

1 **The evolution of neuropeptide signalling: insights from echinoderms**

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17

18 **Abstract**

19 Neuropeptides are evolutionarily ancient mediators of neuronal signalling that  
20 regulate a wide range of physiological processes and behaviours in animals.  
21 Neuropeptide signalling has been investigated extensively in vertebrates and  
22 protostomian invertebrates, which include the ecdysozoans *Drosophila*  
23 *melanogaster* (Phylum Arthropoda) and *Caenorhabditis elegans* (Phylum  
24 Nematoda). However, until recently, an understanding of evolutionary  
25 relationships between neuropeptide signalling systems in vertebrates and  
26 protostomes has been impaired by a lack of genome/transcriptome sequence  
27 data from non-ecdysozoan invertebrates. The echinoderms – a  
28 deuterostomian phylum that includes sea urchins, sea cucumbers and starfish  
29 - have been particularly important in providing new insights into neuropeptide  
30 evolution. Sequencing of the genome of the sea urchin *Strongylocentrotus*  
31 *purpuratus* (Class Echinoidea) enabled discovery of i). the first invertebrate  
32 thyrotropin-releasing hormone (TRH)-type precursor, ii). the first  
33 deuterostomian pedal peptide/orcokinin-type precursors, and iii). NG peptides  
34 – the “missing link” between neuropeptide S (NPS) in tetrapod vertebrates  
35 and crustacean cardioactive peptide (CCAP) in protostomes. More recently,  
36 sequencing of the neural transcriptome of the starfish *Asterias rubens* (Class  
37 Asteroidea) enabled identification of 40 neuropeptide precursors, including the  
38 first kisspeptin and melanin-concentrating hormone (MCH)-type precursors to  
39 be identified outside of the chordates. Furthermore, the characterization of a  
40 corazonin-type neuropeptide signalling system in *A. rubens* has provided  
41 important new insights into the evolution of gonadotropin-releasing hormone  
42 (GnRH)-related neuropeptides. Looking forward, the discovery of multiple

43 neuropeptide signalling systems in echinoderms provides opportunities to  
44 investigate how these systems are used to regulate physiological and  
45 behavioural processes in the unique context of a decentralized, pentaradial  
46 bauplan.

47

48 **Author Biography**

49 Dean Semmens has a BSc in Molecular and Cellular Biology (University of  
50 Bath, 2011), a PhD in Neurobiology (Queen Mary University of London, 2015)  
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52

53 Maurice Elphick studied at Royal Holloway University of London (BSc Biology,  
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56

57 **Key Words**

58 Neuropeptide; evolution; genomics; echinoderms; sea urchin; starfish

59 **I. Neuropeptide signalling systems: evolutionarily ancient**  
60 **regulators of physiology and behaviour**

61 Neuropeptides are intercellular signalling molecules that are secreted by  
62 neurons to act as neurotransmitters, neuromodulators or neurohormones [1].  
63 They are the largest and most diverse class of signalling molecules in the  
64 nervous system [2], ranging in size from just three amino acids (e.g.  
65 thyrotropin-releasing hormone (TRH) [3]) to much longer polypeptides (e.g.  
66 corticotropin-releasing hormone (CRH), which comprises 41 residues [4]).  
67 However, all neuropeptides share the common characteristic of being derived  
68 from larger precursor proteins, which have an N-terminal signal peptide that  
69 targets the precursor protein to the regulated secretory pathway [5]. In  
70 addition, precursor proteins have canonical cleavage sites (e.g. monobasic  
71 and/or dibasic sites recognized by prohormone convertases [6]) and sites for  
72 post-translational modification (e.g. a C-terminal glycine residue is often a  
73 substrate for amidation, which can be crucial for bioactivity [7]).  
74 Neuropeptides, with a few exceptions, typically bind to and activate G-protein  
75 coupled receptors (GPCRs) belonging to the rhodopsin- $\beta$ , rhodopsin- $\gamma$  and  
76 secretin-type receptor families [8].

77 The evolutionary origins of neuropeptides as regulators of physiology  
78 and behaviour are ancient and a number of neuropeptide signalling systems  
79 have been traced back to the common ancestor of bilaterian animals  
80 (Urbilateria) more than 550 million years ago [9, 10]. Furthermore,  
81 neuropeptide signalling pathways are also key components of the nervous  
82 systems in sister phyla to the bilaterians (e.g. cnidarians [11]) and the origins

83 of some peptide signalling pathways may pre-date the emergence of animals  
84 with nervous systems [12].

85 Historically, establishing relationships between neuropeptide signalling  
86 systems in evolutionarily distant phyla was possible for neuropeptides with  
87 highly conserved structures. For example, vasopressin/oxytocin (VP/OT)-type  
88 peptides comprise a characteristic disulphide bridge between cysteine  
89 residues at positions 1 and 6 of the mature peptide that is crucial for  
90 bioactivity and which is conserved in members of this neuropeptide family  
91 throughout the Bilateria [13, 14]. Furthermore, it has been found that VP/OT-  
92 type peptides regulate reproductive behaviour in both vertebrates and  
93 invertebrates, providing evidence of evolutionary conservation of not only  
94 neuropeptide structure but also neuropeptide function [15]. However, perhaps  
95 more typically, there is relatively little sequence similarity shared by related  
96 neuropeptides from different phyla and therefore establishing relationships is  
97 difficult when only the primary amino acid sequence of bioactive  
98 neuropeptides is known. Nevertheless, in the pre-genomic era, evidence of  
99 the evolutionarily ancient origins of neuropeptide signalling systems was  
100 obtained based upon primary sequence similarity [16], cross-immunoreactivity  
101 [17] or functional similarity [18].

102

## 103 *II. Neuropeptide relationships: insights from the first animal* 104 *genome sequences*

105 The turn of twenty-first century heralded the beginning of the post-genomic  
106 era and sequencing of the genomes of the nematode *Caenorhabditis elegans*  
107 in 1998 [19], the fruit-fly *Drosophila melanogaster* in 2000 [20] and *Homo*

108 *sapiens* in 2001 [21] enabled the first comprehensive analyses of genes  
109 encoding neuropeptide precursors and receptors in these species [8, 22, 23].  
110 Subsequently, deorphanization of candidate neuropeptide receptors provided  
111 important new insights into the evolutionary relationships and functional  
112 diversity of neuropeptide signalling systems [24-30]. Furthermore, in some  
113 cases neuropeptide receptor deorphanization revealed unexpected  
114 relationships. This is perhaps best exemplified by the unification of  
115 gonadotropin-releasing hormone (GnRH) and adipokinetic hormone (AKH) as  
116 members of the same neuropeptide family.

117 Insect AKHs are lipid-mobilizing hormones released during flight and  
118 locomotion [31]. In 2002, the receptor for *Drosophila* AKH (pQLTFSPDWG-  
119 NH<sub>2</sub>) was identified and pharmacologically characterized [32, 33].  
120 Interestingly, it was found that insect AKH receptors are structurally and  
121 evolutionarily related to vertebrate GnRH receptors. In mammals, GnRH  
122 controls reproductive maturation and function by stimulating release of  
123 luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the  
124 pituitary gland [34, 35], but mammalian GnRH (e.g. human GnRH is  
125 pQHWSYGLRPG-NH<sub>2</sub>) shares only modest sequence similarity with AKH.  
126 Thus, the discovery of insect AKH receptors enabled unification of a bilaterian  
127 neuropeptide family that hitherto had not been recognized based on primary  
128 sequence similarity or biological activity.

129

130 ***III. Neuropeptide evolution: insights from the genome sequences of***  
131 ***species from an increasing variety of animal phyla***

132 Recently, genome sequence data have been obtained from an increasing  
133 variety of phyla, expanding the scope for genome-wide investigation of  
134 neuropeptide signalling systems beyond the vertebrates and “model”  
135 invertebrates such as *D. melanogaster* and *C. elegans*, which are both  
136 ecdysozoan protostomian invertebrates. For example, analysis of the  
137 repertoire of GPCRs in invertebrate chordates - the urochordate *Ciona*  
138 *intestinalis* [36] and the cephalochordate *Branchiostoma floridae* [37] - has  
139 revealed both loss and expansion of some neuropeptide receptor families. For  
140 example, in *B. floridae* there appears to have been an expansion of  
141 rhodopsin-type receptors related to mammalian neuropeptide FF (NPFF)  
142 receptors [37]).

143         The availability of genome sequence data has also enabled genome-  
144 wide investigation of neuropeptide signalling systems in lophotrochozoan  
145 protostomes, including the mollusc *Lottia gigantea* [38] and the annelids  
146 *Capitella teleta* and *Helobdella robusta* [39]. In 2010, a survey of the genome  
147 of the owl limpet *L. gigantea* identified over 40 neuropeptide precursors [38].  
148 Amongst these were the first homologs of bursicon, proctolin and allatostatin  
149 C (AST-C) to be identified in a molluscan species [38]. Subsequent surveys of  
150 the genomes of the polychaete worm *C. teleta* and the leech *H. robusta*  
151 identified 43 neuropeptide precursors in *C. teleta* and 35 neuropeptide  
152 precursors in *H. robusta* [39]. Interestingly, there were distinct differences  
153 between these two species. For example, *H. robusta* appears to have lost the  
154 bursicon-type and glycoprotein hormone (GPA2/GPB5)-type precursors and  
155 receptors that are present in *C. teleta* [39].



156 In 2013, two independent studies set out to analyse the growing body  
157 of genome sequence data from a range of phyla to investigate neuropeptide  
158 relationships and neuropeptide evolution in the animal kingdom. A core set of  
159 neuropeptide-receptor signalling pathways were traced back to the common  
160 ancestor of the Bilateria [9, 10], revealing relationships between  
161 neuropeptides in protostomes and deuterostomes that were not readily  
162 apparent from comparisons of the primary amino acid sequences of known  
163 bioactive or putative neuropeptides. For example, relationships were  
164 discovered between (i) deuterostomian orexin and protostomian allatotropin;  
165 (ii) deuterostomian neuropeptide S (NPS) and protostomian crustacean  
166 cardioactive peptide (CCAP); (iii) deuterostomian neuropeptide FF (NPFF)  
167 and protostomian SIFamide; (iv) vertebrate gastrin-releasing peptide (GRP)  
168 and endothelin and protostomian CCHamide and (v) deuterostomian galanin  
169 and protostomian allatostatin A (AST-A) [10].

170 Of particular importance in these studies were the analysis of genome  
171 sequence data from lophotrochozoan protostomes (annelids and molluscs)  
172 and non-chordate deuterostomes (the Ambulacraria; hemichordates and  
173 echinoderms). A good example of the importance of the use of  
174 lophotrochozoan and ambulacrarian genome sequence data was the  
175 unification of a bilaterian neuropeptide family that includes allatotropin and  
176 orexin-type precursors. The allatotropins were first identified as peptides  
177 stimulating the synthesis and secretion of juvenile hormone from the *corpora*  
178 *allata* in insects [40, 41]. The orexins were first identified as hypothalamic  
179 neuropeptides that stimulate food intake in mammals [42, 43], but it has  
180 subsequently been discovered that orexins also stimulate wakefulness and

181 energy expenditure [44]. The homology of allatotropins and orexins was not  
182 evident based solely on their primary amino acid sequences. However,  
183 analysis of the genome of the hemichordate *Saccoglossus kowalevskii*  
184 identified an orexin-type precursor with a conserved domain outside of the  
185 putative neuropeptide region [10]. This “cryptic” domain is present in all  
186 protostomian allatotropin-type precursors but had not previously been  
187 identified in orexin-type precursors because this domain appears to have  
188 been lost in the chordates. Therefore, the analysis of genome sequence data  
189 from an ambulacrarian was crucial in unifying a bilaterian neuropeptide family.

190

#### 191 *IV. The echinoderms: “bridging the gap” for reconstruction of* 192 *neuropeptide evolution*

193 The echinoderms are a phylum of marine organisms that together with the  
194 hemichordates form the Ambulacraria. The echinoderms comprise five extant  
195 classes - echinoids (e.g. sea urchins), holothurians (e.g. sea cucumbers),  
196 asteroids (e.g. starfish), ophiuroids (e.g. brittle stars) and crinoids (e.g. sea  
197 lilies/feather stars). The echinoids and holothurians form the echinozoan  
198 clade; the asteroids and ophiuroids form the asterozoan clade, whilst the  
199 crinoids are basal to the echinozoan and asterozoan clades [45, 46].

200 The echinoderms are particularly interesting for comparative and  
201 evolutionary studies on neuropeptide signalling systems for a number of  
202 reasons. The echinoderms are deuterostomian invertebrates and therefore  
203 “bridge” a huge evolutionary gap between the chordates and model  
204 protostomian invertebrates (e.g. *D. melanogaster* and *C. elegans*), providing  
205 key insights into the evolution of neuropeptide systems in the animal kingdom.

206 Furthermore, the echinoderms offer a unique context to investigate the  
207 evolution and diversity of neuropeptide function. The echinoderms exhibit  
208 pentaradial symmetry as adult animals that is derived from a bilateral body  
209 plan both evolutionarily and developmentally and consequently they have a  
210 decentralized nervous system [47, 48]. In addition, there is evidence that  
211 neuropeptides may be involved in mediating neural control of several unusual  
212 biological phenomena in the echinoderms including the ability to autotomize  
213 and then regenerate body parts [49] and the mutability of their collagenous  
214 tissue, which can rapidly change between stiff and soft mechanical states  
215 under the control of the nervous system [50, 51].

216

#### 217 *V. The sea urchin genome yields new insights into neuropeptide* 218 *evolution and diversity*

219 The first extensive analysis of neuropeptide signalling systems in an  
220 echinoderm species was enabled by sequencing of the genome of the sea  
221 urchin *Strongylocentrotus purpuratus* (Class Echinoidea) [52]. Approximately  
222 23,300 genes were identified in *S. purpuratus*, with representatives of nearly  
223 all vertebrate gene families [52]. The sea urchin has long been used as a  
224 model system for developmental and systems biology [53] but sequencing of  
225 the genome allowed exploration of numerous regulatory networks including  
226 the defensome, adhesome and the nervous system [52].

227 An initial analysis of *S. purpuratus* genome sequence data led to the  
228 identification of only a few neuropeptide precursors but a total of 37 candidate  
229 neuropeptide receptors [48, 52]. However, subsequent analysis of 2,026  
230 expressed sequence tags (ESTs) from an *S. purpuratus* radial nerve cDNA

231 library led to the identification of a total of 20 candidate neuropeptide/peptide  
232 hormone precursors in this species [54]. These included homologs of VP/OT,  
233 GnRH, calcitonin and a number of putative neuropeptides that were not  
234 recognized as homologs of known neuropeptides [54]. Below we highlight  
235 some of the more important and interesting discoveries that emerged from  
236 analysis of neuropeptide systems in the sea urchin.

237

238 *The first thyrotropin-releasing hormone (TRH)-type precursor to be*  
239 *discovered in an invertebrate*

240 Thyrotropin-releasing hormone (TRH) was discovered as a hypothalamic  
241 peptide that stimulates the release of thyroid-stimulating hormone (TSH) and  
242 prolactin from the anterior pituitary gland in mammals. TSH then triggers the  
243 release of the thyroid hormones triiodothyronine (T3) and thyroxine (T4) that  
244 stimulate metabolism and thus promote growth and development [55].  
245 However, in mammals, TRH also acts as a neurotransmitter or  
246 neuromodulator in other regions of the brain [56, 57]. Interestingly, in non-  
247 mammalian vertebrates (e.g. amphibians and fish), TRH stimulates the  
248 release of pituitary growth hormone and prolactin but has little or no effect on  
249 the secretion of TSH [58].

250 Analysis of *S. purpuratus* radial nerve cDNA sequence data enabled  
251 discovery of the first TRH-type precursor to be identified in an invertebrate  
252 [54]. This discovery indicated that the origin of the TRH-type neuropeptide  
253 signalling system dates back at least as far as the common ancestor of  
254 deuterostomes. The *S. purpuratus* TRH-type precursor is a 316-residue  
255 precursor protein comprising a predicted 15-residue N-terminal signal peptide

256 and 19 putative TRH-type peptides (see Figure 1). These include 10 copies of  
257 the sequence QYPGG, four copies of the sequence QWPGG and single  
258 copies of the sequences QFPAG, QFPGG, QFVGGELIPSPEL, QWPEV and  
259 QFVGGEALEQESNIN [54]. These putative neuropeptides are predicted to be  
260 subject to post-translational modifications including the conversion of an N-  
261 terminal glutamine residue to a pyroglutamate and use of the C-terminal  
262 glycine as a substrate for amidation, which although not unique to TRH are  
263 nevertheless two characteristic features of vertebrate TRH-type peptides [54].

264 Despite the occurrence of TRH-type receptors in the protostomes [9,  
265 10], the discovery of a TRH-type precursor in a protostomian species had,  
266 until recently, remained elusive. In 2015, it was discovered that  
267 FSEFLGamide is the ligand for a TRH-type receptor in the annelid *Platynereis*  
268 *dumerilii* [59]. It has therefore been proposed that the “EFLGamides” identified  
269 in the lophotrochozoans [60] are orthologous to deuterostomian TRH-type  
270 peptides [59]. Thus, the evolutionary origin of the TRH-type neuropeptide  
271 signalling system dates back to the common ancestor of the Bilateria and the  
272 discovery of the TRH-type precursor in the sea urchin *S. purpuratus* was a  
273 crucial step in providing evolutionary insights into this ancient neuropeptide  
274 family.

275

### 276 *The first pedal peptide/orcokinin-type neuropeptides to be* 277 *discovered in deuterostomes*

278 Pedal peptide (PLDSVYGTHTGMSGFA) was first isolated from the mollusc  
279 *Aplysia californica* as a peptide that causes contraction of pedal muscles [61,  
280 62]. In 2006, the *A. californica* pedal peptide precursor was identified through

281 analysis of transcriptome sequence data, revealing that the precursor  
282 contains 17 copies of pedal peptide as well as two other structurally related  
283 peptides [63]. Furthermore, in *Aplysia*, there are three additional precursors  
284 containing peptides related to pedal peptide [63]. Subsequently, pedal  
285 peptide-type precursors have also been identified in other molluscan species  
286 (e.g. *L. gigantea* [38]) and in annelids (e.g. *P. dumerilii* [60] and *C. teleta* [39]).

287         Analysis of *S. purpuratus* radial nerve cDNA sequence data led to the  
288 discovery of the first pedal peptide-type precursors to be identified in a  
289 deuterostomian invertebrate [54]. This discovery indicated that the origins of  
290 pedal peptide-type signalling dates back to the common ancestor of the  
291 Bilateria. The *S. purpuratus* pedal peptide-type precursor 1 (SpPPLNP1) is a  
292 510-residue protein comprising a 29-residue N-terminal signal peptide and 21  
293 copies of pedal peptide-like peptides (SpPPLN1a-i). The *S. purpuratus* pedal  
294 peptide-type precursor 2 (SpPPLNP2) is a 204-residue protein comprising a  
295 19-residue N-terminal signal peptide and 10 putative pedal peptide-like  
296 peptides (SpPPLN2a-i). Putative pedal peptides derived from both SpPPLNP1  
297 (e.g. SpPPLN1d) and SpPPLNP2 (e.g. SpPPLN2h) share a C-terminal SGFx  
298 motif (where x is a hydrophobic residue) with pedal peptide in *Aplysia*, whilst  
299 also sharing similar characteristics with respect to the number of residues and  
300 distribution of hydrophobic and hydrophilic residues [54].

301         The discovery of SpPPLNP1 and SpPPLNP2 also enabled the  
302 identification of pedal peptide-type precursors in the nematode *C. elegans*  
303 [54] that share sequence similarity with arthropod orcokinin-type peptides [54].  
304 Orcokinin was first isolated from abdominal nerve cord extracts of the crayfish  
305 *Orconectus limosus* on account of its effect in stimulating hindgut myoactivity

306 [64]. Subsequently, orcokinin-type peptides have been identified in a number  
307 of arthropods and attributed a range of functions (e.g. regulation of  
308 ecdysteroidogenesis in the silk moth *Bombyx mori* [65]). The discovery of the  
309 *S. purpuratus* pedal peptide-type precursors provided a crucial step in unifying  
310 lophotrochozoan pedal peptides with ecdysozoan orcokinin-type peptides and  
311 in demonstrating the existence of a bilaterian family of pedal  
312 peptide/orcokinin-type peptides.

313

#### 314 ***NG peptides unify a bilaterian neuropeptide family***

315 A 266-residue protein in the sea urchin *S. purpuratus* comprising a predicted  
316 26-residue N-terminal signal peptide and two tandem copies of the sequence  
317 NGFFFG bounded by dibasic cleavage sites [66] was discovered on account  
318 of sequence similarity that its constituent neuropeptide (NGFFFamide) shares  
319 with NGIWYamide - a myoactive neuropeptide this is a potent inducer of  
320 oocyte maturation and spawning in the sea cucumber *Apostichopus japonicus*  
321 [67, 68]. A surprising feature of the NGFFFamide precursor was the presence  
322 of a C-terminal neurophysin domain [66]. Hitherto, neurophysins were thought  
323 to be a unique feature of VP/OT-type precursors, in which they are required  
324 for axonal transport and secretion of the neurohypophyseal hormones VP and  
325 OT [69].

326 The discovery of the sea urchin NGFFFamide precursor led to the  
327 discovery of the “NG peptide” family in deuterostomian invertebrates; so  
328 called because they have in a common an asparagine (N) – glycine (G) motif  
329 [70]. Interestingly, an NG peptide precursor in the cephalochordate *B. floridae*  
330 comprises two copies of a putative neuropeptide with the sequence

331 SFRNGVamide [70], which is identical to the N-terminal region of  
332 neuropeptide S (NPS) (SFRNGVGTGMKKTSFQRAKS) in humans [71]. NPS-  
333 type peptides are found in the tetrapod vertebrates and have been shown to  
334 have anxiolytic-like effects in humans and rodents [71-73]. Furthermore, NPS  
335 has been identified as the ligand for the human receptor GPR154, which is  
336 paralogous to VP/OT-type receptors [74].

337 A broader phylogenetic analysis revealed that orthologs of NPS-type  
338 receptors are also found in invertebrates [9, 10]. Furthermore, the ligand that  
339 activates the NPS-type receptor in *Drosophila* is crustacean cardioactive  
340 peptide (CCAP; PFCNAFTGCamide) [33], a neuropeptide that controls  
341 ecdysis behaviour in arthropods [75, 76]. NPS and CCAP share very little  
342 sequence similarity and therefore the discovery that their receptors are  
343 orthologous was unexpected. However, it was noted that CCAP shares  
344 superficial sequence similarity with VP/OT-type peptides by virtue of a  
345 disulphide bridge between two cysteine residues [77]. In addition, the finding  
346 that NPS/CCAP-type receptors are paralogous to VP/OT-type receptors  
347 suggested that CCAP and VP/OT-type peptides may have evolved from a  
348 common ancestral molecule [10]. However, the relationship between NPS and  
349 VP/OT-type peptides or CCAP was unclear. In this respect, the discovery of  
350 NG peptides in echinoderms and other deuterostomian invertebrates was  
351 crucial in providing the “missing link” between previously unassociated  
352 neuropeptide signalling systems.

353 Analysis of genome sequence data revealed that NPS/CCAP-type  
354 receptors are also present in deuterostomian invertebrates including in the  
355 sea urchin *S. purpuratus* [10, 78]. In accordance with sequence similarity



356 shared by SFRNGVamide in the cephalochordate *B. floridae* and NPS  
357 (SFRNGVGTGMKTSFQRAKS) in tetrapod vertebrates, it was hypothesized  
358 that the NG peptides may be the ligands for the NPS/CCAP-type receptors in  
359 deuterostomian invertebrates. Crucially, it has recently been shown that the  
360 NG peptide NGFFFamide is present in extracts of the sea urchin *S.*  
361 *purpuratus* and activates a *S. purpuratus* NPS/CCAP-type receptor [79]. This  
362 finding unites a bilaterian family of neuropeptides that includes NPS-type  
363 peptides in tetrapod vertebrates, NG peptides in deuterostomian invertebrates  
364 and CCAP-type peptides in protostomian invertebrates. Furthermore, it  
365 provides support for a scenario of neuropeptide-receptor evolution that has  
366 been postulated based on phylogenetic reconstruction of bilaterian  
367 neuropeptide signalling systems [10, 80]. In this evolutionary scenario, an  
368 ancestral VP/OT-type precursor gene duplicated and one copy retained the  
369 highly conserved features of VP/OT-type precursors. The second copy  
370 diverged through evolution to give rise to genes encoding NPS-type peptides  
371 in vertebrates, NG peptides in deuterostomian invertebrates and CCAP-type  
372 peptides in protostomian invertebrates (see Figure 2).

373 We propose that the bilaterian neuropeptide family comprising  
374 NPS/CCAP-type peptides are collectively known as NG peptides. In support  
375 of this proposal, the NG motif is not only a feature of NPS and NG peptides in  
376 deuterostomes but also a feature of CCAP-type peptides in molluscs. For  
377 example, the NG motif is present in CCAP-type peptides in the owl limpet *L.*  
378 *gigantea* [38] and in other molluscan species, including *Conus villepini* (GI:  
379 325529921) and *A. californica* (GI: 524893759) [79]. Thus, it appears that the  
380 NG motif is a unifying characteristic of this bilaterian family of neuropeptides,

381 but with subsequent loss or substitution of the glycine residue in some CCAP-  
382 type peptides (see Figure 3).

383

#### 384 *VI. Starfish neural transcriptome provides new insights into* 385 *neuropeptide evolution and diversity*

386 As highlighted above, analysis of the genome/transcriptome of the sea urchin  
387 *S. purpuratus* (Class Echinoidea) has demonstrated the importance of  
388 echinoderms in providing key insights into neuropeptide evolution. Analysis of  
389 transcriptome sequence data for neuropeptide-related transcripts has  
390 subsequently been extended to species belonging to other echinoderm  
391 classes. For example, analysis of the transcriptome of the sea cucumber *A.*  
392 *japonicus* (Class Holothuroidea) resulted in the identification of 17  
393 neuropeptide/neurohormone precursors [81]. More recently, transcriptome  
394 sequence data obtained for the brittle star *Ophionotus victoriae* (Class  
395 Ophiuroidea) and the feather star *Antedon mediterranea* (Class Crinoidea)  
396 [82] has enabled identification of SALMFamide precursors in these species,  
397 providing new insights into the evolution of the SALMFamide family of  
398 neuropeptides in echinoderms [82].

399 The most extensive analysis of echinoderm neuropeptide signalling  
400 systems to date has been enabled by sequencing of the radial nerve cord  
401 transcriptome from the common European starfish *Asterias rubens* (Class  
402 Asteroidea) [83]. This led to the identification of 40 neuropeptide precursors  
403 including the first tachykinin, somatostatin, pigment-dispersing factor (PDF)  
404 and corticotropin-releasing hormone (CRH)-type precursors to be discovered  
405 in the echinoderm/ambulacrarian clade of the animal kingdom [83]. Amongst

406 the most interesting findings from this analysis, which are highlighted below,  
407 were the discovery of the first kisspeptin and melanin-concentrating hormone  
408 (MCH)-type precursors to be identified outside of the chordates [83].  
409 Furthermore, identification of the precursors of two GnRH-like peptides in *A.*  
410 *rubens* provided a basis for functional characterisation of receptors for these  
411 neuropeptides, which has provided new insights into the evolution of GnRH-  
412 related neuropeptide signalling systems, as also discussed below.

413

#### 414 ***The first kisspeptins to be discovered in a non-chordate***

415 Kisspeptins are a family of structurally related neuropeptides derived from  
416 differential proteolytic processing of a precursor protein encoded by the KiSS-  
417 1 gene. The most abundant is kisspeptin-54, which can be cleaved to 14, 13  
418 and 10 residue kisspeptins that share a common C-terminal RFamide motif  
419 [84]. Kisspeptins regulate reproductive maturation in humans and other  
420 mammals [85] by triggering the hypothalamic secretion of GnRH, which  
421 stimulates the release of gonadotropins from the pituitary gland [86]. The role  
422 of kisspeptin in regulating reproductive maturation has also been described in  
423 non-mammalian vertebrates [87, 88], whilst a kisspeptin-type precursor was  
424 recently discovered in the cephalochordate *B. floridae* [10].

425 Analysis of the *A. rubens* neural transcriptome identified a 149-residue  
426 precursor protein comprising two putative kisspeptin-type peptides (ArKP1-2;  
427 see Figure 1) [83]. ArKP1 shares a C-terminal NxxSxxLxF-NH<sub>2</sub> motif with  
428 human kisspeptin. However, unlike human kisspeptin, ArKP1 has two  
429 cysteine residues in its N-terminal region that may form a disulfide bridge -  
430 this feature of ArKP1 also occurs in a putative kisspeptin-type peptide in the

431 sea urchin *S. purpuratus*, and therefore it may be a characteristic of  
432 echinoderm kisspeptins [83]. ArKP2 is similar to ArKP1 but it lacks the N-  
433 terminal pair of cysteine residues present in ArKP1 and has additional  
434 residues in the C-terminal region of the putative neuropeptide.

435 The discovery of the *A. rubens* kisspeptin-type precursor is consistent  
436 with the occurrence of kisspeptin-type receptors in non-chordates [9, 10],  
437 although both kisspeptin-type precursors and receptors appear to have been  
438 lost in urochordates and ecdysozoans [10]. The discovery of ArKP1 and  
439 ArKP2 provides an exciting opportunity to investigate the physiological roles  
440 of kisspeptins in an invertebrate for the very first time.

441

442 *The first melanin-concentrating hormone (MCH)-type neuropeptide*  
443 *to be discovered in a non-chordate*

444 Melanin-concentrating hormone (MCH) was first discovered in teleost fish on  
445 account of its effect of inducing a change in body colour [89, 90]. MCH-type  
446 peptides have subsequently been identified throughout the vertebrates [91-93]  
447 and have been implicated in a range of physiological roles including the  
448 regulation of feeding, sleep and reproduction [94, 95].

449 Analysis of the *A. rubens* neural transcriptome identified an 88-residue  
450 precursor protein with a predicted 28-residue MCH-type peptide (ArMCH; see  
451 Figure 1) [83]. The location of the putative MCH-type peptide in the C-terminal  
452 region of the precursor is likewise a characteristic of MCH-type precursors in  
453 vertebrates [96]. Furthermore, vertebrate MCH-type peptides have a  
454 conserved pair of cysteine residues that form a disulphide bridge and,  
455 accordingly, the presence of two cysteine residues in ArMCH indicates that

456 the starfish peptide also has a disulphide bridge [97]. Identification of the *A.*  
457 *rubens* MCH-type precursor also facilitated identification of MCH-type  
458 precursors in the sea urchin *S. purpuratus* and the hemichordate *S.*  
459 *kowalevskii* [83].

460 The discovery of the *A. rubens* MCH-type precursor is consistent with  
461 the occurrence of MCH-type receptors in non-chordates including the  
462 cephalochordate *B. floridae* and the hemichordate *S. kowalevskii* [9, 10].  
463 However, to date, MCH-type precursors and receptors have not been found in  
464 protostomes, which indicates that MCH-type neuropeptide signalling may be  
465 restricted to the deuterostomian branch of the animal kingdom [9, 10]. Thus,  
466 the discovery of a putative MCH-type peptide in *A. rubens* provides a unique  
467 opportunity to investigate the physiological roles of a MCH-type peptide in an  
468 invertebrate for the first time.

469

470 *Starfish reveal the evolutionary origins of paralogous*  
471 *gonadotropin-releasing hormone (GnRH) and corazonin (CRZ)*  
472 *signalling pathways*

473 Gonadotropin-releasing hormone (GnRH) is widely known as a regulator of  
474 reproductive maturation in the vertebrates [34, 35]. It has also been  
475 discovered that homologs of GnRH occur in invertebrates. These include  
476 adipokinetic hormone (AKH), red pigment concentrating hormone (RPCH)  
477 [31-33], corazonin (CRZ) [33, 98] and AKH/CRZ-related peptide (ACP), which  
478 are found in insects and other arthropods [99, 100]. The AKHs are a family of  
479 lipid-mobilizing hormones released during flight and locomotion in insects [31].  
480 CRZ was discovered on account of its stimulatory effect on heart rate in

481 cockroaches [101] but has been implicated in a range of functions in the  
482 arthropods (e.g. initiating ecdysis in moths via the release of pre-ecdysis-  
483 triggering hormone (PETH) and ecdysis-triggering hormone (ETH) ) [102].  
484 ACP is a paralog of AKH that arose in a common ancestor of the arthropods.  
485 However, despite insights into its evolutionary origins, the function of ACP  
486 remains unclear [103]. Recently, there has been debate as to the relationship  
487 of CRZ to AKH, ACP and GnRH. For example, it has been proposed that  
488 AKH/ACP and CRZ neuropeptides are both orthologous to vertebrate GnRH  
489 [9, 29, 30]. However, other studies have been inconclusive in establishing this  
490 relationship [10, 100].

491 A GnRH-like peptide (pQILCARAFTYTHTW-NH<sub>2</sub>) that activates one of  
492 two CRZ-type receptors has been identified in the cephalochordate *B. floridae*  
493 based upon analysis of genomic sequence data [104]. However, insect AKH  
494 also activates the same *B. floridae* CRZ-type receptor [105] and therefore it  
495 was unclear whether or not there are distinct GnRH-type and CRZ-type  
496 neuropeptide signalling systems in deuterostomes.

497 The identification of precursors of two GnRH-like peptides in *A. rubens*  
498 [83] has provided new insights into this issue because it has been found that  
499 one of the peptides (pQIHYNPVGWPG-NH<sub>2</sub>; structure confirmed by mass  
500 spectrometry) activates an *A. rubens* GnRH-type receptor and the other  
501 peptide (HNTFTMGGQNRWKAG-NH<sub>2</sub>; structure confirmed by mass  
502 spectrometry ) activates an *A. rubens* CRZ-type receptor (see Figure 1) [106].  
503 Importantly, no cross-activation between the two ligand-receptor pairs was  
504 observed, demonstrating the existence of two distinct signalling systems  
505 [106]. These findings indicate that the evolutionary origin of the paralogous

506 GnRH-type and CRZ-type signalling systems can be traced back to gene  
507 duplication in a common ancestor of the Bilateria.

508

### 509 *VII. Conclusions and directions for future research*

510 Genome-wide studies have begun to unravel the evolutionarily ancient origins  
511 of neuropeptide signalling systems [9, 10] and analysis of neuropeptide  
512 systems in echinoderms has provided some key insights. Thus, identification  
513 of ligand-receptor pairs in the sea urchin *S. purpuratus* and the starfish *A.*  
514 *rubens* has revealed how ancient gene duplications gave rise to the bilaterian  
515 NG peptide [79] and GnRH/CRZ [106] neuropeptide families, respectively.  
516 Looking ahead, echinoderm genome/transcriptome sequence data presents  
517 us with many more interesting questions. For example, the presence of  
518 neuropeptide Y (NPY) and galanin-type receptors in the sea urchin genome  
519 indicates the presence of NPY and galanin-type peptides, but these have yet  
520 to be identified [9, 10]. Addressing these issues may be aided by analysis of  
521 sequence data from other echinoderms, including brittle stars (Class  
522 Ophiuroidea) and sea lilies/feather stars (Class Crinoidea) [82].

523 In conclusion, the availability of sequence data has provided a  
524 molecular phylogenetic framework to probe how orthologous neuropeptide  
525 systems are used to regulate physiological and behavioural processes in  
526 evolutionarily distant phyla. Looking forward into an era of post-genomic  
527 functional analysis of neuropeptide signalling, we anticipate that by virtue of  
528 their phylogenetic position as non-chordate deuterostomes, echinoderms will  
529 continue to provide us with many more missing pieces in the “jigsaw puzzle”

530 of neuropeptide evolution. Furthermore, with the unique perspective of a  
531 decentralized and pentaradial bauplan [47, 48], we expect some surprises!

532



533 **Key Points**

- 534 • Neuropeptides are evolutionarily ancient mediators of neuronal signalling  
535 controlling a range of physiological processes and behaviours.
- 536 • Genomic/transcriptomic analysis of neuropeptide signalling systems in  
537 echinoderms has recently provided key insights into neuropeptide  
538 evolution.
- 539 • Sequencing of the sea urchin *Strongylocentrotus purpuratus* genome  
540 enabled discovery of the first invertebrate thyrotropin-releasing hormone  
541 (TRH)-type precursor, the first deuterostomian pedal peptide/orcokinin-  
542 type precursors and the unification of a bilaterian NG peptide family.
- 543 • Sequencing of the starfish *Asterias rubens* neural transcriptome enabled  
544 identification of 40 novel neuropeptide precursors, including the first  
545 kisspeptin and melanin-concentrating hormone (MCH)-type precursors to  
546 be discovered outside of the chordates and the discovery of the first  
547 corazonin-type neuropeptide receptor to be deorphanized in a  
548 deuterostome.
- 549 • Discovery of neuropeptide signalling systems in echinoderms provides  
550 opportunities to investigate neuropeptide function in the unique context of  
551 a decentralized and pentaradial bauplan.

552

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556

557

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899 **Figure Legends**

900

901 **Figure 1. Echinoderm neuropeptides that have provided new insights**  
902 **into the evolution of neuropeptide signalling systems.** The sequences of  
903 sea urchin (*Strongylocentrotus purpuratus*) and starfish (*Asterias rubens*)  
904 representatives of six selected neuropeptide types are shown. Predicted or  
905 confirmed post-translational modifications, including conversion of an N-  
906 terminal glutamine (Q) to a pyro-glutamyl (pQ) residue and conversion of a  
907 C-terminal glycine (G) to an amide group (-NH<sub>2</sub>), are depicted and cysteine  
908 (C) residues that form or are predicted to form a disulphide bridge are  
909 underlined. Numbers in parentheses represent the number of copies of the  
910 neuropeptide in the corresponding precursor if this is greater than one. The  
911 image of *S. purpuratus* was obtained from  
912 <https://openclipart.org/detail/170807/sea-urchin-silhouette>, whilst the image of  
913 *A. rubens* was created by M. Zandawala (Stockholm University). **Key:** TRH:  
914 thyrotropin-releasing hormone; MCH: melanin-concentrating hormone; GnRH:  
915 gonadotropin-releasing hormone. **References:** (a) [54]; (b) [66]; (c) [83]; (d)  
916 [107]; (e) [106].

917

918 **Figure 2. Evolution of the VP/OT-type and NG peptide signalling**  
919 **systems.** The diagram shows how duplication of a vasopressin/oxytocin  
920 (VP/OT)-type neuropeptide signalling system in the common ancestor of the  
921 Bilateria gave rise to the highly conserved VP/OT-type (red boxes) and the  
922 divergent neuropeptide S (NPS) (blue boxes), NG peptide (purple boxes) and  
923 crustacean cardioactive peptide (CCAP)-type signalling systems (green

924 boxes) in extant bilaterians. Phyla where neuropeptide ligand-receptor pairs  
925 have been pharmacologically characterised are labelled with a yellow  
926 asterisk. A blue cross (and white box) represents loss of the NPS-type  
927 signalling system in the urochordates, whilst a red cross (and white box)  
928 represents loss of the CCAP-type signalling system in the nematodes. The  
929 image of *S. purpuratus* was obtained from  
930 <https://openclipart.org/detail/170807/sea-urchin-silhouette>, whilst images of  
931 other representative species from each phylum were obtained from  
932 <http://phylopic.org> or were created by the authors or by M. Zandawala  
933 (Stockholm University). **References: (a)** [108]; **(b)** [109]; **(c)** [110]; **(d)** [111];  
934 **(e)** [112]; **(f)** [113]; **(g)** [59]; **(h)** [114]; **(i)** [115]; **(j)** [116]; **(k)** [71]; **(l)** [79]; **(m)**  
935 [117]; **(n)** [118].

936

937 **Figure 3. The NG peptide family.** Schematic showing an alignment of  
938 putative or confirmed neuropeptide(s) derived from neuropeptide S (NPS), NG  
939 peptide and crustacean cardioactive peptide (CCAP)-type precursors in  
940 representative species from phyla across the Bilateria. The conserved NG  
941 motif of NPS, NG peptides and CCAP-type peptides is highlighted in red and  
942 cysteine (C) residues that form or are predicted to form a disulphide bridge  
943 are underlined. A red cross represents loss of the NPS-type signalling system  
944 in the urochordates (e.g. *C. intestinalis*) or CCAP-type signalling system in the  
945 nematodes (e.g. *C. elegans*). Numbers in parentheses represent the number  
946 of copies of the neuropeptide in the precursor if this is greater than one. The  
947 image of *S. purpuratus* was obtained from  
948 <https://openclipart.org/detail/170807/sea-urchin-silhouette>, whilst images of

949 other representative species from each phylum were obtained from  
950 <http://phylopic.org> or were created by the authors or by M. Zandawala  
951 (Stockholm University). **Key:** *H. sapiens*: *Homo sapiens*; *C. intestinalis*: *Ciona*  
952 *intestinalis*; *B. floridae*: *Branchiostoma floridae*; *S. kowalevskii*: *Saccoglossus*  
953 *kowalevskii*; *S. purpuratus*: *Strongylocentrotus purpuratus*; *L. gigantea*: *Lottia*  
954 *gigantea*; *A. californica*: *Aplysia californica*; *P. dumerilii*: *Platynereis dumerilii*;  
955 *T. castaneum*: *Tribolium castaneum*; *C. elegans*: *Caenorhabditis elegans*.  
956 **References:** (a) [71]; (b) [70]; (c) [66]; (d) [38]; (e) [60]; (f) [119]; (g) [120].



*Strongylocentrotus purpuratus*



*Asterias rubens*

**TRH**

pQFVGGELIPSEL  
pQFVGGEALEQESNIN  
pQYPG-NH<sub>2</sub> (x10)  
pQWPG-NH<sub>2</sub> (x4)  
pQFPA-NH<sub>2</sub>  
pQFPG-NH<sub>2</sub>  
pQWPEV

(a)

pQYPPGGAPIGLD-NH<sub>2</sub>  
pQWYT-NH<sub>2</sub> (x11)

(c)

**NG peptide**

NGFFF-NH<sub>2</sub> (x2)

(b)

NGFFY-NH<sub>2</sub> (x2)

(d)

**Kisspeptin**

SRCRGRQCRNVGGLNPNANLRPLPF-NH<sub>2</sub>  
GRTKNRIRERVPFLPF-NH<sub>2</sub>

(c)

SGRCRSGTKCIMRGNPNTASRVLPF-NH<sub>2</sub>  
GRGPPKNSRARGGRTLLPF-NH<sub>2</sub>

(c)

**MCH**

SRSGRKLRFCMDVIRNTWRLCRNTRSN

(a, c)

DRPNRREVTYCMDWIHNTWRPCRGRKAG

(c)

**GnRH**

pQVHHRFSGWRPG-NH<sub>2</sub>

(a)

pQIHYNPGWGPG-NH<sub>2</sub>

(c, e)

**Corazonin**

HNTFSFKGRSYFP-NH<sub>2</sub>

(a, c)

HNTFTMGGQNRWKAG-NH<sub>2</sub>

(c, e)



