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Patsy S. Dickinson  
*Bowdoin College*

Xuan Qu  
*Bowdoin College*

Meredith E. Stanhope  
*Bowdoin College*

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# Neuropeptide modulation of pattern-generating systems in crustaceans: comparative studies and approaches

Patsy S Dickinson<sup>1</sup>, Xuan Qu<sup>2</sup> and Meredith E Stanhope<sup>2</sup>



Central pattern generators are subject to modulation by peptides, allowing for flexibility in patterned output. Current techniques used to characterize peptides include mass spectrometry and transcriptomics. In recent years, hundreds of neuropeptides have been sequenced from crustaceans; mass spectrometry has been used to identify peptides and to determine their levels and locations, setting the stage for comparative studies investigating the physiological roles of peptides. Such studies suggest that there is some evolutionary conservation of function, but also divergence of function even within a species. With current baseline data, it should be possible to begin using comparative approaches to ask fundamental questions about why peptides are encoded the way that they are and how this affects nervous system function.

## Addresses

<sup>1</sup> Biology and Neuroscience, Bowdoin College, 6500 College Station, Brunswick, ME 04011, USA

<sup>2</sup> Neuroscience, Bowdoin College, 6500 College Station, Brunswick, ME 04011, USA

Corresponding author: Dickinson, Patsy S ([pdickins@bowdoin.edu](mailto:pdickins@bowdoin.edu))

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## Introduction

Pattern-generating networks in crustaceans are extensively modulated by amines and neuropeptides, with the number of identified neuropeptides in many species nearing or exceeding 100 (e.g. [1<sup>\*</sup>,2–5]). While the functions of many neuropeptides remain unknown, some peptides are able to modulate pattern-generating networks; these modulatory inputs enable the circuits to produce a plethora of different motor patterns. However, the comparative studies that would enable us to understand the relationships between the effects of different peptides within a species as well as the extent to which those effects are conserved across species are limited.

Physiological studies of modulation in the crustaceans have focused almost exclusively on two networks in the decapods: the stomatogastric nervous system, which controls the rhythmic movements of the foregut, and the cardiac ganglion (CG), which controls rhythmic contractions of the neurogenic heart. For want of truly comparative and evolutionary studies, we can only begin to answer important questions regarding the evolution of peptidergic modulation of pattern generators in the Crustacea, including why so many modulatory neuropeptides exist in these relatively simple systems, and whether there are fundamental differences between highly conserved peptides and those that are much more evolutionarily variable. This review will thus first address the evolutionary dispersion of peptides and the extent to which they are conserved, as well as what is currently known about neuropeptide receptors. We then review recent functional studies of peptide modulation in a comparative light.

## Identity and distribution of neuropeptides across species and tissues

Complete characterization of the first several invertebrate neuropeptides to be identified, such as red pigment concentrating hormone (RPCH) [6] and proctolin [7], required large pools of tissue and multiple biochemical techniques. In recent years, two newer techniques have replaced extensive biochemical sequencing to identify peptides in crustaceans: mass spectrometry and transcriptomics.

## Mass spectrometry

Multiple types of mass spectrometry are used to identify neuropeptides. Matrix-assisted laser desorption/ionization (MALDI) instruments are commonly coupled with time-of-flight (TOF) or Fourier transform (FT) mass analyzers and electrospray ionization (ESI) instruments are often coupled with quadrupole (Q)-TOF hybrid mass spectrometers. Tandem mass spectrometry (MS/MS) is used to determine amino acid sequences and to identify post-translational modifications. More recently, multifaceted mass spectrometric approaches have allowed researchers to identify peptides spanning a wider mass range, as well as to apply this technology in innovative ways. Thus, recent research has not only generated new peptidomes or enlarged known peptidomes, but has also begun to detect spatial and temporal distributions of peptides [8,9,10<sup>\*</sup>,11] and to detect peptides in hemolymph (e.g. [8,9,10<sup>\*</sup>,11,12]).

Ye *et al.* [13<sup>\*</sup>] pioneered the use of multiple mass spectrometry platforms to identify and map the distribution of peptides within individual ganglia; they localized five different peptides within the brain of *Panulirus interruptus*. Although peptides related to those they localized are known to modulate pattern-generating networks in decapod species, Ye *et al.* did not examine tissues in which identified pattern generating networks are localized. Members of the same laboratory adapted other mass spectral techniques to examine the distribution of peptides [14,15] within the stomatogastric ganglion in *Callinectes sapidus*. Before the development of mass spectrometric mapping techniques, distribution of peptides was determined primarily using immunohistochemistry (e.g. [16–18]). Immunohistochemistry still provides far better spatial resolution than mass spectrometry, but most antibodies are not able to distinguish between different peptide isoforms. Thus, the availability of multiple techniques to map the distribution of neuropeptides within the nervous system using mass spectrometry, or combining mass spectrometry and immunohistochemistry, should facilitate comparative studies that will allow us to determine the relative distributions of individual members of the same peptide family. Since key questions in understanding the evolutionary and functional significance of peptide diversification are the extent to which members of a given peptide family are localized and released together and the extent to which they exert common effects, these techniques could be important in enabling them to be addressed.

#### Analysis of peptidomes using transcriptomics

Within the last few years, transcriptomics has emerged as a powerful tool for understanding gene expression in organisms, such as crustaceans, that do not have a fully sequenced genome. Translated mRNA sequences can be assessed for biological markers and homology to known peptide sequences from related species. This technique is particularly useful when it is impossible to gather the large quantities of tissue needed for biochemical techniques, such as when proteins are expressed exclusively in one small tissue type. Like mass spectrometry, however, transcriptomics may not have complete coverage of the proteins produced in the tissue, leaving some transcripts partial or missing, particularly those transcribed in low abundance. Moreover, errors in sequences can be artifacts of the transcriptome assembly process, and post-translational modifications are merely predictions. Some studies counter this dilemma by combining transcriptomics and mass spectrometry approaches to confirm some of the mined sequences [19,20<sup>\*</sup>,21]. Studies such as these, although interesting in their own right, are largely lacking significant physiological relevance.

Nevertheless, transcriptomics is an important methodology in the study of crustacean nervous systems. Many such studies have analyzed transcriptomes for the sole

purpose of elucidating entire peptidomes of species, and have predicted numerous neuropeptides in a wide range of crustacean species (e.g. [22–29]).

#### Conservation of peptides: highly conserved peptides versus extensive peptide families

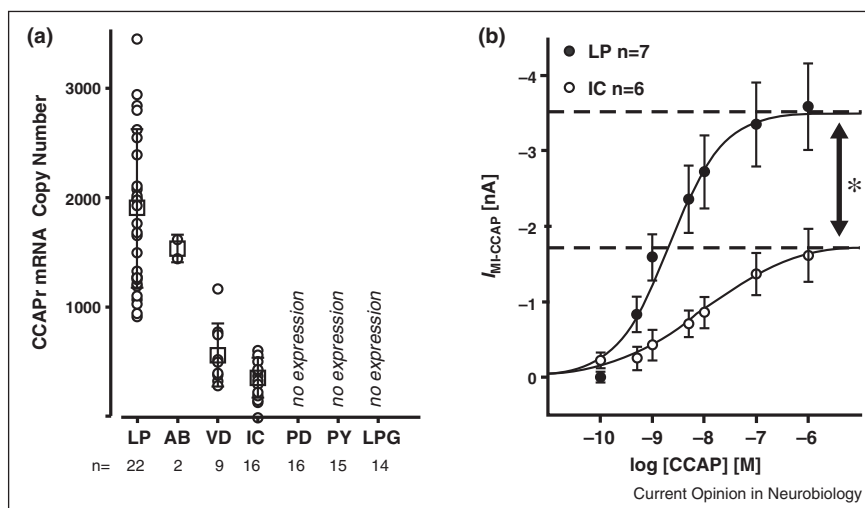
Studies that have focused on single species as well as those that have compared the structure and/or distribution of single peptides across species (e.g. [30]) indicate that the extent to which peptides are conserved varies widely between peptide families. Some peptides, such as proctolin, crustacean cardio-active peptide (CCAP), and red pigment concentrating hormone (RPCH), appear to be highly conserved, with a single sequence conserved across virtually all crustaceans. Others show minor differences (e.g. one amino acid) between species; such peptides include SIFamide [13<sup>\*</sup>,30–32], tachykinin-related peptides [31,33], and myosuppressin (pQDLDHVFLRFamide [30]), a member of the larger RFamide family that is found on its own transcript [1<sup>\*</sup>]. Interestingly, three isoforms of the peptide allatostatin-C were recently identified from a *Carcinus* transcriptome. One of the three is highly conserved across arthropod species (mostly insects); the others are somewhat less conserved, but have not yet been examined in other decapods [34]. Other peptides, such as the A-type allatostatins, the pyrokinins, and the other RFamides, including the FLRFamides, are much more variable, and often have multiple isoforms even within a single precursor transcript [1<sup>\*</sup>,35].

Together, these studies raise important, but still unanswered questions. Why are some peptides so highly conserved while others are extraordinarily variable, both within and across species? Do different isoforms have different functions? Do they bind to the same receptors? Are they perhaps differentially susceptible to peptidases? Does the level of conservation of a peptide correlate with its functions in modulating the output of neuronal networks? Understanding the answers to these questions will be important in understanding the role peptidergic signaling plays in modulating patterned behavior. Unfortunately, as of the present time, no large-scale comparative physiological studies have been conducted, and relatively few smaller studies have been published.

#### Identity and distribution of neuropeptide receptors

To understand the roles played by neuromodulators, it is critical not only to know the identity and distribution of the modulators themselves, but also to understand the distributions of receptors and the interactions of peptides with their receptors. However, relatively little is known about peptide receptors and their distributions in crustaceans, particularly with respect to pattern generators. One recent study [1<sup>\*</sup>] identified 41 transcripts encoding putative neuropeptide receptors in a *Homarus americanus*

Figure 1



(a) Levels of expression of CCAPr in individual pyloric neurons in the crab *Cancer borealis*, determined using quantitative PCR, not only vary among individuals (circles), but also differ among pyloric neurons. The expression in the lateral pyloric (LP) neuron, for example, is much higher than that in the inferior cardiac (IC) neuron. Other neurons do not express this receptor at all. Squares indicate mean; error bars denote SEM. (b) Correlated with the differences in CCAPr expression, the magnitude of the  $I_{MI}$  current activated by CCAP is higher in the LP neuron than in the IC neuron. Error bars denote SEM: \*,  $p < 0.05$ . Modified from [37\*\*].

neural transcriptome that included the stomatogastric and cardiac ganglia. However, the distributions of these receptors within the nervous system are not known. In a study in the spiny lobster *Sagmariasus verreauxi*, Buckley *et al.* [36\*] identified 85 G-protein coupled receptors (GPCRs). Although the transcriptomes used in this study were not nervous system specific, they were able to identify sequences for many expected neuropeptide receptors. Both of these studies, however, identified transcripts solely on the basis of homology to known receptors from other species; studies examining binding specificity and affinities of peptides to the receptors identified in crustaceans are badly needed.

One recent study has begun to tie receptor distribution and function together. In the crab *Cancer borealis*, Garcia *et al.* [37\*\*] identified a putative CCAP receptor. CCAP modulates both the gastric mill and pyloric patterns in this species, activating an identified current,  $I_{MI}$ , in many, but not all, stomatogastric neurons. Garcia *et al.* [37\*\*] showed that the distribution of CCAP receptors paralleled the distribution of electrophysiological responses to CCAP. In the one neuron type that expressed CCAP receptor transcripts but did not respond to CCAP by activating  $I_{MI}$ , they found that CCAP instead modulated synaptic currents. Moreover, expression levels of CCAP receptors (CCAPr) varied not only between animals, but also between neurons (Figure 1a). Intriguingly, in a comparison of two pyloric neurons, higher receptor expression correlated with a larger  $I_{MI}$  response and greater sensitivity to CCAP (Figure 1b). Additionally, Garcia *et al.* [37\*\*] elegantly

showed that there are differences in the proportions of  $I_{MI}$  channels that are activated by CCAP between two neuron types with different levels of CCAPr expression. The implications of this study for modulation of pattern generators, particularly in the presence of more than one modulator, whether as a result of co-transmission, the release of multiple hormones, or the interactions of hormonal and local modulation, are profound: depending on receptor distribution, different neurons may respond more or less strongly to the addition of an additional modulator [37\*\*]. Similar studies of other receptors and species are badly needed.

### Comparative physiological studies of modulators affecting crustacean pattern generators

The pattern generators of the stomatogastric nervous system and CG have been studied physiologically in a number of species, including clawed lobsters and crayfish (*Homarus americanus*, *Homarus gammarus*, *Procambarus clarkii*), several crab species (*Cancer* species, *Carcinus maenas*), and spiny lobsters (primarily *Panulirus interruptus*). However, although these species span several infraorders, most studies have targeted a single species. Nonetheless, one comparative study found that peptidergic modulation of the pyloric pattern in the kelp crab, *Pugettia producta*, whose diet is largely limited to kelp, is considerably reduced compared to that recorded in other crustacean species studied (e.g. *Cancer* crabs); of four peptides examined in *Pugettia* [proctolin, RPCH, tachykinin-related

peptide, and crustacean cardioactive peptide (CCAP)], only proctolin regularly activated the pyloric pattern [38].

Additionally, several studies have compared the effects of related peptides on a single pattern generator or the effects of the same peptides on multiple pattern generators within a species. Others [39–41] have examined this question at a single current level, finding that multiple modulators can activate the same current ( $I_{MI}$ ).

#### Members of a single peptide family can exert similar effects on pattern generators across species

As early as 1988, comparisons of red pigment concentrating hormone (RPCH) and a number of structurally related adipokinetic hormones (AKHs) suggested that structurally similar peptides can exert similar effects on crustacean pattern generators [42]. While the native peptide RPCH had a somewhat lower threshold for its effects on the pyloric pattern in *Cancer borealis*, the effects of RPCH were very similar to those of three different AKH isoforms, all of which were originally identified in insects. All resulted in an increase in the pyloric cycle frequency and in the relative burst duration and number of spikes in the lateral pyloric (LP) neuron when applied at concentrations of  $10^{-7}$  M. Interestingly, although a number of isoforms of AKH have been identified in the insects, all of the decapod crustaceans that have been examined appear to express authentic RPCH [35,43]. Another early study found that two FMRFamide-related peptides, TNRNFLRFamide and SDRNFLRFamide, exerted effects similar to one another on both the pyloric and gastric mill patterns in the crab (*Cancer borealis*) [44]. A more recent study found that two sulfakinins (pEFDEY<sub>(SO<sub>3</sub>H)</sub>GHMRFamide and GGGEY<sub>(SO<sub>3</sub>H)</sub>DDY<sub>(SO<sub>3</sub>H)</sub>GHLRFamide) identified as native to the lobster (*H. americanus*) exerted similar effects on the cardiac neuromuscular system, although the effects of the longer isoform were more pronounced [45]).

#### Effects of FMRFamide-like peptides (FLPs) on the cardiac neuromuscular system

One of the larger crustacean neuropeptide families is the FLPs, whose effects on the cardiac system have been examined across species. In the crab *Callinectes sapidus*, the effects of three FLPs, including a native FLP (GYNRSFLRFamide), were examined in whole and semi-intact heart preparations, in the isolated CG, and in a stimulated muscle preparation [46]. More recently, the effects of two native FLPs (GYSNRNYLRFamide and SGRNFLRFamide) in the lobster *H. americanus* were compared using similar preparations [47\*\*]. Interestingly, all three native peptides increased contraction frequency and amplitude when perfused through the whole heart at low concentrations ( $\sim 10^{-9}$  M); they also increased the amplitude of contractions at the level of the muscle or neuromuscular junction. However, none of the peptides

increased cycle frequency in the isolated CG, suggesting that the increased frequency in the whole heart was indirect, mediated through the stretch feedback system [47\*\*,48]. Qualitative differences between the effects of GYSNRNYLRFamide and SGRNFLRFamide suggest that the peptides do not all act identically on the multiple sites affected. Based on the range of effects of the FMRFamide-like peptides and their different thresholds, Dickinson *et al.* [47\*\*] suggested that, in addition to effects on the CG and the muscle/neuromuscular junction, they might alter the balance of positive (stretch) and negative (nitric oxide [49]) feedback systems that are known to exist within the cardiac neuromuscular system.

When the whole heart is stretched in either the longitudinal or transverse direction, contraction amplitude increases; the extent of this increase is enhanced by the FLPs [50]. Interestingly, this effect is anisotropic; that is, GYSNRNYLRFamide enhanced amplitude only to transverse stretch, while SGRNFLRFamide increased the effects of longitudinal stretch. These results suggest that neuropeptides in this family interact with the length-tension characteristics of the heart to create a more flexible rhythmic heart contraction.

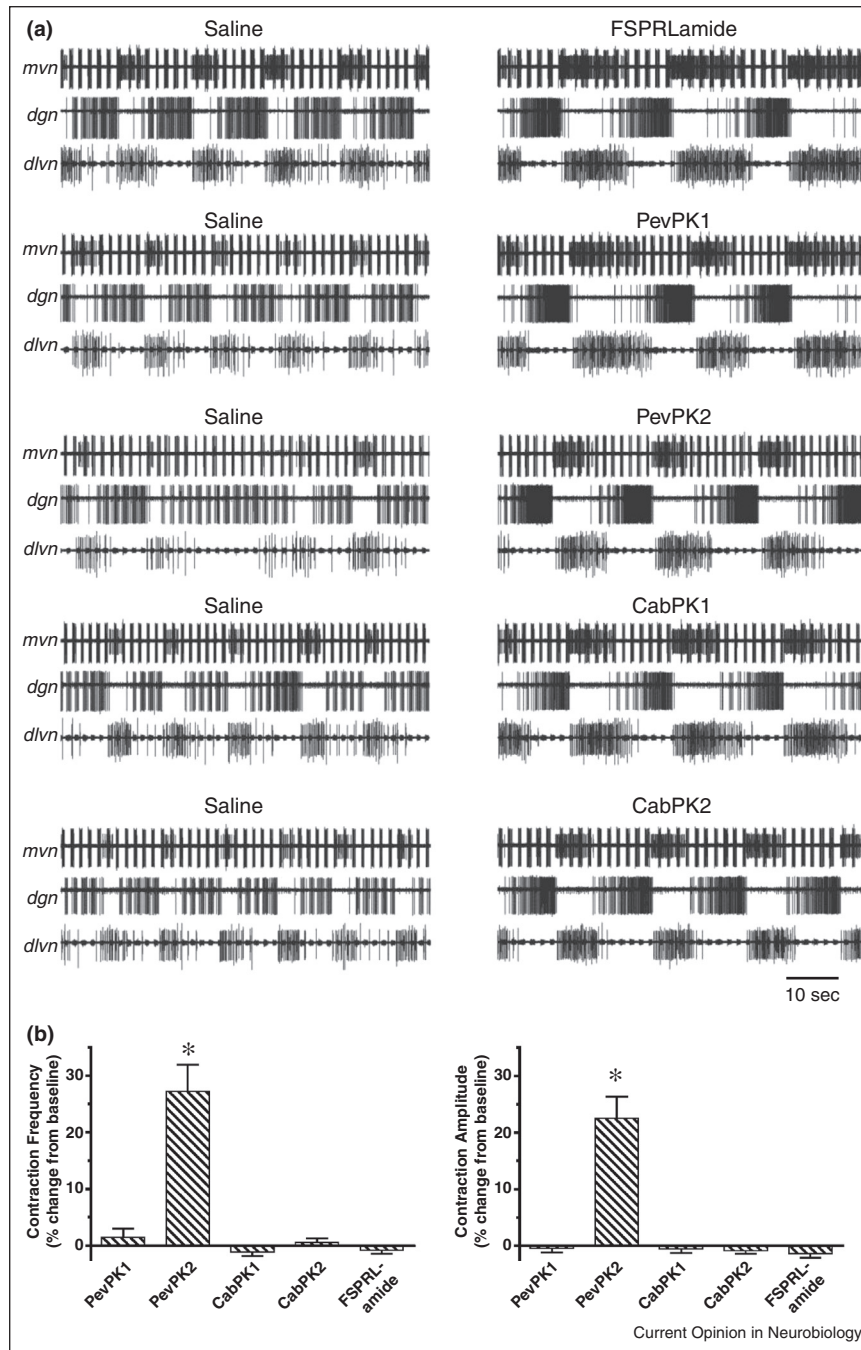
Finally, while it is clear that many of the effects of FLPs are shared across peptides and species, it is also clear that there are differences in the effects of peptides within the family in each species. What is not clear is whether these differences are due to different binding affinities for the peptides on the same receptor or whether the peptides activate different receptors. Thus far, only one (*Homarus* [1\*]) or two (*Sagmariasus verreauxi* [36\*]) FMRFamide-like receptors have been predicted by transcriptomic studies.

#### Pyrokinin family peptides

As is the case with the FLPs acting on the cardiac system, members of the pyrokinin peptide family appear to exert similar effects on stomatogastric pattern generators in two crustacean species, *C. borealis* and *H. americanus*. In particular, pyrokinin peptides native to crab, shrimp and cockroach all have equivalent actions in the stomatogastric ganglion in crabs, where they excite the gastric mill, but not the pyloric, pattern [51]. Likewise, the conserved pyrokinin fragment FSPRamide [1\*,5], and four other crustacean pyrokinins similarly excited only the gastric mill pattern in *Homarus* (Figure 2a) [52\*\*].

The mechanisms underlying pyrokinin modulation have not yet been examined, but the similarity of effects of multiple peptides in two species suggests a highly conservative modulatory function of the peptides in this family. Interestingly, in the crustacean species that have been examined, there appear to be at least two pyrokinin isoforms. Transcriptome analysis suggests, for example, that there are seven pyrokinins in *Homarus* [1\*]. However,

Figure 2



The effects of crustacean pyrokinins are similar when activating patterns in the stomatogastric ganglion, but differ from one another when activating the cardiac neuromuscular system in the same species. **(a)** Five different crustacean pyrokinins, including the fragment FSPRLamide, which had been previously identified from the lobster, activate the gastric mill rhythm, leading to patterns that do not differ from one another when superfused over the stomatogastric ganglion at a concentration of  $10^{-6}$  M. The left column shows patterns recorded in saline just before the application of each peptide; all recordings are from the same individual. The higher frequency bursts recorded on the *mvn* are from the ventricular dilator (VD) neuron, which is active in the pyloric pattern, and is not altered by any of these peptides. *mvn*: medial ventricular nerve; *dgn*: dorsal gastric nerve; *dlvn*: dorsal lateral ventricular nerve. **(b)** When the same 5 pyrokinins were applied to the cardiac system of the lobster, only one, PevPK2, had any significant effect. It increased both contraction amplitude and frequency when applied at a concentration of  $10^{-6}$  M, mimicking local release. Pyrokinins are one of the relatively few peptides that have been localized to the cardiac ganglion. Modified from [52\*\*] and [53\*\*].

Table 1

**Sequences of pyrokinins tested on the *Homarus americanus* cardiac system, together with the more recently identified native *Homarus* pyrokinin sequences. None of the native sequences have the FNPRLamide C-terminus that characterizes PevPK2, the only pyrokinin that enhanced cardiac activity. It is not currently known whether these native peptides exert modulatory effects on the cardiac system.**

Peptide acronym	Peptide sequence	Species	Reference
CabPK1	TNFAFSPRLamide	<i>Cancer borealis</i>	Ma <i>et al.</i> , 2010
CabPK2	SGGFAFSPRLamide	<i>Cancer borealis</i>	Ma <i>et al.</i> , 2010
Conserved fragment	FSPRLamide	<i>Homarus americanus</i>	Ma <i>et al.</i> , 2008
PevPK1	DFAFSPRLamide	<i>Litopenaeus vannemei</i>	Torfs <i>et al.</i> , 2001
PevPK2	ADFAFNPRamide	<i>Litopenaeus vannemei</i>	Torfs <i>et al.</i> , 2001
HoapK1	GDDITNEELAY <sub>(SO<sub>3</sub>H)</sub> DDNLTSEYLRDDNNDYLPEEL-TEDVTEMSSPEMLSESAAALVGKNSVSVFIPRLamide	<i>Homarus americanus</i>	Christie <i>et al.</i> , 2015
HoapK2	DSEDSSVESRNTKTQASIPRPamide	<i>Homarus americanus</i>	Christie <i>et al.</i> , 2015
HoapK3	GDGFAFSPRLamide	<i>Homarus americanus</i>	Christie <i>et al.</i> , 2015
HoapK4	GADFAFSPRLamide	<i>Homarus americanus</i>	Christie <i>et al.</i> , 2015
HoapK5	SDFAFSPRLamide	<i>Homarus americanus</i>	Christie <i>et al.</i> , 2015
HoapK6	SLFSPRLamide	<i>Homarus americanus</i>	Christie <i>et al.</i> , 2015
HoapK7	AYFSPRLamide	<i>Homarus americanus</i>	Christie <i>et al.</i> , 2015

at present, no physiological experiments have examined any of these native peptides. Interestingly, these peptides are encoded on two different transcripts, suggesting that at least some of them can be differentially released [1<sup>\*</sup>].

#### Related peptides can exert divergent effects on different pattern generators in a single species

In contrast to the similar effects of multiple pyrokinins on stomatogastric pattern generators across species, the effects of different pyrokinins on the cardiac system of the lobster are strikingly different from one another. Notably, only one of the peptides tested, PevPK2, had any effect on the heart; it increased both the frequency and amplitude of heart contractions (Figure 2b) [53<sup>\*\*</sup>]. The mechanisms that underlie the differential responses of the stomatogastric and cardiac systems to pyrokinins are not yet known. PevPK2 is not highly similar to any of the native pyrokinins thus far identified in *Homarus* (Table 1), suggesting the possibility that it is acting on an unrelated receptor.

Alternatively, its secondary structure may enable it to bind to a pyrokinin receptor. If so, one possibility is the CPG-specific distribution of multiple pyrokinin receptors with differential sensitivities. Other possibilities include differences in the second messenger systems activated by the different peptides in the two ganglia [54]. Follow-up studies examining the responses of both the cardiac and stomatogastric systems to the seven native *Homarus* pyrokinins could shed light on the extent to which the differential responses of these two networks reflect evolutionary and physiologically important differences.

#### Convergent effects of modulators can result in divergent modulatory outcomes

Previous studies [41] in the crab stomatogastric system have shown that a number of peptide modulators converge to activate the same ionic current,  $I_{MI}$ . Moreover,

by activating these currents in different neurons, these peptides can alter CPG output in distinct ways [41]. It was also postulated [39] that activation of the current on the same neuron could lead to different output patterns when the timing of peptide release differs. Recently, mathematical modeling showed that convergent targeting of  $I_{MI}$  by hormonally released CCAP and the rhythmic synaptic release of CabTRPIa would differentially alter opposing phases of the gastric mill pattern [55<sup>\*</sup>]. Thus, evolutionary changes in CPG modulation could increase plasticity and robustness of a network by acting on either the distribution of receptors on neurons within the CPG or the timing of release of modulators that target a single current, rather than adding new modulated currents. However, although it seems likely that similar currents are activated in other species, these networks have not yet been studied at the same level as those in the crab.

Additionally, it is likely that the different peptides that activate the same receptor are differentially susceptible to the proteases present in the ganglion, with the consequence that the peptides will reach the receptors at different concentrations, and will persist in the ganglion for different periods of time, thereby resulting in divergence of their effects even when activating the same currents. Indeed, Wood and Nusbaum [56] found that an aminopeptidase present in the STG [57] is partly responsible for the differential effects of a single peptide (proctolin) released from different projection neurons in the crab.

#### Conclusions

Although a number of recent studies have examined the effects of the same or related peptides on more than one species, on the comparative roles of the same peptide family in two systems within the same species, or on the function of convergent neuromodulators on the same system, relatively few studies have approached modulation

from a truly comparative perspective. Thus, many questions remain. Little is known about the timing and control of peptide release. This is particularly important for modulators that target the same current in a given neuron, and should have profound effects on the interactions of modulators. Additionally, comparative studies [52<sup>••</sup>,53<sup>••</sup>] of the different effects of pyrokinins on the cardiac and the stomatogastric systems in a single species suggest both different mechanisms and functions in these two pattern generators, but the mechanism that underlies such a differential response remains unknown. Future studies using a molecular approach to determine both the receptors and second messenger pathways used by these peptides may shed light on this finding. More comparisons of this nature will help to determine whether this is an unusual arrangement or if it is shared by other peptides.

One of the biggest evolutionary questions remaining derives from the observation that some peptides are highly conserved, with only one member identified, and only one isoform in the peptide transcript, whereas others exist in multiple isoforms. Some of these are on the same transcript, so are likely released together, while others are on separate transcripts, so could be differentially released. We still do not know whether there are qualitative differences in the roles or modes of release of highly conserved versus highly variable peptides. Moreover, some peptides are highly conserved functionally across species, while others may produce multiple effects and target several pattern generators within a species. Additional questions to be answered focus on more integrated and comparative aspects of modulation: what are the interactions among neuromodulators when present at the same time? How are they regulated? Why do systems respond differently to the same neuromodulators? And how do these components collectively contribute to the flexibility as well as the stability and robustness of the systems?

## Conflict of interest statement

Nothing declared.

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