The evolution of neuropeptide signalling: insights from echinoderms

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Abstract

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

Author Biography

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Key Words

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

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

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

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

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

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

II. Neuropeptide relationships: insights from the first animal genome sequences

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

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

Insect AKHs are lipid-mobilizing hormones released during flight and locomotion [31]. In 2002, the receptor for *Drosophila* AKH (pQLTFSPDWG-NH_2) was identified and pharmacologically characterized [32, 33]. Interestingly, it was found that insect AKH receptors are structurally and evolutionarily related to vertebrate GnRH receptors. In mammals, GnRH controls reproductive maturation and function by stimulating release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gland [34, 35], but mammalian GnRH (e.g. human GnRH is pQHWSYGLRPG-NH_2) shares only modest sequence similarity with AKH. Thus, the discovery of insect AKH receptors enabled unification of a bilaterian neuropeptide family that hitherto had not been recognized based on primary sequence similarity or biological activity.

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

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

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

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

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

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

IV. The echinoderms: "bridging the gap" for reconstruction of neuropeptide evolution

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

The echinoderms are particularly interesting for comparative and evolutionary studies on neuropeptide signalling systems for a number of reasons. The echinoderms are deuterostomian invertebrates and therefore "bridge" a huge evolutionary gap between the chordates and model protostomian invertebrates (e.g. *D. melanogaster* and *C. elegans*), providing 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 (PLDSVYGTHGMSGFA) 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 (SFRNGVGTGMKKTSFQRAKS) 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 villepinii* (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,

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

VI. Starfish neural transcriptome provides new insights into neuropeptide evolution and diversity

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

The most extensive analysis of echinoderm neuropeptide signalling systems to date has been enabled by sequencing of the radial nerve cord transcriptome from the common European starfish *Asterias rubens* (Class Asteroidea) [83]. This led to the identification of 40 neuropeptide precursors including the first tachykinin, somatostatin, pigment-dispersing factor (PDF) and corticotropin-releasing hormone (CRH)-type precursors to be discovered 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_2 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

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

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

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

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

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

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

A GnRH-like peptide (pQILCARAFTYTHTW-NH_2) that activates one of two CRZ-type receptors has been identified in the cephalochordate *B. floridae* based upon analysis of genomic sequence data [104]. However, insect AKH also activates the same *B. floridae* CRZ-type receptor [105] and therefore it was unclear whether or not there are distinct GnRH-type and CRZ-type neuropeptide signalling systems in deuterostomes.

The identification of precursors of two GnRH-like peptides in *A. rubens* [83] has provided new insights into this issue because it has been found that one of the peptides (pQIHYKNPGWGPG-NH_2; structure confirmed by mass spectrometry) activates an *A. rubens* GnRH-type receptor and the other peptide (HNTFTMGGQNRWKAG-NH_2; structure confirmed by mass spectrometry) activates an *A. rubens* CRZ-type receptor (see Figure 1) [106]. Importantly, no cross-activation between the two ligand-receptor pairs was observed, demonstrating the existence of two distinct signalling systems [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

Key Points

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

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Figure Legends

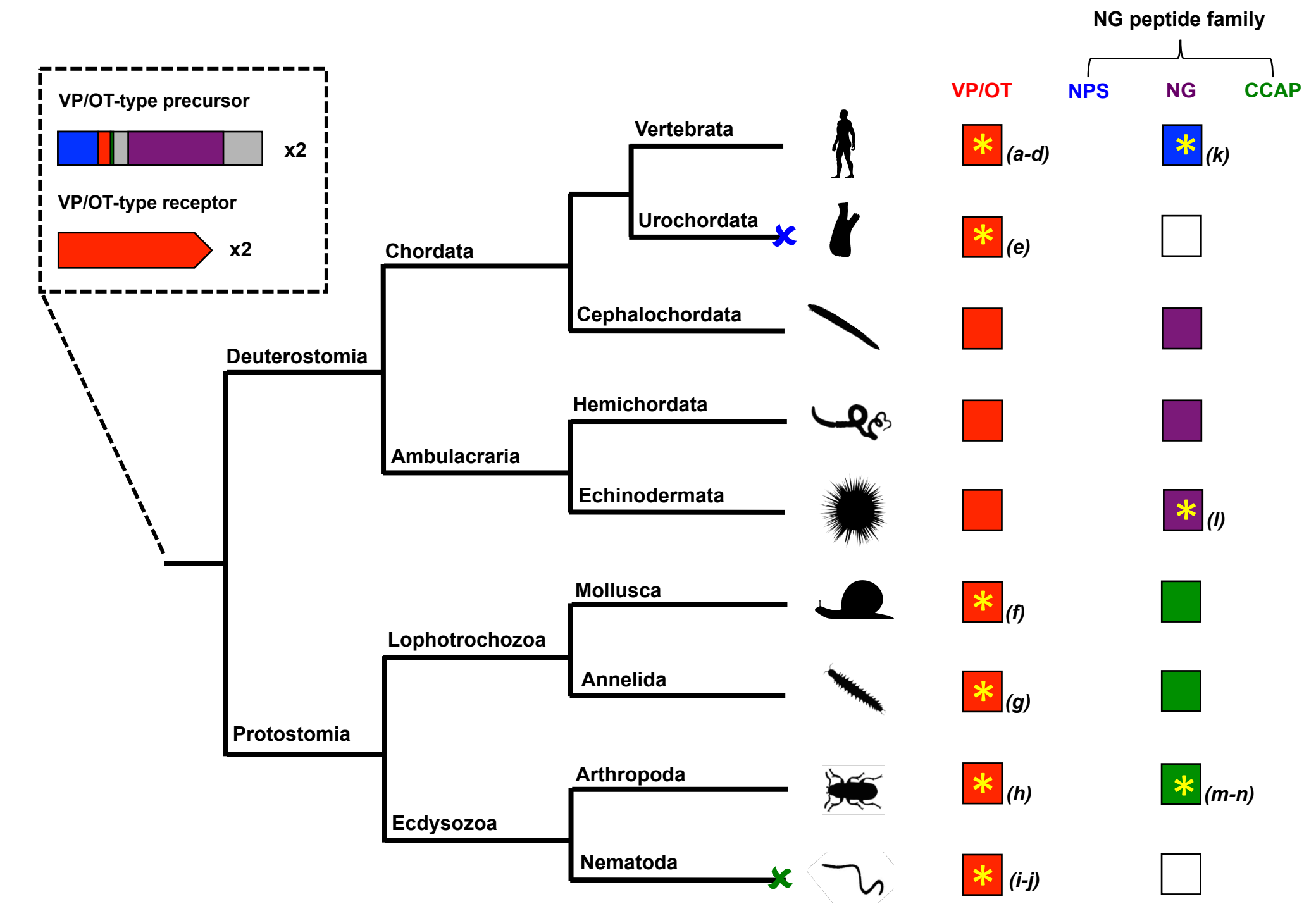
Figure 1. Echinoderm neuropeptides that have provided new insights into the evolution of neuropeptide signalling systems. The sequences of sea urchin (*Strongylocentrotus purpuratus)* and starfish (*Asterias rubens*) representatives of six selected neuropeptide types are shown. Predicted or confirmed post-translational modifications, including conversion of an N-terminal glutamine (Q) to a pyro-glutaminyl (pQ) residue and conversion of a C-terminal glycine (G) to an amide group (-NH_2), are depicted and cysteine (C) residues that form or are predicted to form a disulphide bridge are underlined. Numbers in parentheses represent the number of copies of the neuropeptide in the corresponding precursor if this is greater than one. The image of *S. purpuratus* was obtained from https://openclipart.org/detail/170807/sea-urchin-silhouette, whilst the image of *A. rubens* was created by M. Zandawala (Stockholm University). **Key:** TRH: thyrotropin-releasing hormone; MCH: melanin-concentrating hormone; GnRH: gonadotropin-releasing hormone. **References: (a)** [54]; **(b)** [66]; **(c)** [83]; **(d)** [107]; **(e)** [106].

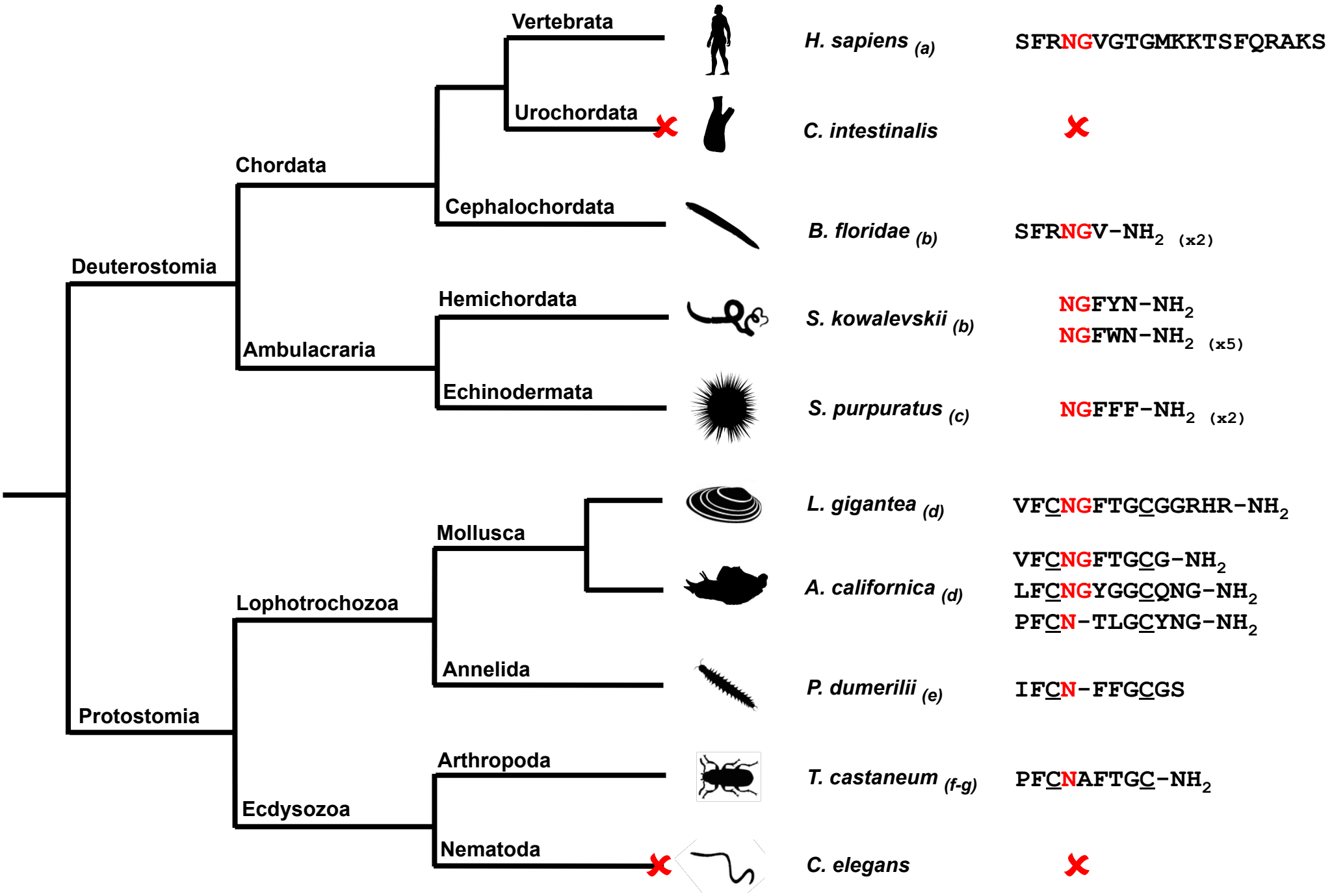
Figure 2. Evolution of the VP/OT-type and NG peptide signalling systems. The diagram shows how duplication of a vasopressin/oxytocin (VP/OT)-type neuropeptide signalling system in the common ancestor of the Bilateria gave rise to the highly conserved VP/OT-type (red boxes) and the divergent neuropeptide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

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

	 *Strongylocentrotus purpuratus*	 *Asterias rubens*
TRH	**pQFVGGELIPSPEL** **pQFVGGEALEQESNIN** **pQYPG-NH_2 (x10)** **pQWPG-NH_2 (x4)** **pQFPA-NH_2** **pQFPG-NH_2** **pQWPEV** ***(a)***	**pQYPGGAPIGLD-NH_2** **pQWYT-NH_2 (x11)** ***(c)***
NG peptide	**NGFFF-NH_2 (x2)** ***(b)***	**NGFFY-NH_2 (x2)** ***(d)***
Kisspeptin	**SRCRGRQCRNVGGLNPNANLRPLPF-NH_2** **GRTKNRIRERVPHFLPF-NH_2** ***(c)***	**SGRCRSGTKCIMRGPNPNTASRVLPF-NH_2** **GRGPPKNSRARGGRTLLPF-NH_2** ***(c)***
MCH	**SRSGRKLRFCMDVIRNTWRLCRNTRSN** ***(a, c)***	**DRPNRREVTYCMDWIHNTWRPCRGRKAG** ***(c)***
GnRH	**pQVHHRFSGWRPG-NH_2** ***(a)***	**pQIHYKNPGWGPG-NH_2** ***(c, e)***
Corazonin	**HNTFSFKGRSYFP-NH_2** ***(a, c)***	**HNTFTMGGQNRWKAG-NH_2** ***(c, e)***


NG peptide family
VP/OT
NPS
NG
CCAP
VP/OT-type precursor
x2
VP/OT-type receptor
x2
Vertebrata
Urochordata
Chordata
Cephalochordata
Deuterostomia
Hemichordata
Ambulacraria
Echinodermata
Mollusca
Lophotrochozoa
Annelida
Protostomia
Arthropoda
Ecdysozoa
Nematoda
(a-d)
(k)
(e)
(f)
(l)
(g)
(h)
(m-n)
(i-j)


Vertebrata
Urochordata
Chordata
Cephalochordata
Deuterostomia
Hemichordata
Ambulacraria
Echinodermata
Mollusca
Lophotrochozoa
Annelida
Protostomia
Arthropoda
Ecdysozoa
Nematoda
H. sapiens (a)
SFRNGVGTGMKKTSFQRAKS
C. intestinalis
B. floridae (b)
SFRNGV-NH2 (x2)
S. kowalevskii (b)
NGFYN-NH2
NGFWN-NH2 (x5)
S. purpuratus (c)
NGFFF-NH2 (x2)
L. gigantea (d)
VFCNGFTGCGGRHR-NH2
A. californica (d)
VFCNGFTGCG-NH2
LFCNGYGGCQNG-NH2
PFCN-TLGCYNG-NH2
P. dumerilii (e)
IFCN-FFGCGS
T. castaneum (f-g)
PFCNAFTGC-NH2
C. elegans