Neuropeptide signalling in echinoderms: from "physiologic activity of nerve extracts" to neuropeptidomics and beyond.

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Introduction

An abstract with a mysteriously vague title - "Physiologic activity of nerve extracts" - was published in 1959 on pages 407 and 408 of volume 117 of *The Biological Bulletin* (Chaet and McConnaughy, 1959). The authors of the abstract, A.B. Chaet and R.A. McConnaughy from the Department of Biology at The American University in Washington, DC., reported the observation that intracoelomic injection of an extract of radial nerve cords from the starfish *Asterias forbesi* triggers spawning in reproductively mature starfish. The active component was named gamete shedding substance (GSS) and subsequently Chaet and colleagues demonstrated that GSS is a polypeptide (Chaet, 1966a). Thus, the 1959 abstract heralded the beginnings of research on neuropeptides in echinoderms.

Chaet's rationale for selecting starfish as an experimental model was the recognition that these animals had largely been neglected as a source of eggs and sperm for studies in reproductive and developmental biology. He attributed this to the difficulty in obtaining mature and synchronized gametes from starfish. In sea urchins, mature gametes can be obtained by simply injecting ripe animals with potassium chloride solution, but this method does not work in starfish (Chaet, 1964). Therefore, Chaet investigated whether an endogenous gonadotropic substance present in the nerve cords would trigger release of mature gametes – and thus GSS was discovered. Furthermore, this was an important novel contribution to the emerging field of neuroendocrinology, which had been pioneered by Enrst and Berta Scharrer in the 1940s (Oksche, 1997)

So what is the rationale for using echinoderms as experimental animals for neuroendocrinology? A primary justification is the phylogenetic position that echinoderms occupy in the animal kingdom. Bilaterian animals are sub-divided into two major clades – the deuterostomes and the protostomes (Figure 1). The vertebrates are deuterostomes, whereas the majority of invertebrates are protostomes, including arthropods (e.g. *Drosophila melanogaster* and other insects), nematodes (e.g. *Caenorhabditis elegans*), molluscs and

annelids. There are, however, some invertebrate deuterostomes, including the chordate sub-phyla that are closely related to vertebrates – the urochordates and cephalochordates – and the ambulacrarians – hemichordates and echinoderms (Dunn et al., 2014; Halanych, 2004; Holland, 2011). Neuroendocrinologists have generally focused on vertebrates and selected protostomes (arthropods, molluscs and more recently *C. elegans*), largely for practical reasons and/or for applied research (Schoofs et al., 2017; Taghert and Nitabach, 2012). Until recently, however, the lack of data from invertebrate deuterostomes has hindered efforts to reconstruct the evolutionary history of neuroendocrine systems and to determine orthologous relationships between neuropeptide signalling systems in vertebrates and in protostomes. But with the availability of transcriptomic/genomic sequence data from echinoderms and other invertebrate deuterostomes, important new insights into the evolution of neuropeptide signalling systems are now being obtained (Elphick et al., 2018; Jekely, 2013; Mirabeau and Joly, 2013; Semmens and Elphick, 2017), as discussed in more detail below.

Echinoderms are not, however, solely of interest in providing "missing pieces" in the "jigsaw puzzle" of neuropeptide evolution. These animals are also of intrinsic interest from a physiological perspective. Echinoderms are unique amongst the Bilateria in exhibiting radial symmetry (typically pentaradial) as adult animals. Consequently, echinoderms do not have an anterior brain but instead they have a central nervous system comprising a circumoral nerve ring and five or more radial nerve cords (Mashanov et al., 2016; Pentreath and Cobb, 1972). Therefore, it is of interest to investigate how neuropeptide signalling systems function to regulate physiological processes and behaviour in the context of a radial body plan. Furthermore, there are many fascinating aspects of echinoderm physiology. Perhaps most notable is the mutable collagenous tissue (MCT) of echinoderms that changes its mechanical state rapidly under the control of the nervous system (Wilkie, 2005), with neuropeptides having been identified as candidate regulators of MCT (Birenheide et al., 1998). Another

intriguing property of echinoderms is the ability to autotomise and then regenerate body parts; for example, the arms of starfish and brittle stars and the visceral organs of sea cucumbers (Wilkie, 2001). Again there is evidence that neuropeptides are involved in regulating these processes (Mladenov et al., 1989).

The first neuropeptides to be sequenced in an echinoderm were two peptides isolated from the nerve cords of the starfish species *Asterias rubens* and *A. forbesi*, which were named SALMFamide-1 (S1) and SALMFamide-2 (S2) (Elphick et al., 1991a). In keeping with the experimental approaches widely employed at the time of their discovery, these peptides were isolated on account of their cross-reactivity with antibodies to a molluscan FMRFamide-like peptide. Thus, nothing was known about the physiological roles of S1 and S2 in starfish when they were first discovered. The same is true of the plethora of putative neuropeptides that are now being discovered in echinoderms using the modern techniques of transcriptomics, genomics and proteomics (Rowe et al., 2014; Rowe and Elphick, 2012; Semmens et al., 2016; Suwansa-Ard et al., 2018; Zandawala et al., 2017). So as we approach the sixtieth anniversary of the publication of Chaet and McConnaughy's 1959 abstract reporting the discovery of GSS, we are spoilt for choice with the abundance of novel echinoderm neuropeptides that have been discovered recently. The challenge for Chaet in 1959 was to determine the molecular identity of a gonadotropic peptide. Now in the "omics" era, the challenge is to find out what are the actions and physiological roles of the many neuropeptides that have been identified in starfish and other echinoderms.

The purpose of this review is two-fold – to provide an overview of our current knowledge of neuropeptide signalling systems in echinoderms and to look ahead in identifying the emerging areas of enquiry in this field of research. To review the literature on echinoderm neuropeptides, I will consider each of the five extant classes sequentially: Asteroidea (starfish), Ophiuroidea (brittle stars and basket stars), Echinoidea (sea urchins), Holothuroidea (sea cucumbers) and Crinoidea (featherstars and sea lilies). Chaet and

McConnaughy's discovery of GSS in 1959 is the justification for starting with starfish and the sequence that follows reflects phylogenetic relationships. Thus, the Asteroidea and Ophiuroidea are sister classes in the clade Asterozoa and the Echinoidea and Holothuroidea are sister classes in the clade Echinozoa. Collectively, the Asterozoa and Echinozoa form the clade Eleutherozoa, with the Crinoidea occupying a basal position with respect to the Eleutherozoa (Figure 2) (O'Hara et al., 2014; Telford et al., 2014).

Neuropeptide signalling systems in the Asteroidea

From GSS to RGP: the long road to identification of a starfish gonadotropic neuropeptide

The discovery of gamete shedding substance or gonad stimulating substance (GSS) (Chaet and McConnaughy, 1959) heralded the beginning of a programme of research that continues to this day. Chaet proceeded to investigate the properties of GSS in the 1960s and summarized his findings in two review articles published in 1966 (Chaet, 1966a; Chaet, 1966b). Subsequently, the Japanese scientists H. Kanatani and H. Shirai became the leading researchers on GSS (Kanatani, 1979; Shirai, 1986), progressing with investigations of the chemical nature of GSS and its mechanism of action. More recently, M. Mita, also based in Japan, has been the leading researcher on GSS and in 2009, fifty years after GSS was first discovered, Mita and colleagues successfully determined the structure of GSS as a heterodimeric polypeptide related to the mammalian reproductive hormone relaxin (Mita et al., 2009) (Figure 3A). With this discovery, Mita renamed GSS as relaxin-like gonadotropic peptide or RGP (Mita et al., 2015) and henceforth I will refer to GSS as RGP. Research on RGP is on-going, and reviews summarizing the latest findings have been published recently (Mita, 2013; Mita, 2016). It is beyond the scope of this chapter to review the complete history of research on RGP and for this readers are referred to the review articles highlighted above.

Instead the focus here will be to summarise the mechanisms by which RGP exerts its effects as a gonadotropic neuropeptide and to discuss what is known about the physiological mechanisms of RGP release in starfish.

RGP triggers gamete maturation and spawning in starfish (Figure 3B). The effect of RGP in causing gamete maturation is indirect and mediated by the maturation-inducing substance or meiosis-inducing substance (MIS) 1-methyl-adenine (1-MeAde), which is produced by associated follicle cells (Kanatani et al., 1969). RGP triggers 1-MeAde production in follicle cells via G-protein-mediated stimulation of cAMP synthesis by adenylyl cyclase (Mita and Nagahama, 1991). Furthermore, progress has been made recently in elucidating the molecular basis of the development of gonadal responsiveness to the effects of RGP. Thus, RGP triggers 1-MeAde production in follicle cells from mature ovaries but not from young ovaries and this is explained by changes in the expression of a G-protein $G_s\alpha$-subunit, which is expressed in the follicle cells of mature ovaries but not in follicle cells of young ovaries (Mita et al., 2013; Mita et al., 2012). The G-protein coupled receptor that mediates the effect of RGP on follicle cells has yet to be identified, but it is likely that it is evolutionarily related to G-protein coupled receptors that mediate the effects of relaxins in vertebrates (Halls et al., 2015). Thus, discovery of the RGP receptor represents an important goal for future research on RGP.

Whilst our knowledge of the molecular mechanisms by which RGP exerts its gonadotropic effects in starfish has advanced significantly, as discussed above, we still know very little about the mechanisms by which RGP is released physiologically. The concentration of RGP in the coelomic fluid of starfish increases prior to spawning, indicating that RGP acts as a hormone (Kanatani and Ohguri, 1966; Kanatani and Shirai, 1969). Because RGP was originally isolated from the radial nerve cords of starfish there has been an assumption that the radial nerve cords are the physiological source RGP in the context of its role as a gonadotropin. With the molecular identification of RGP it has become feasible to investigate

its pattern of expression in starfish. Analysis of the expression of the RGP precursor in the starfish *A. rubens* using mRNA *in situ* hybridisation methods revealed the presence of transcripts in cells located in the ectoneural region of the radial nerve cords and circumoral nerve ring, consistent with original isolation of RGP from this tissue. Furthermore, a dense population of RGP-expressing cells was also revealed at the tips of the arms in close association with sensory organs – the terminal tentacle and the optic cushion (Lin et al., 2017b) (Figure 3C-F). The detection of RGP-expressing cells in the arm tips is intriguing because it is suggestive of a mechanism by which environmental cues for spawning, such as changes in day length, temperature and/or release of gametes by conspecifics, could be detected by sensory cells and relayed to nearby RGP-expressing cells. A key question that remains to be addressed is whether the RGP-expressing cells in the arm tips have processes that project to sites where RGP could be released directly into the coelomic fluid. Recently, antibodies to RGP were generated and used to quantify RGP expression in tissues/organs of the starfish *Patiria pectinifera* using radioimmunoassays and enzyme-linked immunosorbent assays (ELISAs). Consistent with the distribution of RGP precursor transcripts, RGP was detected in the radial nerve cords (1.54±0.09pmol/mg) and the circumoral nerve rings (0.87±0.04pmol/mg) but not in other cells/tissues/organs analysed, which included the pyloric stomach, pyloric caeca, tube feet, ovaries, testes, and ovarian follicle cells (Mita and Katayama, 2017; Yamamoto et al., 2017). With the development and characterization of antibodies to RGP, there now exist opportunities to use these antibodies to examine the distribution of RGP in starfish using immunohistochemical methods and to identify environmental cues that trigger release of RGP into the coelomic fluid.

Interestingly, analysis of transcriptome sequence data has revealed the presence of a second relaxin-like peptide precursor in *A. rubens* (ArRLPP2) (Semmens et al., 2016) and subsequently an ortholog of ArRLPP2 was discovered in the crown-of-thorns starfish *Acanthaster planci* (Smith et al., 2017). Currently, nothing is known about the physiological

roles of this peptide in starfish and therefore this will be an important question to address in the future. Thus, does the second relaxin-like peptide in starfish also act as gonadotropic hormone like RGP or does it have other functions?

SALMFamides: the first echinoderm neuropeptides to be sequenced

A detailed review of twenty-five years of research on SALMFamides was published in 2014 (Elphick, 2014) and it would be superfluous to replicate that here. Therefore, here I will summarise key discoveries and then go on to briefly review a few papers that have been published since 2014.

Two peptides that were found to be immunoreactive with antibodies to a FMRFamide-like peptide were isolated from the starfish species *A. rubens* and *A. forbesi* and identified as structurally related peptides – the octapeptide GFNSALMFamide and the dodecapeptide SGPYSFNSGLTFamide. The C-terminal pentapeptide of the octapeptide, SALMFamide, was coined as a name for the peptides and the octapeptide was named SALMFamide-1 (S1) and the dodecapeptide was named SALMFamide-2 (S2) (Elphick et al., 1991a; Elphick et al., 1991b). Investigation of the expression of S1 and S2 in *A. rubens* using immunohistochemistry revealed widespread patterns of neuronal expression in larval and adult animals (Moore and Thorndyke, 1993; Moss et al., 1994; Newman et al., 1995a; Newman et al., 1995b). Furthermore, examination of the pharmacological actions of S1 and S2 in *A. rubens* revealed that both peptides act as muscle relaxants (Elphick et al., 1995; Melarange and Elphick, 2003; Melarange et al., 1999). At the level of whole-animal behaviour, starfish feed by everting their stomach out of their mouth and over the digestible parts of prey (e.g. mussels) and, interestingly, both S1 and S2 cause relaxation and eversion of the stomach in *A. rubens*. Therefore, SALMFamides may be involved in the neural mechanisms that control extra-oral feeding in starfish (Elphick and Melarange, 2001; Melarange et al., 1999).

Transcriptome sequencing has revealed that S1 and S2 are derived from different precursor proteins in *A. rubens* (Semmens et al., 2016) and the larval expression pattern of transcripts encoding these precursors has been reported (Mayorova et al., 2016). S1 is derived from a precursor that comprises six other related peptides that have in common with S1 a C-terminal LxFamide motif (where x is variable) and hence the S1 precursor is referred to as an L-type SALMFamide precursor (Figure 4). Interestingly, some of the other peptides derived from the S1 precursor have an Amino Terminal Cu(II), Ni(II) Binding (ATCUN) motif, and it has been shown that these peptides can bind Cu(II) and form metal linked dimers; however, the functional significance of this property of S1 precursor-derived SALMFamides remains to be determined (Jones et al., 2016). Like S1, S2 has a C-terminal LxFamide motif but it is atypical of the precursor it is derived from, which comprises seven SALMFamides with a C-terminal FxFamide motif and hence is referred to as an F-type SALMFamide precursor (Figure 4). The functional significance of the presence of the L-type SALMFamide S2 in an F-type SALMFamide precursor is not known, but it appears to be an evolutionarily ancient characteristic because it is also observed in orthologous F-type SALMFamide precursors in other starfish and in other echinoderms (Elphick et al., 2013; Elphick et al., 2015) (Figure 4). Further insights into the functional significance of the occurrence of the neuropeptide "cocktails" derived from SALMFamide precursors could be obtained if the receptors that mediate their effects are identified. Therefore, discovery of SALMFamide receptors is a key objective for the future.

Transcriptomic/genomic identification of neuropeptide precursors in starfish: from genes to "physiologic activity"

The fifty years that separate the discovery of GSS and its molecular identification as a relaxin-type peptide are indicative of how challenging the purification and molecular identification of bioactive peptides can be (Chaet and McConnaughy, 1959; Mita et al., 2009).

Now in an age of high-throughput transcriptome/genome sequencing it is far easier to first identify transcripts/genes encoding putative neuropeptide precursors and then proceed toward molecular and functional characterization of the associated neuropeptides. Thus, sequencing of the neural transcriptome of *A. rubens* recently enabled identification of forty neuropeptide precursors (Semmens et al., 2016). Similarly, analysis of transcriptomic and genomic sequence data from the crown-of-thorns starfish *Acanthaster planci* enabled identification of orthologs of the precursors found in *A. rubens* as well as several other novel candidate neuropeptide precursors. In addition mass spectroscopic analysis of *A. planci* tissue extracts enabled determination of the structures of some of the neuropeptides derived from the precursor proteins (Smith et al., 2017). Identification of neuropeptide precursors in starfish has provided a valuable new resource for neuropeptide research in these animals. Furthermore, in some cases discovery of starfish neuropeptide precursors has provided important insights into the evolution of neuropeptide signalling systems, as discussed below.

Several of the neuropeptide precursors identified in *A. rubens* were the first to be identified outside the phylum Chordata; for example, a precursor of kisspeptin-type neuropeptides and a precursor of a melanin-concentrating hormone (MCH)-type neuropeptide. Thus, new insights into the evolutionary history of kisspeptin-type and MCH-type signalling were obtained (Semmens and Elphick, 2017; Semmens et al., 2016). Another important finding to emerge from *A. rubens* neural transcriptome data was the identification of two precursors of gonadotropin-releasing hormone related peptides – ArGnRH1 and ArGnRH2 (Semmens et al., 2016). Analysis of *A. rubens* transcriptome sequence data also revealed an ortholog of vertebrate GnRH-type receptors (ArGnRHR) and an ortholog of insect corazonin receptors (ArCRZR), which are closely related to GnRH receptors. Functional characterization of ArGnRHR and ArCRZR revealed that ArGnRH1 is the ligand for ArGnRHR and ArGnRH2 is the ligand for ArCRZR. Therefore, ArGnRH1 was renamed ArGnRH and ArGnRH2 was renamed ArCRZ (Figure 5A, B). ArGnRHR and ArCRZR were the first

neuropeptide receptors to be pharmacologically characterized in starfish. Furthermore, the discovery of ArCRZ was of broader significance because it is the first ligand for a corazonin-type receptor to be identified in a deuterostome. Thus, it was established that the evolutionary origin of paralogous GnRH-type and CRZ-type signalling pathways can be traced back to the common ancestor of protostomes and deuterostomes, but with subsequent loss of CRZ-type signalling in some taxa (e.g. vertebrates and nematodes) (Tian et al., 2016; Zandawala et al., 2018).

Molecular identification of novel neuropeptide signalling systems in *A. rubens* has provided a basis for investigation of their physiological roles in starfish. Here I will highlight the progress that has been made so far in the form of published outputs, starting with the GnRH-type and CRZ-type signalling systems introduced above. Analysis of the expression of the ArGnRH and ArCRZ precursors in *A. rubens* using immunohistochemistry and/or mRNA *in situ* hybridisation revealed that both precursors are widely expressed but with differences in their patterns of expression. Informed by these findings, the *in vitro* pharmacological effects of ArGnRH of ArCRZ on neuromuscular systems were examined. Both ArGnRH and ArCRZ were found to be myoactive, causing contraction of apical muscle, tube foot and cardiac stomach preparations. However, ArGnRH was more potent/effective than ArCRZ in its effect on cardiac stomach preparations, whereas ArCRZ was more potent/effective than ArGnRH in its effect on apical muscle and tube foot preparations (Tian et al., 2017). As this was the first study to compare the expression and bioactivity of paralogous GnRH-type and CRZ-type neuropeptides in a deuterostome, new insights into the evolution of neuropeptide function in the animal kingdom were obtained from experimental studies on starfish.

Another starfish neuropeptide that causes contraction of cardiac stomach preparations from *A. rubens* is the amidated pentapeptide NGFFYamide, which is an ortholog of neuropeptide-S (NPS) in tetrapod vertebrates and crustacean cardioactive peptide (CCAP) in protostomes (Semmens et al., 2015; Semmens et al., 2013) (Figure 5C), as discussed below in

more detail with respect to NGFFFamide, a sea urchin homolog of NGFFYamide. Investigation of the *in vivo* effects of NGFFYamide revealed that it causes reversal of magnesium chloride-induced eversion of the cardiac stomach in *A. rubens* (Semmens et al., 2013). Thus, NGFFYamide may act physiologically to trigger cardiac stomach retraction in starfish. It is noteworthy that ArGnRH was found not to cause reversal of magnesium chloride-induced eversion of the cardiac stomach in *A. rubens* (Tian et al., 2017). Thus, although both NGFFYamide and ArGnRH cause dose-dependent contraction of *in vitro* cardiac stomach preparations from *A. rubens*, only NGFFYamide triggers stomach retraction *in vivo*. This may at least in part reflect the fact that NGFFYamide is more potent than ArGnRH as a cardiac stomach contractant *in vitro*. However, differences in the patterns of expression of ArGnRH and NGFFYamide in the cardiac stomach may also be relevant here and therefore investigation of the expression pattern of NGFFYamide in *A. rubens* (and other starfish) represents an important objective for future work.

Discovery of pedal peptide/orcokinin-type neuropeptides as muscle relaxants in starfish.

As highlighted above, the first neuropeptides to be identified in starfish were the SALMFamide-type neuropeptides S1 and S2, which act as muscle relaxants (Elphick, 2014). Recently, other neuropeptides that act as muscle relaxants in starfish have been identified. Employing use of the apical muscle as a bioassay system for myoactive peptides, extracts of the starfish *P. pectinifera* were found to contain a peptide that acts as a muscle relaxant. Purification of this peptide revealed that it is a hexadecapeptide with the amino-acid sequence Phe-Gly-Lys-Gly-Gly-Ala-Tyr-Asp-Pro- Leu-Ser-Ala-Gly-Phe-Thr-Asp and it was named starfish myorelaxant peptide (SMP). A cDNA encoding the SMP precursor revealed the presence of twelve copies of SMP and seven copies of other SMP-like peptides. Furthermore, comparative sequence analysis revealed that SMP and the other SMP-like peptides in *P. pectinifera* belong to a bilaterian family of neuropeptides that include molluscan

neuropeptides known as pedal peptides (PPs) and arthropod neuropeptides known as orcokinins (OKs) (Kim et al., 2016). SMP also acts as a relaxant of tube foot and cardiac stomach preparations from *P. pectinifera* (Kim et al., 2016) and analysis of the effects of an SMP-like peptide in *A. rubens* has revealed that it likewise causes dose-dependent relaxation of apical muscle, tube foot and cardiac stomach preparations from this species (Lin et al., 2017a). Furthermore, analysis of the distribution of SMP-type peptides in *A. rubens* using mRNA *in situ* hybridization and immunohistochemistry has revealed expression in the cell bodies and axonal processes of neurons that innervate muscles (Lin et al., 2017a). Thus, combining the findings from *P. pectinifera* and *A. rubens*, it appears that SMP-type peptides may act as inhibitory neuromuscular transmitters or modulators throughout the class Asteroidea.

Interestingly, a second precursor of PP/OK-type neuropeptides has been identified in *A. rubens* (Semmens et al., 2016), which is now referred to as *A. rubens* pedal peptide-like neuropeptide precursor 2 (ArPPLNP2) so as to distinguish it from the SMP precursor, which is also referred to as ArPPLNP1 (Lin et al., 2017a). Investigation of the actions of a peptide derived from ArPPLNP2 has revealed that it also causes relaxation of cardiac stomach preparations, but it has no effect on apical muscle and tube foot preparations (Lin et al., 2018). The pattern of expression of ArPPLN2 in *A. rubens* is similar to that of the SMP precursor (ArPPLNP1); however, there are differences in the expression patterns of ArPPLNP1-derived and ArPPLNP2-derived neuropeptides in *A. rubens*, consistent with the differences in bioactivity (Lin et al., 2018).

The discovery and functional characterization of PP/OK-type neuropeptides in starfish has provided important new insights into the comparative physiology of this family of neuropeptides. Hitherto, the pharmacological actions of PP/OK-type neuropeptides had been characterized only in protostomes, with excitatory effects on muscle preparations being a common theme (Hall and Lloyd, 1990; Stangier et al., 1992). Thus, this contrasts with

inhibitory effects of PP/OK-type neuropeptides on starfish neuromuscular systems that have been discovered recently. It would be interesting, therefore, to investigate the physiological effects of PP/OK-type neuropeptides in other echinoderms to determine if inhibitory effects on muscle systems are common features of these neuropeptides throughout the phylum Echinodermata.

Surprisingly, nothing is known about the molecular identity of the receptors that mediate the effects of PP/OK-type neuropeptides in any bilaterian. Therefore, if the receptors that mediate the effects of PP/OK-type neuropeptides in starfish or other echinoderms were to be identified, this would have a broad impact in providing insights into the mechanisms by which PP/OK-type neuropeptides exert their effects and the relationships of PP/OK-type neuropeptides with other types of neuropeptides.

The starfish enterprise: novel neuropeptides in search of a mission

Only a handful of the novel neuropeptides that have been identified in starfish based upon analysis of genome/transcriptome sequence data (Semmens et al., 2016; Smith et al., 2017) have been functionally characterized thus far, as illustrated in the examples discussed above. Therefore, there are numerous opportunities ahead to investigate neuropeptide function in starfish. Representatives of many bilaterian neuropeptide families (vasopressin/oxytocin, calcitonin, thyrotropin-releasing hormone, orexin and others (Semmens et al., 2016); Figure 5) have yet to be functionally characterized in starfish. Furthermore, there are other starfish neuropeptides that do not appear to belong to any of the known bilaterian neuropeptide families. For example, “AN peptides”, which are characterized by an N-terminal Ala-Asn motif and were first identified in sea urchins (Rowe and Elphick, 2012) but have subsequently been found in other echinoderms, including starfish (Semmens et al., 2016; Smith et al., 2017; Zandawala et al., 2017).

Investigation of the patterns of neuropeptide expression in adult starfish have provided an anatomical basis for investigation of their physiological roles, as discussed above with reference to specific studies [e.g. ArGnRH and ArCRZ (Tian et al., 2017)]. Accordingly, analysis of the anatomical expression patterns and pharmacological actions of the many other neuropeptides that have been identified recently in starfish (Semmens et al., 2016; Smith et al., 2017) may shed light on their physiological roles. However, it should be noted here that investigation of neuropeptide function in starfish need not be restricted to the adult stage of the life cycle. As highlighted above with reference to SALMFamides, neuropeptides are also expressed by neurons in the nervous systems of the bilaterally symmetrical larvae of starfish. Thus, using mRNA *in situ* hybridization techniques, the expression of neuropeptide precursors has been examined in the bipinnaria and brachiolaria stage larvae of *A. rubens* (Mayorova et al., 2016). Eight neuropeptide precursors were analysed – the SALMFamide-type S1 precursor and S2 precursor, as highlighted above, and precursors of a vasopressin/oxytocin-type peptide ("asterotocin") (Figure 5D), NGFFYamide (Figure 5C), ArGnRH (Figure 5A), thyrotropin-releasing hormone (TRH)-type peptides (Figure 5F), a calcitonin-type peptide (ArCT; Figure 5G)) and a corticotropin-releasing hormone-type peptide (ArCRH). Expression of the S1, S2 and NGFFYamide precursors was revealed in bipinnaria larvae but expression of all eight precursors was revealed in brachiolaria stage larvae. Furthermore, expression of the precursors was observed to be associated with the attachment complex, which enables larval attachment to a substratum prior to the metamorphic transition into pentaradially symmetrical juvenile starfish. Therefore, neuropeptides are likely to be involved in signalling processes associated with the process of larval attachment prior to metamorphosis. Interestingly, several of the neuropeptide precursors were also found to be expressed in cells associated with the ciliary bands, which mediate larval locomotion and generation of currents for feeding on plankton (Mayorova et al., 2016). Recently, high resolution methods for analyzing the currents generated by the

ciliary bands of starfish larvae have been developed (Gilpin et al., 2016) and therefore exciting opportunities lie ahead to use these techniques to investigate the physiological roles of neuropeptides as regulators of ciliary activity in starfish larvae.

Neuropeptide signalling systems in the Ophiuroidea

SALMFamide-type neuropeptide signalling in brittle stars

The discovery of the SALMFamides in starfish (Elphick et al., 1991a) facilitated investigation of the occurrence and functions of related neuropeptides in other echinoderms, including ophiuroids. Using antibodies to S1 the anatomical distribution of S1-like immunoreactive peptides was investigated in brittle star species (de Bremaeker et al., 1997; Ghyoot et al., 1994). More recently, antibodies to the sea cucumber SALMFamide neuropeptide GFSKLYFamide have also been used to for analysis neuropeptide expression in brittle stars (Zueva et al., 2018). Furthermore, modulatory effects of S1 and S2 on light production in bioluminescent brittle stars have also been reported (Bremaeker et al., 1999). However, the low potency of S1 or S2 in exerting these effects probably reflects the use of starfish neuropeptides that are not native to brittle stars. Immunocytochemical investigation of SALMFamide expression in brittle stars has also been extended to larvae, with patterns of expression indicative of roles in regulation of swimming, feeding and gut activity (Cisternas and Byrne, 2003).

Opportunities for further investigation of the physiological roles of SALMFamides in brittle stars have been facilitated recently with the discovery of transcripts encoding SALMFamide precursors in ophiuroid species (Elphick et al., 2015; Zandawala et al., 2017). As in starfish, there are two SALMFamide precursors in ophiuroid species. One precursor is orthologous to the *A. rubens* S1 precursor, comprising peptides with a LxFamide motif ("L-

type") and the other precursor is orthologous to the *A. rubens* S2 precursor, largely comprising peptides with a FxFamide motif ("F-type") but also containing an L-type peptide (Figure 4). Availability of transcriptome sequence data from over fifty brittle star species has enabled evolutionary analysis of SALMFamide precursor structure with reference to a molecular-based ophiuroid phylogeny, revealing examples of clade-specific gain or loss of SALMFamide neuropeptides (Zandawala et al., 2017). The evolutionary and functional significance of changes in the composition of the neuropeptide "cocktails" derived from SALMFamide precursors in ophiuroids is currently unknown. As with the occurrence of SALMFamide "cocktails" in starfish and other echinoderms (see below), insights into the functional significance of evolutionary changes in neuropeptide precursor composition may emerge if the receptors that mediate the effects of SALMFamides are discovered. Furthermore, the availability of SALMFamide precursor sequences from a variety of brittle star species (Elphick et al., 2015; Zandawala et al., 2017) has also provided a basis for detailed investigations of the anatomical expression patterns and physiological roles of SALMFamides in ophiuroids, building upon earlier studies that relied on use of antibodies to heterologous peptides and pharmacological tests with starfish SALMFamides (Bremaeker et al., 1999; de Bremaeker et al., 1997).

Transcriptomic identification of other neuropeptide precursors in brittle stars

Analysis of transcriptome sequence data from ophiuroids (Zandawala et al., 2017) has enabled identification of orthologs of many of the neuropeptide precursors previously identified in starfish (Semmens et al., 2016; Smith et al., 2017); for example, vasopressin/oxytocin-type, NG peptide-type, GnRH-type and corazonin-type neuropeptide precursors (Figure 5). However, in some cases multiple precursors of related neuropeptides were identified in ophiuroids. For example, a single precursor of a somatostatin-type neuropeptide was identified starfish (Semmens et al., 2016) but analysis of ophiuroid

sequence data revealed two precursors of somatostatin-type peptides - SS1 (an ortholog of the putative neuropeptide original identified in starfish) and SS2 (a novel peptide). Likewise, two precursors of cholecystokinin (CCK)-type peptides were identified in ophiuroids – one precursor comprising three CCK-type peptides and another comprising a single CCK-type peptide. Another notable finding was the discovery of four different corticotropin-releasing hormone (CRH)-type precursors in ophiuroid species, contrasting with the single CRH-type precursor previously found in starfish (Zandawala et al., 2017).

Perhaps the most important finding to emerge from analysis of ophiuroid sequence data was the discovery of representatives of bilaterian neuropeptide families that hitherto had not been identified in starfish or echinoderms. Thus, precursors of neuropeptide-Y (NPY)-type peptides were identified in ophiuroid species and this then facilitated the discovery of orthologous precursors in starfish species (Zandawala et al., 2017). Another noteworthy finding to emerge from analysis of ophiuroid sequence data was the identification of echinoderm homologs of the insect neuropeptide eclosion hormone (EH). Hitherto, EH-type peptides had only been identified in arthropods so the discovery of precursors of EH-type peptides in a deuterostome provided the first evidence that the evolutionary origin of this neuropeptide may date back to the common ancestor of the Bilateria.

Analysis of the expression and functions of neuropeptides in brittle stars

The molecular characterization of many types of neuropeptide precursors in ophiuroids (Zandawala et al., 2017), as discussed above, has provided a superb resource for investigations of neuropeptide expression and function in brittle stars. Thus far, there has been no progress in this regard. However, it should be noted that recently a very detailed analysis of the anatomy of the ophiuroid nervous system has been reported, utilizing electron microscopy, molecular markers and 3D reconstructions (Zueva et al., 2018), extending and re-evaluating earlier anatomical studies (Cobb and Stubbs, 1981; Cobb and Stubbs, 1982; Stubbs

and Cobb, 1981) and studies that that employed use of dye-filling of neurons in combination with electrophysiological recordings of neuronal activity (Cobb, 1985). These anatomical studies provide a valuable framework for analysis of the patterns of neuropeptide expression in ophiuroids, as revealed by use of techniques such as mRNA *in situ* hybridization and immunohistochemistry. In particular, it will be interesting to compare the expression patterns of orthologous neuropeptides in ophiuroids and asteroids to investigate the conservation and/or diversification of neuropeptide function in the asterozoan clade of the phylum Echinodermata. For example, are neuropeptide types that are expressed by motoneurons in the hyponeural region of the starfish nervous system (see above) likewise expressed by motoneurons in the hyponeural region of the brittle star nervous system? Furthermore, if the success of J.L.S. Cobb in making electrophysiological recordings from ophiuroid nervous systems (Cobb, 1985) can be replicated, then the effects of neuropeptides on neuronal activity in brittle stars could be examined. Other areas of interest for further investigation are the roles of neuropeptides as candidate regulators of arm autotomy and regeneration in brittle stars (Czarkwiani et al., 2013; Czarkwiani et al., 2016; Dylus et al., 2016) and the roles of neuropeptides in mechanisms of neural control of whole-animal locomotory behaviour (Kano et al., 2017).

Neuropeptide signalling systems in the Echinoidea

SALMFamide-type neuropeptide signalling in sea urchins

As with ophiuroids, the availability of antibodies to the starfish SALMFamides S1 and S2 enabled investigation of the occurrence of related peptides in echinoids. Immunocytochemical studies on larvae of the sand dollar *Dendraster excentricus* revealed the distribution of S1-like immunoreactivity (Thorndyke et al., 1992). Subsequently, the distribution of SALMFamide-like immunoreactivity in larvae of the sea urchin *Psammechinus*

miliaris was reported (Beer et al., 2001). Furthermore, efforts were made to purify and sequence SALMFamides from the extracts of the sea urchin *Echinus esculentus*, employing radioimmunoassays for S1 and S2. However, only a partial N-terminal sequence (MRYH) of one purified peptide was determined (Elphick and Thorndyke, 2005). As discussed in more detail below, it was the sequencing of the genome of the sea urchin *Strongylocentrotus purpuratus* that transformed opportunities for determination of the sequences of sea urchin neuropeptides (Burke et al., 2006; Sodergren et al., 2006). Analysis of *S. purpuratus* transcriptome/genome sequence data revealed two SALMFamide precursors. The first to be identified was an F-type precursor comprising seven peptides with a C-terminal FxFamide motif (Elphick and Thorndyke, 2005) (Figure 4). Then an *S. purpuratus* L-type precursor was identified that comprises two SALMFamide neuropeptides (Rowe and Elphick, 2010) (Figure 4), including MRLHPGLLFamide - a homolog of the partially sequenced (MRYH) peptide that was purified from *E. esculentus* (Elphick and Thorndyke, 2005)

Little is known about the anatomical distribution of SALMFamide-type neuropeptide expression in adult echinoids, although recently it was reported that antibodies to the sea cucumber SALMFamide neuropeptide GFSKLYFamide label processes in the ectoneural region of the radial nerve cords in the sea urchin *Echinometra lucunter* (Mashanov et al., 2016). Likewise, little is known about the physiological roles of SALMFamides in echinoids. However, *in vitro* pharmacological tests with the starfish SALMFamides S1 and S2 revealed that both peptides cause relaxation of tube foot preparations from the sea urchin *E. esculentus* (Elphick and Thorndyke, 2005). Thus, SALMFamides act as muscle relaxants not only in starfish but also in other echinoderms. With the availability of the sequences of SALMFamide precursors from *S. purpuratus* and other sea urchin species, there now exist opportunities to investigate the physiological roles of SALMFamides in sea urchins more extensively, both in larval and adult animals.

Transcriptomic/genomic identification of neuropeptide precursors in sea urchins

The genome of the sea urchin *S. purpuratus* was the first to be sequenced in an echinoderm. A large number of candidate G-protein coupled neuropeptide receptors were identified from analysis of the genome sequence data, but only a few neuropeptide precursors were identified. These included precursors of a vasopressin/oxytocin-type neuropeptide ("echinotocin"), bursicon-type neuropeptides and glycoprotein hormone-type polypeptides (Burke et al., 2006). Subsequently, a systematic analysis of neural transcriptome sequence data from *S. purpuratus* enabled identification of transcripts encoding twenty neuropeptide precursors (Rowe and Elphick, 2012). For example, a precursor of peptides that exhibit sequence similarity with vertebrate thyrotropin-releasing hormone (TRH) was a noteworthy finding because this was the first TRH-type precursor to be discovered in an invertebrate. Other vertebrate neuropeptides for which homologs have been identified in *S. purpuratus* include calcitonin, CCK, GnRH, kisspeptin, orexin, melanin-concentrating hormone and somatostatin (Jekely, 2013; Mirabeau and Joly, 2013; Rowe and Elphick, 2012; Zandawala et al., 2017) (Figure 5).

From a different perspective, analysis of sea urchin sequence data also enabled discovery of homologs of neuropeptides that hitherto had only been identified in protostomes. For example, two precursors (SpPPLNP1, SpPPLNP2) of pedal peptide/orcokinin (PP/OK)-type neuropeptides – a family of neuropeptides that was first discovered in molluscs and arthropods. Thus, the discovery of PP/OK-type neuropeptides in an echinoderm revealed that the evolutionary origin of this neuropeptide family can be traced to a bilaterian common ancestor of protostomes and deuterostomes (Rowe and Elphick, 2012). Currently, nothing is known about the physiological roles of PP/OK-type peptides in sea urchins; however, progress has been made in the functional characterization of PP/OK-type peptides in starfish, as discussed above and in (Kim et al., 2016; Lin et al., 2017a; Lin et al., 2018). Another example of a neuropeptide identified in sea urchins that belongs to a

neuropeptide family that hitherto had only been found in protostomes is luqin (Figure 5E). The neuropeptide luqin was originally discovered in the mollusc *Aplysia californica* (Aloyz and DesGroseillers, 1995) and subsequently related peptides have been identified in other protostomes, including arthropod RYamide-type neuropeptides (Collin et al., 2011; Ida et al., 2011). Comparative analysis of genomic sequence data enabled identification of a protein in the sea urchin *S. purpuratus* comprising a luqin/RYamide-type neuropeptide and a C-terminal domain containing two cysteine residues that are highly conserved amongst luqin/RYamide-type precursors (Jekely, 2013). The discovery of this precursor was consistent with the presence of luqin/RYamide-type receptors in echinoderms and hemichordates (Jekely, 2013; Mirabeau and Joly, 2013). Thus, the discovery of a luqin-type neuropeptide precursor and receptor in the sea urchin *S. purpuratus* established that luqin/RYamide-type signalling originated in a common ancestor of protostomes and deuterostomes, but with subsequent loss in chordates (Jekely, 2013). Furthermore, discovery of the luqin/RYamide-type precursor in *S. purpuratus* facilitated discovery of luqin/RYamide-type precursors in other echinoderms, including sea cucumbers (Rowe et al., 2014), starfish (Semmens et al., 2016; Smith et al., 2017) and brittle stars (Zandawala et al., 2017). However, currently nothing is known about the physiological roles of luqin/RYamide-type neuropeptides in sea urchins or other echinoderms.

In addition to precursors of neuropeptides that are homologs of known neuropeptides from other phyla, other sea urchin precursor proteins comprise peptides that do not appear to exhibit sequence similarity with any known neuropeptides. For example, Spnp13, which comprises a peptide with the amino-acid sequence LPANLARE (Rowe and Elphick, 2012). However, progress has been made in establishing relationships for some of the neuropeptides identified in *S. purpuratus*. For example, we now know that the sea urchin precursor originally designated as “Spnp12” (Rowe and Elphick, 2012) is the precursor of a peptide that is an

ortholog to protostome corazonin-type neuropeptides (Tian et al., 2016; Zandawala et al., 2018).

Discovery of the sea urchin neuropeptide NGFFFamide and its cognate receptor provides new insights into neuropeptide evolution in the Bilateria

One of the most interesting discoveries to emerge from analysis of the *S. purpuratus* genome/transcriptome sequence data was the discovery of a precursor comprising two copies of the neuropeptide NGFFFamide (Elphick and Rowe, 2009). This precursor was discovered on account of the sequence similarity that NGFFFamide shares with the myoactive neuropeptide NGIWYamide, which had been discovered previously in the sea cucumber *Apostichopus japonicus* (see below and (Iwakoshi et al., 1995); Figure 5C). Surprisingly, analysis of the sequence of the NGFFFamide precursor revealed the presence of a C-terminal neurophysin domain, which was an unexpected finding because neurophysins hitherto had only been found in the precursors of vasopressin/oxytocin-type neuropeptides (including the sea urchin "echinotocin" precursor), where they are participate in intracellular transport of the mature vasopressin/oxytocin-type neuropeptides (De Bree, 2000; De Bree and Burbach, 1998). The presence of a neurophysin domain in the *S. purpuratus* NGFFFamide precursor suggested a close evolutionary relationship with vasopressin/oxytocin-type precursors (Elphick and Rowe, 2009). Subsequently, it was discovered that the NGFFFamide precursor is one of a family of precursor proteins in invertebrate deuterostomes that comprise neuropeptides that have an Asn-Gly (NG) motif ("NG peptides") and, like the NGFFFamide precursor, have a C-terminal neurophysin domain (Elphick, 2010). Notably, the NG peptide precursor of the cephalochordate *Branchiostoma floridae* comprises two copies of the peptide SFRNGVamide, which is identical to the N-terminal region of the vertebrate neuropeptide "neuropeptide-S" (NPS). Thus, a relationship between NG peptides and vertebrate NPS was established and a relationship with vasopressin/oxytocin-type signalling was again revealed

because the NPS receptor is closely related to vasopressin/oxytocin-type receptors (Xu et al., 2004). Furthermore, NPS receptors are orthologous to the receptors for crustacean cardioactive peptides (CCAPs) in arthropods, which exhibit some structural similarity with vasopressin/oxytocin-type peptides. Therefore, it was postulated that NG peptides are ligands for NPS/CCAP-type receptors in invertebrate deuterostomes (Mirabeau and Joly, 2013; Valsalan and Manoj, 2014). To test this hypothesis, the NPS/CCAP-type receptor from *S. purpuratus* was cloned and expressed heterologously in Chinese hamster ovary (CHO) cells so that NGFFFamide could be tested as a ligand for this receptor. Importantly, it was discovered that NGFFFamide is a potent ligand for the *S. purpuratus* NPS/CCAP-type receptor, with an EC_{50} of 0.4 nM (Semmens et al., 2015). Furthermore, based on this finding it was inferred that NGFFYamide and NGIWYamide are ligands for NPS/CCAP-type receptors in the starfish *A. rubens* and the sea cucumber *A. japonicus*, respectively (Semmens et al., 2015).

The discovery that NG peptides are ligands for NPS/CCAP-type receptors in echinoderms provided key evidence in support of a scenario of neuropeptide evolution in the Bilateria. Thus, following duplication of a vasopressin/oxytocin-type signalling system in a common ancestor of the Bilateria, one copy of the system retained the ancestral features and gave rise to the highly conserved vasopressin/oxytocin-type peptides and receptors that occur throughout the Bilateria. In contrast, the other copy of the system diverged but this took different courses in protostomes and deuterostomes. In the protostomes, the neurophysin domain was lost from the precursor protein but the neuropeptide derived from the precursor (CCAP) retained a vasopressin/oxytocin-like feature – the presence of a disulphide bridge. In the deuterostomes, the neurophysin domain was retained (although it was subsequently lost in vertebrates and holothurians) but the neuropeptide(s) derived from the precursor (NG peptides and NPS) lost the disulphide bridge that is characteristic of vasopressin/oxytocin-type peptides (Semmens et al., 2015).

The retention of a neurophysin domain in the sea urchin NGFFFamide precursor (and starfish and brittle star NG peptide precursors) is interesting because its functional significance remains unknown. As highlighted above, in vasopressin/oxytocin-type precursors the neurophysin domain binds vasopressin or oxytocin and is required for targeting of vasopressin/oxytocin-type peptides to the regulated secretory pathway (De Bree, 2000; De Bree and Burbach, 1998). Therefore, it is possible that neurophysins derived from deuterostome NG peptide precursors also have this role. However, there is a 1:1 stoichiometry in the interaction between neurophysins and vasopressin/oxytocin type peptides, which reflects the occurrence of single copies of neurophysins and vasopressin/oxytocin-type peptides in the precursor proteins (De Bree, 2000; De Bree and Burbach, 1998). In contrast, in sea urchin, starfish, brittle star and cephalochordate NG peptide precursors there are two NG peptide copies combined with a single copy of neurophysin (Semmens et al., 2015). Therefore, if neurophysins bind NG peptides in a manner similar to the interaction between vasopressin/oxytocin-type peptides, then a 2:1 stoichiometry would be expected. However, the occurrence of an NG peptide precursor in the hemichordate *Saccoglossus kowalevskii* comprising a single neurophysin in combination with six NG peptides represents a challenge to this hypothesis. Furthermore, the loss of neurophysin in the sea cucumber NGIWYamide precursor and in vertebrate NPS precursors suggests that neurophysins are not essential for biosynthesis of NG peptides/NPS in deuterostomes. If this is the case, then what is the function of neurophysin in NG peptide precursors that have retained neurophysins? This represents an interesting line of enquiry for further research on the sea urchin NGFFFamide precursor and other neurophysin-containing NG peptide precursors.

Functional analysis of neuropeptide signalling sea urchins

Currently, very little is known about the physiological roles of neuropeptides in echinoids. To the best of my knowledge, papers reporting the effects of echinotocin (Figure 5D) and NGFFFamide (Figure 5C) in causing contraction of sea urchin tube foot and oesophagus preparations (Elphick and Rowe, 2009) and the effects of the starfish neuropeptides S1 and S2 in causing relaxation of sea urchin tube foot preparations (Elphick and Thorndyke, 2005) are the only studies that have examined the pharmacological actions of neuropeptides in echinoids. Furthermore, with the exception of studies reporting the presence of SALMFamide-type neuropeptides in larval (Beer et al., 2001; Thorndyke et al., 1992) and adult (Mashanov et al., 2016) echinoids (see above), little is known about the patterns of neuropeptide expression in echinoids. With the discovery of many novel neuropeptides in echinoids, as discussed above, there is tremendous scope for further work in this area of enquiry. An indication of the possibilities was a recent report revealing the expression of insulin-like peptides in the digestive system of the pluteus larvae of *S. purpuratus* (Perillo and Arnone, 2014). Thus, insulin-like peptides may be involved in regulation of digestive physiology in larval echinoids.

Another aspect of larval echinoid biology that is likely to be regulated by neuropeptides is the ciliary system that mediates both feeding and locomotion. Studies on other larval marine invertebrates such as the annelid *Platynereis dumerilii* and the brachiopod *Terebratalia transversa* have revealed that neuropeptides are involved in neural control of swimming depth and predator avoidance behaviour (Conzelmann et al., 2011; Thiel et al., 2017). The few studies that have examined neuropeptide expression in sea urchins have revealed the presence of neuropeptide immunoreactivity in fibre tracts associated with the ciliary bands of sea urchin larvae (Beer et al., 2001; Thorndyke et al., 1992). Therefore, as discussed above with respect to starfish larvae, there are opportunities for studies examining the effects of neuropeptides on echinoid larval behaviour.

Neuropeptide signalling systems in the Holothuroidea

SALMFamide-type neuropeptide signalling in sea cucumbers

The use of antibodies to a molluscan FMRFamide-like neuropeptide to monitor purification of the starfish SALMFamides S1 and S2 established a methodology that was then applied to other echinoderms. Two SALMFamide-type peptides were purified from the sea cucumber *Holothuria glaberrima* and identified as GFSKLYFamide and SGYSVLYFamide (Díaz-Miranda et al., 1992). Investigation of distribution of GFSKLYFamide in *H. glaberrima* using immunohistochemistry revealed a widespread pattern of neuronal expression, with immunolabelled cells and/or processes detected in the radial nerve cords, body wall and digestive system (Díaz-Miranda et al., 1995). Furthermore, consistent with relaxing actions of S1 and S2 in starfish, GFSKLYFamide was found to cause relaxation of intestinal and body wall longitudinal muscle preparations from *H. glaberrima* (Díaz-Miranda and García-Arrarás, 1995). Other members of the SALMFamide neuropeptide family were discovered in the sea cucumber *Apostichopus japonicus* as part of a systematic effort to identify bioactive peptides that exert effects on neuromuscular preparations (Ohtani et al., 1999). Two SALMFamides that cause relaxation of *A. japonicus* intestine preparations were purified and identified as GYSPFMFamide and FKSPFMFamide. The discovery of these two peptides revealed for the first time the occurrence of F-type (FxFamide) SALMFamides in echinoderms. More recently, the sequence of the precursor protein that GYSPFMFamide and FKSPFMFamide are derived from has been determined, revealing that it also comprises six other SALMFamide neuropeptides, including L-type (LxFamide) SALMFamides (Elphick, 2012) (Figure 4). A second SALMFamide precursor in *A. japonicus* that comprises three L-type SALMFamides has also been identified (Elphick et al., 2013) (Figure 4). Furthermore, the sequences of SALMFamide precursors have also been determined recently in *H. glaberrima* and *H. scabra* as

part of a comprehensive transcriptomic analysis of neuropeptide precursors in these species (Suwansa-Ard et al., 2018).

With the identification of the two types of SALMFamide precursors in several sea cucumber species, each comprising multiple neuropeptides, there is now scope to investigate the expression of these precursors and the actions of peptides derived from them in both larval and adult sea cucumbers. In particular, nothing is known about the expression pattern and pharmacological actions of peptides derived from the L-type SALMFamide precursor in sea cucumbers and so this represents an interesting line of enquiry for the future.

Discovery of neuropeptides that act as regulators of muscle and/or collagenous tissue in the sea cucumber A. japonicus

As highlighted above, purification of components of extracts of the sea cucumber *A. japonicus* that cause relaxation of *in vitro* preparations of intestinal tissue from this species led to the identification of two F-type SALMFamide neuropeptides (Ohtani et al., 1999). Furthermore, many other myoactive neuropeptides were identified using the intestine and the body wall radial longitudinal muscle (RLM) for bioassays. These included peptides that cause intestinal contraction (GLRFA, holokinin, KHTAYTGIamide) and sixteen peptides that potentiate or inhibit electrically-evoked contraction of the RLM (Iwakoshi et al., 1995; Ohtani et al., 1999). More recently, the precursor proteins that give rise to many these bioactive peptides have been identified by analysis of transcriptome sequence data (Elphick, 2012). Importantly, this has revealed that most of the peptides are derived from proteins with an N-terminal signal peptide, consistent with the notion that these peptides are secreted signalling molecules. One exception, however, is the peptide holokinin, which was found to be derived from collagen (Elphick, 2012). Since the discovery of these myoactive peptides in *A. japonicus*, in most cases nothing more has been learnt about their expression and actions in this species.

However, two of the peptides (stichopin and NGIWYamide) have been investigated in more detail, as discussed below.

A peptide named stichopin was purified from extracts of *A. japonicus* on account of its effect in causing inhibition of electrically-evoked contraction of RLM preparations (Iwakoshi et al., 1995). Stichopin is a 17-residue peptide with two cysteine residues that form a disulphide bridge and it is derived from a 39-residue precursor protein that simply comprises an N-terminal signal peptide and the stichopin sequence (Elphick, 2012). Further investigation of the pharmacological actions of stichopin revealed that it also causes inhibition of acetylcholine-induced stiffening of *in vitro* preparations of the collagenous body wall of *A. japonicus* (Birenheide et al., 1998). This effect of stichopin provided a basis for investigation of the expression of this peptide in *A. japonicus* using immunohistochemistry. Consistent with the effects of stichopin on collagenous body wall tissue, stichopin was found to be present in collagenous tissue associated with a variety of different organ systems. More specifically, stichopin was found to be expressed in two types of cells: neuron-like cells with processes and non-neuronal oval shaped cells without processes. Informed by these findings, it is thought that stichopin acts as a neuropeptide derived from neurons and as a hormone derived from oval-shaped secretory cells to regulate the mechanical properties of mutable collagenous tissue in sea cucumbers (Tamori et al., 2007).

An amidated pentapeptide with the amino-acid sequence Asn-Gly-Ile-Trp-Tyr-NH_2 (NGIWYamide; Figure 5C) was purified from extracts of *A. japonicus* on account of its effect in causing contraction of RLM preparations (Iwakoshi et al., 1995). Subsequently, it was found that NGIWYamide also causes stiffening of *in vitro* preparations of the collagenous body wall of *A. japonicus* (Birenheide et al., 1998), contraction of tentacle preparations and inhibition of spontaneous contractile activity of intestine preparations (Inoue et al., 1999). Consistent with these pharmacological actions of NGIWYamide, immunohistochemical analysis of the expression of this peptide in *A. japonicus* revealed that it is present in neuronal cell bodies

and/or processes located in the radial nerve cords, circumoral nerve ring, tube feet, tentacles, intestine and body wall dermis (Inoue et al., 1999).

The sequence of the *A. japonicus* NGIWYamide precursor has been determined from analysis of transcriptomic sequence data, revealing that it comprises five copies of the NGIWYamide (Elphick, 2012). Similarly, a precursor comprising five copies of NGIWYamide has been identified in *Holothuria glaberrima*, whereas in *Holothuria scabra* the NGIWYamide precursor comprises four copies of NGIWYamide and one copy of the structurally similar peptide NGIWFamide (Suwansa-Ard et al., 2018) (Figure 5C). As discussed above, NGIWYamide belongs to a family of neuropeptides in invertebrate deuterostomes that are characterized by an Asn-Gly motif – "NG peptides" (Figure 5C) -, which include NGFFFamide in sea urchins (Elphick and Rowe, 2009), NGFFYamide in starfish (Semmens et al., 2013) , both NGFFFamide and NGFFYamide in brittle stars (Semmens et al., 2015; Zandawala et al., 2017), NGFYNamide and NGFWNamide in the hemichordate *Saccoglossus kowalevskii* (Elphick, 2010) and SFRNGVamide in the cephalochordate *Branchiostoma floridae* (Elphick, 2010). As also discussed above, a unifying feature of these peptides is that they are derived from precursor proteins that have C-terminal neurophysin domain, which reflects an evolutionary relationship with the neurophysin-containing precursors of vasopressin/oxytocin-type neuropeptides (Elphick, 2010). Interestingly, the NGIWYamide precursor in *A. japonicus* and other sea cucumber species has lost the neurophysin domain (Elphick, 2012; Suwansa-Ard et al., 2018), but the functional significance of this loss remains to be investigated.

Discovery of gonadotropic peptides in sea cucumbers

Sea cucumbers are used as foodstuffs in China, Japan and other Asian countries and consequently they have high economic value. With the depletion of natural populations, methods for aquaculture of economically important sea cucumber species have been

established (Chen, 2004). However, there are challenges associated with sea cucumber aquaculture, including methods for reliably obtaining gametes. A widely used method to induce spawning is thermal and/or mechanical shocking but there are concerns regarding the quantity and quality of viable gametes obtained using this approach (Lovatelli, 2004). Therefore, efforts to purify and identify endogenous regulators of gamete release in sea cucumbers have been initiated.

In 2009 Kato et al. reported the purification of a peptide named cubifrin that triggers oocyte maturation and ovulation of ovarian tissue *in vitro* (Kato et al., 2009). Determination of the structure of cubifrin revealed that it is identical to the neuropeptide NGIWYamide that had previously been identified in *A. japonicus* as a muscle contractant [see above and (Inoue et al., 1999; Iwakoshi et al., 1995)]. Interestingly, a synthetic analog of NGIWYamide, NGLWYamide, was found to be 10-100 times more potent than NGIWYamide. Furthermore, injection of NGIWYamide or NGLWYamide into sexually mature animals induced a characteristic spawning behaviour, where animals raise and shake their anterior (oral) region prior to releasing gametes from the genital pore. In addition to the identification of NGIWYamide as a gonadotropic peptide, Kato et al. also purified a second gonadotropic peptide from *A. japonicus* that was identified as the amidated heptapeptide QGLFSGVamide. However, QGLFSGVamide was found to be much less potent than NGIWYamide.

In parallel with the discovery of NGIWYamide and QGLFSGVamide as gonadotropic peptides in *A. japonicus* (Kato et al., 2009), another paper reporting the identification of a gonadotropic peptide in *A. japonicus* was published in 2009 (Katow et al., 2009). Radial nerve cord extracts were found to contain a peptide or peptides that induce germinal vesicle breakdown in oocytes. Efforts to purify the bioactive peptide led to partial sequencing of a 4.8 kDa polypeptide comprising the amino acid sequence AEIDDLAGNIDY. Furthermore, a partial cDNA sequence was determined that comprised an open reading frame encoding the AEIDDLAGNIDY sequence. Based on these sequence data a 43-residue peptide was

synthesized, tested for gonadotropic activity and found to cause germinal vesicle breakdown in 50% of immature ovarian oocytes at a concentration of 6 μM. Furthermore, a synthetic peptide corresponding to the N-terminal 21 residues of the 43-residue peptide was also found to exhibit gonadotropic activity. However, BLAST analysis of *A. japonicus* transcriptome sequence data using the amino acid sequence AEIDDLAGNIDY as a query reveals that it is part of a myosin heavy chain (M.R. Elphick, unpublished data). Therefore, the physiological significance of the gonadotropic activity of the peptide identified by Katow et al. is unclear.

Further studies are now required to investigate the occurrence and properties of other gonadotropic peptides in sea cucumbers, in addition to NGIWYamide and QGLFSGVamide. Recently, analysis of transcriptome sequence data from the sea cucumbers *Holothuria scabra* and *Holothuria glaberrima* revealed the presence of a transcript encoding a precursor of a relaxin-like peptide that is closely related to starfish RGP (Suwansa-Ard et al., 2018). Therefore, it will be of interest to investigate if the RGP-like molecule derived from this precursor acts as a gonadotropic peptide in sea cucumbers.

Transcriptomic identification of neuropeptide precursors in sea cucumbers

Generation of transcriptome sequence data from the sea cucumber *A. japonicus* (Du et al., 2012) enabled identification of transcripts encoding a number of neuropeptide precursors, including precursors of TRH-type, calcitonin-type, pedal peptide-type, luqin-type, glycoprotein hormone-type and bursicon-type peptides (Rowe et al., 2014) (Figure 5). More recently, precursors of other neuropeptides have been identified in *A. japonicus*, including CCK-type, corazonin-type, kisspeptin-type, orexin-type, pigment dispersing factor (PDF)-type, somatostatin-type and vasopressin/oxytocin-type neuropeptides (Suwansa-Ard et al., 2018; Zandawala et al., 2017). Furthermore, two transcripts encoding calcitonin-type precursors were identified in *A. japonicus* – one precursor comprising two calcitonin-type peptides (AjCT1 and AjCT2; Figure 5G) and second precursor comprising a single copy of AjCT2.

Comparison of the sequence of these two precursors indicates that they are products of the same gene, but with inclusion or exclusion of AjCT1 determined by alternative splicing of transcripts (Suwansa-Ard et al., 2018; Zandawala et al., 2017). Furthermore, opportunities to identify genes encoding neuropeptide precursors and neuropeptide receptors have emerged recently with the sequencing of the genome of *A. japonicus* (Zhang et al., 2017). With the availability of this important resource, it will now be possible to identify the complete complement of neuropeptide-related genes in a sea cucumber species for the first time. Furthermore, analysis of gene structure and gene synteny may provide new insights into relationships between neuropeptides in sea cucumbers and neuropeptides in other echinoderms and neuropeptides in other phyla.

Transcriptomic identification of neuropeptide precursors has also been extended to two other sea cucumber species – *Holothuria scabra* and *Holothuria glaberrima* (Suwansa-Ard et al., 2018). Furthermore, the expression of three neuropeptide precursors was investigated specifically in *H. scabra*. Thus, analysis of expression of transcripts encoding both the long isoform (including HsCT1 and HsCT2) and the short isoform (including only HsCT2) of calcitonin-type precursors revealed that both isoforms are expressed in the circumoral nerve ring, radial nerve cords, intestine and longitudinal body wall muscle. Similarly, a homolog of the *A. japonicus* precursor of the neuropeptide GN-19 (Elphick, 2012) was found to be expressed in the radial nerve cords, intestine and longitudinal body wall of *H. scabra*, whereas expression of a homolog of the *A. japonicus* precursor of the neuropeptide GLRFA (Elphick, 2012) was found to be restricted to the circumoral nerve ring and radial nerve cords (Suwansa-Ard et al., 2018). These findings provide a taster of what now needs to accomplished – a comprehensive analysis of the expression patterns of all the neuropeptide precursors in sea cucumbers, employing use of mRNA *in situ* hybridization and immunohistochemical methods that will reveal more specifically the cell populations that express neuropeptide precursors. This will provide an anatomical basis for investigation of

the physiological roles of neuropeptides using *in vitro* and *in vivo* pharmacological methods, as has already been accomplished successfully in starfish species as discussed above. And there are several aspects of sea cucumber physiology and behaviour where neuropeptides may have important regulatory roles. The importance of neuropeptides as regulators of gamete release in sea cucumbers has already been discussed above. Other processes that are likely to require complex neurochemical control mechanisms are the eversion of Cuvierian tubules and/or evisceration that occur as a defense against predation in some sea cucumber species (Byrne, 2001; Demeuldre et al., 2017). Both of these processes involve changes in the mechanical state of mutable collagenous tissue (MCT) in sea cucumbers and progress has been made recently in elucidating the mechanisms underpinning MCT mechanical adaptability (Mo et al., 2016). However, there is still much to be learnt about how changes in MCT stiffness are controlled by the nervous system. Therefore, the recent discovery of a plethora of novel neuropeptides in sea cucumbers, some of which appear to be unique to sea cucumbers or echinoderms (Elphick, 2012; Rowe et al., 2014; Suwansa-Ard et al., 2018), provides a rich resource for future studies in this fascinating area of echinoderm biology.

Neuropeptide signalling systems in the Crinoidea

SALMFamide-type neuropeptide signalling in feather stars

Evidence of the occurrence of SALMFamide-type neuropeptides in crinoids was first obtained using immunohistochemical methods. Antibodies to the starfish SALMFamide neuropeptide S2 revealed immunoreactivity in neuronal somata and their processes in the brachial nerve of the feather star *Antedon bifida* (Heinzeller and Welsch, 1994). More recently, sequencing of the transcriptome of the featherstar *Antedon mediterranea* enabled determination of the sequence of the first SALMFamide precursor to be identified in a crinoid (Elphick et al., 2015). The *Antedon* SALMFamide precursor comprises fourteen putative

neuropeptides, ranging in length from eight to twenty-four residues. Furthermore, the C-terminal regions of these peptides have a variety of motifs, including L-type (LxFamide) and F-type (FxFamide), as in other echinoderms, LxLamide, FxMamide and others (Figure 4). Because just one SALMFamide precursor has been identified in *A. mediterranea* that exhibits similarity with both L-type and F-type SALMFamide precursors in other echinoderms, it has been proposed that this precursor may represent an ancestral type that predates a gene duplication event that gave rise to the L-type SALMFamide precursors and F-type SALMFamide precursors in other echinoderm classes. However, there remains the possibility that a second SALMFamide precursor exists in *A. mediterranea* but has yet to be discovered due to sequence divergence or incomplete transcriptome coverage.

Transcriptomic identification of other neuropeptide precursors in crinoids

Sequencing of the transcriptome of the featherstar *A. mediterranea* has enabled identification of transcripts encoding precursors of a variety of neuropeptides, including calcitonin-type, CCK-type, luqin-type, orexin-type, melanin-concentrating hormone-type and vasopressin/oxytocin-type (M.R. Elphick et al., unpublished data). Further insights into the diversity of neuropeptide precursors in crinoids would be obtained if the genome of a crinoid species is sequenced. Informed by identification of neuropeptide precursors in crinoids, there are exciting opportunities ahead to investigate the expression and functions of neuropeptides in both larval and adult crinoids. One aspect of adult crinoids that makes them of interest from a comparative perspective is that the ectoneural and hyponeural regions of the nervous system are less prominent than in other echinoderms and it is the entoneural (apical or aboral) region of the nervous system that is predominant both anatomically and functionally (Mashanov et al., 2016). Furthermore, immunohistochemical studies on *A. mediterranea* have revealed the presence of L-glutamate in the brachial nerves of the entoneural nervous system and pharmacological studies have revealed that L-glutamate induces rhythmic contractions of

arm muscles and arm autotomy in this species (Wilkie et al., 2013; Wilkie et al., 2010). These studies are illustrative of experimental approaches that could be employed to investigate the physiological roles of neuropeptides in crinoids.

General conclusions and looking ahead

At the outset of this chapter I highlighted two perspectives that make echinoderms of particular interest for research on neuropeptide signalling. Firstly, a phylogenetic perspective, with echinoderms providing "missing pieces" in the "jigsaw puzzle" of neuropeptide evolution by virtue of their status as non-chordate deuterostomes that occupy an "intermediate" evolutionary position with respect to the well-studied vertebrates and selected protostomes (e.g. *C. elegans*, *D. melanogaster*). Secondly, a functional perspective, with the pentaradial symmetry and other unusual properties (e.g. mutable collagenous tissue) of adult echinoderms providing unique contexts for gaining insights into the physiological roles of neuropeptides and the evoluton of neuropeptide function in the animal kingdom. Furthermore, it will be apparent from reading this chapter that there is also a third perspective on neuropeptide signalling in echinoderms – a developmental perspective.

Here in the final concluding section of this chapter, I will summarise progress in investigating the biology of neuropeptides in echinoderms from these three perspectives. Furthermore, I will highlight some of the opportunities that lie ahead for investigation of neuropeptide signalling in echinoderms.

Reconstructing neuropeptide evolution: insights from echinoderms

A review article summarising progress in this aspect of echinoderm neuropeptide biology was published recently (Semmens and Elphick, 2017) and I refer readers to this article for a more detailed overview of this topic. A unifying theme is that insights into the evolutionary origins of neuropeptide signalling systems have been obtained with the

discovery of echinoderm orthologs of neuropeptide-related genes/proteins that hitherto had only been identified in vertebrates/chordates or in protostomes (Elphick et al., 2018; Semmens and Elphick, 2017). Definitive proof of orthology has been obtained by experimental demonstration that neuropeptides act as ligands for their candidate cognate receptors. The discovery that NGFFFamide is the ligand for the *S. purpuratus* NPS/CCAP-type receptor is an important example of this because it provided the key "missing link" between the NPS signalling system in vertebrates and the CCAP signalling system in protostomes (Semmens et al., 2015). Similarly, the disovery of a corazonin-type signalling system in *A. rubens* illustrates how research on an echinoderm species (Tian et al., 2016) has had a broad impact in changing our perspective on the evolution of a neuropeptide signalling system (Zandawala et al., 2018). Are there other instances where identification of ligand-receptor partnerships in echinoderms could provide important insights into neuropeptide evolution? Here I will highlight some examples.

The first putative precursor of TRH-like peptides to be discovered in an invertebrate was identified in the sea urchin *S. purpuratus* (Rowe and Elphick, 2012) but the receptor(s) for these peptides have yet to be characterised experimentally. However, peptides that act as ligands for TRH-type receptors have been identified recently in two protostomes – the annelid *Platynereis dumerilii* and the nematode *C. elegans* (Bauknecht and Jékely, 2015; Van Sinay et al., 2017). Interestingly, alignment of these peptides, and related peptides from other protostomes, has revealed structural similarities with the TRH-like peptides in sea urchins and other echinoderms, providing further evidence of orthology. Furthermore, investigation of the roles of TRH-type signalling in *C. elegans* has revealed an evolutionarily ancient role in regulation of growth (Van Sinay et al., 2017). In this context it will be interesting to gain insights into the physiological roles of TRH-type signalling in sea urchins and/or other echinoderms because it will provide a "missing link" between protostomes and vertebrates.

The MCH-type and kisspeptin-type precursor proteins that were identified in the starfish *A. rubens* and in other echinoderms were the first to be identified outside the phylum Chordata (Semmens et al., 2016; Smith et al., 2017; Suwansa-Ard et al., 2018; Zandawala et al., 2017) and therefore functional characterisation of the neuropeptides derived from these precursors and the receptors they act on would provide important insights into the evolution of MCH-type and kisspeptin-type signalling. Conversely, there are a number of neuropeptide signalling systems that were discovered in protostomes but which are not present in the vertebrate/chordate lineage. These include corazonin-type and pedal peptide/orcokinin-type neuropeptides and functional characterisation of these neuropeptides in starfish has provided new insights into the evolution of neuropeptide function in the animal kingdom (Lin et al., 2017a; Lin et al., 2018; Tian et al., 2017). However, the receptors that mediate the effects of pedal peptide/orcokinin-type peptides have yet to be discovered in any animal. Therefore, this represents an area of research where echinoderms could expand significantly our knowledge of the mechanisms of neuropeptide signalling. Other bilaterian neuropeptide signalling systems that have been lost in chordates but retained in echinoderms are the luqin-type and PDF-type signalling systems. Therefore, investigations of the physiological roles of the luqin and PDF signalling systems in echinoderms are interesting research objectives for the future. It is noteworthy that PDF signalling has an important role in mediating circadian control of behaviour in insects (Mezan et al., 2016; Renn et al., 1999), so it will be of particular interest to discover if this role extends to echinoderms.

Discovering neuropeptide function in adult echinoderms

The discovery of a gonadotropic peptide in starfish heralded the beginnings of research on neuropeptide function in adult echinoderms (Chaet and McConnaughy, 1959; Chaet, 1964). Furthermore, the identification of this neuropeptide as a relaxin-like peptide provided evidence that relaxin-type peptides are evolutionarily ancient regulators of

reproductive processes (Mita et al., 2009). It remains to be determined, however, if other relaxin-type peptides that have been identified in starfish and in other echinoderms also act as gonadotropins. The discovery that NGIWYamide (Iwakoshi et al., 1995), an ortholog of the vertebrate neuropeptide known as neuropeptide-S (Semmens et al., 2015), acts as a gonadotropin in the sea cucumber *A. japonicus* (Kato et al., 2009) may be evidence of divergence in the mechanisms of neuroendocrine regulation of spawning in different echinoderm classes. In this regard, it should be noted that the common ancestor of asterozoans (starfish and brittle stars) and echinozoans (sea cucumbers and sea urchins) is estimated to have lived ~480 mya (Pisani et al., 2012) and over this geological timescale there has been huge scope for divergence of physiological mechanisms. In this context it will be interesting to obtain further insights into the mechanisms of neuropeptide-mediated control of gamete maturation and spawning in echinoderm species belonging to each of the five extant classes. Likewise, as highlighted elsewhere in this chapter, comparative analysis of neuropeptide function in species belonging to each of the echinoderm classes will also be of interest for investigation of other aspects of echinoderm physiology. For example, is the action of pedal peptide/orcokinin-type neuropeptides as muscle relaxants in starfish (Kim et al., 2016; Lin et al., 2017a; Lin et al., 2018) applicable to other echinoderms? However, each of the five extant echinoderm classes may provide differing opportunities for investigation of neuropeptide function, as discussed below.

A unique aspect of echinoderm biology is the presence of mutable collagenous tissue (MCT) and progress in elucidating the mechanisms of MCT has largely been made through studies on sea cucumbers, which have a voluminous and easily accessible layer of MCT in their body wall (Wilkie, 2005). Thus, the molecular identification of peptides and proteins that regulate or mediate mechanisms of MCT has been accomplished in sea cucumbers (Birenheide et al., 1998; Takehana et al., 2014; Tipper et al., 2002). Building upon these important advances and recent insights into the mechanisms of MCT in the sea cucumber

body wall (Mo et al., 2016), there are opportunities ahead to utilise the power of transcriptomics and proteomics to obtain more comprehensive molecular insights into how changes in the mechanical state of the sea cucumber body are regulated by the nervous system through the release of neuropeptides and other neurotransmitters/neuromodulators. In other echinoderm classes MCT may be less abundant and accessible than in the body wall of sea cucumbers, but species from other echinoderm classes have nevertheless been valuable model systems for research on MCT. For example, the compass depressor ligament in sea urchins (Ribeiro et al., 2011), the interossicular ligaments of crinoids (Motokawa et al., 2004) and the inner dermis of some starfish species (Motokawa, 2011). Using these experimental preparations, it will be interesting to determine if the mechanisms of neuropeptidergic control of MCT are conserved between the echinoderm classes.

Another aspect of the biology of echinoderms that has attracted interest is their capacity to autotomise arms or eviscerate internal organs as a defense against predation and then regenerate the lost arms or organs (Wilkie, 2001). There is evidence that neuropeptides may trigger arm autotomy in starfish (Mladenov et al., 1989), but the identity of these molecules remains to be determined and therefore this represents a fascinating objective for the future. Furthermore, immunohistochemical evidence that SALMFamide-type neuropeptides may be involved in regulation of arm regeneration in starfish has also been reported (Moss et al., 1998); now there is scope for studies of this kind to be extended to the many other neuropeptides that have been identified in starfish (Semmens et al., 2016). Arguably the most impressive capacity for regeneration in echinoderms is seen in brittle star species such as *Amphiura filiformis* (Figure 2), which frequently lose arms due to predation and then very rapidly regrow new arms (Dupont and Thorndyke, 2006). For this reason *A. filiformis* is an emerging model system in regenerative biology and insights into the molecular mechanisms of regeneration in this species have been obtained recently (Czarkwiani et al., 2013; Czarkwiani et al., 2016). Furthermore, as in starfish, the recent discovery of multiple

neuropeptide precursors in *A. filiformis* (Zandawala et al., 2017) has provided an opportunity to investigate the roles of neuropeptide signalling systems in regenerative processes in this species.

With a more specific focus on starfish, an intriguing aspect of their biology is the mechanism of feeding, which in many species involves eversion of the stomach out of the mouth and over prey (Anderson, 1954). For example, the common European starfish *A. rubens* feeds on mussels and other bivalves and therefore in order that the everted stomach can gain access to soft and digestible tissues, a gap between the valves of their prey has to be created by employing the pulling power of the tube feet on the underside of each arm. By way of contrast, the crown-of-thorns starfish *A. planci* feeds on coral so here the stomach simply has to be everted from mouth so that external digestion of soft polyp tissue can commence. Investigation of the physiological roles of the first neuropeptides to be discovered in starfish, the SALMFamides S1 and S2, revealed that intracoelomic injection of these peptides induces stomach eversion in *A. rubens* but with S2 being more effective than S1 (Melarange et al., 1999). Transcriptomic identification of neuropeptide precursors in *A. rubens* (Semmens et al., 2013; Semmens et al., 2016) has enabled investigation of other neuropeptides as candidate regulators of stomach eversion or retraction. Thus, the NG peptide NGFFYamide was identified as a potent stimulator of stomach contraction (*in vitro*) and retraction (*in vivo*) (Semmens et al., 2013). However, it has been found that neuropeptides that trigger stomach relaxation or contraction *in vitro* do not necessarily trigger stomach eversion or retraction *in vivo*. For example, GnRH-type and corazonin-type neuropeptides both trigger contraction of *in vitro* preparations of the stomach from *A. rubens* but, unlike NGFFYamide, neither peptide was observed to cause stomach retraction (Tian et al., 2017). Conversely, neuropeptides derived from two pedal peptide/orcokinin-type precurors in *A. rubens* (ArPPLNP1, ArPPLNP2) were found to cause relaxation of stomach preparations *in vitro* but did not trigger stomach eversion when injected *in vivo* (Lin et al., 2017a; Lin et al., 2018). It is clear that

neuropeptidergic control of stomach activity in starfish is highly complex and there is much to be learnt about the roles of the many neuropeptide signalling systems that are present in the starfish stomach and in other regions of the digestive system. Furthermore, gaining deeper insights into the mechanisms of neuropeptidergic regulation of feeding in starfish may have applications as part of the effort to identify ways of controlling species such as *A. planci*, which is contributing to the loss of coral on the Great Barrier Reef and on other reefs in the Indo-Pacific region (Hall et al., 2017; Leray et al., 2012).

Finally, the discovery of multiple neuropeptide signalling systems in echinoderms provides new opportunities to gain insights into the functional anatomy of echinoderm nervous systems in the unique context of a pentaradially symmetrical body plan. Use of molecular markers has provided valuable insights into the neuroarchitecture of echinoderm nervous systems (Mashanov et al., 2016; Zueva et al., 2018) and with the development of a growing toolkit of neuropeptide-specific antibodies there are exciting opportunities ahead to gain new neuroanatomical insights from the perspective of neuropeptide signalling. An example of how immunohistochemical analysis on neuropeptide expression has already provided such insights is the localisation of pedal peptide/orcokinin-type neuropeptides in the lateral motor nerves of starfish (Lin et al., 2017a). The pioneering studies of J. Eric Smith using classical histological staining methods provided remarkably detailed accounts and illustrations of the neuroanatomy of the starfish nervous system and one feature of the nervous system that Smith described for the first time was the lateral motor nerves (Smith, 1937; Smith, 1946; Smith, 1950). The detection of neuropeptides derived from the ArPPLN1 precursor in the lateral motor nerves has provided the first evidence that these nerves contain the axons of peptidergic neurons. Furthermore, it also enabled immunohistochemical visualisation of axonal processes in branches of the lateral motor nerves that innervate interosscicular muscles or project into the circular muscle layer of the coelomic lining of the arms (Lin et al., 2017a). Analysis of neuropeptide expression in *A. rubens* has also revealed

that some neuropeptides (e.g. ArPPLN1, ArPPLN2, ArGnRH) are expressed in cell bodies of hyponeural motoneurons in the radial nerve cords and circumoral nerve ring, whereas other neuropeptides (e.g. ArCRZ) are not (Lin et al., 2017a; Lin et al., 2018; Tian et al., 2017). Looking ahead it may be possible to analyse the neuropeptide expression profile of hyponeural neurons to identify sub-populations of neurons that innervate different regions of the starfish body, which would transform our knowledge of the neuroanatomy of motor systems in starfish. Likewise, the same approach could be employed for other echinoderms, with the intriguing potential prospect of identifying sub-populations of homologous motoneurons in different echinoderm classes.

Developmental analysis of neuropeptide function: echinoderms as model systems

The sequencing of the genome of the sea urchin *S. purpuratus* was founded upon a long history of using this species as a model system in developmental biology (Sodergren et al., 2006). However, very little is known about neuropeptide expression and function during the embryonic and larval development of this species and other echinoderms. Prior to the sequencing of the *S. purpuratus* genome, use of antibodies to SALMFamides enabled immunocytochemical visualisation of neuropeptides in the larvae of echinoids (Beer et al., 2001; Thorndyke et al., 1992). Now with the identification of many neuropeptide precursors in *S. purpuratus* (Rowe and Elphick, 2012; Zandawala et al., 2017) there are opportunities ahead to investigate neuropeptide expression and function in sea urchin larvae more comprehensively. The recent analysis of the expression of eight neuropeptide precursors in larvae of the starfish *A. rubens* using mRNA *in situ* hybridisation methods has provided an indication of the insights that can be obtained (Mayorova et al., 2016). Now these studies need to be extended to a wider range of neuropeptides and to other echinoderms, employing use of both mRNA *in situ* hybridisation methods and immunocytochemistry for anatomical studies. Furthermore, pharmacological testing of the effects of neuropeptides combined with gene-

knockdown (using morpholino antisense oligonucleotides) and gene-knockout (CRISPR/Cas9) techniques (Mellott et al., 2017; Oulhen et al., 2017) could be used to reveal the functions of neuropeptides in larvae. Thus, in echinoderms there exists a unique opportunity amongst extant bilaterian phyla to discover the physiological roles of neuropeptides in both the pre-metamorphic bilaterally symmetrical nervous systems of the larval stage and the post-metamorphic pentaradially symmetrical nervous systems of juvenile and adult animals.

And so we have come full circle. Alfred Chaet's rationale sixty years ago for using radial nerve cord extracts to induce spawning in starfish was to establish a method for obtaining eggs and sperm for studies in reproductive and developmental biology (Chaet and McConnaughy, 1959; Chaet, 1964; Chaet, 1966a; Chaet, 1966b). Now in the "omics" era we have the resources to not only trigger spawning in starfish using RGP or its downstream effector 1-MeAde (Mita, 2013; Mita, 2016) but also to examine the expression and functions of multiple neuropeptide signalling systems during the embryonic and larval development of starfish and other echinoderms. We have come a long way since that first report of the "physiologic activity of nerve extracts" but there is so much more to be discovered.

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Figure legends

Figure 1. Animal phylogeny. Diagram showing the phylogenetic position of the phylum Echinodermata with respect to other selected animal phyla and sub-phyla. The Metazoa comprise bilaterian phyla and non-bilaterian phyla. The bilaterians comprise two super-phyla: the deuterostomes, which include chordates and echinoderms, and the protostomes, which include ecdysozoans (e.g. the arthropod *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*) and lophotrochozoans (e.g. the mollusc *Aplysia californica*). The non-bilaterians include phyla that lack nervous systems (Porifera, Placozoa) and phyla that have nervous systems (Ctenophora, Cnidaria). Note that the branch lengths in the tree are arbitrary. This figure is an adapted version of Figure 1 from (Elphick et al., 2018).

Figure 2. Phylogenetic relationships of extant echinoderm classes. The phylum Echinodermata comprises five extant classes: Asteroidea (starfish), Ophiuroidea (brittle stars), Echinoidea (sea urchins), Holothuroidea (sea cucumbers) and Crinoidea (featherstars and sea lilies). The phylogenetic relationships of the five classes are shown on the left, based on the findings of (O'Hara et al., 2014; Telford et al., 2014). Photographs of species belonging to each class are illustrated and include *Asterias rubens* (taken by Ray Crundwell), *Amphiura filiformis* (taken by Paola Oliveri), *Strongylocentrotus purpuratus* (taken by Maurice Elphick), *Apostichopus japonicus* (taken by Ding Kui) and *Antedon mediterranea* (taken by Dario Fassini).

Figure 3. Relaxin-like gonadotropic peptide (RGP) in the starfish *Asterias rubens*. (A) Sequence of the *A. rubens* RGP (ArRGP) precursor protein. The N-terminal signal peptide is shown in blue, dibasic cleavage sites are shown in green and the A chain and B chain peptides

are shown in pink and orange, respectively. The A chain and B chain dimerise to form mature RGP, which has two interchain disulphide bridges and a single intrachain disulphide bridge in the A chain. **(B)** Ovary dissected from a female specimen of *A. rubens*; the inset shows the effect of synthetic ArRGP in triggering release of eggs from an ovary fragment *in vitro*. **(C)** Photograph of a living specimen of *A. rubens* showing the arm tip region viewed under a microscope. The most prominent feature is the pigmented optic cushion, which is located at the base of the terminal tentacle. The terminal tentacle and optic cushion are bounded on each side by spines and rows of tube feet can be seen adjacent to the optic cushion. **(D)** Localisation of ArRGP precursor expression in a transverse section of the arm tip region of *A. rubens*, using mRNA *in situ* hybridisation methods with antisense probes. Stained cells expressing ArRGP precursor transcripts (arrow heads) can be seen in the body wall epithelium lining a cavity that surrounds the terminal tentacle and the pigmented optic cushion. **(E)** Localisation of ArRGP precursor expression in a transverse section of the distal region of the arm tip beyond the terminal tentacle in *A. rubens*, using mRNA *in situ* hybridisation methods with antisense probes. Stained cells (arrow heads and rectangle) can be seen in the body wall epithelium at the base of two adjacent spines; the region highlighted with a rectangle is shown in panel F. The inset shows absence of staining (arrow head) in a section of the arm tip adjacent to the section shown in the main panel, which was incubated with sense probes instead of the anti-sense probes, demonstrating the specificity of staining observed with anti-sense probes. **(F)** Detail of the region highlighted with a rectangle in panel E, showing stained cells with processes (arrowheads) at high magnification. Abbreviations: Ep, epithelium of body wall; OC, optic cushion; TF, tube foot; Sp, spine; TT, terminal tentacle. Scale bars: C, 400 μm; D, 100 μm; E, 50 μm; E inset, 100 μm; F, 10 μm. This figure was adapted from figures shown in (Lin et al., 2017b).

Figure 4. The occurrence and properties of SALMFamide precursors and SALMFamide peptides in species representing each of the five extant echinoderm classes. (A) SALMFamide precursors are shown in a phylogenetic diagram in accordance with phylogeny shown in Figure 2, with crinoids basal to the Echinozoa (Holothuroidea and Echinoidea) and the Asterozoa (Asteriodea + Ophiuroidea). The estimated divergence times for the nodes (labelled with numbers in pentagons) according to (O'Hara et al., 2014) are: 1. 501-542 Ma, 2. 482-421 Ma, 3. at least 479 Ma, 4. 464-485 Ma. *A. rub* is the starfish *Asterias rubens* (Asteroidea), *O. vic* is the brittle star *Ophionotus victoriae* (Ophiuroidea), *S. pur* is the sea urchin *Strongylocentrotus purpuratus* (Echinoidea), *A. jap* is the sea cucumber *Apostichopus japonicus* (Holothuroidea), and *A. med* is the feather star *Antedon mediterranea* (Crinoidea). Signal peptides are shown in blue and dibasic or monobasic cleavage sites are shown in green. L-type SALMFamides with a C-terminal LxFamide motif or with an L-type-like motif (e.g. IxFamide) are shown in red. F-type SALMFamides with a FxFamide motif or with an F-type-like motif (e.g. YxFamide) are shown in yellow. SALMFamides with a FxLamide-type motif are shown in orange and SALMFamides with LxLamide-type motif are shown in dark red. Peptides that do not conform with any of the four colour-coded categories are shown in white (e.g. GVPPYVVKVTYamide in *A. japonicus* and SRLPFHSGLMQamide in *O. victoriae*). The diagram shows how in a presumed ancestral-type precursor in crinoids the majority of the putative peptides have a FxLamide-type motif or a LxLamide-type motif and there is only one L-type SALMFamide and one F-type SALMFamide. However, as a consequence of specialisation following a presumed duplication of the ancestral-type gene in a common ancestor of the Echinozoa and Asterozoa, two types of SALMFamide precursor have evolved: one that is predominantly comprised of L-type SALMFamides (red) and another that is exclusively or predominantly comprised of F-type SALMFamides (yellow). **(B)** C-terminal alignments of SALMFamide neuropeptides derived from the precursor proteins shown in A. The C-terminal regions of each peptide are colour-coded according to the key shown in A. This

figure is an adapted version of Figure 4 from (Elphick et al., 2015), with sequence data from *Asterias rubens* (Semmens et al., 2016) replacing sequence data from *Patiria miniata*.

Figure 5. Sequences of echinoderm representatives of selected neuropeptide families

Neuropeptides identified in species from four echinoderm classes are shown: Class Asteroidea (A.rub, *Asterias rubens*), Class Ophiuroidea (O.vic, *Ophionotus victoriae*), Class Echinoidea (S.pur, *Strongylocentrotus purpuratus*) and Class Holothuroidea (H.sca, *Holothuria scabra*; A.jap, *Apostichopus japonicus*). Amino acid residues that are conserved in the majority of neuropeptides are shown in red; post-translational modifications include amidation (a) and conversion of glutamine to pyroglutamate (pQ). Numbers in parentheses indicate that multiple peptides are derived from the same precursor protein. Numbers without parentheses indicate that related peptides are derived from different precursor proteins. The sequences shown are taken from the following publications: (Semmens et al., 2016; Suwansa-Ard et al., 2018; Zandawala et al., 2017). Note that for the TRH-type peptides only the sequences of the most abundant peptide in each precursor are shown. Note also that the numbering of the *Holothuria scabra* orexins is based on sequence similarity with the two orexin types identified in other echinoderms and is different from the numbering in (Suwansa-Ard et al., 2018).

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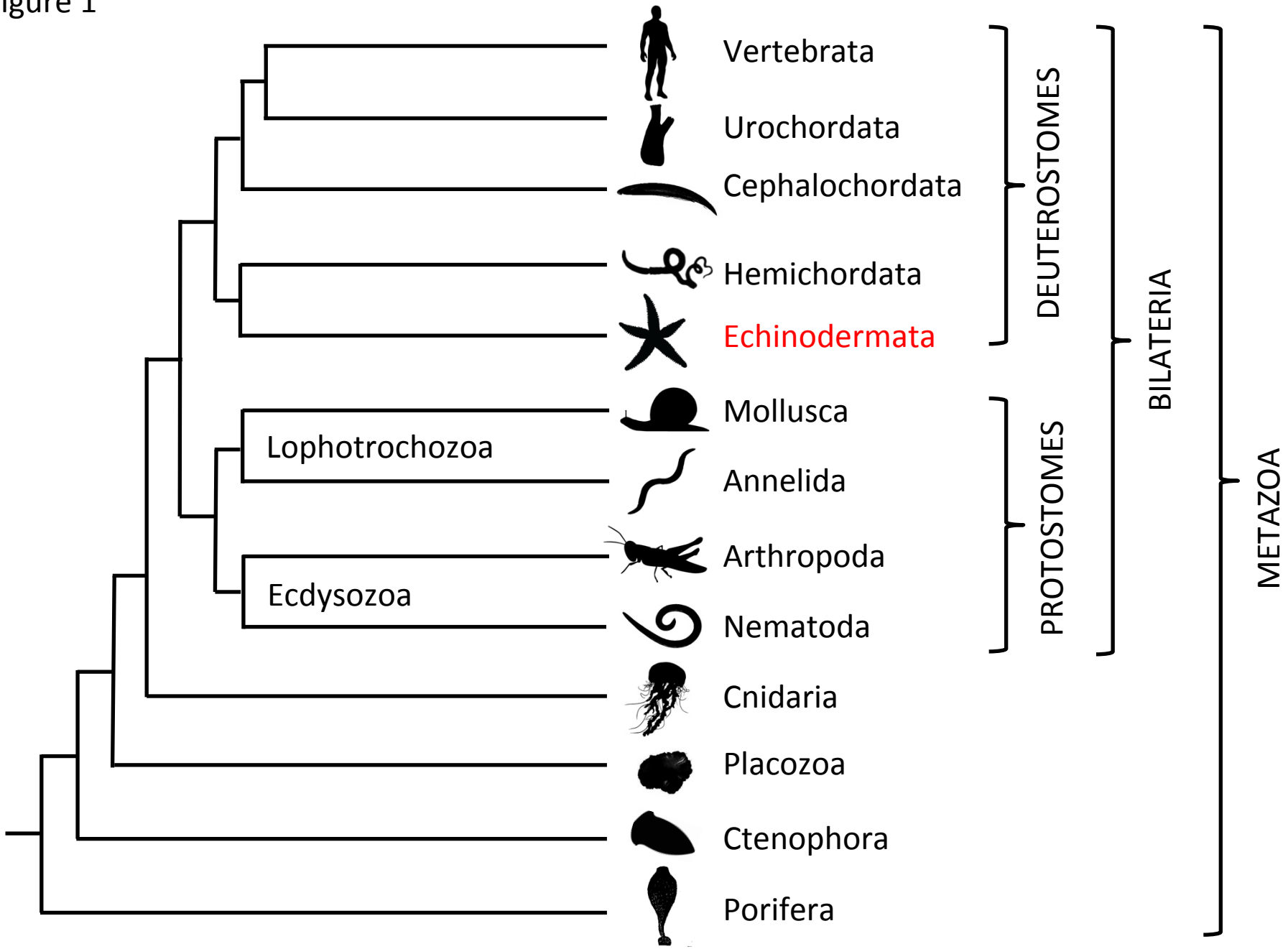
Figure 1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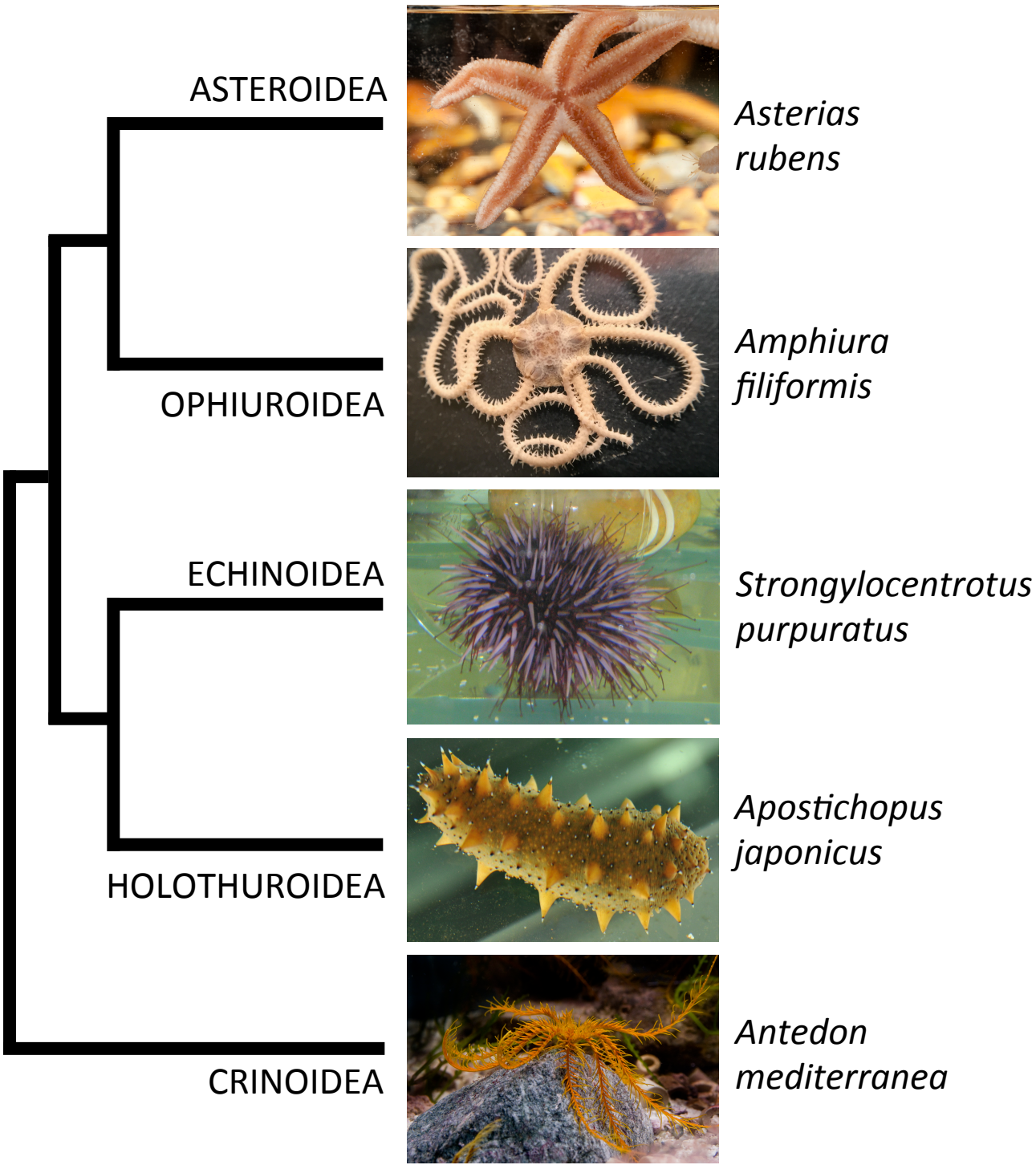
DEUTEROSTOMES
PROTOSTOMES
BILATERIA
METAZOA

Figure 2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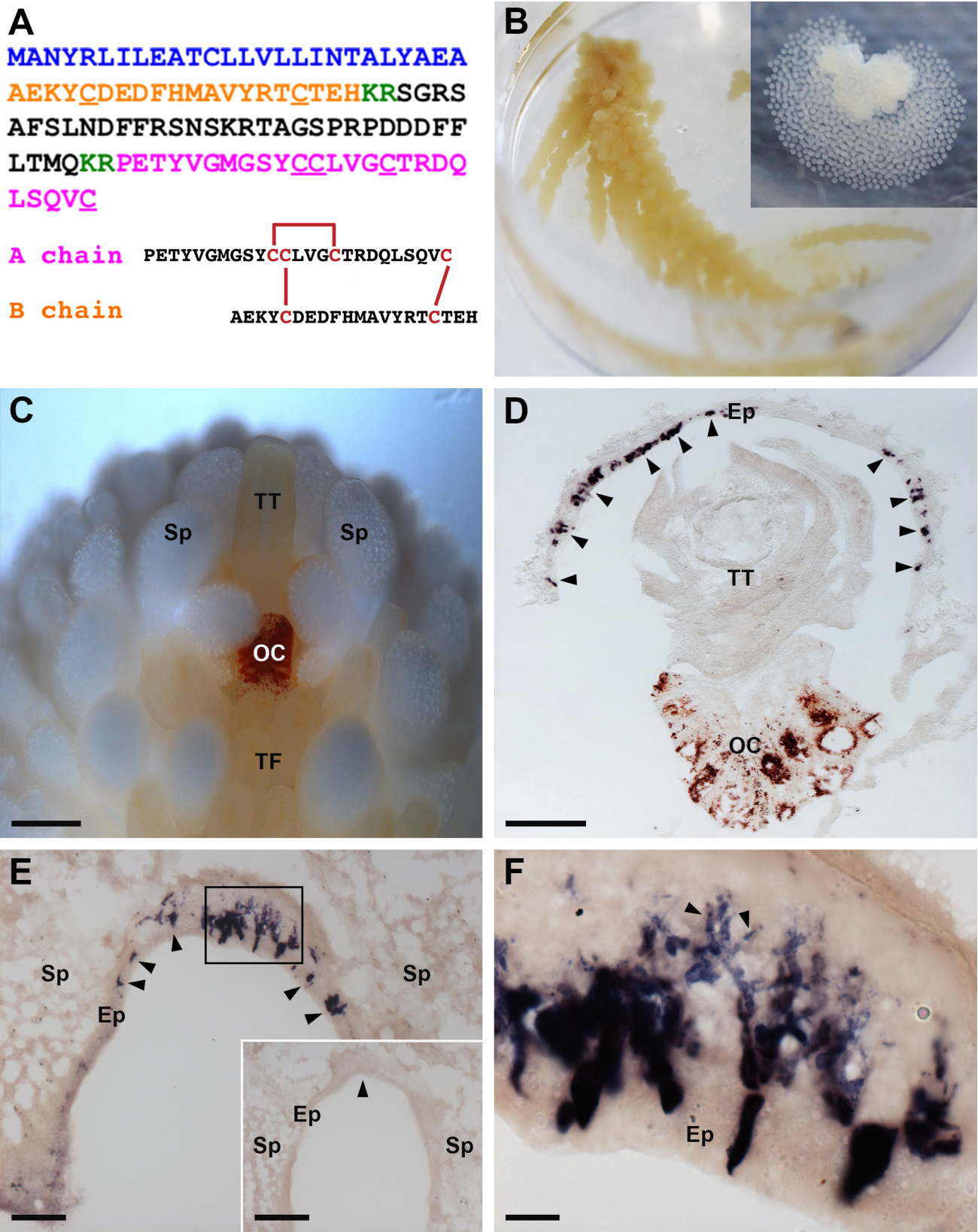
Apostichopus japonicus
Antedon mediterranea


A
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B
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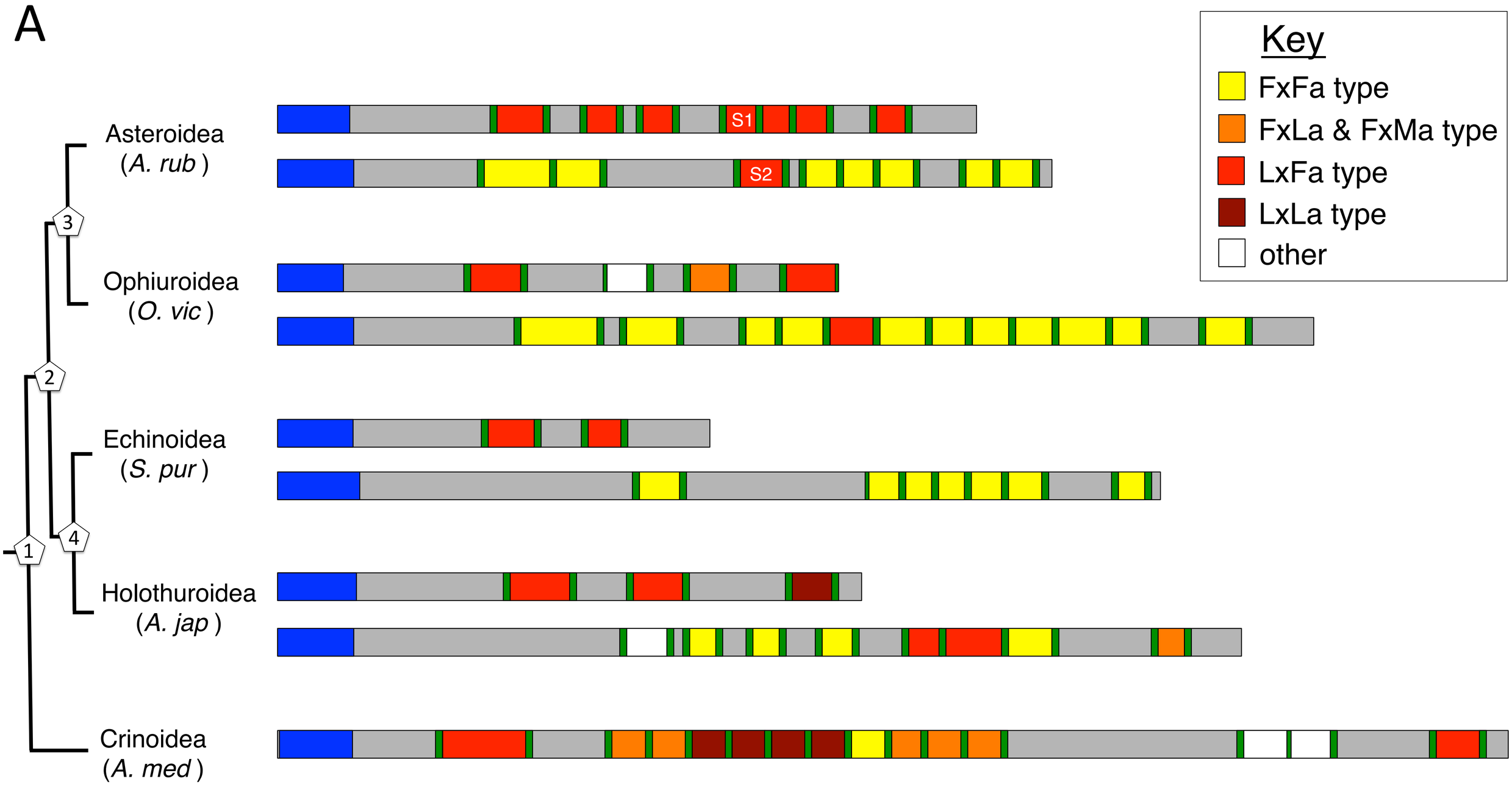

B

Putative L-type precursor-derived peptides

A. rubens

PAGASAFHSALSYa
AYHSALPFa
AYHTGLPFa
GFNSALMFa
LHSALPFa
GYHSALPFa
GYHSGLPFa

O. victoriae

SGRRNPSLNSGLIFa
SRLPFHSGLMQa
SRPQFHTGFMMa
KAGQRLRFSDGMLFa

S. purpuratus

NMGSIHSHSGIHFa
MRLHPGLLFa

A. japonicus

VVSRAWSPLVGQTGIAFa
TRSRSMFGNTALPFa
MGFTGNTGILLa

Putative F-type precursor-derived peptides

A. rubens

QAVRPGGGAPMNVPVKMSGFSFa
SAGATPSKLAGFAFa
GAMDAFAFa
PSGDPMSAFSFa
RNPMNSLSALAFa
AGMDPNSLNAFNFa
RDPLSAFSFa
GMDSLSAFNFa
GRDHLSAFSFa
GRNPMNGLSAFDFa
GGMDAFAFa
GYENGLSGYAFa

O. victoriae

QQSDREREVEAAQTQFYPYa
TDPRKASGGFTFa
SGPYSFNSGLTFa
NIFGSYDFa
GMGVSSFSFa
AFGDFSFa
NNGLSSFTFa

S. purpuratus

PPVTTRSKFTFa
DAYSAFSFa
GMSAFSFa
AQPSFAFa
GLMPSFAFa
PHGGSAFVFa
GDLAFAFa

A. japonicus

GVPPYVVKVTYa
FKSPFMFa
GYSPFMFa
ARYSPFTFa
GGYSALYFa
VPELAESDGGQSKLYFa
GHRGGQFSQFKFa
FKSSFYLa

A. mediterranea putative SALMFamide precursor-derived peptides

ANTSSEPINNWIRALPVLHRGLYFa
NPALSEFMLa
DPSFSSYMLa
NPRLSDLMLa
DPRLSDLMLa
DPRLSDLMLa
DPRLSDLMLa
DPGFSDFTFa
DALGDFMMa
EARLSDYIMa
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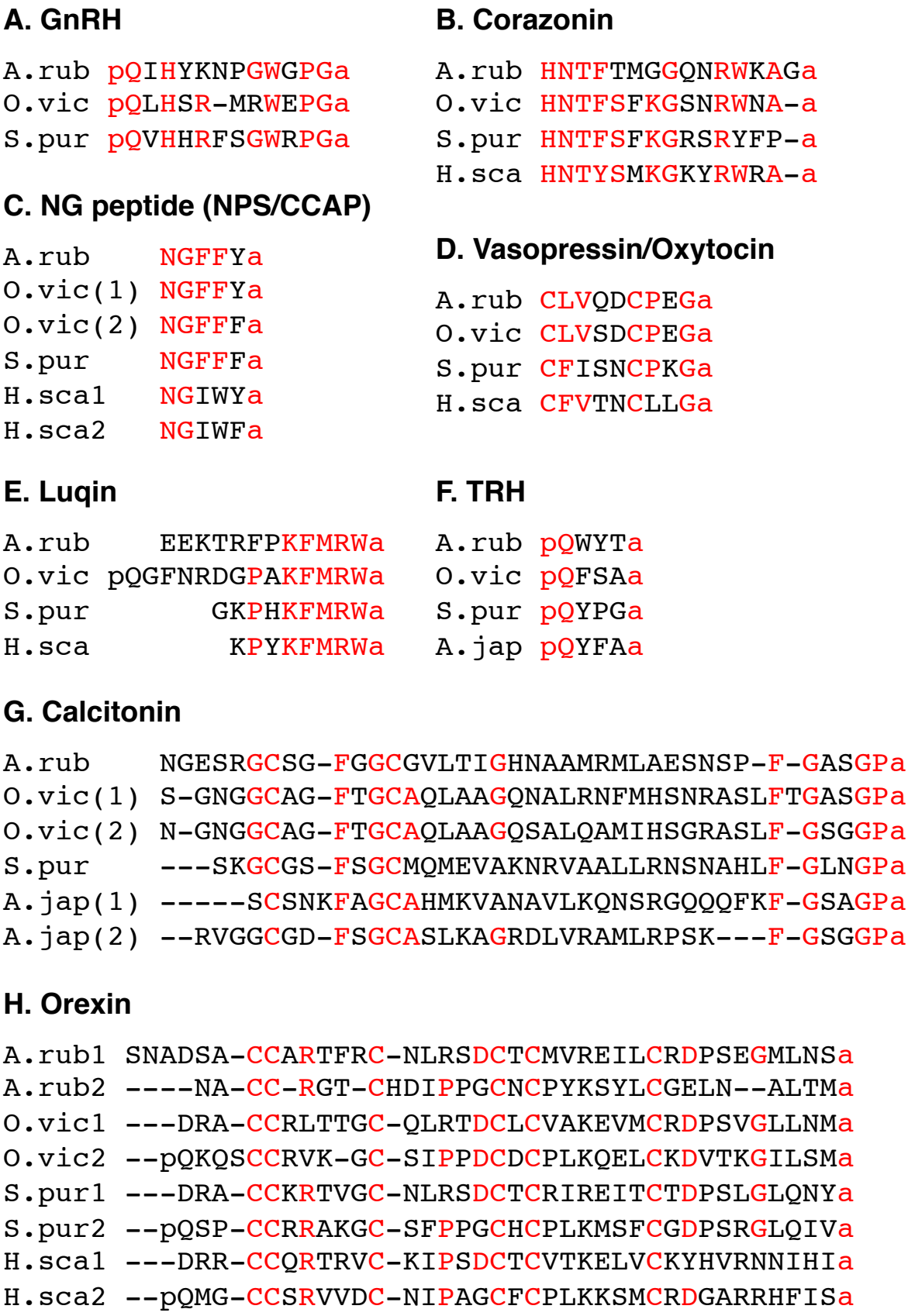
KAKFQRPVYPGNa
TPSQIWDTFGAa
FPPAALHKGLYFa

Figure 5


A. GnRH
A.rub pQIHYKNPGWGPGa
O.vic pQLHSR-MRWEPGa
S.pur pQVHHRFSGWRPGa
B. Corazonin
A.rub HNTFTMGGQNRWKAGa
O.vic HNTFSFKGSNRWNA-a
S.pur HNTFSFKGRSRYFP-a
H.sca HNTYSMKGKYRWRA-a
C. NG peptide (NPS/CCAP)
A.rub NGFFYa
O.vic(1) NGFFYa
O.vic(2) NGFFFa
S.pur NGFFFa
H.sca1 NGIWYa
H.sca2 NGIWFa
D. Vasopressin/Oxytocin
A.rub CLVQDCPEGa
O.vic CLVSDCPEGa
S.pur CFISNCPKGa
H.sca CFVTNCLLGa
E. Luqin
A.rub EEKTRFPKFMRWa
O.vic pQGFNRDGPAKFMRWa
S.pur GKPHKFMRWa
H.sca KPYKFMRWa
F. TRH
A.rub pQWYTa
O.vic pQFSAa
S.pur pQYPGa
A.jap pQYFAa
G. Calcitonin
A.rub NGESRGCSG-FGGCGVLTIGHNAAMRMLAESNSP-F-GASGPa
O.vic(1) S-GNGGCAG-FTGCAQLAAGQNALRNFMHSNRASLFTGASGPa
O.vic(2) N-GNGGCAG-FTGCAQLAAGQSALQAMIHSGRASLF-GSGGPa
S.pur ---SKGCGS-FSGCMQMEVAKNRVAALLRNSNAHLF-GLNGPa
A.jap(1) -----SCSNKFAGCAHMKVANAVLKQNSRGQQQFKF-GSAGPa
A.jap(2) --RVGGCGD-FSGCASLKAGRDLVRAMLRPSK---F-GSGGPa
H. Orexin
A.rub1 SNADSA-CCARTFRC-NLRSDCTCMVREILCRDPSEGMLNSa
A.rub2 ----NA-CC-RGT-CHDIPPGCNCPYKSYLCGELN--ALTMa
O.vic1 ---DRA-CCRLTTGC-QLRTDCLCVAKEVMCRDPSVGLLNMa
O.vic2 --pQKQSCCRVK-GC-SIPPDCDCPLKQELCKDVTKGILSMa
S.pur1 ---DRA-CCKRTVGC-NLRSDCTCRIREITCTDPSLGLQNYa
S.pur2 --pQSP-CCRRAKGC-SFPPGCHCPLKMSFCGDPSRGLQIVa
H.sca1 ---DRR-CCQRTRVC-KIPSDCTCVTKELVCKYHVRNNIHIa
H.sca2 --pQMG-CCSRVVDC-NIPAGCFCPLKKSMCRDGARRHFISa