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REVIEW

Crustacean neuropeptides

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Abstract Crustaceans have long been used for peptide research. For example, the process of neurosecretion was first formally demonstrated in the crustacean X-organ–sinus gland system, and the first fully characterized invertebrate neuropeptide was from a shrimp. Moreover, the crustacean stomatogastric and cardiac nervous systems have long served as models for understanding the general principles governing neural circuit functioning, including modulation by peptides. Here, we review the basic biology of crustacean neuropeptides, discuss methodologies currently driving their discovery, provide an overview of the known families, and summarize recent data on their control of physiology and behavior.

Keywords Transcriptomics · Mass spectrometry · Peptidergic neuromodulation · Neurotransmitter · Neurohormone · Peptidomics · Stomatogastric ganglion · Cardiac ganglion

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General biology of neuropeptides

The largest class of signaling molecules used by nervous systems are peptides, short strings of α-amino acids linked by amide bonds. Like all peptides, neuropeptides are encoded within genomes as larger precursor proteins, known as pre/prepro-hormones (Fig. 1). After transcription and translation, a neuropeptide-containing precursor protein is directed into the secretory pathway via a signal sequence present at its amino (N)-terminus. Within this pathway, post-translational processing takes place, and the peptides within the precursor are packaged into secretory vesicles. In some cases, a single peptide is contained within a precursor protein (here termed a pre-hormone), liberated by signal peptidase cleavage of the signal sequence. More commonly, multiple peptides are encoded within a prepro-hormone, each surrounded by sites for cleavage by enzymes such as prohormone convertase (Fig. 1). Following cleavage from the pro-hormone (the precursor protein minus its signal sequence), many peptides undergo extensive post-translational processing, which can result in modifications including, but not limited to, carboxy (C)-terminal amidation, cyclization of N-terminal glutamine/glutamic acid residues, disulfide bridging between cysteines, and sulfation of tyrosines (Fig. 1). The presence of these post-translational modifications is often responsible for a peptide assuming its bioactive conformation.

Once packaged and processed to its mature conformation, a peptide is released from the neuron synthesizing it to exert its effects on a target. These targets can be the neuron releasing the peptide itself (autocrine functioning), tissues in direct apposition/close proximity to the point of release (paracrine actions), or tissues distantly located from the locus of release, where the peptide is delivered via the circulatory system (hormonal delivery). In crustaceans,

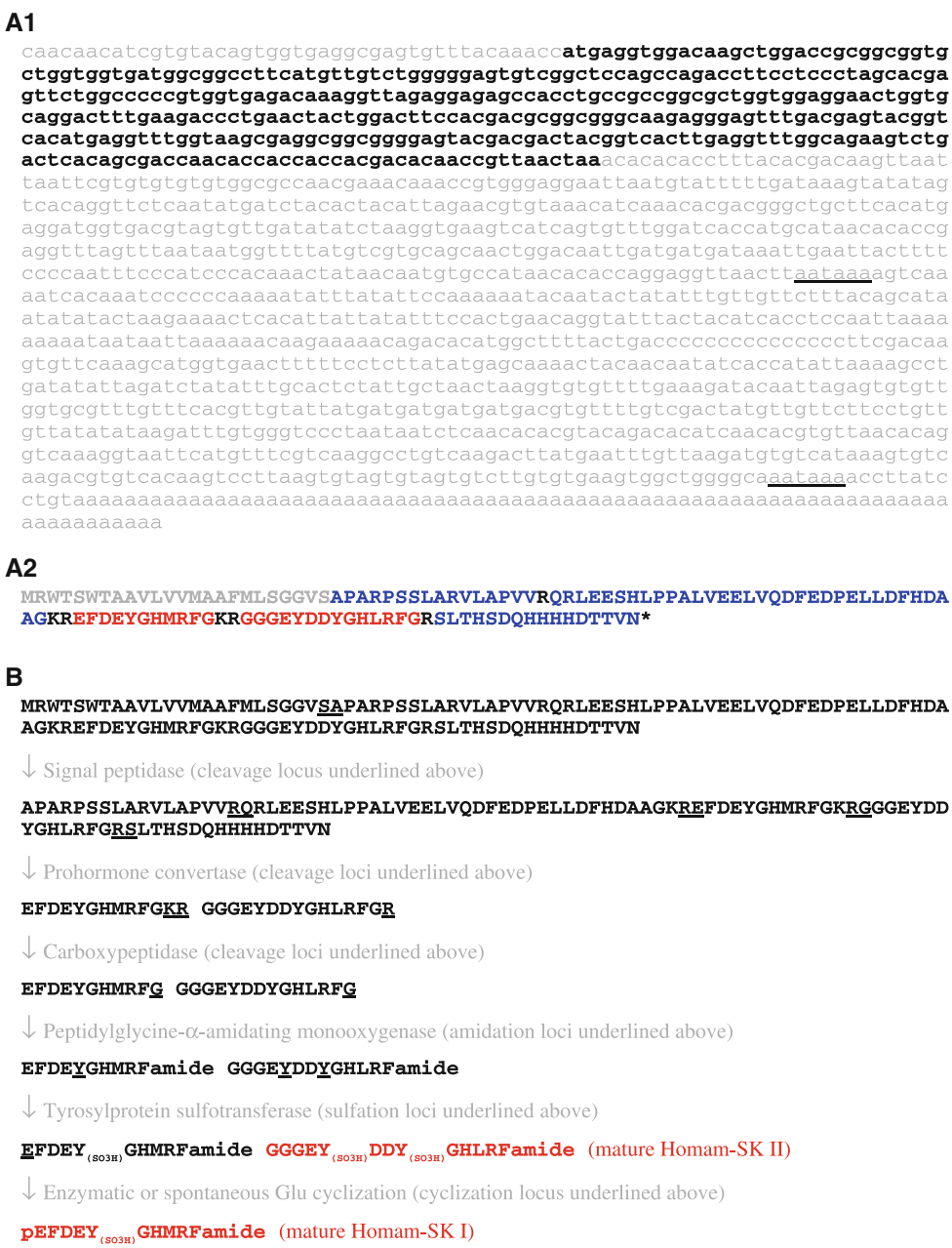

Fig. 1 Nucleotide and deduced amino acid sequences of *Homarus americanus* (Homam) prepro-sulfakinin. *A1* Nucleotide sequence of *Homam-prepro-sulfakinin* cDNA (accession no. EF418605). The open reading frame of the cDNA, including the stop codon, is shown in *black font*, with two 3′ polyadenylation signal sequences indicated by *underline in black*. *A2* Deduced amino acid sequence of Homam-prepro-sulfakinin. The signal peptide is shown in *grey*, with prohormone convertase cleavage loci shown in *black*. The two encoded sulfakinin isoforms are shown in *red*, with additional precursor-related peptides shown in *blue*. The *asterisk* indicates the position of the stop codon. *B* Putative processing scheme resulting in the production of the two isoforms of Homam-sulfakinin from its precursor protein. The mature conformations of the two sulfakinin isoforms (Homam-SK I and II) are colored *red*. Figure modified from Dickinson et al. [158]

a single peptide frequently serves both autocrine/paracrine and hormonal roles within a nervous system [1]. Moreover, crustacean neurons often synthesize and release multiple peptides [2], either concurrently or differentially. In fact, the multiplicity of the co-transmitters produced by and released from crustacean neurons has been postulated to allow the generation of complex behavioral output from the "simple," "hard-wired" neural networks that control behavior in this group of animals.

Unlike classical neurotransmitters, where release is generally limited to the synapse, it is believed that neuropeptides can be secreted at essentially any point along the length of a neuron, and, unlike the synapse, there are no morphological correlates that can be used a priori to define a putative peptide release site. In spite of this, peptides tend to be sequestered within varicose-like terminals located in central and peripheral regions of the nervous system, and, in crustaceans, these regions are generally recognized as areas of release (Fig. 2).

A number of factors determine the sphere of influence of a peptide once it is released from a neuron. Clearly, the presence/absence and relative distribution/concentration of receptors for a given peptide play a critical role in determining whether or not it exerts modulatory activity on a target, as does the quantity of peptide released from the neuron. Moreover, for both locally acting and circulating

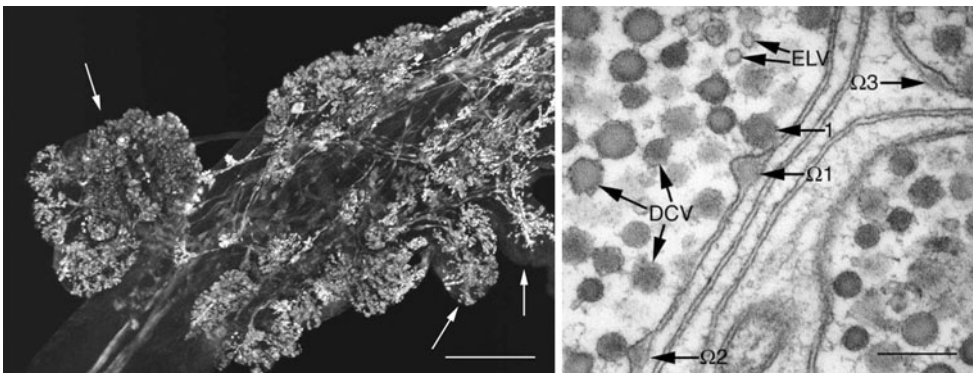

Fig. 2 General organization of a crustacean neuroendocrine organ; the anterior cardiac plexus (ACP) of *Cancer productus* is used as an example. (*Left panel*) The *Cancer productus* ACP is composed of nerve terminals contained within blister-like protuberances of the anterior cardiac nerve sheath; these are in direct contact with the circulatory system. The confocal image shown illustrates the presence of FMRFamide-like immunoreactivity within these protuberances, several prominent ones indicated by *arrows*. *Scale bar* 100 μm. (*Right panel*) Transmission electron micrograph illustrating morphological correlates of hormone secretion in the *Cancer productus* ACP. Both dense-core vesicles (*DCV*), which are likely peptidergic, and electron-lucent vesicles (*ELV*) are present in these terminals. In this image, one DCV (*1*) is docked to the plasma membrane, while several others have fused with the membrane and are in the process of exocytosing their contents, creating characteristic ultrastuctural features, i.e., omega (*Ω*)-figures, on the plasma membrane (*Ω2*, *Ω3*). The docked DCV and the three *Ω*-figures visible in this micrograph create a pseudo-time course of peptide hormone secretion. A DCV first docks to the plasma membrane (*1*), then fuses with it, releasing its dense-core and forming an *Ω*-figure (*Ω1*). The membrane of the DCV is rapidly incorporated into the plasma membrane of the terminal, and the *Ω*-figure subsides (*Ω2* and *Ω3*). *Scale bar* 200 nm. Figure modified from Christie et al. [8]

peptides, the local concentrations of peptidases that can act on a given peptide and the distance that the peptide must diffuse to a target can regulate the concentration of peptide reaching its receptors. Finally, physical barriers within a local release area, or impenetrable ensheathment of a tissue in the case of a hormonally delivered substance, can limit access of a peptide to its receptors on a potential target.

Neural sources of crustacean peptides

The crustacean central nervous system (CNS), like that of all arthropods, is a distributed one, generally consisting of a chain of ganglia interconnected by a longitudinal nerve cord. In decapods, the neural circuits responsible for mediating many behavioral outputs are located within the ganglia of the CNS. For example, the ventilatory rhythms of the gills are generated by a neural network located in the thoracic nervous system [3], while the swimmeret system of the tail is controlled largely by neural circuitry present in the abdominal ganglia [4]. In addition, several offshoots of the CNS are ganglionated, e.g., the stomatogastric nervous system (STNS) and the cardiac ganglion (CG), with the resident somata synapsing within the local neuropil to form the circuitry controlling the rhythmic movements of the foregut and the neurogenic heart, respectively [3, 5, 6]. Peptides released locally within the neuropil of these and other ganglia, in some cases from the circuit elements themselves, are capable of reconfiguring the resident neural circuits, thereby modulating their outputs.

In addition to regions of synaptic interactions, the crustacean nervous system gives rise to a number of neuroendocrine structures, defined as regions of the nervous system in which secretory nerve terminals have direct access to the hemolymph. In decapods, these neuroendocrine sites vary from loosely associated clusters of release terminals located along the ventral nerve cord or in peripheral nerves to highly organized neuroendocrine organs. Two neuroendocrine organs appear to be ubiquitously conserved in decapods [7]: the X-organ-sinus gland (XO-SG) system, typically located in the eyestalk, and the pericardial organ (PO), situated along the lateral walls of the pericardial chamber surrounding the heart. Others, such as the post-commissural organ [7], the anterior cardiac plexus [8] (Fig. 2), and the anterior commissural organ [9], all located within or near the STNS, may be more limited in their phylogenetic conservation. Like locally released modulators, peptide hormones released from these neuroendocrine sites act as powerful modulators of physiology and behavior.

Recent advancements in the methodology for crustacean neuropeptide discovery

The first invertebrate neuropeptide to be fully characterized was red pigment concentrating hormone (RPCH) from the shrimp *Pandalus borealis* [10]. This peptide was isolated chromatographically/biochemically from a large pool of starting tissue [11] and, upon its bioassay-directed

purification, the structure of the peptide was determined by a combination of proteolytic cleavage, Edman analysis, and mass spectrometry [10]. This strategy was commonly employed for peptide discovery in crustaceans for the next quarter century [12–17].

While a number of techniques, including ones similar to those used for the isolation and characterization of RPCH, continue to be employed for peptide discovery [18–20], the emphasis has shifted from the purification and structural elucidation of targeted neuropeptides to an emphasis on peptidomics, the qualitative and quantitative characterization of the complement of endogenous bioactive peptides present in a species. When genomic/transcriptomic information is available, it is possible to use bioinformatics techniques to identify neuropeptide genes and apply processing models to predict neuropeptide sequences. Peptide profiling and de novo sequencing via mass spectrometry (MS) have also played an important role in these efforts. For crustaceans, no genome information is available for public use; thus, the application of genomic analysis is not yet possible. In contrast, many data are available for in silico transcriptome mining and biological mass spectrometry, and these methods are now at the forefront of crustacean peptidomics.

Transcriptome mining

With the advent of new molecular and sequencing technologies, it has become possible to produce expressed sequence tags (ESTs) for mRNA/cDNA libraries from neural and other tissues, or, in some cases, from whole organisms. For an ever-growing number of crustacean species, extensive collections of ESTs have been generated and deposited in publicly accessible databases. These data provide a rich resource for mining transcripts encoding proteins of interest, including neuropeptide precursors. Moreover, on-line software programs available to translate and predict the post-translational processing of the deduced pre/prepro-hormones make transcriptome mining a rapid and readily accessible approach for neuropeptide discovery (Table 1).

In crustaceans, a common strategy has been used for transcriptome mining [21–25]. Specifically, known pre/prepro-hormone sequences are used as queries to search the public database for ESTs that encode putatively orthologous proteins. The BLAST program used for these analyses, tblastn, which searches the translated nucleotide database using a protein query, can be searched for transcripts in a general sense or restricted to a desired subset of animals. Positive hits are translated and checked for sequence identity/similarity to the target query. If the hit seems likely to represent a viable transcript, post-translational processing of the deduced protein is subsequently predicted (Fig. 1). Using this protocol, the extant publicly accessible data have recently been used for several taxon-wide surveys of crustacean peptide-encoding ESTs [24, 26], as well as for targeted searches from individual crustacean species [22, 23, 25]. As ESTs are continuously being added to the public database, periodic mining of this resource will certainly reveal additional peptide-encoding transcripts, thereby continuing to expand our knowledge of crustacean peptidergic signaling.

Transcriptome mining offers both pros and cons with respect to other methods of peptide discovery currently in vogue. First, many peptides are present in nervous systems in very small quantities, and thus for many isolation/characterization regimes, large pools of tissue are needed to obtain sequence data. Given this need, standard biochemical/mass spectral sequencing is often not practical, particularly for minute species, such as planktonic crustaceans. Similarly, the rarity of an organism and/or its geographic range can hinder peptide discovery using techniques that require large quantities of tissue. In contrast, transcriptomics is not hampered by a need for large pools of tissue. Moreover, once deposited into the public database, EST sequences provide a stable resource for mining proteins from a given species. In addition, the data obtained from transcriptome mining allow for the unambiguous determination of all amino acids, whereas in other methods, such as some mass spectral platforms, ambiguity occurs for amino acids that are isobaric, e.g., leucine and isoleucine.

Counterbalancing the pros of transcriptomics are a number of limitations. Firstly, for all crustaceans with extant ESTs in the public domain, the sequences thus far deposited represent only a small portion of a transcriptome for any given species. Also, because most ESTs are single pass sequences, miss/uncalled nucleotides can lead to errors in the sequence of a deduced protein. Additionally, predictions of the post-translational processing of the resultant proteins are just that, predictions, which may or may not represent the actual biological processing of a precursor. Moreover, by their very nature, transcriptomes represent only a snapshot of the genes being transcribed in an animal, and thus may be biased by age, sex, physiological state, etc. Therefore, although it is a powerful tool, transcriptomics alone is unlikely to provide a complete peptidome for any species.

Biological mass spectrometry

MS-based techniques have revolutionized the field of neuropeptide discovery [27–30], providing the means to probe complex biological samples and generate detailed

Table 1 Major crustacean peptide families and their modes of identification

Family (subfamily)	Example	Identification
A-AST	AGPYSFGLamide	B, T
B-AST	GNWNKFQGSWamide	B, T
C-AST	pQIRYHQCYFNPISCF	B, T
Bursicon α	DECSLRPVIHILSYPGCTSKPIPSFACQGRCTSYVQVSGSKLWQTER SCMCCQESGEREAAITLNCPKPRPGEPKEKKVLTRAPIDCMCRP CTDVEEGTVLAQKIANFIQDSPMDSVPFLK	T
Bursicon β	RSYGVECETLPSTIHISKEEYDDTGRLVRVCEEDVAVNKCEGACVS KVQPSVNTPSGFLKDCRCCREVHLRARDITLTHCYDGDGARLSG AKATQHVKLREPADCQCFKCGDSTR	T
Corazonin	pQTFQYSRGWTNamide	B, T
CCAP	PFCNAFTGCamide	B, T
CHH	pQIYDTSCKGVYDRALFNDLEHVCDDCYNLYRTSYVASACRSNCYS NLVFRQCMDDLLMMDEFDQYARKVQMVamide	B, T
CPRP	RSTQGYGRMDRILAALKTSPMEPSAALAVQHGTTHPLE	B, T
DH (calcitonin-like)	GLDLGLGRGFSGSQAAKHLMGLAAANFAGGPamide	T
ETH	DPSPEPFNPNYNRFRQKIPRIamide	T
EH	AVAANRKVSICIKNCGQCKKMYTDYFNGGLCGDFCLQTEGRFIPDC NRPDILIPFFLQRLE	T
Enkephalin	YGGFM	B
FLP (myosuppressin)	pQDLDHVFLRFamide	B, T
FLP (NPF)	KPDPSQLANMAEALKYLQELDKYYSQVSRPRFamide	T
FLP (sNPF)	APALRLRFamide	B, T
FLP (sulfakinin)	pQFDEY$_{(SO3H)}$GHMRFamide	B, T
FLP (–FLRFamide)	TNRNFLRFamide	B, T
FLP (–YLRFamide)	AYSNLNYLRFamide	B, T
FLP (–FVRFamide)	GYSNKNFVRFamide	B
Insect kinin	DFSAWAamide	B
Neuroparsin	APRCDRHDEEAPKNCKYGTTQDWCKNGVCAKGPGETCGGYR WSEGKCGEGTFCSCGICGGCSPFDGKCGPTSIC	T
Orcokinin	NFDEIDRSGFGFN	B, T
Orcomyotropin	FDAFTTGFamide	B, T
PDH	NSGMINSILGIPRVMTEAamide	B, T
Proctolin	RYLPT	B, T
Pyrokinin	DFAFSPRLamide	B
RPCH	pELNFSPGWamide	B, T
RYamide	pEGFYSQRYamide	B
SIFamide	GYRKPPFNGSIFamide	B, T
TRP	APSGFLGMRamide	B, T

A-AST A-type allatostatin, *B-AST* B-type allatostatin, *C-AST* C-type allatostatin, *CCAP* crustacean cardioactive peptide, *CHH* crustacean hyperglycemic hormone, *CPRP* CHH precursor-related peptide, *DH* diuretic hormone, *ETH* ecdysis triggering hormone, *EH* eclosion hormone, *FLP* FMRFamide-like peptide, *PDH* pigment dispersing hormone, *RPCH* red pigment concentrating hormone, *TRP* tachykinin-related peptide, *NPF* neuropeptide F, *sNPF* short NPF, *B* sequenced biochemically or via mass spectrometry, *T* predicted via molecular cloning or transcriptome mining

structural information with extraordinary sensitivity. In this section, we present a brief overview of the strengths and limitations of the mass spectrometric instruments that have been applied to crustacean neuropeptide identification and summarize how MS instruments and MS-based strategies have been used for the analysis of crustacean tissues and hemolymph.

MS instrumentation for crustacean neuropeptide identification

Three basic elements define the capabilities of all instruments used for MS-based peptidomics: (1) ionization, the production of ions from the biological sample, (2) the measurement of the mass-to-charge ratio (*m/z*) characteristic

of sample components (MS for direct peptide profiling), and (3) the measurement of *m/z* following the activation and dissociation of isolated ions (MS/MS or MS^n for peptide sequencing). Coupled with the resolution of prior chromatographic separations, the attributes of each step define the capabilities and limitations of MS-derived information. The attributes for instruments directed at crustacean neuropeptide characterization are summarized below. The reader is directed to other, more comprehensive, recent reviews of mass spectrometric techniques [31–35] for more detailed information.

Ionization and mass analysis for peptide profiling Two ionization techniques, matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI), are used extensively for the analysis of peptides in crustacean tissues and tissue extracts [27–30]. Following ionization, many studies have based neuropeptide identification upon the measurement of the *m/z* ratio via direct peptide profiling. For these measurements, the ability to make mass measurements with both high mass resolution and high mass accuracy is critical. Mass spectrometric instruments used for crustacean neuropeptide identification based upon *m/z* measurements include instruments that couple MALDI with time-of-flight (TOF) or Fourier transform (FT) mass analyzers. For instruments using ESI, quadrupole (Q)-TOF hybrid mass spectrometers have been the most common.

TOF mass analyzers are used to determine *m/z* values by accelerating ions down a field-free flight tube and measuring their flight times. This time-based mass analyzer is compatible with the pulsed laser-desorption mode of ion production used for MALDI, and many early applications of mass spectrometry to the analysis of crustacean tissues [36–40] made use of MALDI-TOF instruments. More recently, MALDI-TOF/TOF instruments have been applied to the analysis of crustacean samples [41]. Unique to the field of crustacean neuropeptide analysis, MALDI-FTMS instruments have been applied extensively to the analysis of tissues and tissue and fluid extracts [42–44]. MALDI-FTMS instruments offer ultra-high mass resolution coupled with unique methods of mass calibration, a combination that significantly increases the reliability of *m/z*-based neuropeptide identifications. However, the ions produced using vacuum-UV MALDI-FTMS are often unstable when trapped for the long times ($\sim$10 s) required for FTMS detection, which results in the detection of abundant metastable decay products in the MALDI-FT mass spectra [43]. Later MALDI-FTMS work by Li and co-workers [45] was carried out using a higher pressure MALDI source, which minimizes the problem of metastable decay. Figure 3 shows two representative MALDI-FT mass spectra, drawn from work by Cape et al. [46]. In this study, where direct tissue analysis was one tool used to assess developmental differences in neuropeptides profiles from the lobster *Homarus americanus*, a single embryonic stomatogastric ganglion (STG) yielded high-quality MALDI spectra (Fig. 3a), which could be compared with the profile generated from an adult STG (Fig. 3b).

For instruments relying upon ESI, most work on crustacean peptidomics has made use of hybrid instruments, specifically hybrid Q-TOF mass spectrometers. Q-TOF instruments permit the continuously produced ions generated by the ESI source to be introduced orthogonally into the pulsed TOF mass analyzer. Q-TOF instruments, which can achieve higher resolution and mass accuracy compared with non-hybrid TOF instruments, are extensively used to sequence peptides using MS/MS measurements (described below).

MS/MS for peptide sequencing To structurally characterize novel neuropeptides, and to confirm the assignment of previously established neuropeptide sequences, tandem mass spectrometry or MS/MS is used to isolate a precursor ion from a sample, dissociate the precursor and generate product ions that, upon mass analysis, can be used for structural characterization. The MS/MS spectrum can be used for the de novo determination of amino acid sequences or, in combination with bioinformatics data, can confirm a proposed neuropeptide amino acid sequence. Tandem mass spectrometry can also be used for the identification of post-translational modifications, such as the sulfation of tyrosine residues. In early work, when MALDI-TOF instruments were most common, MS/MS was carried out using the technique of post-source decay (PSD). With instrument evolution, MALDI-TOF/TOF instruments are now used to isolate precursor peptide ions with higher resolution and to dissociate the precursor using high-energy collision-induced dissociation (CID), which produces more peptide backbone cleavages, as well as amino acid informative fragment ions that can be used to distinguish leucine and isoleucine. For example, Fig. 4a shows a representative MALDI-TOF/TOF MS/MS spectrum for the orcokinin family peptide NFDEIDRSGFGFA ([Ala^{13}]-orcokinin) [47]. The MS/MS spectrum yields almost complete sequence information, and the detection of low mass immonium ions provides information helpful for distinguishing isobaric leucine and isoleucine.

MALDI-FTMS instruments have also been used for MS/MS peptide fragmentation because of their ability to isolate a precursor at high resolution and accurately determine the *m/z* values of product ions at high resolution. However, the low energy CID process used on most FTMS instruments (sustained off-resonance irradiation, or SORI) and the fact that the MALDI-produced ions are singly charged result in spectra that are often dominated by small neutral losses, little sequential fragmentation and, consequently, fewer structurally useful sequence ions. For

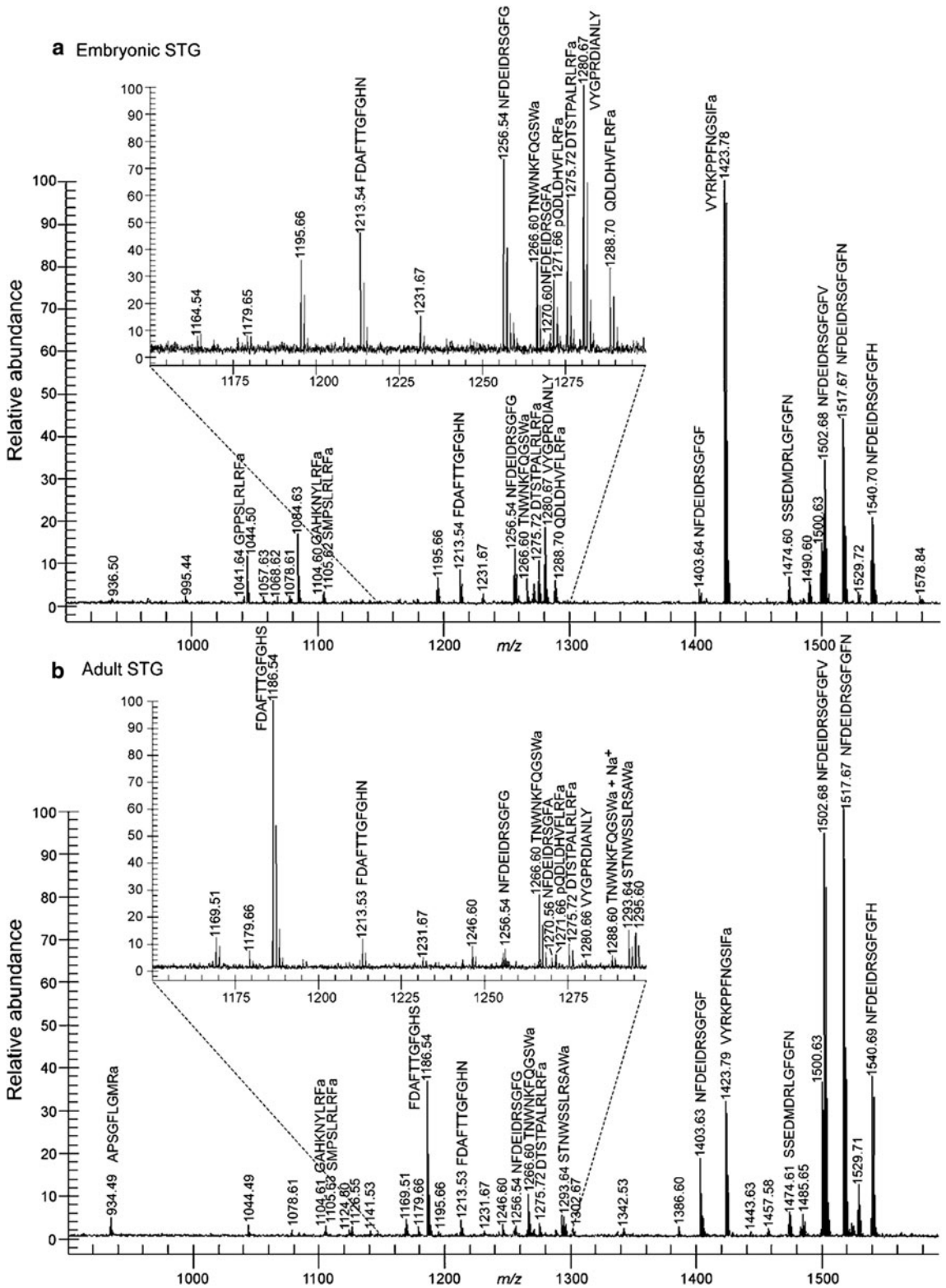

Fig. 3 Direct tissue MALDI-FTMS used for neuropeptide profiling. In this example, a single stomatogastric ganglion (STG) from an **a** embryonic or **b** adult *Homarus americanus* was analyzed. The STG, which is small enough to be analyzed as a whole tissue, was freshly dissected, rinsed in acidified methanol, desalted, and co-crystallized with 2,5-dihydroxybenzoic acid as the MALDI matrix. Figure modified from Cape et al. [46]; used with permission

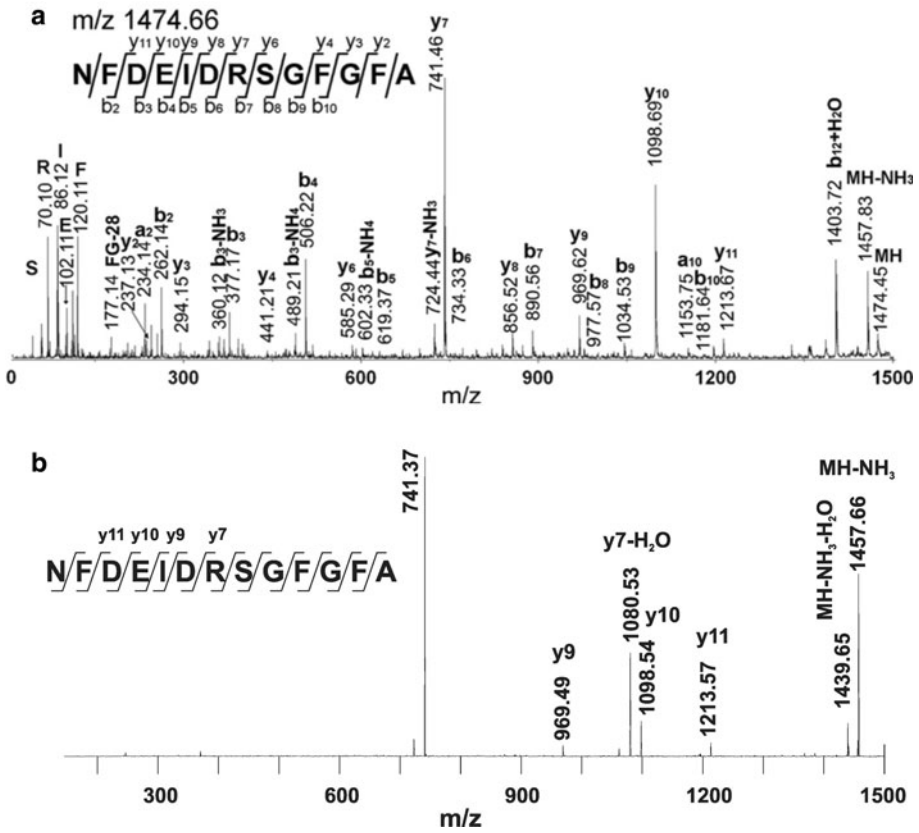

Fig. 4 **a** MS/MS spectrum of [Ala^{13}]-orcokinin from the brain of *Cancer borealis*, measured using a MALDI-TOF/TOF instrument. MS/MS was carried out using air as the collision gas and a 2-kV collision energy. **b** MS/MS spectrum of an [Ala^{13}]-orcokinin standard, measured using a MALDI-FTMS instrument. MS/MS was carried out using SORI-CID with argon as the collision gas and a V_p amplitude of 6.5 V. The lower energy SORI-CID conditions yield spectra dominated by Asp-Xxx cleavages (cleavages C-terminal to aspartate residues) and fewer product ions that can be used for peptide sequencing. Figure 4a modified from Chen et al. [47]; used with permission

example, the MS/MS spectrum of [Ala^{13}]-orcokinin, measured using a MALDI-FTMS instrument (Fig. 4b), shows a spectrum dominated by two y-type ions and fewer fragments that would permit sequencing. With this limitation, confirmation of peptide sequence has often relied upon comparing the MS/MS spectrum of the novel peptide with that of a synthetic reference peptide.

For tissue and hemolymph extracts, ESI-Q-TOF instruments have provided the most powerful tool for peptide sequencing. While MS/MS occurs under lower energy CID conditions, the more highly charged ions produced by ESI yield more detectable fragments that can be used for sequencing. The higher charges, coupled with the rapid, sensitive TOF mass analyzer, have made this technique an effective means for sequencing and identifying large numbers of novel neuropeptides from complex samples.

Examples of MS-based approaches for crustacean neuropeptide identification

In this section, we provide representative examples of MS-based strategies that have been used for crustacean neuropeptides analysis.

Characterizing the crustacean neuropeptidome The vast majority of currently identified crustacean neuropeptides have been determined through large-scale studies directed at the neuropeptidome of particular species. Most studies have focused on the analysis of pooled tissue extracts from a large number of animals, which have then been subjected to off- and on-line chromatographic separations. This peptidomic approach was first applied to tissues from the crab *Cancer borealis*, where ESI-Q-TOF and MALDI-TOF measurements were used to characterize neuropeptides extracted from the brain and thoracic ganglion [48]. A large number of studies, initially relying heavily upon nanoESI-Q-TOF mass spectrometric analysis [23, 46, 49–51], have followed. In all of these studies, the acquisition of MS/MS data and the use of complementary instrumentation, such as MALDI-FTMS with nanoESI-Q-TOF MS, have played a critical role in supporting peptide identifications. More recent studies have further developed this multipronged approach, using a variety of MS ionization and mass analysis techniques (MALDI-FTMS, MALDI-TOF/TOF, nanoESI-Q-TOF) in combination to enhance the number of neuropeptides that can be detected and sequenced [52]. These studies, which have recently included a bioinformatics component [23, 25], have greatly enhanced our understanding of the range of neuropeptides in the crabs *Cancer borealis* [52], *Carcinus maenas* [23], the lobster, *Homarus americanus* [46, 51], and the shrimp, *Litopenaeus vannamei* [25]. Li and co-workers [53] have taken a novel approach to analyze the wealth of data provided by information-rich high-resolution

mass spectrometric analyses by applying bioinformatics approaches to analyze MALDI-FTMS data for the comparison of the peptidome of five crustacean species.

Targeted neuropeptide identification/analysis In contrast with peptidomic approaches, mass spectrometry has been used in studies focused on the detailed identification and characterization of specific novel neuropeptides. For example, MS sequencing of the native neuropeptides, coupled with confirmation using neuropeptide standards, has been used to identify a number of peptides, including the SIFamide VYRKPPFNGSIFamide [54], the tachykinin-related peptide (TRP) TPSGFLGMRamide [55], and the pyrokinins SGGFAFSPRLamide and TNFAFSPRLamide [56] (see "Crustacean neuropeptide families" for descriptions of these peptide families).

Bioinformatics-aided MS peptide identification Early work by Yasuda-Kamatani and Yasuda showed that mass spectrometry, in combination with molecular cloning techniques, provided an efficient strategy for peptide identification [36, 57, 58]. More recently, in silico database searches for putative peptide precursors or molecular cloning approaches, coupled with predictions of peptide processing, have led to the MS confirmation of the structures of novel neuropeptides, including members of the C-type allatostatin (AST) [59–61], orcokinin [62], pigment dispersing hormone (PDH) [63], and SIFamide [64] families (see "Crustacean neuropeptide families" for descriptions of these peptide groups). As mentioned above, bioinformatics-aided MS techniques are also playing an important role in large-scale neuropeptidome studies [23, 25].

Analysis of larger neuropeptides The ability to characterize larger neuropeptides presents challenges for MS/MS-based sequencing, as illustrated by studies of crustacean hyperglycemic hormone (CHH)- and CHH precursor-related peptide (CPRP)-like peptides. Early attempts to sequence CPRP peptides (30+ amino acid residues), which were extracted from the SG of the crayfish *Orconectes limosus*, yielded only 65–76% sequence coverage following nanoLC-Q-TOF MS/MS analysis [65]. For the larger (roughly 70 amino acids) CHH-like peptide, extracted from the PO and SG of *Carcinus maenas*, proteolytic digestion followed by MS/MS sequencing on a Q-TOF instrument still required Edman peptide degradations to determine the complete amino acid sequences [66]. In more recent work, Li and co-workers were able to use a Q-TOF instrument to fully characterize CPRPs from *Cancer productus* [67], as well as from *Cancer borealis* and *Homarus americanus* [49], taking advantage of truncated versions of the full length peptides that were present in the pooled tissue extracts. Most recently, Li and co-workers were able to de novo sequence a full length CHH peptide from the SG of *Cancer borealis*, making use of both "bottom-up" (tryptic digestion followed by tryptic peptide sequencing) and "top-down" (dissociation of the full-length peptide) characterization strategies [68]. Key to complete sequence characterization was the top-down strategy, implemented using electrospray ionization and high field-strength FTMS instruments. Cleavage of the peptide disulfide bonds was critical for establishing the amino acid sequences.

Novel methodologies for crustacean neuropeptide identification and characterization

Imaging mass spectrometry is an emerging technique that offers advantages over immunohistochemical imaging for localizing neuropeptides within tissue samples because labeling is not required and specific information about small variations in peptide structure (post-translational modifications or sequence variations) is available. MALDI-TOF/TOF MS has been applied by Li's group to the two- [41] and three-dimensional [47] mapping of neuropeptides in the PO and brain of *Cancer borealis*. In these studies, MS peptide profiling provided detailed information about peptide localization, high-energy MS/MS experiments were used to confirm neuropeptide identity, and *m/z* intensity maps provided three-dimensional distributions of selected neuropeptides in brain slices.

Other developments in the area of sample preparation and analysis have involved work by Li and co-workers to improve the production of sequence-specific product ions in MS/MS experiments using peptide derivatization (reductive methylation [69] and methyl esterification [45]). Capillary electrophoresis techniques coupled with MALDI-FTMS analysis have been applied to crustacean tissue extracts [70, 71]. Li and co-workers have developed immunoaffinity-based enrichment techniques (immunoprecipitation and immunodot blot screening), coupled with MALDI-FTMS and nanoLC-ESI-Q-TOF MS/MS, for the targeted analysis of FMRFamide-related peptides in the PO of *Cancer borealis* [72].

Finally, quantitative peptidomic strategies are emerging as MS-based techniques that can be used to provide insights into neuropeptide function. MS-based quantitative approaches assess the up- or downregulation of peptide concentrations in response to a physiological change, such as an environmental stress or food deprivation [73]. In recent work, Li and co-workers have applied quantitative peptidomic techniques to assess changes in peptide expression in the brain and PO of fed and unfed *Cancer borealis* [74]. Variations in neuropeptide expression were quantified using stable isotopic labeling of extracted neuropeptides with H_2- or D_2-formaldehyde. In combination with imaging measurements, two potential feeding centers

in the brain (the boundary of the olfactory lobe and the median protocerebrum) were identified.

Crustacean neuropeptide families

While a number of recent review articles have focused on specific families of neuropeptides in crustaceans, there has been no comprehensive review of the extant families of crustacean neuropeptides since that of Keller [75]. Here, we provide a brief overview of each of the neuropeptide families that are currently recognized as existing in crustaceans (Table 1), briefly describing their general structures, and, where possible, their putative modes of action and bioactivities.

A-type allatostatins

The A-type ASTs, first identified in insects, are typified by the C-terminal motif –Y*X*FGLamide, where *X* represents a variable amino acid. In crustaceans, the existence of A-ASTs was first suggested by immunohistochemical labeling in the STNS in *Cancer borealis* [76]. The identification of native A-ASTs from *Carcinus maenas*, e.g., AGPYSFGLamide, followed shortly thereafter [16], with additional isoforms subsequently identified from a number of other decapod species [17, 25, 46, 48, 50–52, 77–79]. A-ASTs have also been identified by transcriptomics from several lower crustaceans, specifically the copepod *Calanus finmarchicus* [80] and the cladoceran *Daphnia pulex* [22]; in *Calanus*, the predicted isoforms exhibit variant –Y*X*FGI/Vamide C-termini, e.g., APYGFGIamide and pQ/QPYNFGVamide [80].

A-ASTs are broadly distributed within the nervous systems of crustaceans, including regions of synaptic neuropil [22, 76, 80, 81] and neuroendocrine organs [1, 22, 80, 81], suggesting that they function as both locally released autocrines/paracrines and circulating hormones.

In crustaceans, the A-ASTs are well-documented inhibitory neuro/myomodulators. The stomatogastric and cardiac neuromuscular systems are two targets of A-type peptides. In the stomatogastric system, A-ASTs decrease the activity of the pyloric neural circuit, which produces the pyloric motor pattern [17, 76], and elicit a decrease in neuromuscular transmission in a number of pyloric and gastric mill muscles [82]. In the CG, whose rhythmic activity drives the heartbeat, A-ASTs decrease cycle frequency, as well as the number and frequency of spikes per burst in cardiac motor neurons [83]. A-type peptides have also been shown to decrease skeletal muscle performance, acting through both pre- and post-synaptic mechanisms [84], and have been implicated in the regulation of methyl farnesoate production by the mandibular organ [85].

B-type allatostatins

Members of the B-type AST family are characterized by the C-terminal motif –WX_6Wamide, where X_6 represents six variable amino acids. Although originally described from insects, B-type peptides, e.g., GNWNKFQGSWamide, have also been identified/predicted from a number of decapod species [21, 23, 25, 50–52], as well as from *Daphnia pulex* [22].

Mass spectral/molecular studies suggest that B-ASTs are broadly distributed within the nervous systems of at least the decapods, functioning both as locally released autocrines/paracrines and as circulating hormones.

At present, investigations into the physiological roles played by crustacean B-ASTs are limited to a single study on the *Cancer borealis* STNS [86], here eliciting a decrease in the activity of the ongoing pyloric motor pattern [86].

C-type allatostatins

Authentic C-type peptide

C-type ASTs are characterized by the presence of a pyroglutamine blocked N-terminus, the C-terminal motif –PISCF, and a disulfide bridge between the Cys residues located at positions 7 and 14. While members of this peptide family were long believed to exist only in holometabolous insects, an authentic C-AST, pQIRYHQCYFNPISCF (disulfide bridging between Cys^7 and Cys^{14}), was recently shown via transcriptomics and mass spectrometry to be broadly conserved within the Decapoda, being predicted/detected in 29 species representing seven infraorders [25, 60, 61].

Mass spectrometry and immunohistochemistry suggest that pQIRYHQCYFNPISCF likely serves as both a circulating hormone (Dickinson and Christie, unpublished) and a locally released autocrine/paracrine in crustaceans [60, 61, 87]. Additionally, it was detected in the midgut epithelium of *Cancer borealis* and *Homarus americanus*, suggesting gut-derived endocrine/paracrine functioning as well [60].

Physiologically, pQIRYHQCYFNPISCF appears to serve as an inhibitory modulator of both the stomatogastric neural circuit, where it decreases the frequency of the pyloric motor pattern [61], and the cardiac neuromuscular system, in which it decreases heart rate (Dickinson and Christie, unpublished).

C-type-like peptide

In addition to the authentic C-AST just described, a C-AST-like peptide, SYWKQCAFNAVSCFamide (disulfide

bridging between Cys^6 and Cys^{13}), also appears to be broadly conserved within the Crustacea. This peptide was first identified via transcriptome analysis from *Daphnia pulex* [22], with subsequent transcriptomic/mass spectral detection in a second cladoceran, *Daphnia carinata* [24], and 25 decapod species [59, 61], including members of five infraorders.

As with the authentic C-AST, mass spectral and immunohistochemical data suggest that SYWKQCAFNAVSCFamide serves as both a locally released autocrine/paracrine [59, 61] and a circulating hormone (Dickinson and Christie, unpublished), at least in decapods.

One target of SYWKQCAFNAVSCFamide is the cardiac neuromuscular system, where application of the peptide modulates the frequency and amplitude of heart contractions [59]. Interestingly, its effects on frequency were mixed, increasing the heart rate in some preparations and decreasing it in others [59]. In addition, the peptide modulates the output of the pyloric motor pattern, eliciting a decrease in cycle frequency [59, 61].

Bursicon

In insects, melanization and sclerotisation of the cuticle following ecdysis are controlled by bursicon, a heterodimeric cysteine knot protein comprised of bursicon α and bursicon β subunit peptides. In crustaceans, the first isoform of each subunit was identified from *Carcinus maenas* [23, 88], i.e., DECSLRPVIHILSYPGCTSKPIPSFACQGRCTSYVQVSGSKLWQTERSCMCCQESGEREAAITLNCPKPRPGEPKEKKVLTRAPIDCMCRPCTDVEEGTVLAQKIANFIQDSPMDSVPFLK (bursicon α [88]) and RSYGVECETLPSTIHISKEEYDDTGRLVRVCEEDVAVNKCEGACVSKVQPSVNTPSGFLKDCRCCREVHLRARDITLTHCYDGDGARLSGAKATQHVKLREPADCQCFKCGDSTR (bursicon β [88]). Isoforms of the α and/or β subunit peptides have subsequently been identified in several other decapods [24, 25, 60, 61], as well as in *Daphnia pulex* [22, 88] and the euphausid *Euphausia superba* [24].

In *Carcinus maenas*, in situ hybridization studies show that bursicon α- and β-producing neurons are limited to the suboesophageal, thoracic, and abdominal ganglia, with all cells that produce one subunit also producing the other [88]. Interestingly, the bursicon-expressing somata also appear to produce crustacean cardioactive peptide (CCAP; see "Crustacean cardioactive peptide") [88]. Based on the known projection patterns of the CCAP cells, the bursicon-containing somata likely project to and innervate the PO, suggesting a hormonal mode of delivery for the peptide [88]. The physiological roles played by bursicon in crustaceans remain unknown.

Corazonin

pQTFQYSRGWTNamide (Arg^7-corazonin) is a well-known insect neuropeptide. In crustaceans, it was first sequenced via mass spectrometry from *Cancer borealis* [38]. Subsequent mass spectral investigations have identified it in several other decapods [23, 25, 51]. Prepro-hormones encoding the peptide have also been identified via transcriptome analysis from *Litopenaeus vannamei* [25] and *Daphnia carinata* [24].

In decapod species, immunohistochemical and/or mass spectral studies suggest that Arg^7-corazonin likely functions as both a circulating hormone and a locally released autocrine/paracrine [23, 25, 51]. Additionally, the *Litopenaeus vannamei* ESTs that encode Arg^7-corazonin were derived from the lymphoid organ [25], suggesting that it may also be produced by non-neural tissues.

The functional roles served by corazonin in crustaceans are currently limited to a single study [89], which suggests an involvement in the control of pigment migration in chromatophores.

Crustacean cardioactive peptide

CCAP

A peptide with the structure PFCNAFTGCamide (disulfide bridging between Cys^3 and Cys^9) was originally identified from *Carcinus maenas* [90]; in this species the peptide was cardioactive and thus was named crustacean cardioactive peptide [90]. Following its original description, authentic CCAP was identified via molecular/mass spectral studies in a number of other decapod species [38, 50, 52, 91, 92], with a variant isoform, PFCNAFAGCamide (Ala^7-CCAP), predicted via transcriptome mining from *Daphnia pulex* and *Daphnia carinata* [22, 24].

In decapods, CCAP is present in both neuroendocrine organs [1, 23, 38, 50–52, 81, 91–95] and regions of the central neuropil [23, 25, 51, 52, 81, 91, 95–97], suggesting it functions as both a circulating hormone and a locally released autocrine/paracrine.

CCAP has been implicated in the control of many physiological processes in decapods. Although the peptide was named for its cardioexcitatory properties [83, 90, 93, 98], it also modulates the stomatogastric neuromuscular system [99–103], induces pigment dispersion in chromatophores [104, 105], induces changes in the light sensitivity of the retina [106], and is implicated in the control of ecdysis [88, 107, 108].

CCAP precursor-related peptides

The known crustacean CCAP-encoding prepro-hormones are predicted to liberate several peptides in addition to

CCAP itself [22, 24, 25, 91]. For example, in the lobster *Homarus gammarus*, four peptides, GPVA, DIGDLLEGKD, SDPSMEGLASSSELDALAKHVLAEAKLWEQLQSKM EMMRSYASRMENHPVY, and STPHTQPRQHLTSTP QQKVETEKQ, are predicted to be produced along with authentic CCAP [91]. DIGDLLEGKD was recently sequenced from the brain and thoracic/abdominal ganglia of *Homarus americanus* using mass spectrometry [23, 25, 51]. Similarly, DIADLLDGKD, which was predicted from a *Litopenaeus vannamei* prepro-CCAP, was sequenced via mass spectrometry from the brain and thoracic/abdominal ganglia of this species [25]. The functional roles played by crustacean CCAP precursor-related peptides are largely unknown, although DIGDLLEGKD is cardioactive in *Homarus americanus*, increasing both the frequency and amplitude of the heartbeat (Wiwatpanit and Dickinson, unpublished data).

Crustacean hyperglycemic hormone superfamily

CHH superfamily

The CHH superfamily is a group of large, 70+ amino acid peptides whose members were originally isolated and characterized from the XO-SG systems of decapods [109–116]; the first CHH to be fully characterized was from *Carcinus maenas*, i.e., pQIYDTSCKGVYDRALFNDLEH VCDDCYNLYRTSYVASACRSNCYSNLVFRQCMDDL LMMDEFDQYARKVQMVamide [117]. Members of the CHH superfamily can be divided into two subgroups, the CHH subfamily and the molt-inhibiting hormone (MIH)/gonad-inhibiting hormone (GIH)/vitellogenesis-inhibiting hormone (VIH)/mandibular organ-inhibiting hormone (MOIH) subfamily (hereafter termed the MIH subfamily), based on their structures and/or the structures of their precursor proteins. Specifically, members of the CHH subgroup are typically 70–72 amino acids in length, possess six identically placed internal Cys residues (which allow for the formation of three stereotypic disulfide bridges), and the prepro-hormones from which they are cleaved include a second, 30+ amino acid peptide (commonly referred to as CHH precursor-related peptide or CPRP) between the CHH isoform and the signal sequence. In contrast, members of the MIH subgroup are typically larger, 77–78 amino acids long, and possess a similar, though not identical arrangement of Cys resides; their precursors lack the presence of a CPRP. Members of both the CHH and MIH subfamilies have been characterized from a large number of decapod species [109–116], as well as from members of several lower crustacean taxa, e.g., the isopod *Armadillidium vulgare* [118, 119] and *Daphnia pulex* [22].

The XO-SG system is a common source of members of the CHH superfamily [120–122]. In addition, isoforms of CHH distinct from those present in the XO-SG have been isolated and characterized from the PO [109–116]. In some species, CHH superfamily members have been identified immunologically in regions of synaptic neuropil, for example, MOIH-like labeling is present throughout the STNSs of several *Cancer* species [123]. Thus, while originally thought of as endocrine signaling agents, at least some members of the CHH superfamily appear likely to serve as locally released autocrines/paracrines. Moreover, CHH has also been found in epithelial endocrine cells of the fore- and hindguts of *Carcinus maenas*, implicating members of this peptide family in gut paracrine/endocrine signaling [124, 125].

Members of the CHH superfamily are highly pleiotropic [109–116]. As their names imply, this group of peptides has been implicated in the control of carbohydrate metabolism, ion transport and water uptake, molting, and reproduction [109–116]. The recent immunohistochemical identification of MOIH in the STG of *Cancer* crabs [123] suggests local paracrine modulation of the neural circuitry involved in the ingestion, chewing, and filtering of food within the foregut as well.

One feature of the CHH superfamily that currently appears to be unique is the existence of chiral variants (L and D) of some family members; in some cases, these variants have been shown to be differentially distributed within the nervous system and to serve distinct functions [109–116].

CPRP

As stated in the "CHH superfamily," the precursors from which CHHs are derived contain a second peptide, CHH precursor-related peptide or CPRP, between the signal sequence and the CHH isoform. In decapods, CPRPs show considerable sequence identity to one another within members of a given infraorder, e.g., RSTQGYGRMDRI LAALKTSPMEPSAALAVQHGTTHPLE and RSAQGM GKMERLLASYRGALEPSTPLGDLSGSLGHPVE in the crabs *Carcinus maenas* [14] and *Cancer pagurus* [121], respectively; more variation is seen between the CPRPs of different infraorders, particularly in their C-termini [14]. Although CPRPs are detectable in the hemolymph [126], where they can persist for a considerable period of time [126], nothing is currently known about the functional roles served by them in any species.

Diuretic hormone

In insects, peptides with structural similarity to vertebrate calcitonins have been identified and implicated in diuresis.

Transcriptome mining has recently identified homologs of calcitonin-like diuretic hormone (CLDH) in several crustaceans including *Daphnia pulex* [22], the copepod *Caligus clemensi* [24], and *Homarus americanus* [127], e.g., GLD LGLGRGFSGSQAAKHLMGLAAANFAGGPamide from the latter species [127].

In *Homarus americanus*, RT-PCR tissue profiling shows that the native CLDH is produced by both neuroendocrine somata and somata likely to contribute to modulation in regions of synaptic neuropil, suggesting that CLDH functions as both a circulating hormone and a locally released autocrine/paracrine [127]. Surprisingly, the CG was one portion of the nervous system in which the CLDH-encoding transcript was identified, making it the first intrinsic peptide identified in the crustacean cardiac neuromuscular system [127]; CLDH is cardioactive in *Homarus* [127].

Ecdysis-triggering hormone

In insects, a group of structurally related peptides possessing –FF*X*K*XX*K*X*VPR*X*amide (where the *X*s represent variable residues) C-termini have been shown to play a critical role in triggering ecdysis. Recently, the first crustacean ecdysis-triggering hormones (ETHs) were predicted via transcriptome mining from *Daphnia pulex*, i.e., DPSP EPFNPNYNRFRQKIPRIamide and GEGIIAEY$_{(SO3H)}$MN SESFPHEGSLSNFFLKASKAVPRLamide [22]. The cellular distribution and functions of these peptides remain unknown.

Eclosion hormone

In insects, eclosion hormones (EHs) play critical roles in adult ecdysis. The known insect isoforms of eclosion hormone possess considerable amino acid identity, including six internal Cys residues that allow for the formation of three disulfide bridges. Via transcriptome mining, EHs have recently been identified from the crab *Callinectes sapidus* [24, 128], the shrimp *Marsupenaeus japonicus* and *Penaeus monodon* [24], and the tadpole shrimp *Triops cancriformis* [24], a branchiopod. The crustacean isoforms, e.g., the *Calinectes* peptide AVAANRKVSICIKNCGQC KKMYTDYFNGGLCGDFCLQTEGRFIPDCNRPDILIPF FLQRLE [24, 128], show significant sequence similarity to the known insect EHs, and like their insect counterparts, possess 6 Cys residues. At present nothing is known about the cellular distributions or physiological roles played by EHs in crustaceans.

Enkephalin

The peptides YGGFM and YGGFL were isolated and characterized from the thoracic ganglia of *Carcinus maenas* [129]; these peptides are identical in structure to the vertebrate opioid peptides Met-enkephalin and Leu-enkephalin, respectively. While *Carcinus maenas* is the only crustacean from which enkephalins have been fully characterized, biochemical/immunohistochemical data suggest they are broadly conserved in the taxon [129–136]; these data also suggest that the enkephalins function both as locally released autocrines/paracrines and circulating hormones in crustaceans.

Enkephalins appear to play a conserved role in the regulation of carbohydrate metabolism; the actions of the enkephalins on this process appear to be species-specific, inducing hypoglycemia in some species and hyperglycemia in others [137–143]. Several lines of evidence suggest that the modulatory activity of enkephalin on carbohydrate metabolism results from their involvement in the regulation of CHH release from the SG. For example, both δ- and β-opioid receptors have been identified in the eyestalk ganglia of crustaceans [144], opioid-binding sites have been localized to CHH-containing terminals of the SG [132], the hypo-/hyperglycemic actions of enkephalins are absent in eyestalk-ablated animals [139–143], and the peptides have been shown/implicated in the inhibition of CHH release in animals exhibiting hypoglycemic responses [135, 138]. Additionally, the enkephalins appear to play roles in the control of pigment granule migration in chromatophores, likely mediated via their regulation of release of other peptide hormones, e.g., PDH or RPCH [145–147], and they have been implicated in the modulatory control of both locomotion [146] and ovarian development [146, 148–153].

FMRFamide-related peptides

Myosuppressin

The myosuppressin subfamily of FMRFamide-like peptides (FLPs) possesses the consensus motif –HVFLRFamide. In decapod crustaceans, a single peptide possessing this C terminus has been identified, pQDLDHVFLRFamide [44]. Mass spectrometry suggests that pQDLDHVFLRFamide is broadly, perhaps ubiquitously, conserved within the Decapoda [44].

Mass spectral tissue profiling suggests that pQDLDH VFLRFamide is broadly distributed within decapod nervous systems [23, 25, 51, 52], likely functioning as both a locally released autocrine/paracrine and a circulating hormone.

Physiologically, pQDLDHVFLRFamide is a powerful modulator of the cardiac neuromuscular system [154].

Neuropeptide F

Members of the neuropeptide F (NPF) subfamily of FLPs are typically 36 amino acids in overall length and possess

the C-terminal motif –GRPRFamide, as well as tyrosine residues at positions 10 and 17 from their C-termini. In crustaceans, three NPF-like peptides have recently been predicted via transcriptomics [21, 22], one from *Marsupenaeus japonicus*, i.e., KPDPSQLANMAEALKYLQELD KYYSQVSRPRFamide, and the others from the cladocerans *Daphnia magna* and *Daphnia pulex*, i.e., DGFVMGG GEGGEMTAMADAIKYLQGLDKVYGQAARPRFamide and DGGDVMSGGEGGEMTAMADAIKYLQGLDKVY GQAARPRFamide, respectively. No information is currently available as to the tissue distributions or functional roles played by NPFs in any crustacean.

Short neuropeptide F

A third subfamily of FLPs is the short neuropeptide Fs or sNPFs. Like the NPFs proper, these peptides possess –R*X*RFamide C-termini, where *X* is a variable residue; they are shorter in overall length than are the NPFs, typically being ~10 amino acids long. The first crustacean sNPFs, i.e., APALRLRFamide and DRTPALRLRFamide, were identified from the shrimp *Macrobrachium rosenbergii* [155]. To date, sNPF isoforms have been identified in decapod species encompassing four infraorders [23, 25, 48, 51, 72, 155–157], as well as in the cladoceran *Daphnia pulex* [22]. Interestingly, a peptide appearing to be an intermediate between the sNPFs and the NPFs proper has recently been predicted from the copepod *Lepeoptheirus salmonis*, i.e., LSQIKDFY$_{(SO3H)}$NEAGRPRFamide [24].

Mass spectral tissue profiling suggests that sNPFs are broadly distributed within the decapod CNS [23, 51, 52, 72], serving as both autocrines/paracrines and circulating hormones. At present, the functional roles played by crustacean sNPFs remain unknown.

Sulfakinin

A fourth subfamily of FLPs is the sulfakinins, whose family members are characterized by the C-terminal motif –Y$_{(SO3H)}$GHM/LRFamide. In crustaceans, sulfakinins have been identified from three decapod species, the first being *Penaeus monodon*, where two peptides, pQFDEY$_{(SO3H)}$GH MRFamide and AGGSGGVGGEYDDY$_{(SO3H)}$GHLRFamide, were biochemically characterized [18]. With the exception of the predicted sulfation state of one tyrosine in the latter peptide, an identical set of peptides was subsequently identified from *Litopenaeus vannamei* [20]. In *Homarus americanus*, molecular cloning identified the first crustacean sulfakinin-encoding transcript, with the peptides predicted from it being pEFDEY$_{(SO3H)}$GHMRFamide and GGGEY$_{(SO3H)}$DDY$_{(SO3H)}$GHLRFamide [158].

Immunohistochemistry conducted on the CNS of *Penaeus monodon* suggests that the sulfakinins have a highly restricted distribution within the nervous system, being detected only in approximately ten neurons in the brain [18]. Moreover, large amounts of tissue were needed as starting material for the isolation and purification of the native isoforms from both *Penaeus monodon* and *Litopenaeus vannamei*, suggesting that the sulfakinins are present in low abundance within the CNS [18, 20]. These data are consistent with the sulfakinins serving as locally released modulators rather than hormones in at least penaeid species.

Functionally, the native *Homarus americanus* isoforms are cardioactive, increasing both the frequency and amplitude of ongoing heart contractions in the lobster [158].

Other FLPs

In addition to the subfamilies just described, a number of other FLPs have been identified from decapod crustaceans. Many of these peptides possess the C-terminal motif –FLRFamide [13, 19, 23, 25, 48, 51, 52, 72, 79, 155, 157, 159–161]. In fact, the first FLPs identified from crustaceans contain this structural element, i.e., TNRNFLRFamide and SDRNFLRFamide from *Homarus americanus* [13]. Mass spectral tissue surveys suggest that –FLRFamides function as both locally released autocrines/paracrines and circulating hormones [23, 25, 51, 52, 72]. Studies directed at assessing the physiological roles played by extended –FLRFamides suggest that these peptides are powerful modulators of the cardiac and stomatogastric neuromuscular systems, and of exoskeletal muscles in many decapod species [13, 83, 159, 161–169].

Another C-terminal motif seen in multiple crustacean FLPs is –YLRFamide [23, 25, 50, 51, 79, 157, 170], e.g., AYSNLNYLRFamide from *Penaeus monodon* [157]. Mass spectral tissue profiling suggests that, like most of the other FLP subfamilies, the –YLRFamides are broadly distributed within the nervous system, functioning as both locally release autocrines/paracrines and as circulating hormones [23, 25, 51, 52, 72]. Functionally, –YLRFamides have been shown to modulate the motor outputs of both the cardiac and stomatogastric neuromuscular systems [170].

Recently, two peptides possessing –FVRFamide C-termini were identified from the brain of *Litopenaeus vannamei*, i.e., GYSNKNFVRFamide and GYSNKD FVRFamide [25]. No information on the functional roles played by these peptides is currently available.

Insect kinin

Members of the insect kinin family possess the consensus motif –FX_1X_2WGamide, where X_1 and X_2 represent variable amino acids. In crustaceans, the first members of this peptide family were identified from *Litopenaeus vannamei*,

with some isoforms having an Ala for Gly substitution at their C-terminus [171, 172], e.g., DFSAWAamide. A subset of the *Litopenaeus vannamei* peptides has also been detected via mass spectrometry in *Cancer* crabs [48, 50].

Data on the distribution of insect kinins in crustaceans are limited to members of the Decapoda [48, 50, 171–173], where they have been found both in regions of central neuropil and in neuroendocrine sites, suggesting autocrine/paracrine and hormonal functioning [48, 173].

Physiologically, application of insect kinins to the STG excited the pyloric rhythm, particularly in preparations with slow ongoing motor patterns [173]. In addition, they consistently enhanced activity in the dorsal gastric (DG) neuron, a member of the gastric mill neural circuit, although the peptide did not elicit or alter the full motor program per se [173, 174]. Insect kinins have also been shown to increase the rate of spontaneous hindgut contractions in crustaceans [171].

Neuroparsin

The neuroparsins are a group of large, structurally related peptides that, in insects, were originally identified as antigonadotropic agents, though they have subsequently been shown to be highly pleiotropic. Insect neuroparsins contain 12 cysteine residues, which allow for the formation of 6 disulfide bridges, a hallmark of the family. In crustaceans, neuroparsin-like peptides have recently been predicted via transcriptome mining from several decapods [23–25], as well as from the copepod *Caligus rogercresseyi* [24]. Like their insect counterparts, these peptides contain Cys residues that are likely to result in a similar set of disulfide bridges, e.g., APRCDRHDEEAPKNCKYGTTQDWCKN GVCAKGPGETCGGYRWSEGKCGEGTFCSCGICGGC SPFDGKCGPTSIC from *Carcinus maenas* [23, 25]. At present, nothing is known about the tissue distribution or functional roles played by neuroparsins in crustaceans.

Orcokinin

The peptide NFDEIDRSGFGFN was originally isolated from *Orconectes limosus* [175]; based on its species of origin and myotropic activity on the gut, the peptide was named orcokinin [175]. Since this original description, additional isoforms of orcokinin have been identified from both this and other crustacean species via biochemical, mass spectral and/or molecular analyses [21–25, 36, 37, 39, 40, 43, 48, 50–52, 62, 176, 177]. In most decapods, multiple orcokinin isoforms are present, encoded by a common precursor; for example, 11 orcokinins (seven copies of NFDEIDRSGFGFN, two copies of NFDEIDRSGFGFV and one copy each of NFDEIDRSGFGFA and NFDEID RTGFGFH) are present in the precursor of the crayfish *Procambrarus clarkii* [36]. However, in the shrimp *Marsupenaeus japonicus*, only a single orcokinin appears encoded within its prepro-hormone, i.e., 13 copies of NFDEIDRAGMGFA [21]. Regardless of species, all full-length decapod orcokinins are 13 amino acids long and possess the N-terminal consensus motif NFDEIDR–. Interestingly, in lower crustaceans, i.e., daphnids and copepods, a different situation pertains, namely one or two isoforms per species, with the native peptides being 14 rather than 13 amino acids long, e.g., the *Daphnia pulex* peptides NLDEIDRSNFGTFA and NLDEIDRSDFGRFV, both of which also exhibit a Leu for Phe substitution at position 2 [22], and NFDEIDRAGFGSFM, NFDEIDRAG FGSLI from the copepod *Lernaeocera branchialis* [24].

Biochemical, immunohistochemical, and/or mass spectral studies have shown that members of the orcokinin family are broadly distributed within crustacean nervous systems, and are likely to function as both locally released autocrines/paracrines and circulating hormones [23, 39, 40, 46, 51, 52, 62, 94, 178].

Orcokinin bioactivity has been demonstrated for several tissues in decapods. Specifically, in several species, orcokinins increase both the frequency and amplitude of spontaneous hindgut contractions [62, 175]; interestingly, they have little if any modulatory influence on hindgut contractions in others [62]. Orcokinins also modulate the output of the STNS [39, 40].

Orcomyotropin and other orcokinin precursor-related peptides

A peptide with the structure FDAFTTGFamide was originally sequenced from *Orconectes limosus* [177]. Given its pronounced enhancement of hindgut contractility in this species, the peptide was named orcomyotropin. Orcomyotropin in its authentic form has subsequently been found in a number of other decapod species [37], as have several unamidated, C-terminally extended peptides with significant sequence identity to orcomyotropin, e.g., FDAFTTGFGHN and FDAFTTGFGHS [44, 51]. With the identification of the precursors encoding orcokinin, it became clear that these extended peptides, likely the precursors for orcomyotropin, are encoded (one copy per prepro-hormone) on the same precursor as orcokinins [36, 62].

Mass spectral tissue profiling has shown that orcomyotropin and/or its extended variants are widely distributed within the nervous systems of at least decapods [23, 25, 50–52], suggesting both autocrine/paracrine and hormonal modes of delivery.

At present, investigations into the physiological roles played by crustacean orcomyotropins are limited to a single study where FDAFTTGFamide was shown to be a powerful excitatory modulator of hindgut contractility [177].

In addition to orcomyotropin, several other peptides are encoded on the orcokinin precursor [36, 62]. For example, one copy each of SSEDMDRLGFGFN, GPIKVRFLSAIFI PIAAPARSSPQQDAAAGYTDGAPV, GDY$_{(SO3H)}$DVYPE, VYGPRDIANLY, and SAE are predicted from the *Homarus americanus* prepro-hormone [62]. Mass spectrometry confirmed the presence of SSEDMDRLGFGFN and VYGP RDIANLY in the brain, STNS, and SG of *Homarus americanus*, with desulfated GDYDVYPE detected in the SG [62]. The functional roles served by these and other orcokinin precursor-related peptides remain unknown.

Pigment dispersing hormone

One of the first crustacean neuropeptides to be fully characterized was NSGMINSILGIPRVMTEAamide from the eyestalk ganglia of *Pandalus borealis* [12]. Given its ability to affect light-adapting pigment movements in the retina, the peptide was named light-adapting distal retinal pigment hormone. The peptide was subsequently found to be a potent pigment granule dispersing agent in chromatophores [179], and hence was dubbed pigment dispersing hormone, the name that is commonly used today. With the subsequent identification of the structurally related peptide NSELINSILGLPKVMNDAamide from the crab *Uca pugilator* [180], the *Pandalus* peptide was redesignated α-PDH and the *Uca* isoform *β*-PDH. Since their initial descriptions, other PDH isoforms have been identified biochemically, molecularly and/or via mass spectrometry from a wide variety of decapod species (e.g., [49, 50, 63, 181–189]), with those possessing sequence, acidity, and charge similarity to α-PDH forming one subgroup and those with similarity to *β*-PDH forming a second subfamily [190]. While members of the *β*-PDH subfamily have been identified in species from a number of decapod infraorders, detection of members of the α-PDH subfamily has thus far been limited to members of the Caridea [191]. In many species, multiple isoforms of PDH are present, e.g., NSELINSILGLPKVMNDAamide and NSELINSLLG ISRLMNEAamide in *Cancer productus* [50, 63]. In addition to decapods, *β*-PDHs [191] have been identified from *Armadillidium vulgare*, i.e., NSELINSLLGAPRVL NNAamide [192], and *Daphnia pulex*, i.e., NSELINS LLGLPRFMKVVamide [22].

Immunohistochemical and/or mass spectral data suggest that PDHs are likely to serve as both autocrines/paracrines and hormones in decapods (e.g., [1, 23, 49–52, 63, 191, 193, 194]). It is important to note that in species with multiple PDH isoforms, one isoform may function primarily as a hormone and the other as an autocrine/paracrine. Which isoform is delivered hormonally versus released locally appears to vary from species to species, even in relatively closely related animals. For example, in *Callinectes sapidus*, authentic *β*-PDH has been proposed as the SG hormone, with a second *β*-PDH isoform proposed as a local transmitter in central neuropil [184], whereas in *Cancer productus* the modes of delivery for the two PDHs appear flipped [63]. PDH-like peptides have also been detected in neuropilar processes in the central nervous systems of several lower crustaceans, i.e., *Calanus finmarchicus* [195] and *Daphnia pulex* [22], suggesting at least a local modulatory functioning in these animals.

Members of the PDH family are classically known for their ability to affect pigment granule translocation, specifically pigment dispersion, within a number of cell types in the eye, as well as in epithelial chromatophores [191]. In addition, the presence of PDH-like immunoreactivity in regions of synaptic neuropil suggests that these peptides are likely to function as locally released neuromodulators; however, to the best of our knowledge, there has been no direct demonstration of this function. In fact, in the STNS, where immunoreactivity is present, *β*-PDH shows no bioactivity [194]. Anecdotal evidence suggests that the PDH system in crustaceans may also be involved in the generation of circadian rhythmicity [196–198].

Proctolin

A peptide with the structure RYLPT, originally isolated from the cockroach *Periplaneta americana* and named proctolin, has been identified in authentic form from many decapod crustaceans [23, 25, 38, 50–52, 199–201], the first being the crab *Cardisoma carnifex* [201]. Recently, the first crustacean proctolin-encoding transcript was identified from *Litopenaeus vannamei* [25].

The distribution of proctolin in crustacean tissues has been the focus of numerous studies. In decapods, proctolin is widely distributed within the nervous system [23, 38, 50–52, 92, 94, 200, 202–211], and likely serves both as an autocrine/paracrine and as a hormone. In addition, immunohistochemical data indicate that proctolin is present in the central nervous system of members of several lower crustaceans, i.e., the isopod *Porcellio scaber* [212] and *Daphnia pulex* [22], suggesting at least a local modulatory role for it in these animals.

Proctolin has widespread modulatory actions in crustaceans. The decapods have received by far the most extensive investigation, and here, proctolin has been shown to modulate exoskeletal muscles/neuromuscular junctions [213–216], the cardiac neuromuscular system [98, 217–222], the stomatogastric neuromuscular system [92, 103, 204, 223–228], the ventilatory system [229], the neural circuitry controlling the swimmerets [230, 231], mechanosensory neurons [232–234], and hindgut contractility [235]. In non-decapods, proctolin has been shown to be a potent

myomodulator [236, 237] and to modulate cardiac output [238].

Pyrokinin

Members of the pyrokinin family exhibit the C-terminal motif –F*X*PRLamide, where *X* represents a variable residue. In decapods, several pyrokinin isoforms have been identified [23, 25, 51, 52, 56, 239], e.g., DFAFSPRLamide and ADFAFNPRLamide from *Litopenaeus vannamei* [25, 239].

In decapods, pyrokinins are broadly distributed within the nervous system [23, 25, 51, 52, 56], suggesting both autocrine/paracrine and hormonal functioning.

Assessment of the physiological roles served by pyrokinins in crustaceans is limited to a single study, where their actions on the *Cancer borealis* stomatogastric neural circuits were examined [56]. Interestingly, and unlike most peptide modulators, pyrokinins had little effect on the pyloric motor pattern, but consistently activated the gastric mill rhythm.

Red pigment concentrating hormone

The first invertebrate neuropeptide to be fully characterized was pELNFSPGWamide, which was isolated from the eyestalk of *Pandalus borealis* [10]. Based on its ability to affect color change via the aggregation of pigment granules within epithelial erythrophores, this peptide is commonly referred to as red pigment concentrating hormone (RPCH). Since its initial description, authentic RPCH has been identified via a variety of techniques from many other decapod species [92, 240–243]. RPCH was also detected using biochemistry/mass spectrometry in the CNS of *Porcellio scaber* [244]. While it was long thought that pELNFSPGWamide was the sole RPCH isoform present in crustaceans, recent transcriptome mining has shown that in at least two daphnids, *Daphnia magna* and *Daphnia carinata*, a variant isoform, pQVNFSTSWamide, is present [21, 24].

The classic source of RPCH in members of the Decapoda is the XO-SG system [10, 11]. Biochemical, immunohistochemical, and/or mass spectral studies, however, have shown that it is also present in other areas of the decapod nervous system, including other neuroendocrine organs [1, 107] and regions of synaptic neuropil [223, 245–247], suggesting dual endocrine and autocrine/paracrine function. In lower crustaceans, RPCH-like immunoreactivity has been reported in the CNS of *Daphnia pulex* [22], suggesting at least local modulatory functioning here.

Although RPCH was originally identified based on its ability to affect the concentration of pigment granules in erythrophores, it has subsequently been shown to be highly pleiotropic, modulating the central pattern generating networks present in the STNS [223, 228, 246, 248–252] and CG [83], as well as the motor output of the swimmeret system [247]. Recently, RPCH was implicated in the mobilization of energy stores in *Porcellio scaber* [244].

RYamide

A family of peptides exhibiting –RYamide C-termini has recently been identified in members of the Decapoda [23, 25, 38, 50, 253], e.g., pEGFYSQRYamide [253].

Mass spectral data suggest that RYamides are present in neuroendocrine stuctures and in regions of central neuropil, serving as both hormones and locally released autocrines/paracrines [23, 25, 38, 50, 52, 253]. Nothing is known about the bioactivity of RYamides in Crustacea.

SIFamide

Members of the SIFamide family of neuropeptides typically exhibit the structure *X*YRKPPFNGSIFamide, where *X* represents a variable residue. In most decapod crustaceans, Gly^1-SIFamide appears to be the sole isoform present [24, 25, 44, 57, 64, 157]. However, in homarid lobsters, Gly^1-SIFamide has been replaced by a Val^1 variant [44, 51, 54, 64]. In several mass spectral studies, the peptide PPFNGSIFamide has also been detected, though it is likely a breakdown product of the full-length peptide [64]. In *Daphnia pulex*, transcriptome mining predicts the SIFamide variant TRKLPFNGSIFamide [254].

Immunohistochemistry and mass spectral tissue profiling suggest that SIFamide is widely distributed within the nervous systems of decapod species [23, 25, 51, 52, 54, 79, 255], but is not present in any neuroendocrine release site. Based on these data it appears that SIFamide functions solely as a locally released autocrine/paracrine. However, SIFamide has also been identified in epithelial endocrine cells of the midgut, suggesting that gut-derived endocrine functioning is possible, as is local autocrine/paracrine modulation of the midgut [256].

While much is known about the identity of the SIFamide isoforms present in decapods [44], comparatively little is known about the physiological roles served by members of this peptide family. In fact, only two direct functional studies currently exist. In *Homarus americanus*, Val^1-SIFamide is a potent modulator of the pyloric neural circuit [54, 64], while in *Macrobrachium rosenbergii*, injection of the Gly^1 isoform increases the level of aggressive behavior in males, and thus appears to play a role in the establishment of dominance hierarchies [257]. Although indirect, anatomical studies have suggested other neuromodulatory roles for the SIFamides in decapods,

implicating them in both visual and olfactory control [57, 79, 258].

Tachykinin-related peptide

A family of peptides possessing the C-terminal motif –FX_1GX_2Ramide, where X_1 and X_2 represent variable residues, is broadly conserved in invertebrates. Given their sequence similarity to members of the vertebrate tachykinins, these peptides are commonly referred to as tachykinin-related peptides. The first crustacean TRP identified was APSGFLGMRamide from *Cancer borealis* [15]. APSGFLGMRamide was subsequently identified in many other decapod species [44] and for some time was believed to be the sole TPR present in members of this crustacean taxon [58]. Recently a second decapod TRP, TPSGFLGMRamide, was identified [55]. Although this peptide is present in a number of decapods [21, 23, 51, 55], it appears to be less broadly conserved than its Ala^1 counterpart [44]. Additionally, mass spectral analyses conducted on *Litopenaeus vannamei* identified several other TRPs, i.e., APAGFLGMRamide, APSGFNGMRamide and APSFGLDMRamide [25], bringing the current number of known decapod isoforms to five. TRPs have also been predicted via transcriptomics from the isopod *Eurydice pulchra*, i.e., APSGFLGMRamide, VPRRFLGIRamide, APASFLGMRamide, APSAFLGMRamide, and ARSSFL GMRamide [21].

TRPs are widely distributed within the CNSs of decapods, including both synaptic neuropil and neuroendocrine sites [1, 9, 23, 25, 51, 52, 131, 134, 259–262]. In addition, TRPs have been shown to be present in and released from midgut epithelial endocrine cells in several species [55, 256, 263]. Collectively, these data suggest that TRPs function as both locally released autocrines/paracrines and circulating hormones in the Decapoda.

TRP bioactivity has been demonstrated for the decapod stomatogastric and cardiac neuromuscular systems [9, 15, 55, 83, 228, 251, 263–267]; TRP is also implicated in the modulation of photoreceptor sensitivity [268].

Other peptides

CFITNCPPGamide

A peptide with the sequence CFITNCPPGamide was recently predicted from *Daphnia pulex* [269]; its structure places it within the oxytocin/vasopressin family [269]. The cellular distribution and functional roles played by CFITNCPPGamide remain unknown, and it is unclear how broadly conserved this peptide, or related isoforms, may be in crustaceans.

HIGSLYRamide

HI/LGSI/LYRamide has been identified via mass spectrometry from several decapods [21, 23, 50, 52], the Ile/Leu ambiguity resulting from the isobaric nature of these amino acids. In *Carcinus maenas*, a partial transcript encoding the peptide has also been identified [21], revealing the structure of the peptide to be HIGSLYRamide [21]. Mass spectral tissue profiling suggests that HIGSLYRamide is widely distributed within decapod nervous systems, serving as both a locally released autocrine/paracrine and a circulating hormone [21, 23, 50, 52]. The functional roles played by HIGSLYRamide are unknown.

Physiological effects of neuropeptides in crustaceans

The most extensively studied effects of crustacean neuropeptides are the modulatory effects they exert on pattern generators in the central nervous system. In addition to effects at the central level, these peptides alter behavior by modulating both sensory receptors and muscle contraction. Moreover, hormonally released neuropeptides control a wide variety of other physiological processes, ranging from metabolism and osmoregulation to the synthesis and release of other hormones. Here, we focus on recent studies of neuropeptides in the crustacean cardiac and the stomatogastric neuromuscular systems, examining the multiple neuropeptides that work together to control them.

The cardiac neuromuscular system

Peptidergic modulation of the neurogenic heartbeat of crustaceans involves multiple mechanisms acting at multiple sites

The crustacean heart is neurogenic, with contractions driven by the rhythmic output of a central pattern generator located in the CG, which lies within the single-chambered heart (reviewed in [5]). To alter hemolymph flow, neuropeptides can thus exert modulatory effects on the pattern generator (i.e., the CG itself), on the cardiac muscle and/or neuromuscular junction, and/or on the vessels that carry hemolymph from the heart to the tissues. Because all parts of the circulatory system are constantly bathed with the full array of neuropeptides being used as circulating hormones, neuropeptides, which play a major role in controlling circulation, can exert their effects at all of these sites.

When they are perfused through the isolated whole heart, most neuropeptides that have been examined cause increases in contraction amplitude and frequency (e.g. [93, 127, 158, 159, 162, 164, 169, 221, 270–272]). Only a

few peptides examined in *Homarus americanus*, i.e., myosuppressin [154], the AST-C-like peptide SYWKQCA FNAVSCFamide [59], and the A-AST ASPYAFGLamide (Powers and Dickinson, unpublished), have been shown to result in decreases in either frequency or amplitude.

Modulation of the cardiac central pattern generator

The pattern generator that drives cardiac contractions consists of nine neurons in most crustaceans that have been studied. Four small cells, which have strong endogenous oscillatory properties, drive bursting in the five large motor neurons. In *Cancer borealis*, Cruz-Bermudez and Marder [83, 170] examined the effects of ten neuropeptides on the rhythmic output of the CG. Of these, seven had excitatory effects, one inhibited the rhythmic output of the ganglion, and two had little or no effect. The only peptide that exhibited inhibitory effects in this study was GGSL YSFGLamide, an insect A-type AST, which also inhibits the pattern generators of the stomatogastric system (see below).

Interestingly, although the FLPs and CCAP generally increased cycle frequency, spike frequency within bursts, and duty cycle in *Cancer borealis* [83, 170], studies of these peptides in other species have found more complex effects. In *Callinectes sapidus*, for example, the effects of CCAP on the isolated CG were primarily excitatory, increasing burst duration, duty cycle, and number of spikes/burst [93]; however, cycle frequency did not change. Several FLPs, including TNRNFLRFamide and SDRNFLRFamide and the native *Callinectes* FLP, GYNR SFLRFamide, exerted similar effects in this species [162]. In the isolated CG of *Homarus americanus*, several of the identified FLPs have effects similar to those recorded in crabs. TNRNFLRFamide, for example, causes increased spike frequency within bursts and increased burst duration when bath applied to the isolated CG [273].

In contrast, myosuppressin caused a large decrease in cycle frequency in the isolated CG in both *Procambarus clarkii* [270] and *Homarus americanus* [154]. At the same time, burst duration increased by at least 50%, and the amplitude of the driver potential (slow wave) that underlies the bursts of action potentials in the motor neurons increased.

Nearly all of the neuropeptides that have been shown to modulate the crustacean heart, including those described above, are released from neuroendocrine organs, but are not present in the CG. Recently, however, the mRNA that encodes a CLDH was localized to the large motor neurons of *Homarus americanus* CG itself [127]. This peptide profoundly increases both the frequency and amplitude of heart contractions, indicating that it modulates the cardiac neuromuscular system at one or more sites, likely including the CG, which determines the frequency of the heartbeat. This is the first peptide to be found in the CG [127], suggesting the possibility that it may act as an intrinsic neuromodulator of this system.

Effects on cardiac muscle

The motor behavior that results from activity in any central pattern generator is determined not only by the motor output of that CPG, but also by the way that the output is translated into movement by the muscles, i.e., the neuromuscular transform [274–276]. Thus, effects of neuropeptides on the cardiac muscle will likewise alter the behavioral changes that these peptides provoke.

Effects of neuropeptides on cardiac muscle contraction have been examined for relatively few of the many neuropeptides that influence cardiac activity. All but one of the neuropeptides whose modulatory effects on the periphery have been examined are FLPs; all result in increased muscle contraction. For example, TNRNFLRFamide [273] and myosuppressin [154] cause contraction amplitude to increase in response to controlled trains of stimuli in *Homarus americanus*. Fort et al. [162] found that GYNR SFLRFamide caused an increase in the amplitude of both contractions and excitatory junctional potential amplitudes in response to controlled trains of stimuli in *Callinectes sapidus*. The FLP in these studies could have exerted its effects on either the muscle itself or the neuromuscular junction. In *Homarus americanus*, Wilkens et al. [221] demonstrated that both proctolin and SDRNFLRFamide act directly on the muscle, where they enhance contractions caused by direct stimulation of the cardiac muscles. In addition, Wilkens et al. [221] measured increases in intracellular Ca^{++} concentration when the peptide was present at low concentrations ($\sim 10^{-10}$M); the results of these experiments and experiments using specific channel blockers suggest that the peptides modulate voltage-gated L-type Ca^{++} channels at threshold concentrations and activate sarcolemmal Ca^{++} transporters at higher concentrations.

In addition to effects mediated by changes in heart contractions themselves, hemolymph flow can be altered by changes in the properties of the outflow vessels, particularly changes in the resistance to flow in the arteries. Recordings from a variety of locations within the circulatory system have shown that a number of peptides, including proctolin, TNRNFLRFamide, and CCAP, can increase vascular resistance in both *Homarus americanus* and the achelatan lobster *Jasus edwardsii* [98, 222]. In the dorsal abdominal artery, at least part of this increased resistance is due to changes in the valves that lead from this artery into each of the pairs of lateral arteries within the abdomen [98]. Increasing resistance in these valves

increases the general resistance in the abdomen and tends to force more hemolymph into the other parts of the lobster. Neuropeptides can also cause an increase in resistance in other parts of the circulatory system, such as the anterior arteries. Although the majority of circulatory vessels in the lobsters are not muscular, a recent study [220] showed that *Homarus americanus* arteries contain actin, myosin, and tropomyosin. The dorsal abdominal artery contains striated muscle cells and responds to electrical stimulation. Other arteries respond to proctolin with slow circumferential, but not longitudinal, contractions. The magnitude of these contractions appears sufficient to account for the measured increases in vascular resistance [220].

Effects of neuropeptides on the integrated output of the crustacean heart neuromuscular system

Because the CG sits within the heart, and because most neuropeptides are delivered to the heart hormonally, the CG and heart muscles are virtually always exposed to these neuropeptides in concert. The peptides thus exert their effects simultaneously at multiple levels within these pattern generator-effector systems [93, 154, 162, 277]. Because of the presence of a number of feedback loops (e.g., stretch receptors [5, 278, 279] and nitric oxide [280]), the global effects of any given peptide on the integrated system may or may not be predictable from the simple effects on the CPG and/or the isolated muscle. For example, although cycle frequency does not increase in response to any of the FLPs in the isolated *Callinectes sapidus* CG, frequency increases by as much as 300% when the same peptides are perfused through the whole heart [162]. Similarly, TNRNFLRFamide causes cycle frequency to increase in the whole heart of *Homarus americanus*, but to decrease in the isolated ganglion [273] (Fig. 5). This difference is likely due to indirect effects of the peptides via the feedback loops. The role of both passive stretch and active contraction of the heart muscle has been examined in the CG of the isopod *Ligia pallasii* [279]; here stretch or contraction can phase-advance the next burst, resulting in an increase in cycle frequency.

In contrast, central and peripheral mechanisms appear to act largely in concert, and to reinforce one another's effects, in the response of the whole heart to myosuppressin [154]. Responses in the whole heart are remarkably similar to those recorded in the isolated CG. However, myosuppressin caused a much larger decrease in frequency in the whole heart than in the isolated CG, which drives the contractions. This difference cannot readily be explained by the expected changes in stretch feedback in the whole heart; increased contraction amplitude is predicted to activate stretch receptors and thereby increase, rather than decrease, the heart rate. Thus, in addition to modulating the CG and the muscle/neuromuscular junction, myosuppressin may directly modulate one of the feedback pathways.

Although studies examining the effects of neuropeptides at multiple sites in this integrated neuromuscular system are limited at present, they suggest that the interactions between the central nervous system and the periphery are complex, and are themselves likely to be modulated by neuropeptides. Additional studies examining the effects of the many neuropeptides that act simultaneously at multiple sites are likely to follow and to further enhance our understanding of these important interactions.

The stomatogastric neuromuscular system

Organization of the stomatogastric neuromuscular system

The STNS, a relatively small extension of the central nervous system, consists of four ganglia, the paired commissural ganglia (CoGs), and the unpaired esophageal (OG) and stomatogastric (STG) ganglia, their interconnecting nerves, motor nerves, and a number of integrated sensory organs. The STNS generates the rhythmic motor patterns that control the four major regions of the crustacean foregut: (1) the esophagus, (2) the cardiac sac, which serves largely for food storage, (3) the gastric mill, consisting of three teeth that shred ingested food, and (4) the pylorus, a set of filters responsible for sorting digested or partially digested food particles. Both the gastric mill and the pyloric patterns are generated in the STG, which contains between 25 and 35 neurons, depending on the species.

The core pyloric motor pattern is triphasic, consisting of alternating bursts of action potentials (period $\sim$1–2 s) in the pyloric dilator (PD)/anterior burster (AB) neurons, followed by bursts in two types of constrictor neurons, the lateral pyloric (LP) and then the pyloric (PY) neurons. In the intact animal and in vitro, this pattern is usually constitutively active as long as the inputs from the anterior CoGs and OG are intact. The PD and AB neurons, which are electrically coupled, function as the pacemaker for the pattern, since the AB is an endogenous burster. Additional neurons include the inferior cardiac (IC) neuron, which usually fires in phase with the LP neuron, and the ventricular dilator (VD) neuron, which fires with the PY neurons.

In contrast to the pyloric pattern, there is no single pacemaker neuron for the gastric mill pattern; instead, this pattern results from the interactions of neurons within the network. The resulting pattern is more or less biphasic, with the neurons that control the power and return strokes for each of the two tooth types (medial tooth; paired lateral teeth) firing in alternation due to reciprocal inhibitory connections. Thus, as initially described for the achelatan lobster *Panulirus interruptus* (see [281]), the dorsal gastric

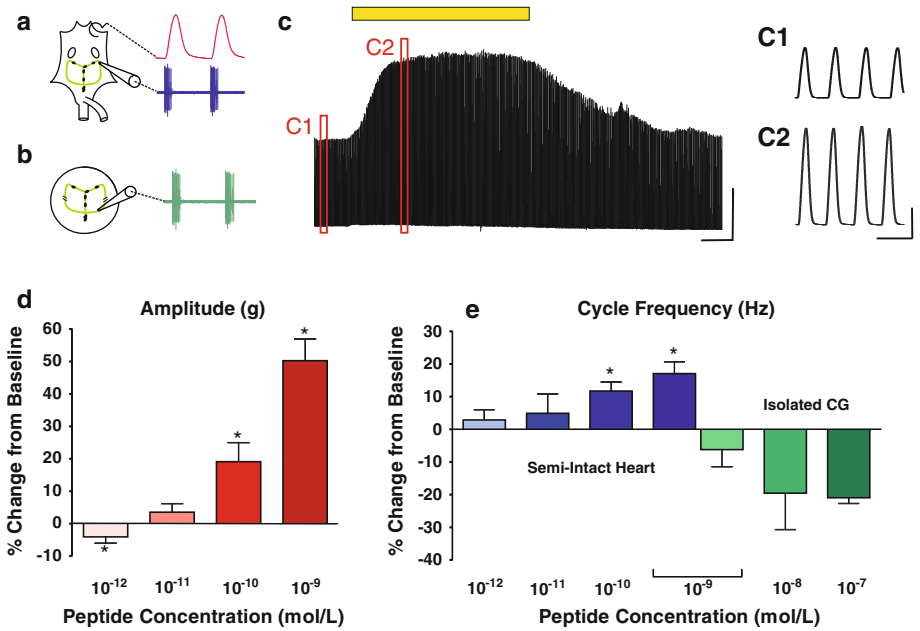

Fig. 5 Modulation of the heartbeat in *Homarus americanus* by TNRNFLRFamide involves complex effects at multiple sites. **a**, **b** Schematic diagrams of the preparations used to record muscle contraction and activity of the cardiac ganglion. **a** Contraction of an isolated whole heart was monitored with a force transducer; motor output of the cardiac ganglion was recorded simultaneously with a suction electrode on a motor nerve. **b** In other preparations, the cardiac ganglion was removed from the heart, and motor output was recorded on the motor nerves in the isolated cardiac ganglion. **c** Perfusion of TNRNFLRFamide through the heart increases both amplitude and frequency of heart contractions. *C1* and *C2* are higher speed recordings from the regions shown in **c**, illustrating the increases in both amplitude and frequency recorded during TNRNFLRFamide perfusion (*yellow bar* in **c**; *C2*). **d** The effect of TNRNFLRFamide on contraction amplitude in the whole heart is dose-dependent, with significant effects at concentrations as low as 10^{-10} M. **e** Cycle frequency of the motor bursts recorded in the whole heart (*blue bars*) increases in response to perfusion with TNRNFLRFamide, with a threshold of $\sim 10^{-10}$ M. In contrast, superfusion of the same peptide over the isolated CG causes a decrease in cycle frequency (*green bars*). Moreover, effects of this peptide on the isolated CG are seen only at much higher concentrations (e.g., 10^{-8} M) than those on the whole heart. *Scale bars* 1.0 g, 100 s in **c**, 2.5 s in *C1* and *C2*. Figure modified from Stevens et al. [273]

(DG) and gastric mill (GM) neurons alternate, and the lateral gastric (LG)/medial gastric (MG) neurons fire in alternation with the lateral posterior gastric (LPG neurons). The gastric mill pattern is considerably slower than the pyloric pattern, with a period of 5–20 s, and is active only intermittently in the intact animal. It is considerably less stereotyped than the pyloric pattern; at least part of that variability is related to the extensive interactions of the pattern generating neurons in the STG with modulatory neurons and other inputs from the CoGs. This has been studied most extensively in *Cancer borealis*, in which numerous projection neurons have been identified and the patterns that result from their activation have been characterized (e.g., [264, 265, 267, 282–287]).

Modulation of the pyloric and gastric motor patterns by neuropeptides: shared mechanisms

The frequency and phasing of the gastric mill and pyloric patterns rely on both the intrinsic membrane properties of the neurons that generate them and the synaptic interactions between those neurons. Thus, alterations of either parameter are likely to modulate the outputs of the pattern generators.

Among the membrane properties that play major roles in the generation of the rhythmic gastric and pyloric patterns are endogenous bursting, plateau potentials (i.e., bi-stability in neuronal membrane potential) and post-inhibitory rebound. These are all modulated by at least one of the neuropeptides present in the STNS. Post-inhibitory rebound in the LP neuron of *Panulirus interruptus*, for example, is enhanced in the presence of RPCH [248]. Weimann et al. [168] showed that SDRNFLRFamide and TNRNFLFRamide enhance the ability of the DG neuron to produce plateau potentials, sometimes even causing it to fire in endogenous bursts. This alteration is accompanied by an activation of the gastric pattern. The endogenous bursting of the AB neuron is modulated by neuropeptides, as was first shown by Hooper and Marder [227], in experiments in which proctolin enhanced AB bursting.

Because the modulation of intrinsic properties is ultimately the result of changes in specific ionic currents, recent studies have focused on the modulation of membrane currents. Golowasch and Marder [288] initially

demonstrated that the peptide proctolin enhances a voltage-dependent inward current that is also sensitive to external Ca^{++} concentrations [288]. Subsequently, Swensen and Marder [289] determined that six different modulators, including five neuropeptides (proctolin, TNRNFLRFamide, APSGFLGMRamide, CCAP, and RPCH), all converge to modulate this same current. Interestingly, application of these five peptides results in five different patterns because different suites of neurons have receptors for different peptides [290]. In fact, by experimentally adding (via the dynamic clamp) the "proctolin current" to the appropriate suite of neurons in the presence of one peptide, Swensen and Marder [289] were able to elicit the pattern typical of a different peptide.

Like intrinsic properties, the strength of chemical synapses can be modulated by neuropeptides. Few examples of peptidergic synaptic modulation at central synapses have been recorded, and their functional significance varies considerably, suggesting that synaptic modulation may play a number of different roles in pattern generating networks.

First, in *Panulirus interruptus*, RPCH increases the amplitude of the post-synaptic potentials from the inferior ventricular (IV) neurons onto a large number of its post-synaptic targets, including neurons in both the gastric [250] and pyloric [248] networks. At the same time, RPCH activates the IV neurons to fire in a bursting pattern that drives the cardiac sac pattern. Thus, synaptic modulation is largely responsible for the complete fusion of the cardiac sac and gastric mill motor patterns; it is also among the mechanisms by which RPCH elicits a pyloric pattern that includes complex temporal variation, also tied to the cardiac sac pattern, of the normally stereotyped pyloric pattern [248].

In *Cancer borealis*, proctolin enhances the strength of the synapse between a pyloric (IC) and a gastric (GM) neuron. Although the functional effects of this change were not examined, the extent to which the gastric and pyloric networks interact is highly variable in this species [291], presenting the possibility that this synaptic enhancement may likewise play a role in modulating the coordination of two networks.

Finally, Thirumalai and Marder [251] showed that RPCH enhances a synapse within the pyloric network in *Homarus americanus*. The inhibitory synapse from the LP to the PD neuron, which provides the major source of feedback from the constrictor neurons (LP/PY) to the pacemaker group (PD/AB), is strongly potentiated by RPCH; bursting in the pacemakers is simultaneously enhanced. Surprisingly, because of its timing, this synaptic potentiation does not alter the pyloric cycle frequency, but may instead serve to stabilize the pattern as dilator bursting is strengthened [252].

Modulation of the stomatogastric motor patterns by neuropeptides: the role of multiple modulators and co-transmission

Most of the modulatory neurons whose transmitter complement has been identified in the STNS contain more than one transmitter, including neuropeptides (reviewed in [286]). Like modulatory neurons, neuroendocrine organs contain many different neurotransmitters, including neuropeptides (e.g., [1, 23, 49–52]). Although the patterns of hormonal co-release of different peptides remain unknown, varicosities within these organs frequently show co-localized peptides [1]. Given the large number of peptides localized to these organs, it seems inevitable that multiple peptides will, at times, be released more or less together, so that neurons of the STG will be simultaneously exposed to multiple neuropeptides.

This raises several questions, including (1) whether and how modulatory neurons using some of the same co-localized transmitters are able to exert different effects on their target networks and (2) whether the effects of different peptides are simply additive or interact in more complex ways. The first question has been studied extensively in the *Cancer borealis* STNS, in which three different neurons, all of which modulate the gastric and/or pyloric patterns, contain proctolin as a co-transmitter [2]. The motor patterns that result from the activation of these neurons are distinct, with different patterns being triggered by activity in each of the neurons (e.g., [2]). The mechanisms by which co-transmission in the crab is regulated and by which modulatory neurons containing different complements of co-transmitters exert their effects have been extensively reviewed (e.g., [285, 286, 292–294]). Nonetheless, it is worth noting that not only are co-transmitters likely to activate distinct receptors, but also that the patterns of firing, and therefore of transmitter release, of neurons containing different co-transmitter complements are likely to differ. Both factors may play a role in determining the differential effects of these modulatory neurons. Moreover, even neurons that respond to a given co-transmitter when it is bath-applied may not respond to simulation of the neuron containing that peptide. Possible explanations include the distribution of synapses and the segregation of transmitters within a modulatory neuron.

The second question, regarding the ways in which different peptides interact within a given circuit, may involve the same issues discussed above. However, an early study showed that the influence of proctolin on the cardiac sac network differs dramatically when it was applied alone and when it was applied either shortly after RPCH or in the presence of low (sub-threshold for overt effects) levels of RPCH. Alone, proctolin had no obvious effect; with RPCH, it, like higher concentrations of RPCH alone,

activated the cardiac sac pattern in *Panulirus interruptus* [295]. Although no mechanism was proposed, the subsequent demonstration [289] that RPCH and proctolin converge to activate the same inward current in many STG neurons suggests a possible site for their interactions.

Modulation of sensory feedback within the stomatogastric system

Both the anterior gastric receptor (AGR) and the gastropyloric receptor (GPR) neurons monitor stretch in stomach muscles, providing feedback to the stomatogastric pattern generators. Neuropeptides, including TNRNFLRFamide and SDRNFLRFamide, excite these receptors. These effects are more complex than a simple increase in firing frequency, presenting possible mechanisms for the coding of more information. The dendrites of the AGRs in *Homarus gammarus* fire tonically in the absence of stretch and increase their firing frequency in response to stretch [296]. Exogenously applied TNRNFLRFamide causes them to reversibly switch from a tonic-firing mode to a bursting mode of firing in the absence of stretch. When stretched, the bursting pattern is altered, so that stretch in the presence of the peptide is encoded by three parameters: increased burst frequency, decreased burst duration, and increased spike frequency within bursts [296].

Although TNRNFLRFamide does not cause the firing mode of the GPRs to switch from tonic to bursting in *Cancer borealis*, these receptors can spontaneously fire in a bursting mode. When they do so, TNRNFLRFamide increases the burst rate, even in the absence of a stretch stimulus [297]. When these receptors are not in bursting mode, TNRNFLRFamide increases the tonic firing rate, as well as the response to stretch.

Although most neuropeptides known to modulate sensory organs in crustaceans result in increases in sensory responses to the same stimulus, one peptide, an A-AST, causes an average decrease in tonic spike frequency as well as in the response to stretch in the GRP [297]. These modulatory effects are both amplitude and history-dependent. Specifically, the relative effects of the peptide are greater when spike frequency in the receptor is low. Thus, the peptide decreases the response to smaller stretches more than that to large stretches (Fig. 6). Moreover, because these receptors adapt to repeated stimulation, response amplitude decreases with repeated stimulation. Consequently, the effects of the peptide tend to be more pronounced in a preparation that has been subject to repeated stimulation.

In addition to decreasing spike frequency, the A-ASTs have more subtle effects on the timing of spikes in the GPRs [298]. The precision of spike timing increases; that is, there is less jitter in the times at which the spikes occur during a changing stimulus. Thus, if a neuron getting postsynaptic input from the GPRs has a short time constant and is integrating information from multiple sources, the change in jitter could, like the change in average firing frequency, play an important role in information transfer.

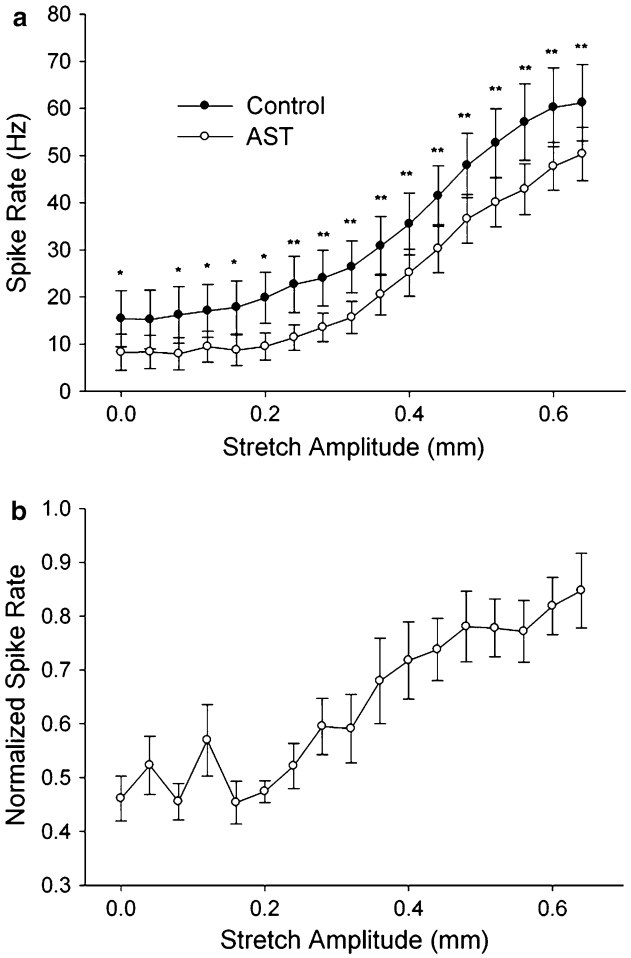

Fig. 6 A-type allatostatin (A-AST) decreases the responses of the gastro-pyloric stretch receptor (GPR) in the *Cancer borealis* stomatogastric system. **a** Spike frequency in response to stretch increases as a function of stretch amplitude, but is lower at all amplitudes in the presence of AST. **b** Spike frequency in A-AST is shown normalized to the response in control saline at each stretch amplitude, showing that the effects of A-AST are greater at larger stretch amplitudes. Figure modified from Birmingham et al. [297]; used with permission

Other factors determining the outcome of peptidergic modulation: state dependence, life history, and evolutionary considerations

One of the earliest principles to come from studies of peptidergic modulation of the STNS is the concept of state-dependent modulation [299, 300]. Initially, it was shown that the effects of excitatory peptides or the activity of modulatory neurons was stronger when the starting pattern

was weak than when it was strong. It has subsequently become clear that the same is true for inhibition: the inhibitory ASTs are likewise more effective on an initially weak pattern [61, 76, 86]. While the "state" is clearly a complex function of ion channels present in many neurons within the system, the factors determining channel distribution are considerably less clear. This question becomes particularly interesting when one considers the large number of peptides present in the STNS, and then asks how and whether all of them are effective modulators of the system. While it is clear that many of the modulatory peptides located in the STG do alter the patterns produced there, it is equally clear that, at least under some conditions, a number of peptides within the ganglion have little or no effect on the activity of the pyloric and gastric mill patterns. Thus, for example, the recently identified *Homarus americanus* CLDH, which alters the output of the CG, is found in the STG, but appears to have little or no effect on the patterns generated there (Dickinson, unpublished). Likewise, pEGFYSQRYamide is found within the STG, but does not appear to alter the patterns produced there (Dickinson, unpublished data).

In the short term, the presence of other neuromodulators is a major factor in determining the level of activity and thus the state of the system. The experiments on *Panulirus interruptus* described above, in which proctolin activates the cardiac sac pattern in the presence of low concentrations of RPCH or just after RPCH application, but not in its absence [295], illustrate one such example. This is also seen in decentralization, when inputs to the STG are removed; in most species, this leads to a cessation of the gastric pattern and either a decrease or cessation of the pyloric pattern. In *Homarus americanus*, the PD neurons continue to burst strongly, but at a decreased frequency [301]. If, however, the preparations are maintained in this state, deprived of all neuromodulatory inputs, for hours to days, activity returns to something resembling normal in many species, e.g., *Jasus lalandii* [302, 303], *Cancer borealis* [304, 305], and *Homarus gammarus* [301]. The mechanisms underlying this change, and the role played by neuropeptides in it, have recently been examined in *Cancer borealis* [306, 307]. These studies showed that the alterations in ionic currents that underlie recovery after decentralization, including decreased co-regulation of specific currents, are controlled not only by activity in the pattern generator, but also by neuromodulators, notably proctolin. Interestingly, the currents whose expressions were altered by decentralization and whose coordinated expression was regulated over the time (hours to days) involved in the recovery from decentralization were not the currents that are acutely modulated by proctolin, suggesting the possibility that neuropeptides have longer term, more subtle effects than have previously been studied.

It is also worth considering and speculating on the determinants of the extent to which peptides alter activity on longer time scales: developmentally, over the course of seasons and years, and evolutionarily.

A number of studies in *Homarus americanus* [101, 228, 266, 308, 309] and *Homarus gammarus* [310, 311] have examined the roles of neuropeptides in the development of the pattern generators of the STNS, at least partly by assessing the effects of the peptides at different developmental stages (for review see [308, 312, 313]). Interestingly, the full complement of STG neurons and modulatory projection neurons is present very early in development. However, modulatory transmitters are acquired only gradually. Thus, some peptides, including FLRF-like peptides, proctolin and RPCH, are all present by the mid-embryonic stage, whereas A-ASTs and TRPs are acquired only near or after the end of the embryonic stage (for review see [312, 313]). Moreover, although all of the STG neurons are present, the motor patterns that are generated in the embryonic and larval stages differ dramatically from those generated in the adult. Specifically, the embryonic STG produces only a single rhythm, in which all of the STG neurons participate, whereas clearly distinct pyloric and gastric mill patterns are generated by the adult STG [310, 311]. A number of experiments suggest that the differential organization is at least partly a function of modulation, including peptidergic effects. Thus, blocking all modulation eliminates rhythmic activity in the embryonic nervous system and eliminates most rhythmic activity in the adult nervous system. Adding back a modulator (oxotremorine in the published experiments) restores activity, but results in the generation of two patterns with different frequencies in both the embryo and the adult, suggesting that the specific pattern of modulators released in the embryo is responsible for maintaining the single embryonic pattern. In spite of this, a number of peptides have different effects on the embryonic and adult motor patterns, while the effects of others are remarkably similar at these different life stages. For example, in recordings from neurons that show pyloric activity in the adult, CCAP activates a pyloric-like triphasic pattern, albeit one with a relatively low cycle frequency, in the embryonic STNS [101]. RPCH likewise produces similar patterns in the adult and the embryo [228] (Fig. 7). In contrast, SIFamide activates different classes of neurons in the embryo and the adult [228] (Fig. 7). Thus, while peptides may play an important role in sculpting the pattern generators and the resultant stomatogastric patterns during development, it is clear that developmental stage partly determines the responses of the STG pattern generators to peptides.

After metamorphosing to their adult forms, crustaceans continue to undergo periodic changes in their internal as well as external environments. Perhaps the most

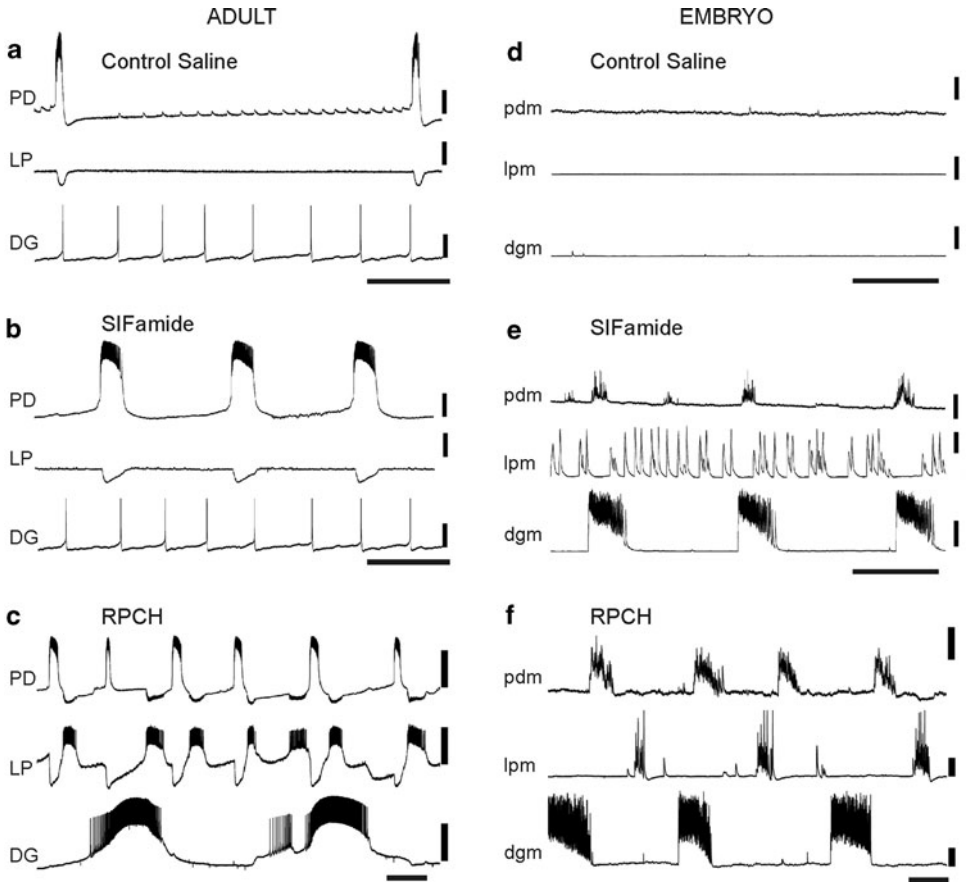

Fig. 7 Peptides modulate the same system differently in adults (**a**, **b**, **c**) and embryos (**d**, **e**, **f**) of *Homarus americanus*. (**a**) When inputs from the anterior ganglia are blocked in the adult, the only rhythmically active neurons in the STG are the PD neurons, which fire in regular bursts at a low cycle frequency. **b** Superfusion of the isolated STG with SIFamide activates bursting in the PD neurons, but has little effect on the other two neurons shown, the LP neuron of the pyloric network, and the DG neuron from the gastric mill network. **c** RPCH causes an increase in activity in all three neurons shown. **d** In the embryo, recordings are from muscles innervated by the same neurons whose activity is shown in the adult neuronal recordings. In control saline, when modulatory inputs are blocked, none of the neurons are rhythmically active. **e** Superfusion with SIFamide activates all three neurons, with the PD and DG innervated muscles (*pdm*, *lpm*) firing in bursts that are more or less synchronous, while the LP-innervated muscle (*dgm*) is activated more or less tonically. This contrasts with the adult, in which only one of the neurons (i.e., PD) is activated. **f** RPCH activates activity in all three muscles; however, in contrast to what is seen in the adult, they all fire with the same cycle frequency. **c** and **f** are from different preparations than **a/b** and **d/e**, respectively; note also that they are on slightly different time scales. *Scale bars* 5 mV, 5 s. Figure modified from Rehm et al. [228]; used with permission

physiologically demanding change is the periodic molt cycle. As the cuticle is removed in ecdysis, crustaceans swallow water to increase their size, which might be expected to alter functioning of the foregut, although only one study has examined molting. Clemens et al. [314] found that the pyloric rhythm continued relatively unchanged except for a brief time during ecdysis itself. They likewise found that the depression of the pattern that occurred during this time could be largely accounted for by a hypoxia induced by molting. Nonetheless, it is known that peptide levels, for example CCAP, change significantly during the molt cycle [108], suggesting the possibility that the effects of peptides may change as a function of the molt cycle. For example, given that CCAP strongly activates the pyloric pattern between molts, how is it that this pattern remains relatively constant as CCAP levels increase with the approaching molt? The extent to which, and the mechanism by which, the molt cycle influences peptide effects on the STNS as well as on other systems (e.g., the heart) are areas of investigation that are likely to provide insight into fundamental aspects of the function of peptides in physiological control.

On an evolutionary time scale, the structure and the required function of the stomatogastric system as a whole have undergone significant changes. These are reflected in the structure of the foregut and of the STNS. Nonetheless, the pattern generators of the STNS remain remarkably similar between species, with changes most evident in the

strengths of the synaptic connections (both electrical and chemical) between neurons within the system (for review see [313]). However, peptidergic modulation appears to be less conserved, and homologous neurons often contain different peptide complements (for review see [313]). Additionally, even when the same modulatory peptides are present, the ability of the pattern-generating neurons of the STNS to respond to them may change over evolutionary time. For example, virtually the same complement of peptides is present in the STNS and neuroendocrine organs of *Cancer* crabs and the kelp crab *Pugettia producta*, which has a much more restricted diet than *Cancer* species [92]. However, the *Pugettia* STNS appears to be much less sensitive to many of these peptides, including both CCAP and the TRP APSGFLGMRamide [92].

Conclusions and future directions

Much has changed in the 15 years since the last comprehensive review of crustacean neuropeptides [75] was undertaken. The tools currently being used for peptide discovery have changed substantially; consequently, in contrast to the fewer than two dozen neuropeptides characterized from the entirety of the crustacean taxon, more than two dozen peptide families have now been characterized from many individual species. For some peptide families, over two dozen isoforms have been characterized from a single animal. The functional roles played by peptides in members of the Crustacea continue to expand; most, if not all, peptides now appear to be highly pleiotropic within this taxon. Clearly much work remains to be done before we will understand fully the roles served by the diverse set of crustacean neuropeptides, and undoubtedly many more peptides remain to be discovered.

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