REVIEW

SALMFamide *salmagundi*: the biology of a neuropeptide family in echinoderms

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Abstract

The SALMFamides are a family of neuropeptides that occur in species belonging to the phylum Echinodermata. The prototypes for this neuropeptide family (S1 and S2) were discovered in starfish but subsequently SALMFamides were identified in other echinoderms. There are two types of SALMFamides: L-type, which have the C-terminal motif SxLxFamide, and F-type, which have the C-terminal motif SxFxFamide. They are derived from two types of precursor proteins: an L-type SALMFamide precursor, which comprises only L-type or L-type-like SALMFamides and an F-type SALMFamide precursor, which contains several F-type or F-type-like SALMFamides and, typically, one or more L-type SALMFamides. Thus, SALMFamides occur as heterogeneous mixtures of neuropeptides - a SALMFamide *salmagundi*. SALMFamides are produced by distinct populations of neurons in echinoderm larval and adult nervous systems and are present in the innervation of neuromuscular organs. Both L-type and F-type SALMFamides cause muscle relaxation in echinoderms and, for example, in starfish this effect of SALMFamides may mediate neural control of cardiac stomach eversion in species that feed extra-orally (e.g. *Asterias rubens*). The SALMFamide S1 also causes inhibition of neural release of a relaxin-like gonadotropin in the starfish *Asterina pectinifera*. An important issue that remains to be resolved are the relationships of SALMFamides with neuropeptides that have been identified in other phyla. However, it has been noted that the C-terminal SxLxFamide motif of L-type SALMFamides is a feature of some members of a bilaterian neuropeptide family that includes gonadotropin-inhibitory hormone (GnIH) in vertebrates and SIFamide-type neuropeptides in protostomes. Similarly, the C-terminal FxFamide motif of F-type SALMFamides is a feature of vertebrate QRFP (26RFa)-type neuropeptides. These sequence similarities may provide a basis for molecular identification of receptors that mediate effects of SALMFamides. Furthermore, analysis of the actions of the heterogeneous mixtures of SALMFamides that occur in echinoderms may provide new insights into the physiological significance of the general phenomenon of precursor proteins that give rise to neuropeptide “cocktails”.

Key words: neuropeptide; echinoderm; SALMFamide; starfish; sea urchin; sea cucumber; brittle star; FMRFamide

1. Introduction

Twenty-five years ago a paper reporting "FMRFamide-like immunoreactivity in the nervous system of the starfish *Asterias rubens*" was published in *Biological Bulletin* [27]. When the paper was submitted for peer review, the feedback from reviewers was supportive but the tone leaned towards "yet another paper reporting FMRFamide-like immunoreactivity in an invertebrate!" This was not unreasonable because by 1989, twelve years after FMRFamide was identified as a cardioexcitatory neuropeptide in molluscs [61], there was already a long list of species and phyla in which the presence of FMRFamide-like immunoreactivity had been reported [60]. In fact, a paper reporting the *absence* of FMRFamide-like immunoreativity in starfish would have been more surprising! What made the paper of interest was that it was the first to reveal the anatomical distribution of any neuropeptide(s) in the nervous system of an echinoderm. Furthermore, it laid the foundations for discovery of the first neuropeptides to be identified in echinoderms, SALMFamide neuropeptides, which are the focus of this review article.

The review is divided into five main sections corresponding to the five classes of extant echinoderms. The Asteroidea (starfish) lead the review because it was in species belonging to this class (*Asterias rubens* and *Asterias forbesi*) that SALMFamide neuropeptides (S1 and S2) were first identified [30]. The Holothuroidea follow because soon after the discovery of S1 and S2, two SALMFamide neuropeptides were identified in the sea cucumber *Holothuria glaberrima* [20], providing the first evidence that SALMFamides may occur throughout the phylum Echinodermata. Then come the Echinoidea, which through analysis of genome/transcriptome data from the sea urchin *Strongylocentrotus purpuratus* provided the first insights into the diversity of SALMFamides that occur in an echinoderm species [32, 64]. Lastly the Ophiuroidea and Crinoidea, the two echinoderm classes for which least is currently known but which have the potential to provide fascinating insights into the evolution and physiological roles of SALMFamide neuropeptides.

Before proceeding, perhaps an explanation for the title of this review is necessary. The word *salmagundi* is thought to originate from the French word *salmigondis*, which translates as "an assortment" or "a collection containing a variety of things". In English the word salmagundi has become associated with a 17th century salad dish comprising a rich variety of ingredients including meats, seafood, nuts, fruit, vegetables etc. However, like its French counterpart, salmagundi also has the more general meaning of a "heterogeneous mixture". As described in more detail below, genome sequence data and/or transcriptome sequence data have revealed that there are indeed heterogeneous mixtures of SALMFamide neuropeptides in echinoderms. Thus, there are both L-type SALMFamides and F-type SALMFamides; L-type SALMFamides are derived from L-type SALMFamide precursors and F-type SALMFamides are derived from F-type SALMFamide precursors but in some cases F-type SALMFamide precursors also give rise to L-type SALMFamides. Furthermore, there are SALMFamides that are not strictly L-type but are L-type-like and there are SALMFamides that are not strictly F-type but are F-type-like [26]. This is the SALMFamide *salmagundi*; a lexiconic marriage just waiting to be happen!

2. Asteroidea

2.1 FMRFamide-like immunoreactivity in the nervous system of the starfish Asterias rubens

In order that patterns of neuropeptide expression in starfish and other echinoderms can be described, it is necessary to first briefly outline the architecture of the nervous systems in these animals. The organisation of the nervous system in adult starfish reflects its pentaradial body plan; there are five radial nerve cords that extend along the midline of each arm linked by a circumoral nerve ring in the central disk. The radial nerve cords control the activity of rows of tube feet that enable locomotor activity. The radial nerve cords and the circumoral nerve ring comprise two parts, the ectoneural and the hyponeural, which are separated by a basement membrane. The ectoneural division comprises sensory, inter- and motor neurons, and is continuous with an extensive basiepithelial nerve plexus underlying the body wall surface. The hyponeural division is considered

to be purely motor. In visceral organs such as the cardiac stomach and the associated digestive glands (pyloric caecae), bipolar neuronal somata are located in the mucosal epithelium and have processes that form a basiepithelial nerve plexus. Neurons are also located within the coelomic epithelium of the gut and their processes innervate an underlying muscle layer, which is separated from the basiepithelial plexus by a basement membrane [11, 12, 35, 38, 58].

Immunocytochemical studies using antibodies to the molluscan neuropeptide FMRFamide revealed immunoreactivity in the radial nerve cords and circumoral nerve ring of the starfish *Asterias rubens* [27]. The immunostaining was localised in cell bodies and axonal fibres in both the ectoneural and hyponeural parts of the nerve cords and nerve ring. Furthermore, immunoreactive fibres were also evident in the basiepithelial nerve plexus of the tube feet, indicating a potential role for the immunoreactive peptides in control of tube foot activity. These findings were of interest because they provided the first insight into the neuroanatomical organisation of peptidergic signalling systems in the nervous system of an echinoderm. Furthermore, although by the time this study was published FMRFamide-like immunoreactive peptides had been identified in vertebrates and a variety of protostomian invertebrates, FMRFamide-like peptides had not been identified in any deuterostomian invertebrate species. A pattern was beginning to emerge, with peptides sharing the motif FxRFamide (where x is variable) with FMRFamide only being found in protostomian invertebrates. Accordingly, it was proposed that there is a family of orthologous FMRFamide-related peptides (FaRPs) in protostomians, with other FMRFamide-like peptides that have a C-terminal RFamide motif being more widely distributed phylogenetically (e.g. in cnidarians and vertebrates) [60]. It was against this backdrop that it was of particular interest from an evolutionary perspective to determine the molecular identity of the peptides responsible for the FMRFamide-like immunoreactivity detected in the starfish *A. rubens*.

2.2 Discovery of the starfish SALMFamide neuropeptides S1 and S2

The detection of FMRFamide-like immunoreactivity (ir) in the nervous system of *A. rubens*, as discussed above, provided a basis for efforts to purify and identify the peptide(s) responsible for this immunoreactivity. Initially a radioimmunoassay (RIA) employing antibodies to FMRFamide was used to screen extracts of nerves from *A. rubens* and *A. forbesi* that had been fractionated using high-performance liquid chromatography (HPLC). However, subsequently it was found that an antiserum (Q2) to a leucine-containing FMRFamide-like peptide (pQDPFLRFamide) detected more immunoreactivity in starfish nerve extracts and therefore Q2 was used to monitor purification of immunoreactive peaks [30]. Four peaks (B-E) were purified to homogeneity and sequenced. Peak E was identified as the amidated octapeptide GFNSALMFamide, peak C was identified as the oxidised form of the peak E peptide and peak B was identified as a C-terminal fragment (SALMFamide) of the peak E peptide. Peak D was identified as the amidated dodecapeptide SGPYSFNSGLTFamide, which shares sequence similarity (underlined) with the peak E peptide (GFNSALMFamide). Interestingly, the presence of the LxFamide motif in both peptides provided an explanation for why antibodies to pQDPFLRFamide detected more immunoreactivity in starfish nerve extracts than antibodies to FMRFamide. However, the two starfish peptides differ from FMRFamide-like peptides identified in invertebrates and vertebrates because they do not have an arginine residue in the penultimate position from the C-terminal amide. Thus, the starfish peptides are not strictly "RFamide-type" neuropeptides and therefore they were designated as founding members of a new family of neuropeptides - the SALMFamides. The octapeptide GFNSALMFamide was designated as SALMFamide-1 (or S1) and the dodecapeptide SGPYSFNSGLTFamide was designated as SALMFamide-2 (or S2) [30, 31]. S1 and S2 were the first neuropeptides to be identified in a species belonging to the phylum Echinodermata and therefore it was of interest to investigate the physiological roles of these neuropeptides in starfish. To facilitate investigation of the physiological roles of S1 and S2, antibodies to these two peptides were generated and characterised using RIA methods [29].

2.3 Localisation of SALMFamide neuropeptides in starfish larvae

The development of antibodies to S1 and S2 enabled the first investigations of the expression of native neuropeptides in echinoderm nervous systems. In the life of an echinoderm there are two nervous systems – first the larval nervous system and then the post-metamorphic nervous system of juvenile and adult animals. Accordingly, taking a chronological approach, the larval nervous system will be discussed in this section and then the adult nervous system will be discussed in section 2.4 below.

The first developmental studies of SALMFamide expression in starfish analysed S1-ir in the planktotrophic larvae of three species - *Asterias rubens*, *Pisaster ochraceus* and *Patiriella regularis* [7, 52]. The most comprehensive analysis of larval S1-ir has been reported for *P. regularis* [7] and therefore this is described below. S1-ir is first observed in early bipinnarian larvae, expressed by neurons in a bilaterally symmetrical pair of dorsolateral ganglia located anterior to the mouth. As development proceeds the number of cells in each ganglion increases and in 6-day old bipinnaria a meshwork of S1-immunoreactive neuronal processes derived from the ganglia can be seen innervating the anterior dorsal region. These S1-immunoreactive processes also innervate the adoral and pre-oral ciliated bands, where they intermingle with fibres derived from S1-immunoreactive cells in the epithelium of the ciliated bands. An S1-immunoreactive nerve tract connects the pre-oral ciliated band with a network of S1-immunoreactive fibres associated with post-oral ciliated band. By 3 weeks the network of S1-immunoreactive cells and processes becomes more prominent, particularly those associated with the pre-oral and post-oral ciliated bands, and bilaterally symmetrical S1-immunoreactive fibre tracts that project into the posterior region of the larva are evident. By the brachiolaria stage at 8 weeks a larval attachment complex has formed anteriorly and a dense meshwork of associated S1-immunoreactive fibres derived from the ganglia is apparent. Fibres from the adoral nerve plexus can be seen innervating the oesophagus and S1-immunoreactive cells and processes are also present in the stomach.

186 The patterns of immunoreactivity observed with S1 antibodies in the nervous system of
187 planktotrophic starfish larvae suggest that SALMFamides may modulate ciliary activity associated
188 with swimming and feeding. S1-immunoreactive fibres in the oesophagus and stomach may be
189 involved in regulation of visceral muscle activity and the dense S1-immunoreactive innervation of
190 the brachium in brachiolaria suggests a potential role in larval settlement. However, as yet, no
191 experimental studies that investigate the effects of SALMFamides on starfish larval behaviour have
192 been reported. Interestingly, in species belonging to the genus *Patiriella* that have non-feeding
193 (lecithotrophic) larvae (e.g. *P. calcar* and *P. exigua*), there is no bipinnaria stage and neural systems
194 associated with feeding are not present. However, S1-immunoreactive fibres innervating the
195 brachium are present in the brachiolaria larvae of these species. Thus, there appear to be distinct
196 developmental programs for SALMFamidergic systems associated with control of feeding
197 (bipinnaria) and settlement (brachiolaria) in starfish larvae [8].
198
199 *2.4 The distribution of SALMFamide neuropeptides in adult starfish*
200 Antibodies to S1 and S2 have been used to both measure (using RIA) and map (using
201 immunocytochemistry) the distribution of these peptides in adult specimens of *A. rubens* [29]. RIA
202 analysis of tissue extracts revealed, not surprisingly, that the highest concentrations of the two
203 peptides were present in the radial nerve cords (S1; 265 pmol/g; S2 417 pmol/g). However, S1-ir
204 and S2-ir was also detected at lower concentrations in a wide range of other organs/tissues, which
205 included the cardiac stomach (S1; 31 pmol/g; S2 121 pmol/g), pyloric stomach (S1; 24 pmol/g; S2
206 55 pmol/g), pyloric caecae (S1; 11 pmol/g; S2 66 pmol/g), body wall (S1; 14 pmol/g; S2 49
207 pmol/g) and ovaries (S1; 2 pmol/g; S2 20 pmol/g). In addition S2-ir (1.4 pmol/g), but not S1-ir, was
208 detected in the perivisceral coelomic fluid, suggesting a potential hormonal role for S2 in starfish.
209 The widespread detection of S1-ir and S2-ir in starfish provided a basis for detailed
210 immunocytochemical investigations of the distribution of S1- and S2-expressing cells throughout
211 the starfish body.

Abundant S1-ir was revealed in the radial nerve cords and circumoral nerve ring of *A. rubens*, localised in neuronal cell bodies and in nerve fibres in both the ectoneural and hyponeural parts of these nerve tracts [51]. The pattern of immunstaining observed was very similar to that originally reported using FMRFamide antibodies [27], with FMRFamide-immunoreactive somata and S1-immunoreactive somata located in very similar positions in the radial nerve cords. Likewise, similar to findings with FMRFamide antibodies, a dense network of S1-immunoreactive fibres was revealed in the basiepithelial nerve plexus of the tube feet. However, it is possible that some of the immunostaining detected with FMRFamide antibodies is not attributable to S1. Consistent with the RIA data, S1-immunoreactive neuronal somata were also detected in mucosal epithelia throughout the digestive system (oesophagus, cardiac stomach, pyloric stomach, pyloric caecae) with an associated network of S1-immunoreactive fibres in the basiepithelial nerve plexus. In the pyloric caecae S1-ir was also revealed in nerve fibres underlying the coelomic epithelium. In the body wall, S1-ir was revealed in the subepithelial plexus as well as in the nerve plexi underlying the coelomic epithelum, associated with circularly and longitudinally oriented muscle layers.

Analysis of the distribution of S2-ir in adult specimens of *A. rubens* revealed a pattern of expression broadly similar to that of S1. For example, in the cardiac stomach S2-immunoreactive cells are present in the mucosa and a dense meshwork of immunostained fibres are present in basiepithelial nerve plexus, as illustrated in Fig. 1A. However, double-labelling studies showed that S1 and S2 appear to be expressed in different populations of neurons. Furthermore, unlike with S1, no S2-ir neuronal somata were observed in the hyponeural part of the radial nerve cords [54, 55].

Subsequent to the original reports of SALMFamide expression in *A. rubens*, other starfish species have been analysed using antibodies to S1 and/or S2. For example, the distribution of S1-ir in the radial nerve cords and tube feet of adult specimens of *P. regularis* was found to be very similar to that seen in *A. rubens* [7]. Likewise, analysis of both S1-ir and S2-ir in the radial nerve cords and cardiac stomach of *Marthasterias glacialis* revealed patterns of expression similar to *A. rubens* [71]. Interestingly, S1-ir has also been observed in the innervation of the gonads in the

starfish *Asterina pectinifera*, consistent with RIA-based detection of S1 in extracts of gonads from *A. rubens* [49]. However, when specimens of *A. rubens* were analysed immunocytochemically, no S1-ir was observed in the gonads [51]; this may be due to seasonal variation in SALMFamide expression in gonadal tissues.

2.5 Pharmacological effects of SALMFamides in adult starfish

The detection of S1-ir and S2-ir in the innervation of a variety of neuromuscular organs (digestive system, tube feet, apical muscle) in *A. rubens* (see section 2.4 above) provided a neuroanatomical basis for investigation of the pharmacological effects of SALMFamides on myoactivity. Initial studies revealed that S2, but not S1, causes relaxation of cardiac stomach preparations *in vitro*; no effects of S1 and S2 on tube foot and apical muscle preparations were observed [29]. However, with optimisation of the recording conditions for these experiments, it was subsequently found that both S1 and S2 cause relaxation of cardiac stomach (see Fig. 1B,C,D), tube foot and apical muscle preparations [46, 47]. When tested at the same concentration the magnitude of the relaxing effect of S2 on the three preparations was found to be significantly larger than the relaxing effect of S1 [46, 47]. Furthermore, dose-response data obtained for cardiac stomach and tube foot preparations have revealed that S2 is an order of magnitude more potent than S1 (Fig. 1C) [28, 57].

Feeding in *A. rubens* and many other starfish species is accomplished by eversion of the cardiac stomach over the digestible parts of prey (e.g. mussels, for *A. rubens*). Discovery of the relaxing effect of S1 and S2 on cardiac stomach preparations *in vitro* provided a rationale for investigating if this effect of S1 and S2 causes cardiac stomach eversion *in vivo*. Injection of 100 µl of 1 mM S2 was found to cause cardiac stomach eversion within 5 minutes in 57% of tests, whereas injection of 100 µl of 1 mM S1 caused cardiac stomach eversion within 5 minutes in only 11% of tests [47]. Thus, the effectiveness of S1 and S2 in triggering cardiac stomach eversion *in vivo* correlates with the potency of these peptides *in vitro*. Furthermore, the discovery that

SALMFamides trigger cardiac stomach eversion in *A. rubens* provided the first insight on neurochemical mechanisms underlying the unusual extraoral feeding behaviour of starfish. Recently, a neuropeptide that triggers cardiac stomach contraction *in vitro* and cardiac stomach retraction *in vivo* has been identified as the pentapeptide NGFFYamide [65]. Thus, counteracting neuropeptide systems appear to be involved in controlling the process of cardiac stomach eversion and retraction in starfish.

The pharmacological actions of SALMFamides have also been investigated as potential regulators of hormone release in the starfish *Asterina pectinifera*. Gamete release in starfish is triggered by gonad-stimulating substance (GSS), a neurohormone that is present in starfish radial nerve cords and that is related to the mammalian hormone relaxin [50]. Release of GSS from radial nerve cords can be triggered *in vitro* by KCl-induced depolarisation and S1 causes dose-dependent inhibition of KCl-induced GSS release [49]. Thus, S1 may act as a neurotransmitter in the radial nerve cords of starfish that inhibits release of GSS. This is interesting because it suggests a potentially important role for SALMFamides as regulators of reproductive physiology in starfish. Furthermore, the detection of S1-ir in the gonads of *A. pectinifera* suggests that SALMFamides may regulate reproductive processes peripherally as well as centrally.

2.6 Investigation of a structural basis for the differing potency of S1 and S2 in starfish

Recently, a structural basis for the difference in the potency of S1 and S2 as muscle relaxants in the starfish *A. rubens* has been investigated [57]. The most striking difference between S1 and S2 is that S1 is an octapeptide (GFNSALMFamide) whereas S2 is a dodecapeptide (SGPYSFNSGLTFamide), with four additional N-terminal residues (SGPY). It was hypothesised, therefore, that the presence of these four residues may account for S2's greater potency compared to S1. Synthesis of an N-terminally truncated analog of S2 (short S2 or SS2; SFNSGLTFamide) enabled experimental testing of this hypothesis. However, the results obtained were complex. SS2 caused dose-dependent relaxation of cardiac stomach preparations and comparison of the relaxing

290 actions of S1, SS2 and S2 when tested at 1 μM revealed that SS2 was significantly more effective
291 than S1 but only slightly less effective than S2. These findings indicated that the biological activity
292 of S2 is largely attributable to its C-terminal octapeptide sequence (SFNSGLTFamide). When SS2
293 was tested on tube foot preparations SS2 caused dose-dependent relaxation, but surprisingly the
294 effects of SS2 at 10 μM were consistently larger than the effects of S2 at the same concentration.
295 Conversely, when the effects of S1, S2 and SS2 were compared at 1 μM, SS2 was significantly
296 more effective than S1 but significantly less effective than S2. Thus, results from tests at 10 μM
297 indicate that the presence of the N-terminal SGPY sequence impairs the bioactivity of S2, while
298 results from tests at 1 μM indicate that the presence of the N-terminal SGPY sequence contributes
299 to the bioactivity of S2. Further studies are required to gain understanding of these complex
300 structure-activity relationships, which would be facilitated by identification of the receptors that
301 mediate the effects of SALMFamides in starfish.
302 In parallel with *in vitro* pharmacological studies that compared the bioactivity of S1, S2 and
303 SS2, spectroscopic methods have been employed to compare the solution conformations of these
304 peptides [57]. Use of circular dichroism spectroscopy showed that S1 does not have a defined
305 structure in aqueous solution and this was supported by 2D nuclear magnetic resonance
306 experiments; these findings are consistent with previous studies on other small neuropeptides. In
307 contrast, S2 was found to have a well-defined conformation in aqueous solution. However, this was
308 concentration dependent, with increasing concentration inducing a transition from an unstructured
309 to a structured conformation. This property of S2 was not, however, observed with the N-terminally
310 truncated analog of S2, SS2 (SFNSGLTFamide). Collectively, the data obtained indicate that the N-
311 terminal region of S2 facilitates self-association of this neuropeptide at high concentrations. The
312 functional significance of this property of S2 is not known, but it may have relevance to the
313 biosynthesis and/or bioactivity of S2 *in vivo*. Further investigation of the structure-activity
314 relationships of starfish SALMFamides is now needed following the recent discovery that S1 and

S2 are derived from precursor proteins that contain many other members of this neuropeptide family, as discussed below in section 2.7.

2.7 More than S1 and S2: SALMFamide precursor proteins reveal the diversity of SALMFamides in starfish

When the prototype SALMFamides S1 and S2 were originally isolated from extracts of *A. rubens* and *A. forbesi* using antibodies the FMRFamide-like peptide pQDPFLRFamide, some additional minor peaks of immunoreactivity were detected but these were not identified. With the development of antibodies to S1 and S2 it became possible to screen starfish nerve extracts for putative additional S1-like and/or S2-like neuropeptides. However, when HPLC-fractionated nerve extracts from *A. rubens* were assayed using S1-antibodies only a single peak of immunoreactivity was detected and this was identified as S1 [31].

The development of antibodies to S1 and S2 also enabled purification and identification of SALMFamides from other starfish species. Thus, analysis of nerve extracts from the starfish *Pycnopodia helianthoides* revealed that S1 is also present in this species [31], indicating that this peptide may be conserved amongst starfish species. However, as with the analysis of nerve extracts from *A. rubens*, no additional peaks of S1-like-ir were detected in *P. helianthoides*. Interestingly, however, when HPLC-fractionated nerve extracts from the starfish species *Marthasterias glacialis* were assayed several peaks of S1-like-ir and/or S2-like-ir were detected [72]. Five of these were successfully purified to homogeneity and sequenced. One of the peaks (peak B3) was identified as S1, providing further evidence that the S1 peptide may be conserved amongst starfish species. An S2-like immunoreactive peak (A2) was identified as SGPYSMTSGLTFamide, a dodecapeptide that is similar to the *Asterias* S2 peptide. Thus, this revealed for the first time the occurrence of S2-like peptides in other starfish species but, unlike the conserved S1 peptide, the sequences of S2-type peptides were found to vary between starfish genera. Furthermore, two S1-like immunoreactive peaks (A1 and B1) detected in nerve extracts from *M. glacialis* were identified as the amidated

341 octapeptide AYQTGLPFamide and the S1-like immunoreactive peak B2 was identified as
342 AYHSALPFamide. Thus, it became apparent for the first time that the molecular diversity of
343 SALMFamide neuropeptides in starfish is more complex than just a pair of peptides (S1 and S2).
344 Sequencing of the genome of the starfish *Patiria miniata* has recently provided the first
345 insights into the structure of starfish SALMFamide precursor proteins and the diversity of
346 SALMFamide neuropeptides that occur in starfish species [26]. Genes encoding two SALMFamide
347 precursor proteins were identified. One precursor comprises S1 and six other putative neuropeptides,
348 five of which are like S1 in having the C-terminal motif SxLxFamide or TxLxFamide. The other
349 precursor comprises a putative S2-like peptide (SNGPYSMSGLRSLTFamide) and eight other
350 putative peptides, six of which that have the C-terminal motif SxFxFamide (Fig. 2, 3). Discovery of
351 these precursor sequences provided several important insights on SALMFamides in starfish. Firstly,
352 S1 and the S2-like peptide are derived from different precursor proteins, a finding that is consistent
353 with earlier observations from immunocytochemical studies, which revealed that S1-ir and S2-ir are
354 localised in different populations of neurons in the nervous system of *A. rubens* [54, 55]. Secondly,
355 the occurrence of two types of SALMFamides is evident – L-type SALMFamides, which typically
356 have the C-terminal motif SxLxFamide, and F-type SALMFamides, which typically have the C-
357 terminal motif SxFxFamide. Thirdly, one of the precursor proteins is an L-type SALMFamide
358 precursor, giving rise to S1 and other L-type peptides, some of which are structurally similar to L-
359 type SALMFamides that were identified previously in nerve extracts from *M. glacialis*. Fourthly,
360 the second precursor protein is largely comprised of F-type SALMFamides and therefore it is
361 predominantly an F-type SALMFamide precursor; however, this protein is also the precursor of an
362 S2-like peptide, which is an L-type SALMFamide. Fifthly, some of the putative peptides deviate
363 from the canonical L-type SALMFamide motif, SxLxFamide, or the F-type SALMFamide motif,
364 SxFxFamide. For example, in two of the putative peptides derived from the S1 precursor the serine
365 residue is replaced by a structurally similar amino acid, threonine. Furthermore, in one of the
366 peptides derived from the S1 precursor, the C-terminal phenylalanine is replaced by the structurally

similar amino acid tyrosine. Furthermore, there are "F-type-like" peptides derived from the F-type SALMFamide precursor that deviate from the canonical F-type SALMFamide motif (SxFxFamide) and these include two peptides with the C-terminal pentapeptide sequences PFYPYamide and RSYAFamide.

Taking a broader perspective, what is perhaps most striking from these data is the large number of putative SALMFamide neuropeptides that appear to be present in *P. miniata*; in total there are 16 putative SALMFamides neuropeptides [26]. But is this representative of other starfish? It would appear that it is – we have recently determined the sequences of the SALMFamide precursors from *A. rubens* and have found that S1 is derived from a precursor protein that contains six other L-type or L-type-like SALMFamides and S2 is derived from a precursor protein that contains seven F-type or F-type-like SALMFamides (D. Semmens, M. Pancholi, M. Elphick, unpublished data). Thus, in *A. rubens*, in addition to the prototypes S1 and S2, there are thirteen other putative SALMFamide neuropeptides. As will be discussed in more detail below in section 7, this SALMFamide *salmagundi* invites functional explanations and future work will need to address this issue in starfish. For example, nothing is known about the actions of F-type SALMFamides in starfish; do they act as muscle relaxants like their L-type counterparts? We do, however, have some insights into the actions of L-type SALMFamides in starfish, additional to the well-characterised actions of S1 and S2. When the L-type SALMFamide AYHSALPFamide (also known as MagS3) was identified in *M. glacialis* its effects on cardiac stomach preparations from *A. rubens* were examined. Like S1 and S2, MagS3 caused dose-dependent relaxation of cardiac stomach preparations but with lower efficacy than S1 or S2 when tested at 1 μM [72]. Further studies are now needed in which the effects of all of the SALMFamides present in a starfish species are examined and compared both individually and as mixtures that reflect the natural composition of neuropeptide "cocktails" that are derived from a common precursor protein.

3. Holothuroidea

393 *3.1 FMRFamide-like immunoreactivity in sea cucumbers*

394 The presence of FMRFamide-like ir in sea cucumbers was first reported in an
395 immunocytochemical study of *Holothuria glaberrima* [34]. Immunostained neuronal somata and
396 fibres were observed in the radial nerve cords, oesophagus and both the large and small intestine.
397 Interestingly, many of the FMRFamide-like immunoreactive fibres in the digestive tract were also
398 immunoreactive with antibodies to cholecystokinin (CCK), which shares C-terminal sequence
399 similarity (MDFamide) with FMRFamide. Therefore, this may have reflected cross-reactivity of the
400 immunoreactive peptides with both FMRFamide antibodies and CCK antibodies.

401 More recently, FMRFamide antibodies have been used for an immunocytochemical analysis
402 of the sea cucumber *Holothuria scabra* [1]. As in *H. glaberrima*, immunoreactivity was observed in
403 the radial nerve cords and in nerve plexi of the submucosal and serosal layers of the digestive tract.
404 However, FMRFamide-like-ir was also detected in other organs including the testes, the respiratory
405 trees and the stone canal. Furthermore, efforts were made to characterize the immunoreactive
406 peptides using HPLC and dot-blotting methods but the molecular identity of the FMRFamide-like
407 immunoreactive peptides in *Holothuria scabra* was not determined.

408 Another recent study used FMRFamide antibodies for analysis of the nervous system of the
409 sea cucumber species *Leptosynapta clarki*, which is semi-transparent and therefore amenable for
410 whole-mount immunstaining and imaging using confocal microscopy [41]. Immunoreactive cell
411 bodies were observed in the buccal tentacles, oesophageal region and closely associated with the
412 radial nerve cords. Sensory-like cells in the tentacles project toward the circumoral nerve ring,
413 while cells close to the radial nerve cords have processes that are in close association with muscle
414 and other body wall structures.

415 The molecular identity of the neuropeptides that are responsible for the FMRFamide-like-ir
416 that is observed in sea cucumbers is not known, but it is likely that it is at least partially attributable
417 to SALMFamide-type neuropeptides (see section 3.2 below). However, the possibility remains that
418 other types of neuropeptide are also revealed by FMRFamide antibodies in sea cucumbers (and

other echinoderms). For example, a transcript encoding a putative neuropeptide (PYKFMRWamide) that shares C-terminal sequence similarity with FMRFamide was recently identified in the sea cucumber *Apostichopus japonicus* [63]. This peptide belongs to the luqin neuropeptide family, the prototype for which was originally identified in molluscs [2]. Further studies are now needed to investigate if this luqin-type neuropeptide contributes to the patterns of FMRFamide-like-ir observed in sea cucumbers.

3.2 Discovery of SALMFamide neuropeptides in sea cucumbers.

The identification of the starfish SALMFamides S1 and S2 was enabled by use of antibodies to the FMRFamide-like peptide pQDPFLRFamide to monitor peptide purification [30]. So the same strategy was employed to identify FMRFamide-like peptides in the sea cucumber *H*. *glaberrima*. Two peptides were purified to homogeneity and identified as the amidated heptapeptide GFSKYLFamide and the amidated octapeptide SGYSVLYFamide [20]. Discovery of these peptides revealed for the first time that SALMFamide-type neuropeptides do not only occur in starfish but are also present in other echinoderms. Furthermore, comparison of the sequences of S1, S2 and the two peptides identified in *H*. *glaberrima* revealed a conserved C-terminal motif – SxLxFamide (where x is variable). Thus, the concept of a family of SALMFamide neuropeptides in echinoderms emerged [28].

Completely independent of the discovery of GFSKYLFamide and SGYSVLYFamide in *H*. *glaberrima*, two SALMFamides were identified in another holothurian species, the edible sea cucumber *A*. *japonicus* [56]. Here peptide purification was accompanied by use of bioassays for myoactivity and two peptides that cause muscle relaxation were identified as GYSPFMFamide and FKSPFMFamide. Analysis of the sequences of these two peptides revealed similarities with the two peptides identified in *H*. *glaberrima*, and therefore they were categorised as members of the SALMFamide neuropeptide family. Importantly, however, the two *A*. *japonicus peptides* have a SxFxFamide motif, which contrasts with the SxLxFamide motif of S1, S2 and the two

SALMFamides identified in *H. glaberrima*. Thus, the discovery of the SALMFamides from *A. japonicus* provided the first insight on the existence of two types of SALMFamides in echinoderms: L-type SALMFamides that have the C-terminal SxLxFamide motif and F-type SALMFamides that have the C-terminal SxFxFamide motif [28].

3.3 SALMFamide expression in sea cucumber larvae

To date, there are no published reports of studies employing immunocytochemical methods to analyse SALMFamide expression in sea cucumber larvae. However, an excellent framework for anatomical analysis of SALMFamide expression in sea cucumber larvae has been provided by detailed description of neural development in *A. japonicus* [53]. Furthermore, there are transcriptome data available that indicate that SALMFamides are expressed in sea cucumber larvae. Transcriptome sequence data have been obtained for the gastrula and larval stages of *Parastichopus parvimensis*. These sequence data are available for BLAST analysis at http://www.spbase.org/Pp/ and analysis of these data reveals transcripts for two SALMFamide-type precursor proteins in larvae, but not in gastrulae. Only a partial sequence is available for one of the transcripts (Locus_16236) but analysis of this sequence reveals that it contains the putative F-type SALMFamide ARYSPFMFamide, which is very similar to one of the putative F-type SALMFamides that has recently been identified in *A. japonicus* (ARYSPFTFamide; see section 3.6 below). The second transcript (Locus_15676) encodes a precursor protein comprising three L-type or L-type-like SALMFamides, which shares 95% sequence identity with the L-type SALMFamide precursor that has recently been identified in *A. japonicus* (see section 3.6 below). These data indicate that both L-type and F-type SALMFamides are expressed in sea cucumber larvae. Furthermore, these data provide a basis for investigation of SALMFamide expression in sea cucumber larvae using mRNA *in situ* hybridization methods and/or immunocytochemistry. It will be interesting to compare patterns of SALMFamide expression observed in sea cucumber larvae

with reported patterns of SALMFamide expression in starfish and sea urchin larvae (see sections 2.3 and 4.2).

3.4. Localisation of the SALMFamide neuropeptide GFSKLYFamide in the sea cucumber H. glaberrima

With the discovery of the L-type SALMFamides GFSKYLFamide and SGYSVLYFamide in *H. glaberrima* it was possible to investigate the anatomical distribution of SALMFamide-type neuropeptides in a sea cucumber species for the first time. Antibodies to the peptide GFSKYLFamide were generated and used for immunocytochemical studies [18].

GFSKYLFamide-ir was detected in neuronal somata and fibres in both the ectoneural and hyponeural parts of the radial nerve cords and immunoreactive fibres were evident in the longitudinal and circular muscle layers of the body wall. GFSKYLFamide-ir somata and fibres were also revealed in appendages associated with the body wall of sea cucumbers - the buccal tentacles, which serve as feeding organs, and the locomotory tube feet [18].

Turning to visceral organs, GFSKYLFamide-ir was revealed throughout the digestive system, including the oesophagus, small intestine and large intestine, with prominent immunoreactivity localised in somata and fibres in the serosal layer. Immunoreactive cell bodies were also evident in the mucosal layer of the oesophagus and intestine, and in the oesophagus these gave rise to a network of GFSKYLFamide-immunoreactive fibres in the submucosal nerve plexus. Furthermore, analysis of intestinal tissue at the electron microscopic level revealed that the GFSKYLFamide-ir was localised in dense cord vesicles in both somata and fibres, consistent with the notion that this peptide is a secreted neuropeptide in sea cucumbers. Organs that are closely associated with the digestive system in holothurians are the respiratory trees, which are evaginations of the cloaca; as in the digestive system, a prominent GFSKYLFamide-immunoreactive plexus was observed in the serosal layer of the respiratory trees. Finally, GFSKYLFamide-ir was revealed in the tubular reproductive system of *H. glaberrima*, with

496 immunostaining evident in somata located in the coelomic epithelium and in sub-epithelial fibres in
497 both male and female gonads [18].
498 What is immediately apparent from this overview of the distribution of GFSKYLFamide-ir
499 in *H. glaberrima* is that expression is widespread and associated with the majority of organ systems.
500 In this respect, the findings are similar to findings from analysis of SALMFamide expression in
501 adult starfish (as described in section 2.4 above). Furthermore, these anatomical data provided an
502 expectation for pleiotropic actions of SALMFamides in sea cucumbers.
503
504 *3.5. Pharmacological effects of SALMFamide neuropeptides in sea cucumbers*
505 Consistent with the presence of GFSKLYFamide-ir fibres in the intestine of *H. glaberrima*,
506 application of synthetic GFSKLYFamide to *in vitro* preparations of large intestine from this species
507 caused dose-dependent relaxation at concentrations ranging from 10^{-10} to 10^{-6} M [19]. At 10^{-5} M the
508 relaxing effect of GFSKLYFamide was much smaller than at 10^{-6} M, indicative of desensitisation at
509 high peptide concentrations. Relaxing effects of GFSKLYFamide were observed on longitudinal
510 strips of intestine as well as rings of intestinal tissue, indicating that the peptide acts on both the
511 longitudinally and circularly orientated muscle layers. Furthermore, application of GFSKLYFamide
512 also reversed ACh-induced contraction of intestinal preparations. A dose-dependent relaxing action
513 of GFSKLYFamide was also observed when tested on strips of longitudinal body wall muscle from
514 *H. glaberrima*. Effects were observed at concentrations ranging from 10^{-10} to 10^{-6} M, but the
515 maximal effect was reached with 10^{-8} M [19].
516 The discovery that GFSKLYFamide causes relaxation of both intestinal and body wall
517 muscle preparations from *H. glaberrima* was consistent with the relaxing effects observed with S1
518 and S2 when tested on neuromuscular preparations from the starfish *A. rubens* (see section 2.5
519 above). Furthermore, collectively these findings indicate that SALMFamide neuropeptides may act
520 as muscle relaxants throughout the Phylum Echinodermata [28].

Further evidence that SALMFamides have a general action as muscle relaxants was obtained with the discovery of the first F-type SALMFamides to be identified in an echinoderm: GYSPFMFamide and FKSPFMFamide [56]. Thus, these two peptides were isolated from the sea cucumber *A. japonicus* on account of their relaxing effect on intestine preparations from this species. Interestingly, however, these peptides do not cause relaxation of longitudinal body wall muscle preparations from *A. japonicus*. This contrasts with the relaxing effect of the L-type SALMFamide GFSKLYFamide on longitudinal body wall muscle preparations from *H. glaberrima*. These findings may indicate that L-type SALMFamides and F-type SALMFamides exert effects by binding to different receptor types.

3.6 *SALMFamide precursor proteins reveal SALMFamide diversity in sea cucumbers*

Sequencing of the transcriptome of the sea cucumber *A. japonicus* has enabled identification of SALMFamide precursor proteins in this species. Thus, a transcript was identified that encodes the precursor of the two F-type SALMFamides (GYSPFMFamide and FKSPFMFamide) that were purified from this species on account of their relaxing effects on muscle preparations [24]. The precursor also contains two other putative F-type SALMFamides, ARYSPFTFamide and GHRGGQFSQFKFamide and two F-type-like SALMFamides - GVPPYVVKVTYamide and FKSSFYLamide. Furthermore, this SALMFamide precursor protein also contains two putative L-type SALMFamides (GGSALYFamide and VPELAESDGGQSKLYFamide), which are homologs of the two L-type SALMFamides originally isolated from *H. glaberrima* (GFSKYLFamide and SGYSVLYFamide) (Fig. 2, 3). Thus, this is an F-type SALMFamide precursor but, like the F-type SALMFamide precursor in the starfish *P. miniata*, it also gives rise to a smaller number of L-type SALMFamides. This suggests, therefore, that the presence of L-type SALMFamides in F-type SALMFamide precursors may be an evolutionarily conserved and therefore functionally relevant phenomenon.

In *A. japonicus*, as in the starfish *P. miniata*, there is also a second SALMFamide precursor that contains only L-type SALMFamides. Unlike the *P. miniata* L-type SALMFamide precursor, which contains seven putative SALMFamides, the *A. japonicus* L-type SALMFamide precursor contains only three L-type or L-type-like SALMFamides: TRSRSMFGNTALPFamide, VVSRAWSPLVGQTGIAFamide and MGFTGNTGILLamide (Fig. 2, 3) [26]. Nothing is known about the neuroanatomical expression of the L-type SALMFamide precursor or the pharmacological actions of its putative neuropeptide products. It will be of interest to compare the expression and actions of neuropeptides derived from the L-type SALMFamide precursor with the expression and actions of neuropeptides derived from the F-type SALMFamide precursor, which also contains L-type SALMFamides.

4. Echinoidea

4.1 SALMFamide-like immunoreactive peptides in the sea urchin Echinus esculentus

The development of radioimmunoassays for the starfish SALMFamides S1 and S2, as described above in section 2.2 above, facilitated investigation of the occurrence of structurally related SALMFamides in other echinoderms. With SALMFamides having already been identified in a holothurian species (see section 3.2 above), effort was focused on a species belonging to the class Echinoidea, the sea urchin *Echinus esculentus* [32]. Because it is difficult to dissect nerves from sea urchins, acetone extracts of whole animals were analysed. This revealed four chromatographically distinct peaks of S2-like-ir, which were labelled peaks 1-4. Only one of these peaks (peak 3) was purified to homogeneity and subjected to sequencing. This revealed that peak 3 has the N-terminal sequence Met-Arg-Tyr-His but it was not possible to obtain the complete sequence of this peptide. However, with the availability of SALMFamide precursor sequences from a sea urchin species (see section 4.5 below) it was possible in retrospect to deduce that the *Echinus* peak 3 peptide is probably a homolog of a SALMFamide neuropeptide that is a predicted product of the L-type SALMFamide precursor in *Strongylocentrotus purpuratus* – MRLHPGLLFamide [64].

572 This peptide has the N-terminal tetrapeptide sequence MRLH, which is very similar to the partial
573 sequence obtained for the *Echinus* peak 3 peptide (MRYH).
574
575 *4.2. Distribution of SALMFamide-type neuropeptides in larval echinoids*
576 The first investigation of SALMFamide expression in the larval nervous system of an
577 echinoderm employed use of antibodies to S1 for immunocytochemical analysis of the larvae of the
578 sand dollar *Dendraster excentricus* [67]. S1-immunoreactive fibres first appear in the apical
579 ganglion between 56 h and 72 h. By 6 days (4-6 arm plutei), 2-4 pairs of S1-immunoreactive cell
580 bodies can be seen, and by 21 days (8-arm plutei) there are 9-10 pairs of S1- immunoreactive cell
581 bodies in the apical ganglion. S1-immunoreactive cell bodies are also present in the oral ganglion,
582 first evident in early 4-arm plutei as 2-4 cells and then increasing to 6 pairs by 3 weeks (8-arm
583 plutei). From 12-13 days (6-8 arm plutei) a network of S1-immunoreactive fibres is also present in
584 the oesophagus and by 21 days the process of single S1-immunoreactive cell can be seen encircling
585 the pyloric sphincter of the larval gut.
586 SALMFamide expression has also been analysed in larvae of the sea urchin *Psammechinus*
587 *miliaris* using antibodies to S1 [3], and the patterns of immunoreactivity are similar to those seen in
588 sand dollar larvae (see above). A population of at least 20 pairs of S1-immunoreactive cells are
589 evident in the apical ganglion in mature 8-armed plutei; a smaller population of S1-immunoreactive
590 cells is associated with the lower lip. A plexus of S1-immunoreactive cells and processes develops
591 around the pylorus at the posterior end of the stomach, which is first apparent as a single cell and
592 fibre in 6-arm plutei. In mature larvae S1-ir can also be seen in the adult rudiment, with the
593 SALMFamidergic nerve fibres delineating the five radial nerves that innervate the primary tube feet.
594 A novel feature of the study [3] was the use of antibodies to S2, which revealed a SALMFamidergic
595 system distinct from that revealed by antibodies to S1. Thus, S2-ir was observed in the cell bodies
596 of neurons that are located between the anterolateral and posterodorsal arms and that have processes
597 underlying the ciliated bands.

598 The patterns of SALMFamide expression in echinoid larvae point to roles in neural
599 processing of sensory signals in the apical ganglion and regulation of the ciliary activity required
600 for swimming and feeding. The S1-ir nerve plexus associated with the stomach pylorus is
601 suggestive of a role in regulation of gut muscle activity; possibly a relaxing action, given the now
602 well established effects of SALMFamides as muscle relaxants in adult echinoderms [28].
603 Furthermore, with identification of genes encoding SALMFamides in sea urchins (see section 4.5
604 below), there are now opportunities to experimentally investigate SALMFamide function in
605 echinoid larvae.
606
607 *4.3. Distribution of SALMFamide-type neuropeptides in adult echinoids*
608 Currently, very little is known about the anatomical distribution of SALMFamide
609 neuropeptides in adult echinoids. However, immunocytochemical analysis of the sea urchin *Arbacia*
610 *lixula* using antibodies to the starfish SALMFamide S2 has revealed immunoreactivity in the podial
611 nerve that innervates the tube feet [38]. This finding is consistent with the detection of S1-ir and S2-
612 ir in the basiepithelial plexus of starfish tube feet. Furthermore, it suggests a potential role for
613 SALMFamides in regulation of tube foot motility in sea urchins (see section 4.4 below).
614
615 *4.4 Pharmacological effects of SALMFamide neuropeptides in adult echinoids*
616 The molecular identity of echinoid SALMFamide neuropeptides has only been determined
617 relatively recently, through the analysis of genome/transcriptome sequence data (see section 4.4
618 below). Therefore, as yet there are no published reports of the effects of native SALMFamides on
619 echinoid preparations. However, the effects of the starfish SALMFamides S1 and S2 on tube foot
620 preparations from the sea urchin *Echinus esculentus* have been examined and both peptides cause
621 relaxation,[32], consistent with the detection of S2-like-ir in the innervation of tube feet in the sea
622 urchin *Arbacia lixula* [38]. Furthermore, these findings provide further evidence that
623 SALMFamide-type neuropeptides act as muscle relaxants throughout the phylum Echinodermata.

624

625 *4.5 SALMFamide precursor proteins reveal the diversity of SALMFamides in sea urchins*

626 The sea urchin *Strongylocentrotus purpuratus* was the first echinoderm species to have its
627 genome sequenced and BLAST analysis of the sequence data enabled determination of the
628 sequence of the first SALMFamide neuropeptide precursor to be identified - a precursor comprising
629 seven putative F-type SALMFamides, which were named SpurS1 – SpurS7 [32]. Four of the
630 peptides have the canonical F-type SALMFamide motif SxFxFamide (SpurS1, SpurS2, SpurS3 and
631 SpurS6). However, the serine residue is replaced by a proline residue in two of the peptides (SpurS4
632 and SpurS5) and by a leucine residue in one of the peptides (SpursS7) (Fig. 2, 3).

633 Subsequently, analysis of neural trancriptome sequence data enabled identification of an L-
634 type SALMFamide precursor in *S. purpuratus* [64]. This protein comprises just two putative
635 SALMFamides, NMGSIHSHSGIHFamide (SpurS8) and MRLHPGLLFamide (SpurS9; probably a
636 homolog of the peak 3 peptide purified from *Echinus* - see section 4.1 above). SpurS8 has the C-
637 terminal motif SxIxFamide, which is structurally very similar to the canonical L-type SALMFamide
638 motif (SxLxFamide), whereas in SpurS9 the canonical serine residue is replaced by a proline (Fig. 2,
639 3).

640 Since completion of the *S. purpuratus* genome project, genome sequence data have been
641 obtained from other sea urchin species. Analysis of partial SALMFamide precursor sequences
642 obtained from *Strongylocentrotus franiscanus* reveals peptides that are identical to SpurS1, -S2, -S3,
643 -S4, -S7 and -S8, as might perhaps be expected for species belonging to the same genus (A. Patel
644 and M. Elphick, unpublished observations from data available at http://www.spbase.org/SpBase/).
645 However, analysis of genome sequence data obtained for *Lytechinus variegatus*, a sea urchin
646 species that is more distantly related to *S. purpuratus*, reveals sequence divergence in its two
647 putative L-type SALMFamides and seven putative F-type SALMFamides, with amino acid
648 substitutions per peptide ranging from just one to as many as seven (A. Patel and M. Elphick,
649 unpublished observations from data available at http://www.spbase.org/SpBase/)

5. Ophiuroidea

5.1 Detection of SALMFamide-like immunoreactive peptides in ophiuroids

In parallel with studies using antibodies to S1 and/or S2 to assay for SALMFamide-type neuropeptides in the sea urchin *Echinus esculentus* (see section 4.1 above), extracts of the brittle star *Ophiura ophiura* were analysed using the same methodology. S1-like-ir was detected in a range of HPLC-separated fractions but the levels of immunoreactivity were not sufficient to enable purification and sequencing of the immunoreactive peptides [23]. S1-like-ir and S2-like-ir have also been detected in extracts of the brittle star species *Amphipholis squamata* [4]. To date no brittle star SALMFamide-type neuropeptides have been purified and sequenced. However, as discussed in section 5.5 below, insights on the sequences of SALMFamides in ophiuroids have been obtained from transcriptome sequence data.

5.2 Localisation of SALMFamide-like immunoreactivity in brittle star larvae

Analysis of the development and organisation of the nervous system in brittle star larvae has been facilitated recently through use of antibodies to synaptotagmin. Using this approach neural development has been described in the brittle star species *Amphipholis kochii* and *Amphiura filiformis* [22, 40]. The distribution of larval SALMFamide expression has been examined in the brittle star species *Ophiactis resiliens* using antibodies to S1 [9]. S1-like-ir is first observed in 6-armed ophioplutei, associated with a nerve containing several cell bodies that encircles the stomach. By the 8-armed stage, S1-ir is also evident in fibres innervating the pre-oral ciliated band, the post-oral transverse ciliated band and the adoral ciliated band. A cluster of 2-3 S1-like-immunoreactive cell bodies is present at the base of the antero-lateral arms. In advanced ophioplutei (90 days) the S1-like-immunoreactive neural elements associated with the gut and ciliated bands begin to degenerate but a new group of S1-like-immunoreactive cells appears along the proximal end of the antero-lateral arms. In metamorphosing larvae (98-100 days) S1-like immunoreactive fibres can be

676 seen in the developing rudiment. As with the other echinoderm classes, at present nothing is known
677 about the physiological roles of SALMFamides in brittle star larvae. Nevertheless, the patterns of
678 expression are indicative of roles in regulation swimming, feeding and gut activity. Furthermore,
679 with the molecular identification of SALMFamides in brittle stars (see section 5.5) it may soon be
680 possible to directly address this issue by testing the pharmacological actions of synthetic peptides.
681
682 *5.3 Localisation of SALMFamide-like immunoreactivity in adult brittle stars*
683 Although SALMFamide-type neuropeptides have not been purified from ophiuroids,
684 antibodies to S1 have been used to examine the presence and distribution of SALMFamide-like
685 peptides in adult brittle stars. A detailed immunocytochemical study of the brittle star species
686 *Ophiura ophiura* revealed S1-like immunoreactive neuronal somata and fibres in the ectoneural part
687 of the radial nerves and the circumoral nerve ring [36]. The patterns of immunostaining reflected
688 the segmental organisation of the radial nerve cords, with distinct clusters of immunoreactive cell
689 bodies occupying the same positions in each segment of the radial nerve. The majority of
690 immunostained cell bodies were small (8 – 15 μm); however, in each segment of the nerve cord a
691 single giant neuron (diameter > 25 μm) or occasionally a pair of giant neurons was labelled by S1
692 antibodies. Furthermore, in the two segments proximal to the circumoral nerve ring, the number of
693 immunostained cell bodies was higher than in more distal segments. In the circumoral nerve ring
694 immunostaining was largely localised in fibres, supportive of the view that "the ring cannot be
695 regarded as a central nervous system but only functions as a link between the five segmented nerve
696 cords" [36]. Thus, analysis of SALMFamide-like-ir in *O. ophiura* provided important insights on
697 the functional organisation ophiuroid nervous systems. Furthermore, the data obtained provide a
698 basis for investigation of SALMFamide neuropeptide function in repeatedly identifiable
699 echinoderm neurons; thus, it may be possible to employ electrophysiological recording methods to
700 analyse the properties of the S1-like immunoreactive giant neurons in each segment of the radial
701 nerves.

Subsequently, S1-antibodies were used to examine the distribution of SALMFamide-type neuropeptides in a different brittle star species, the luminescent *Amphipholis squamata*. However, in this study antibodies to S2 were also used in parallel with antibodies to S1 [15]. Similar to findings in *O. ophiura*, S1-immunoreactive cell bodies and fibres were revealed in the ectoneural part of the radial nerve cords, with a segmentally repeating pattern of immunostaining. In addition, a distinct small population of S1-ir neurons and associated processes was revealed in the hyponeural part of the radial nerve cords. S2-ir was found to be less abundant than S1-ir and was restricted to cells and fibres in the ectoneural part of the nervous system. Interestingly, this mirrors findings in *A. rubens* (see section 2.4 above), where S1-ir was detected in both the ectoneural and hyponeural parts of the nervous system and S2-ir was restricted to the ectoneural. Further investigation of the functional significance of these differences in patterns of staining in *A. squamata* would be facilitated if the peptides responsible for S1-ir and S2-ir in this species were identified (see section 5.5 below). Nevertheless, some insights on SALMFamide function in *A. squamata* have been obtained by using the starfish SALMFamides S1 and S2 for pharmacological studies, as described in section 5.4 below.

5.4 Pharmacological effects of SALMFamides on brittle stars

The investigation of SALMFamide expression in the brittle star *A. squamata*, as described above, was conducted in the context of an interest in neural control of luminescence in this species. The classical neurotransmitter acetylcholine (ACh) induces low intensity light flashes from isolated arms of *A. squamata* [16, 17], whereas the depolarizing agent KCl induces higher intensity monophasic light production [45]. It was postulated, therefore, that KCl may induce release of other neurotransmitters or neuromodulators that regulate luminescence alongside ACh. To investigate a potential role for SALMFamides in regulating luminescence in *A. squamata,* the starfish SALMFamides S1 and S2 were tested *in vitro* on isolated arms [4]. Experiments were performed on the two varieties of *A. squamata* that occur naturally – black and brown (also referred to as “clear”).

Application of S1 or S2 did not induce luminescence of isolated arms from either variety. However, pretreatment with S1 significantly increased ACh-induced luminescence in both black and clear specimens, whilst pre-treatment with S2 had little or no effect on ACh-induced luminescence. These data indicate that SALMFamides may act as neuromodulators that regulate light production in *A. squamata*. It interesting that a potentiating effect of S1 on ACh-induced luminescence was observed because hitherto only inhibitory effects of SALMFamides have been reported (e.g. muscle relaxation; inhibition of GSS release in starfish). One possibility is that SALMFamides act to inhibit the release of inhibitory neurotransmitters such as GABA and glycine, which cause inhibition of ACh-induced luminescence in *A. squamata* [4]. Further investigation of the physiological roles of SALMFamides in regulation of luminescence in *A. squamata* would be facilitated if neuropeptides native to this species or other brittle star species are identified (see section 5.5 below).

5.5 SALMFamide precursor proteins in brittle stars

Recently, a paper reporting transcriptome sequencing of the Antarctic brittle star species *Ophionotus victoriae* was published [6]. The sequences of 18,003 contigs were determined and analysis of these data has revealed a contig (7706) encoding a partial protein sequence that contains eleven putative F-type SALMFamides and a single putative L-type SALMFamide (RNPMNSLSALAFamide) that shares sequence similarity with the starfish SALMFamide S2 (SGPYSFNSGLTFamide) (M.R. Elphick, D.C. Semmens & M.S. Clark, unpublished data). Thus, this protein appears to be a brittle star ortholog of the F-type SALMFamide precursors that have been identified in other echinoderms. On-going studies are directed toward determining the complete sequence of this protein as well as investigating the occurrence of an L-type SALMFamide precursor in *Ophionotus victoriae*.

6. Crinoidea

754 Currently, very little is known about SALMFamides in crinoids. An immunocytochemical
755 study of pinnules from the crinoid *Antedon bifida* using antibodies to the starfish SALMFamide
756 neuropeptide S2 revealed groups of S2-like immunoreactive bipolar and roundish neuronal somata
757 (~6 μm diameter) located at the periphery of pinnular sections of the brachial nerve just before the
758 nerve enters the ossicles [37]. These cells have processes that project along the boundary between
759 ossicles and interossicular muscles and ligaments, whilst another process projects into the
760 interossicular segment of the nerve. These anatomical observations suggest a possible role for
761 SALMFamides in regulating the mechanics of interossicular muscles and ligaments in crinoids.
762 However, there are, as yet, no reports of experimental studies investigating the pharmacological
763 activity of SALMFamides in crinoids.
764 Progress in analysis of SALMFamide function in crinoids would be facilitated by
765 identification of genes/cDNAs encoding SALMFamide precursor proteins. Currently, however,
766 there are no genome/transcriptome sequence data available for crinoid species. Discovery of
767 SALMFamide precursors in crinoids would be of great interest, but not only as a basis for
768 experimental studies. Because crinoids occupy a phylogenetic position that is basal to the four other
769 extant echinoderm classes [59], analysis of sequence data from crinoids may provide important
770 insights into the evolution of SALMFamide neuropeptides. For example, do crinoids have both an
771 L-type and an F-type SALMFamide precursor? If they do, then does the F-type SALMFamide
772 precursor also contain one or more L-type SALMFamides, as has been found in starfish, sea
773 cucumbers and brittle stars?
774
775 **7. Conclusions and directions for future research**
776 Looking back over a research programme that was initiated twenty-five years ago, it is
777 timely to assess the broader impact of the discovery of SALMFamide neuropeptides. Perhaps the
778 greatest impact has been providing new tools (in the form of antibodies) for visualisation of
779 echinoderm nervous systems. In particular, antibodies to the starfish SALMFamide neuropeptide S1

780 have been widely used to reveal for the first time the architecture of neuropeptidergic systems in a
781 variety of echinoderms, both as larvae and as adults. These neuroanatomical studies have yielded
782 data that have provided new insights into the organisation of echinoderm nervous systems.
783 However, the more challenging task of determining the physiological roles of SALMFamide
784 neuropeptides in echinoderms has not kept pace with neuroanatomical studies. Although patterns of
785 SALMFamide expression have been revealed in larvae from several echinoderm classes, at present
786 nothing is known about the physiological roles of SALMFamides, or indeed any neuropeptides, in
787 echinoderm larvae. This represents an exciting field of investigation for the future, especially now
788 that SALMFamide precursor transcript sequences have been identified in several echinoderm
789 species. Recently, progress has been made determining the physiological roles of neuropeptides in
790 the larval nervous systems of other marine invertebrates such as the annelid *Platynereis dumerilii*.
791 For example, neuropeptides alter ciliary beat frequency, which affects the vertical distribution of
792 larvae in the water column [13, 14]. Accordingly, similar experimental approaches to those used
793 with *Platynereis* larvae could be employed to investigate neuropeptide function in echinoderm
794 larvae.

795 Progress has been made, as discussed above, in revealing the pharmacological actions of
796 SALMFamide neuropeptides in adult echinoderms and a consistent finding is relaxing effects on
797 muscle systems [28]. Furthermore, the *in vitro* and *in vivo* relaxing effect of SALMFamides on the
798 cardiac stomach of starfish suggests a physiological role in mediating neural control of stomach
799 eversion associated with their extraoral feeding behaviour [47]. However, the discovery of
800 physiological roles is determined by choices of bioassays, and it would be simplistic to conclude
801 that the function of SALMFamides is solely to act as muscle relaxants in echinoderms. The
802 inhibitory effect of S1 on neural release of GSS in starfish [49] points to a more general role for
803 SALMFamides as inhibitory neurotransmitters and as such it is likely that SALMFamides have
804 pleiotropic actions in echinoderms.

Perhaps the most important recent breakthrough in SALMFamide research has been the identification of genes/transcripts encoding the precursors of SALMFamide neuropeptides [26]. This has revealed that a much greater diversity of SALMFamides exists in echinoderm species than had been revealed previously. This feature of SALMFamides was discussed in detail in the class-specific sections of text above and is illustrated in figures 2 and 3. Thus, the currently available data indicate that in echinoderms there are two types of SALMFamide precursors: an L-type SALMFamide precursor and an F-type SALMFamide precursor.

L-type SALMFamide precursors comprise only L-type or L-type-like SALMFamides but the number of putative L-type peptides derived from L-type SALMFamide precursors ranges from just two (*S. purpuratus*; Echinoidea) to seven (*P. miniata*; Asteroidea) (Fig. 2, 3). Furthermore, comparative alignment of L-type SALMFamide precursors suggests that a common ancestral precursor protein may have comprised three L-type SALMFamides (as in *A. japonicus*; Holothuroidea) [26]

F-type SALMFamide precursors comprise variable numbers of F-type or F-type-like SALMFamides and typically (Asteroidea, Holothuroidea, Ophiuroidea) one or more L-type SALMFamides. In fact the first F-type SALMFamide precursor to be identified in the sea urchin *S. purpuratus* is atypical in comprising only F-type SALMFamides (Fig. 2, 3). Therefore, with reference to echinoderm phylogeny [59], a parsimonious explanation based on the sequence data available would be that the occurrence of one or more L-type SALMFamides in the F-type SALMFamide precursors is an ancient characteristic that dates back to the common ancestor of echinoids, holothurians, asteroids and ophiuroids. Accordingly, it would be concluded that there has been loss of L-type peptides in F-type SALMFamide precursors in the echinoid lineage.

The occurrence of multiple SALMFamide isoforms in echinoderms raises questions concerning their functional significance. Neuropeptide “cocktails” derived from common precursor proteins are, of course, not unique to SALMFamides. Indeed, it is a widespread phenomenon in the animal kingdom, particularly in invertebrates [70]. However, in spite of this, its physiological

relevance remains poorly understood. Some studies indicate that neuropeptide isoforms derived from a common precursor are functionally redundant [39], whilst other studies have revealed differential effects [33, 44]. The discovery that heterogeneous mixtures of SALMFamides are derived from common precursor proteins has provided opportunities to use this neuropeptide family as a model system to investigate the physiological significance of neuropeptide "cocktails".

Ultimately, an understanding of the physiological relevance of the SALMFamide *salmagundi* will require identification and characterisation of receptors that mediate the effects of these neuropeptides. Relevant to this issue, it was recently postulated that L-type SALMFamides may belong to a bilaterian family of neuropeptides that include gonadotropin-inhibitory hormone (GnIH) in vertebrates and SIFamide-type neuropeptides in protostomian invertebrates [25]. The rationale for this hypothesis is that some SIFamide-type neuropeptides have a C-terminal SxLxFamide motif, as found in L-type SALMFamides. Further evidence can be found in the physiological roles of GnIH-type and SIFamide-type neuropeptides, which inhibit reproductive processes in vertebrates and *Drosophila*, respectively [68, 69]. In this respect there are similarities with the L-type SALMFamide S1, which causes inhibition of neural release of the relaxin-like gonad stmulating substance in starfish [49]. Furthermore, GnIH also stimulates feeding behaviour and it has been proposed that GnIH functions as a "molecular switch" between reproduction and feeding in vertebrates [10] It is intriguing, therefore, that SALMFamides trigger cardiac stomach eversion in starfish, indicative of a physiological role in neural control of feeding behaviour [47]. Thus, it is tempting to speculate that GnIH/SALMFamide/SIFamide-type neuropeptides may be an evolutionarily ancient family of neuropeptides that stimulate feeding and inhibit reproduction in bilaterian animals. Support for this hypothesis would be obtained if it is found that SALMFamide receptors are orthologs of the GnIH/SIFamide-type receptors that have been identified in vertebrates and in *Drosophila* [42, 43, 48].

It is also noteworthy that the C-terminal FxFamide motif of F-type SALMFamides is a feature of vertebrate QRFP (26RFa)-type neuropeptides, which regulate food intake in mammals by

857 stimulating intake of a high fat diet [62]. Accordingly, F-type SALMFamides may exert their
858 effects by binding to echinoderm QRFP-type receptors. Testing this hypothesis is now feasible with
859 the availability of echinoderm transcriptome/genome sequence data [5, 21, 66].
860 This review has looked back over a period of twenty-five years of research on
861 SALMFamide neuropeptides, which began with a paper published in 1989 reporting FMRFamide-
862 like immunoreactivity in the starfish *A. rubens*. Looking ahead, key objectives for the future are to:
863 i). determine the evolutionary relationships of echinoderm SALMFamides with neuropeptides in
864 other phyla, which could be achieved by identifying the receptors that mediate effects of
865 SALMFamides ii). examine more widely the physiological roles of SALMFamides in larval and
866 adult echinoderms and iii). investigate the evolutionary and functional significance of the
867 "cocktails" of neuropeptides that are … the SALMFamide *salmagundi*.

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Fig 1

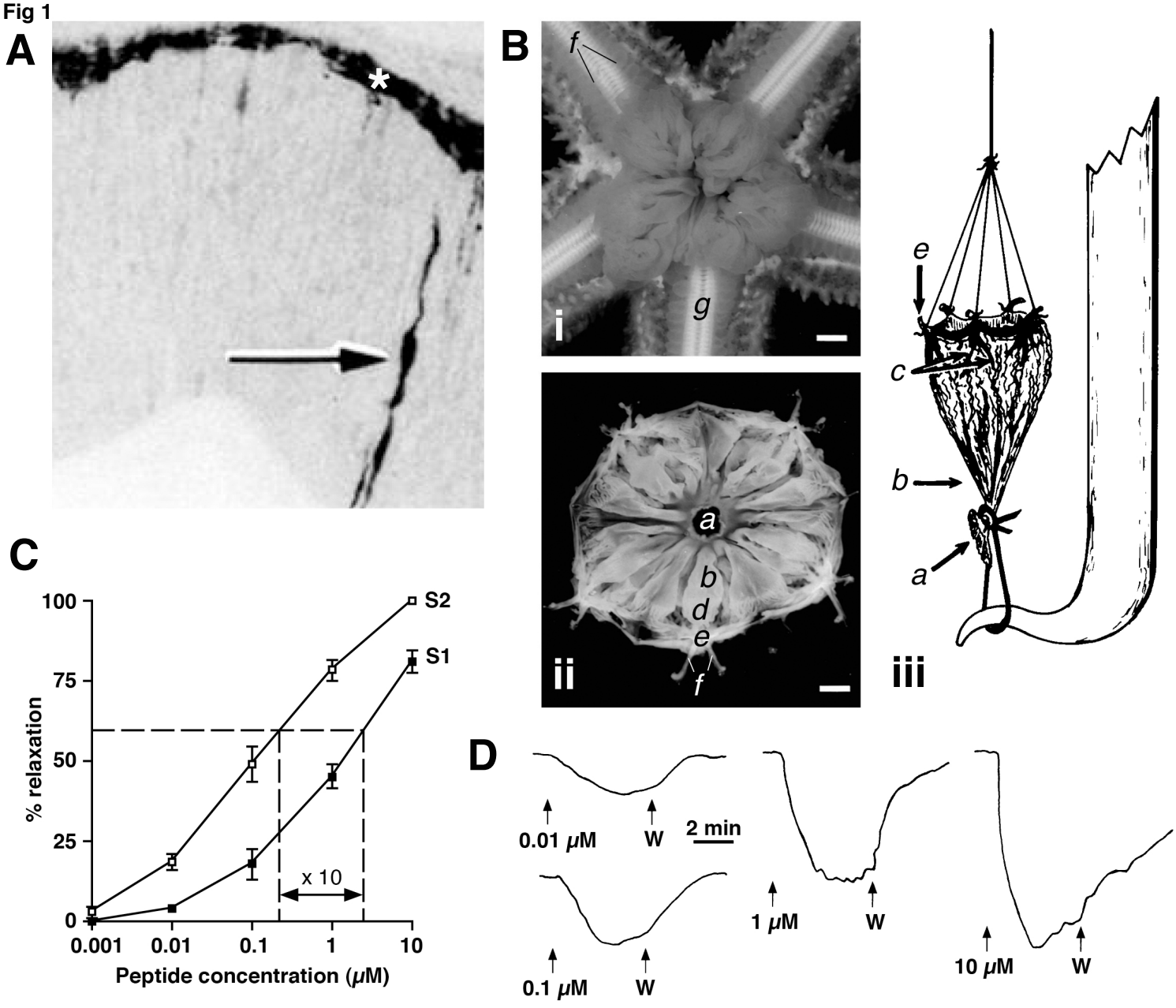
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Fig 2

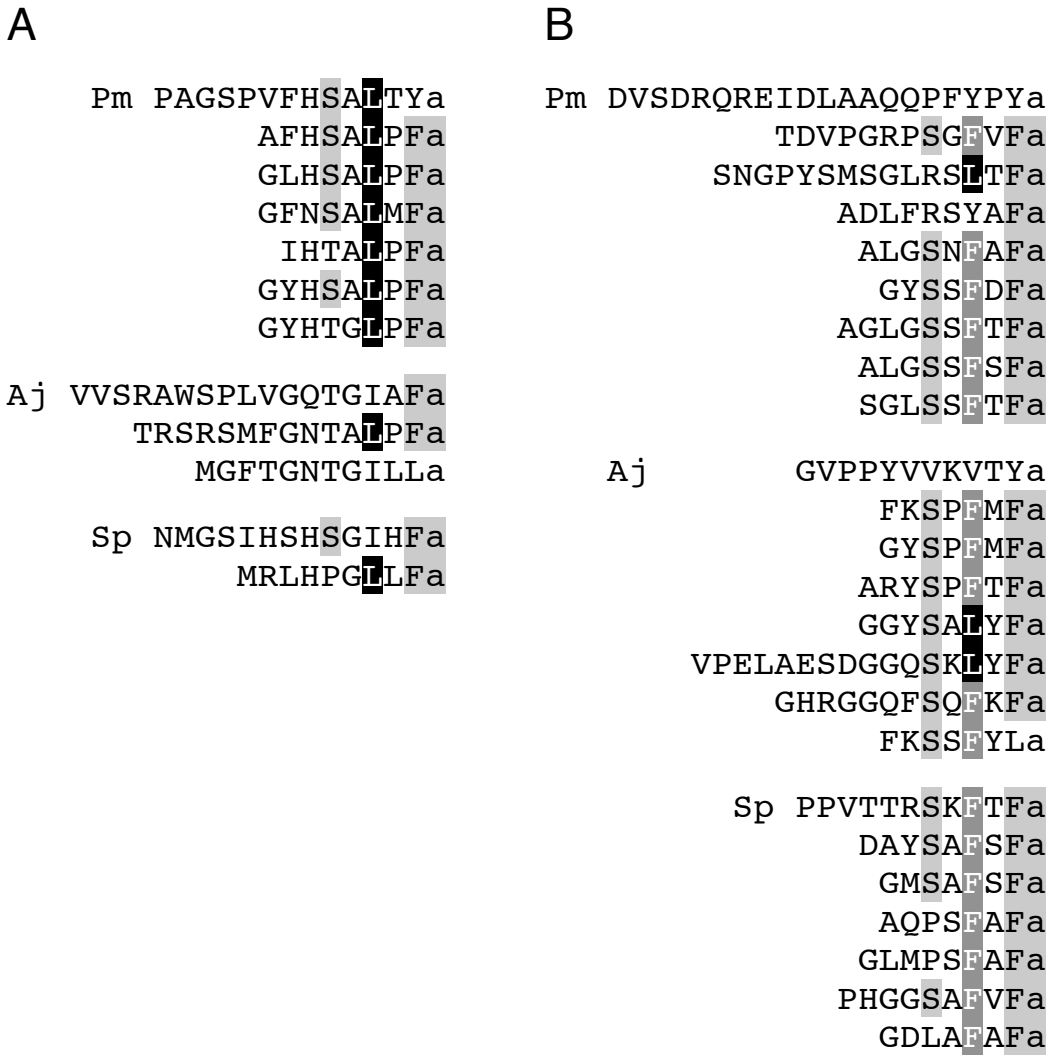
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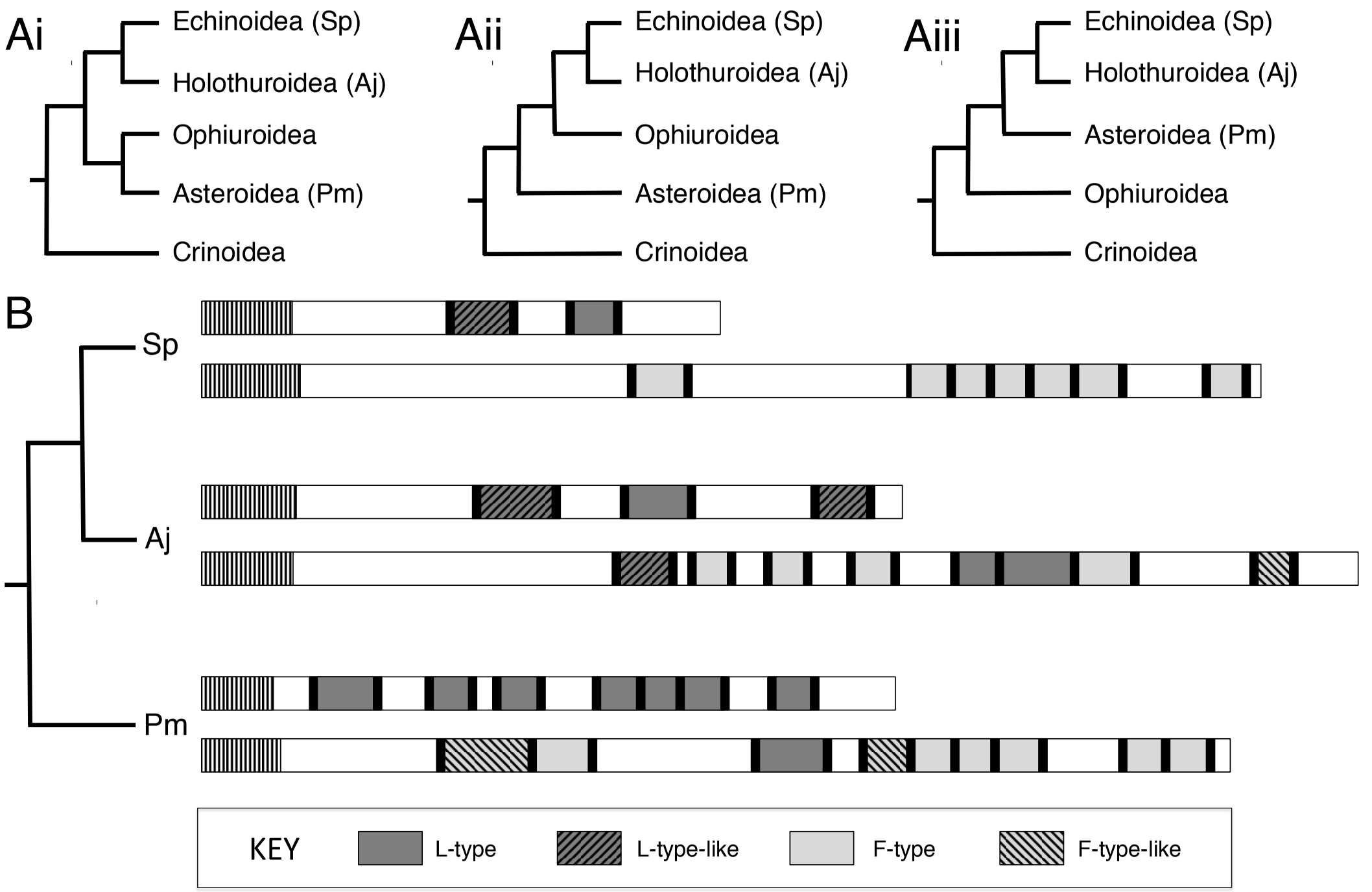
Fig 3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KEY
L-type
L-type-like
F-type
F-type-like

Revised manuscript showing changes

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