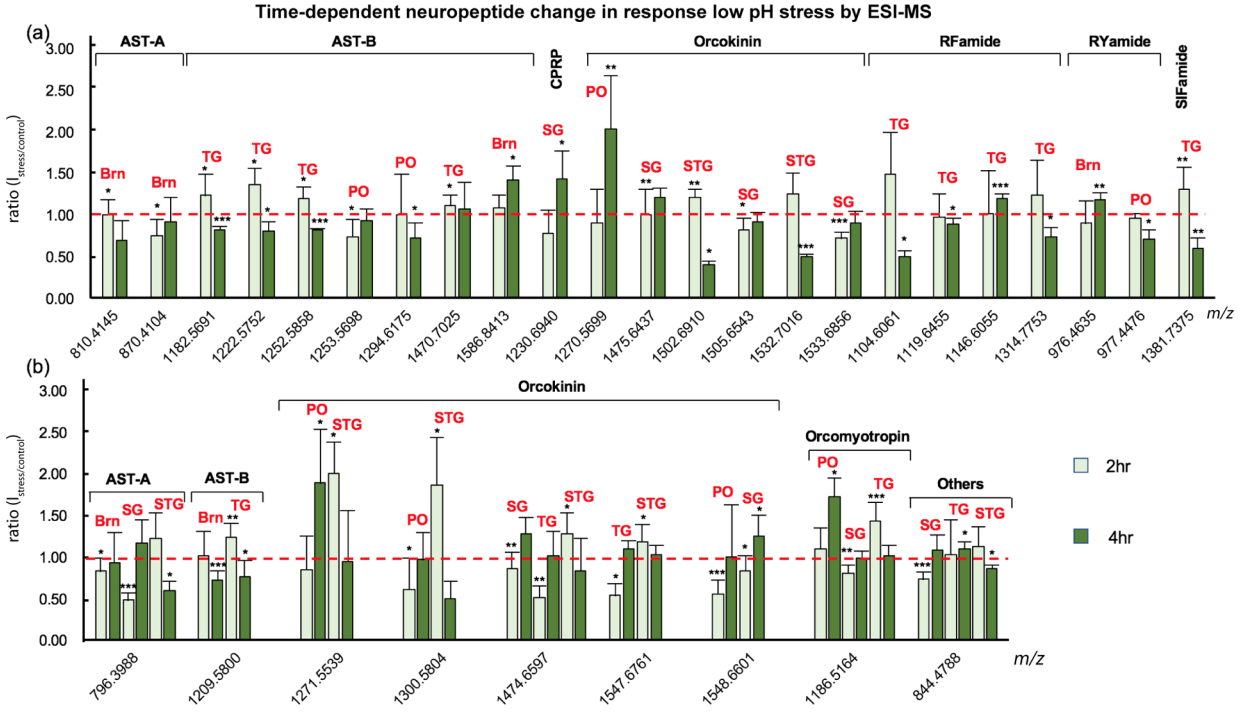

Figure 5. Neuropeptide changes identified by ESI−MS at different time points in a single tissue (a) indicating the stress-related function of the peptide and in multiple tissues (b), indicating the hormonal role of the peptide when pH declined. *X-axis*, exact mass of neuropeptides ($M + H^+$); *Y-axis*, peak intensity ratio of stress over control; *Error bar*, standard deviation; *Red dash line* (a ratio of one) indicated no change in peptide levels. Student's *t*-test was applied to evaluate the significance of neuropeptide level changes upon stress compared to control groups. $*p < 0.05$; $**p < 0.005$; $***p < 0.001$. *Brn*, brain.

matrix, whereas some other peptides would generate multiply charged ions more easily, resulting in preferential detection by either ionization method.

As stated above, ESI and MALDI together contributed to a broadened coverage of the neuropeptidome in our study. Furthermore, consistency in expression level changes should be demonstrated in the overlaps of MALDI− and ESI−MS

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Table 1. Neuropeptide Changes in the Hemolymph in Response to 2 h low pH Stress[a]

source	family	mass	sequence	$ave_{s/c}$	$std_{s/c}$	tissue	$ave_{s/c}$	$std_{s/c}$
ESI	AST-B	997.5003	AWSALHGAWamide	0.76	0.22			
	others	1254.8144	AVLLPKKTEKK	0.29***	0.06			
	CPRP	3838.0028	RSAEGLGRMGRLLASLKSDTVTPLRGFEGETGHPLE	1.00	0.32	PO***	0.656	0.05
						SG	0.840	0.21
						TG	1.345	0.53
MALDI	proctolin	649.3668	RYLPT	2.02	0.66	brain	1.07	0.12
						PO*	0.63	0.31
						SG	0.68	0.37
						TG	0.79	0.34
						STG	0.89	0.16
						OG**	4.75	0.76
	RFamide	966.5268	DRNFLRFamide	0.04*	0.02	PO	0.38	0.38
						TG	0.69	0.38
	SIFamide	1161.6527	RKPPFNGSIFamide	0.03***	0.02			
	actin	1209.6837	LRVAPEESPVL	0.61	0.31			
	RFamide	1253.6538	HDSPHVFLRFamide	0.12*	0.04			

[a]*p < 0.05; **p < 0.005; ***p < 0.001.

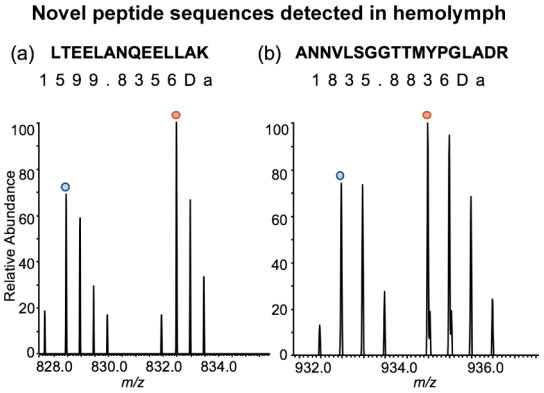

Figure 6. Mass spectra (full scan) of two novel peptides detected in the hemolymph using ESI–MS. *Blue circle*, control; *Red circle*, 2 h acidification stress.

analysis. As shown in Figure 3, most neuropeptides that were detected in both MALDI and ESI exhibited similar trends in response to 2 h acidification stress. However, several outliers were found, such as AST-B GNWNKFQGSWamide (m/z 1222.5752) in SG, AST-B AWSNLGQAWamide (m/z 1031.5057) and RFamide QDLDHVFLRFamide (m/z 1288.6797) in PO. The discrepancy may result from different data analysis methods applied to the two data sets. When identifying neuropeptides from MALDI–MS data using accurate mass matching, we search through the in-house database which contained exact masses calculated by neuropeptide sequences (pyroglutamine (pQ) and C-terminus amidation were involved in calculation if indicated in sequences). Other PTMs (*e.g.*, methionine oxidation and dehydration) were included in PEAKS search against ESI–MS data. Some neuropeptides with C-terminus amidation were also accompanied by their nonamidation isoforms. In fact, PEAKS applied statistical methods to determine the intensity ratio from multiple isoforms and charge states of a neuropeptide. This result suggests that peptides with different modifications may play distinct roles in physiological regulation.

Neuropeptide Changes in Neuronal Tissues upon 2 h Water Acidification Stress

Neuropeptides can be classified into different families according to the common sequence motif they shared. Distinct motifs among neuropeptide families are expected to give rise to their different functional roles in physiological modulation.[14,15,55−58] In this study, several neuropeptide families were found, throughout the crustacean neuronal tissues, to respond to the low pH stress. RFamides in the brain (Figure 3a), proctolin in the PO (Figure 3b), and allatostatin A-type (AST-A) in the SG (Figure 3c) were all found to be down-regulated, while the expression levels of tachykinin and orcomyotropin in the TG (Figure 3d) were up-regulated. The diversity in the response further illustrated that each neuropeptide family contributed differently to the adaptation to the environmental stress.

We also discovered that isoforms in a neuropeptide family, although having similar sequences, may still differ from each other in their response to the stimulus. For example, in the brain, orcokinin NFDEIDRSGFA (m/z 1270.5699) decreased significantly (MALDI: s/c, 0.57; p, 0.0475; ESI: s/c, 0.45; p, 0.0240) due to the acidification; however, another isoform NFDEIDRSSFGFA (m/z 1504.6703) exhibited significant increasing trend (MALDI: s/c, 1.50; p, 0.0042; ESI: s/c, 1.31). In the PO, level of AST-B AGWSSMRGAWamide (m/z 1107.5153; MALDI: s/c, 1.30; ESI: s/c, 1.14) and SGDWSSLRGAWamide (m/z 1220.5807; MALDI: s/c, 1.37; ESI: s/c, 1.26) were elevated when the animal was exposed to low pH stress. In contrast, the expression level of VPNDWAHFRGSWamide (m/z 1470.7025) was reduced (MALDI: s/c, 0.61; ESI: s/c, 0.7). The disparity of response within the same neuropeptide family suggested that the modulatory function of a single isoform may differ from each other. Each isoform likely has its unique role in physiological regulation.[59,60]

Among the neuronal tissues collected in this study, many peptides were found in common (Figure 4c). Interestingly, it was found that an identical neuropeptide may exhibit different expression trend in response to low pH stress in different tissues. Orcokinin NFDEIDRSSFGF (m/z 1433.6332) detected by MALDI–MS (Figure 4b), was significantly reduced in the SG (s/c, 0.64; p, 0.0085) and OG (s/c, 0.35; p, 0.0083), while its expression level was elevated by nearly one and half-fold in the

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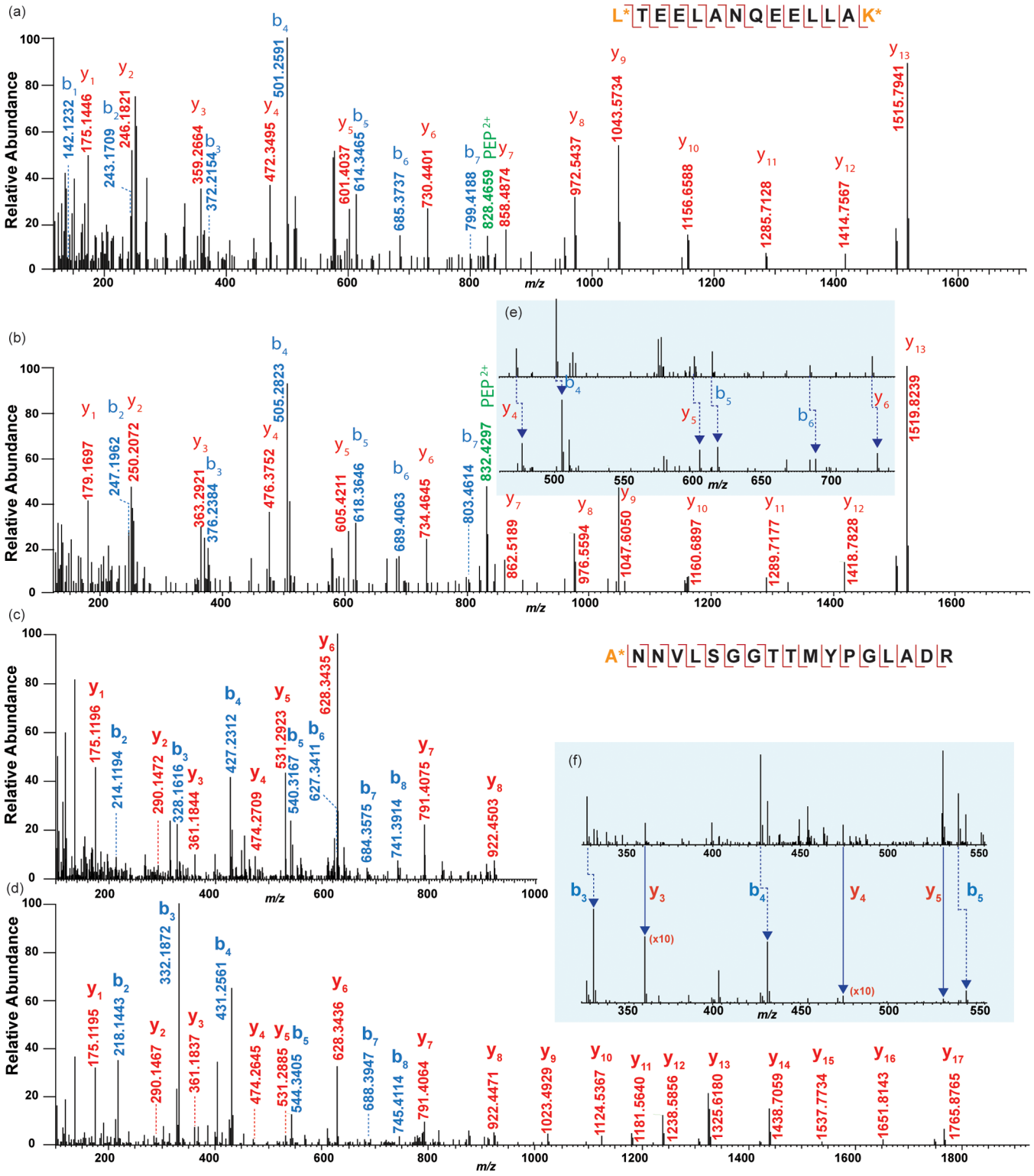

Figure 7. Tandem mass spectra of novel peptides (LTEELANQEELLAK and ANNVLSGGTTMYPGLADR) acquired from ESI−MS for control (a, c) and 2 h stressed (b, d) hemolymph samples. Dimethyl labeling was applied for quantification. Amino acid residues with asterisks showed the position of dimethyl groups added. Mass difference caused by isotopic labeling was illustrated in (e, f).

TG (s/c, 1.45; *p*, 0.0262). In the PO, this neuropeptide remained unaffected (s/c, 1.05; *p*, 0.7688) by the acidification. Similar phenomenon was also observed in the ESI−MS studies (Figure 4a). The level of AST-B AWSNLGQAWamide (*m/z* 1031.5057) was reduced in the STG (s/c, 0.75; *p*, 0.0179), increased in the SG (s/c, 1.55; *p*, 0.0505), yet kept approximately the same in other tissues (brain: s/c, 0.91; *p*, 0.1918; PO: s/c, 1,01; *p*, 0.9879; TG: s/c, 0.96; *p*, 0.5494). It was noted that neuropeptides detected in both STG and OG followed the same trend in response to the stress. The disparity and the consistency in neuropeptide expression in different tissues may reflect that, under environmental stress, the role of a

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neuropeptide could be excitatory or inhibitory depending on the location of the neuropeptide and the position of the tissue within the nervous system. POs release neuropeptides directly into the circulatory system in the region of heart; thus, the dynamic changes of neuropeptides in the PO in response to the stress may indicate their modulation of cardiac activity. The TG has extended nerve cords connecting to the limbs of crabs, therefore, it is reasonable to consider that the TG, to some degree, controls the movements of crustaceans. It has been shown that blue crabs got fatigued in a shorter period and their pull force decreased faster in the addition of CO_2 at 50% air saturation treatment when compared to 100% air saturation group.[39] Hence, neuropeptide changes in the TG may provide clues to unveil the physiological functions in locomotion control during the low pH stress. The STG contains the motor neurons which manage the movements of the muscles of pyloric regions of the stomach.[16] The OG contains descending modulatory neurons that can influence activity of the STG[2]. Accordingly, neuropeptides in the STG and the OG may act as modulators in pyloric rhythms.[61] The multiple directions of a single neuropeptide being regulated under environmental stress may elucidate the multifunctions and distinct roles of the neuropeptide in different neuronal regions.

Another possible explanation to the distinct trends of identical neuropeptide in six different tissues could be that, in addition to their local functions, neuropeptides were also released from neurons and neuroendocrine tissues as circulating hormones.[55,62−64] For example, after 2 h incubation in acidified conditions, SIFamide GYRKPPFNGSIFamide (m/z 1381.7375) was measured to be significantly reduced in the brain (ESI: s/c, 0.69; p, 0.0037), STG (ESI: s/c, 0.65; p, 0.0464) and OG (ESI: s/c, 0.67; p, 0.0400), meanwhile its expression level was up-regulated in the PO (ESI: s/c, 1.94; p, 0.0050) and the TG (ESI: s/c, 1.31; p, 0.0030). This may indicate that SIFamide was released from the brain, STG and OG, followed by transporting via circulating fluid, and exerted influence on the PO and TG. Neuropeptides in the brain were found to be unaffected by temperature elevation.[9] In this study, orcokinin NFDEIDRSGFG (m/z 1256.5542, s/c, 1.01; p, 0.9373), NFDEIDRSSFG (m/z 1286.5648, s/c, 0.94; p, 0.4126) and NFDEIDRSGFGF (m/z 1403.6226, s/c, 1.07; p, 0.8089), detected by MALDI−MS, orcomyotropin FDAFTTGFGHS (m/z 1186.5164, MALDI: s/c, 1.01; p, 0.8771; ESI: s/c, 1.06; p, 0.1406), and others HL/IGSL/IYRamide (m/z 844.4788, MALDI: s/c, 0.91; p, 0.1922; ESI: s/c, 0.91; p, 0.2409), detected by both MALDI−MS and ESI−MS, remained constant after exposure to the low pH stress. While it might be tempting to conclude that neuropeptides in the brain were irrelevant to the adaptation of acidification, this may also suggest that the fluctuation of neuropeptide levels in the brain was balanced out by those in the peripheral neuronal organs. For example, orcokinin NFDEIDRSGFG (m/z 1256.5542) exhibited statistically significant increase in the PO (MALDI: s/c, 1.42; p, 0.0115) and decrease in the SG (MALDI: s/c, 0.65), respectively, while in the TG (MALDI: s/c, 0.95; p, 0.3165) and STG (MALDI: s/c, 1.14; p, 0.5366), the orcokinin level had minimal change. The opposite trends of this orcokinin isoform in surrounding tissues may contribute to the relatively stable expression level in the brain, since it acts as a central control of the entire nervous system.

Stress Development Analysis under 2 and 4 h Incubation

In order to investigate how the low pH stress developed in the crustacean and how the neuropeptides modulated the accommodation to the stimulus, we also collected tissue from crabs incubated for 4 h in an acidified environment. The neuropeptide expression changes were calculated against the control groups using the same method applied to 2 h stress. The stress-to-control ratio of the same peptide was compared between 2 and 4 h stress incubation (Figure 5a). Several neuropeptides, such as AST-B TSWGKFQGSWamide (m/z 1182.5691) in the TG, orcokinin NFDEIDRSGFGFV (m/z 1502.6910) in the STG, and SIFamide GYRKPPFNGSIFamide (m/z 1381.7375) in the TG all significantly increased at 2 h measured point (s/c, 1.23; p, 0.0165; s/c, 1.21; p, 0.0059; s/c, 1.31; p, 0.0030, respectively) but dropped remarkably after 4 h incubation (s/c, 0.81; p, 0.0009; s/c, 0.40; p, 0.0032; s/c, 0.60; p, 0.0025, respectively). This observation may suggest that the regulation of these neuropeptides was different when the animals encountered with acute or long-term stress. The decrease of peptide expression level may indicate either degradation by protease within the tissue or released externally to other targets, being involved in physiological processes. Further studies concerning the circulating fluid will be needed to prove the secretion of peptides and the targeted function of these peptides as hormones. Similarly, AST-B NDWSKFGQSWamide (m/z 1253.5698) in the PO and orcokinin DFDEIDRSSFGFA (m/z 1505.6543) in the SG recovered to normal levels (s/c, 0.93; p, 0.2407; s/c, 0.92; p, 0.0625, respectively) by the end of the 4 h stress duration after a dramatic change (s/c, 0.74; p, 0.0239; s/c, 0.82; p, 0.0120, respectively) during the halfway point. Neuropeptides were likely produced or used up to regulate the stress at the beginning. As the self-adjustment of the animal brought the physiological environment toward normal condition, the level of peptide also recovered close to normal level. Some other neuropeptides, like AST-B (m/z 1294.6175), orcokinin (m/z 1270.5699), and RYamide (m/z 977.4476) in the PO, remained unaffected in the first 2 h (s/c, 1.00; p, 0.7233; s/c, 0.91; p, 0.3444; s/c, 0.96; p, 0.1473, respectively) but deviated from normal level significantly (s/c, 0.73; p, 0.0248; s/c, 2.03; p, 0.0037; s/c, 0.71; p, 0.0460, respectively) by the end of the 4 h incubation, exhibiting that the initiation of stress-induced response of these neuropeptides was delayed for at least 2 h. Studies on blue crab locomotion have revealed that the onset of fatigue would be delayed because of the additional CO_2 to hypoxia induced by N_2 only.[39] This could likely be explained by the CO_2-specific effect on blue crab hemocyanin, the affinity to oxygen of which would largely increase to overcome the negative effect due to acidification.[39,41] In another circulating fluid study, a lag for the initiation of any active response to the hemolymph pH change was also found when the blue crab was put in water bubbled with CO_2.[40] Here, the neuropeptidomic analysis provided deeper insight into the mechanisms of how crustaceans accommodated to low pH stress at the molecular level and facilitated functional discovery of neuropeptides.

Stress development was also monitored across different tissues (Figure 5b). Again, tissue-specific response was observed. For instance, at 2 h time point, orcokinin DFDEIDRSGFA (m/z 1271.5539) was present at the standard level in the PO (s/c, 0.84; p, 0.1274), but was up-regulated in the STG (s/c, 2.00; p, 0.0156). When it came to the end of 4 h stress treatment, the level of this orcokinin peptide in the STG returned to normal level (s/c, 0.94; p, 0.5291) while being elevated in the PO

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significantly (s/c, 1.88; p, 0.0078). The transmission pathway could likely be described as when the animal initially encountered the stimulus, the orcokinin was generated and accumulated in the STG; with time development and self-accommodation, the neuropeptide was released from the STG and transported to the PO, indicating the cardiac activity as a modulation target in this period.

Quantification of Neuropeptide Levels in Circulating System

In the crustacean, hemolymph fills the hemocoel of the body and surrounds all the organs and cells. When encountered with acidification, several physiological aspects are affected by the stress, such as immune system and acid−base balance.[65−67] Neuropeptides are secreted upon stimuli from neuronal tissue into hemolymph and exert influence on target tissue, which provides critical information for mapping the peptidomic regulatory pathway. In the ESI−MS analysis (Table 1), CPRP RSAEGLGRMGRLLASLKSDTVTPLRGFEGETGHPLE (m/z 3838.0028) remained at constant level in the hemolymph after 2 h exposure to low pH stress, while it was either up or down-regulated in the PO, SG, and TG. Donors and receptors delivered and received, respectively, similar amount of peptides, yielding a relatively stable concentration in the hemolymph during stress incubation. CPRP is a circulating hormone with unknown function.[63] Our study may reveal potential regulatory role of this CPRP in pH stress adaptation. Similarly, proctolin RYLPT (m/z 649.3668) was found to have stable expression level in the brain and the TG, while decreased level in the PO, SG, and STG, yet dramatically elevated in the OG. Its relative concentration in the hemolymph was increased significantly, suggesting its secretion into the circulating system and possible hormonal role.

In general, we expect to observe opposite trends of neuropeptide level changes within the neuroendocrine or secretory tissue and circulating fluid assuming hormonal role of secreted peptides. However, RFamide DRNFLRFamide (m/z 966.5268) detected by MALDI−MS and others such as AVLLPKKTEKK (m/z 1254.8144) observed via ESI−MS were measured to be down-regulated in both tissue and hemolymph. One possible explanation would be that normally the source of peptides in hemolymph was the secretion from tissues. However, when the animal was exposed to stress, fewer neuropeptides were produced in neurons or the produced neuropeptides were enzymatically degraded, therefore, fewer neuropeptides were released into the circulating system or a particular function of the peptide was suppressed due to stress.

Two novel peptides were found to be up-regulated due to the decrease of pH, Pep1599 LTEELANQEELLAK (m/z 1599.8356) and Pep1835 ANNVLSGGTTMYPGLADR (m/z 1835.8836) (Figure 6). With dimethyl derivatization, the fragmentation was relatively complete, accounting for most of the b- and y- ions (Figure 7a−d). In the isotopic labeling, two methyl groups were added to primary amine groups, such as N-terminus and lysine residue (K). For Pep1835, dimethyl labeling on the N-terminus resulted in mass increment of 28.0313 and 32.0564 Da, by two CH_2 or two CD_2, respectively. In the tandem mass spectrum, a 4.0251 Da mass shift was observed in all b-ions, while y-ions showed good alignments (Figure 7f). For Pep1599, isotopic formaldehyde reacted with both the N-terminus and the lysine on the C-terminus, yielding mass increment by 56.0626 and 64.1128 Da. Therefore, a 4.0251 Da mass shift occurred on both b- and y-ions (Figure 7e).

Pep1599 and Pep1835 do not belong to any known neuropeptide family based on our current knowledge. It has been reported that in blue crabs, hemolymph proteins (mainly hemocyanin) are involved in pH adjustment.[39,41] As for other species, hemolymph protein levels altered under hypercapnia in both shallow-water crabs and deep-sea crabs.[68] In brine shrimp, proteins related to metabolic process, stress response, immune defense, cytoskeletal, and signal transduction were examined to change once hatched and raised in acidification environments.[69] Therefore, we performed a BLAST search against the protein database using the sequence of Pep1599 and Pep1835. There was no match of any known proteins found with Pep1599, indicating this peptide could be produced via cleavage from an unidentified protein related to pH stress. Pep1835 was searched to align with actin in several other crustacean species, such as *Daphnia magna* and *Homarus americanus*. Another actin fragment detected in this study was LRVAPEESPVL (m/z 1209.6838), which was found to be down-regulated in MALDI−MS analysis. This actin fragment was found to increase in the hemolymph of Jonah crab, *Cancer borealis*, upon feeding.[53] Actin typically participates in vital cellular process, such as muscle contraction and cell signaling. Our current study may reveal its additional function related to stress adaptation in crustaceans.

CONCLUSIONS

In this study, we quantitatively analyzed the influence of low pH stress on neuropeptide changes in Atlantic blue crab, *Callinectes sapidus*, both in tissue and in body fluid. Different ionization methods were adopted, and the results were combined to provide more comprehensive coverage. It has been demonstrated that neural organs, brain, PO, SG, TG, STG, and OG, were involved in stress-related modulation. Each neuropeptide family and each isoform within the family has possibly distinct functions depending on its sequence and the tissue from which it belongs. The discovery of identical neuropeptides in multiple tissues and the circulating fluid also indicated those peptides as hormonal molecules, being released from neuroendocrine organs upon stimulus, traveling through the circulatory system, and exerting effects on targeted organs. The time course study of low pH stress revealed dynamic changes of neuropeptides, indicating the pathway of self-adjustment and adaptation in the animal over time. Collectively, our study offers a more comprehensive view of the neuropeptidome as key molecular players in the physiological regulation in response to environmental stress in crustacean model system. Future directions of this study should focus on extending to more severe pH change and longer incubation period. The neuropeptidome analysis under only hypoxia stress should be investigated to facilitate better understanding of the CO_2-induced low pH stress.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.9b00026.

MALDI−MS spectra of labeled control tissues (Figure S1) (PDF)

Neuropeptide level changes in response to 2 and 4 h low pH stress (Tables S1−S11) (XLSX)

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Notes

The authors declare no competing financial interest.

Representative mass spectrometry data have been deposited to the ProteomeXchange Consortium[70] via the PRIDE partner repository with the data set identifier PXD013536.

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ABBREVIATIONS

PO, pericardial organ; SG, sinus gland; TG, thoracic ganglia; STG, stomatogastric ganglion; OG, esophageal ganglion; CHH, crustacean hyperglycemic hormone; CPRP, CHH precursor-related peptide; AST-A, A-type allatostatins; AST-B, B-type allatostatins; s/c, stress/control; MALDI, matrix-assisted laser desorption/ionization; ESI, electrospray ionization; LC, liquid chromatography; MS, mass spectrometry.

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