SHORT COMMUNICATION

From gonadotropin-inhibitory hormone to SIFamides: are echinoderm SALMFamides the "missing link" in a bilaterian family of neuropeptides that regulate reproductive processes?

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Abstract

Gonadotropin-inhibitory hormone (GnIH) belongs to a family of vertebrate neuropeptides with a C-terminal PxRFamide motif, which exert effects by activating the G-protein coupled receptors NPFF1 and/or NPFF2. Comparative genomics has revealed that orthologs of NPFF1/NPFF2-type receptors occur throughout the bilateria and the neuropeptide ligand that activates the *Drosophila* NPFF1/NPFF2-type receptor has been identified as AYRKPPFNSIFamide ("SIFamide"). Therefore, SIFamide-type neuropeptides, which occur throughout protostomian invertebrates, probably share a common evolutionary origin with vertebrate PxRFamide-type neuropeptides. Based on structural similarities, here SALMFamide neuropeptides are identified as candidate ligand components of this ancient bilaterian peptide-receptor signaling system in a deuterostomian invertebrate phylum, the echinoderms (e.g. starfish, sea urchins). Furthermore, functional studies provide evidence that PxRFamide/SALMFamide/SIFamide-type neuropeptides have evolutionarily conserved roles in regulation (typically inhibitory) of reproductive processes.

Key words: GnIH; NPFF; SIFamide; SALMFamide; FMRFamide

Thirty years ago the pentapeptide LPLRFamide was identified in extracts of chicken brain on account of its immunoreactivity with antibodies to the molluscan cardioexcitatory neuropeptide FMRFamide [5]. It was the first FMRFamide-like immunoreactive peptide to be discovered in a vertebrate species. Discovery and functional characterisation of an N-terminally extended homolog of LPLRFamide from quail brain (SIKPSAYLPLRFamide) revealed that this peptide acts as a gonadotropin-inhibitory hormone (GnIH) by inhibiting pituitary gonadotropin release [37, 39].

Avian GnIH is derived from a precursor protein that contains two other related peptides, GnIH-RP1 and GnIH-RP2, which share with GnIH the C-terminal motif LPxRFamide (where x is L or Q) [35]. GnIH-like neuropeptides with the C-terminal motif LPxRFamide have also been identified in humans and other mammals [17, 23] and evidence that these peptides suppress reproductive activity in mammals has also been obtained [1]. The receptor that mediates effects of GnIH-type neuropeptides has been identified as the G-protein coupled receptor GPR147 or NPFF1 [2, 23, 29]. Furthermore, consistent with the physiological actions of GnIH, NPFF1 is expressed in the hypothalamic-pituitary axis as well as in other brain regions [14, 38]. A paralog of the GnIH receptor, GPR74 or NPFF2 [2, 13, 29], is activated by two RFamide-type neuropeptides (NPFF and NPAF; [44]) that are derived from a different precursor protein to GnIH-like neuropeptides but which have a C-terminal motif (PQRFamide) similar to GnIH [32, 43]. The NPFF2 receptor is expressed in several regions of the central nervous system, including the dorsal horn of the spinal cord, and consistent with its expression in the dorsal horn, NPFF and NPAF attenuate morphine-induced anti-nociception in mammals [2, 43, 44].

Sequencing of the genome of the insect *Drosophila melanogaster* revealed a gene (CG10823) encoding an NPFF1/NPFF2-like receptor [16] and the endogenous ligand for this receptor has been identified as SIFamide, an amidated dodecapeptide (AYRKPPFNGSIFamide) [3, 18, 20]. Furthermore comparative analysis of genome sequence data has revealed that SIFamide-type peptides are also present in a variety of protostomian invertebrates (arthropods, nematodes, molluscs, annelids) and are derived from a family of orthologous precursor proteins [19, 26, 41, 42].

It is noteworthy that the sequence similarity shared between protostomian SIFamide-type neuropeptides and GnIH/NPFF-type neuropeptides is limited to a C-terminal Phe-NH_2 motif. However, because the *Drosophila* SIFamide receptor is an ortholog of vertebrate NPFF-type receptors, it has been proposed that protostomian SIFamide-type neuropeptides and vertebrate GnIH/NPFF-type neuropeptides may share a common evolutionary origin as ligand components of an ancient bilaterian peptide-receptor signaling system [19, 26].

Further insights on the evolution and diversification of GnIH/NPFF/SIFamide-type neuropeptide signaling could be obtained by identifying related peptides in deuterostomian invertebrates. Recently, two precursors of GnIH/NPFF-like neuropeptides have been identified in the invertebrate chordate *Branchiostoma floridae* (sub-phylum Cephalochordata) [26, 33]. One of these precursors (XP_002596281) comprises five putative neuropeptides that have a C-terminal motif PxRFamide. The other precursor (XP_002609543) contains nine putative neuropeptides, seven of which have a GnIH-like C-terminal LRFamide motif. Thus, the evolutionary origin of GnIH/NPFF-like peptides can be traced back to the common ancestor of the chordates.

What is now needed to "bridge the gap" between chordate GnIH/NPFF-type neuropeptides and protostomian SIFamide-type neuropeptides are data from non-chordate deuterostomes (i.e. echinoderms and/or hemichordates). Here I have addressed this issue by comparing the sequences of chordate GnIH/NPFF-type neuropeptides and protostomian SIFamide-type neuropeptides with the sequences of neuropeptides that have been identified in echinoderms [6, 7, 34].

No neuropeptides that have a PxRFamide motif were identified in echinoderms. Importantly, however, members of the echinoderm SALMFamide neuropeptide family [7] were found to share sequence similarity with several protostomian SIFamide-type neuropeptides. Thus, echinoderm SALMFamide neuropeptides have a C-terminal SxLxFamide motif (L-type SALMFamides) or SxFxFamide motif (F-type SALMFamides) and SIFamide-type neuropeptides with an L-type SALMFamide motif are present in the mollusc (limpet) *Lottia gigantea* (GINPDMSSLFFamide; [41]) and in the annelid (polychaete) *Capitella telata* (DPLEDHLPETSGLFFamide; [42]). To

98 further investigate a potential relationship between echinoderm SALMFamides and chordate
99 GnIH/NPFF-type neuropeptides and protostomian SIFamide-type neuropeptides, representative
100 peptide sequences for each of these three types of neuropeptides were aligned C-terminally (Fig. 1).
101 This revealed that three SIFamide-type neuropeptides in the nematode *Caenorhabditis elegans* have
102 the C-terminal sequence SGGMYamide, which is structurally similar to the echinoderm
103 SALMFamides. Similarly, one of the NPFF-like peptides in *Branchiostoma floridae* has the C-
104 terminal sequence SPNRFamide, which also shares sequence similarity with echinoderm
105 SALMFamides. Furthermore, as highlighted above, seven predicted *Branchiostoma floridae*
106 neuropeptides have a GnIH-like LxFamide motif, which is also a feature of L-type SALMFamides
107 [7]. Lastly, another shared feature of several GnIH/NPFF-type neuropeptides, SALMFamide
108 neuropeptides and SIFamide-type neuropeptides are one or two proline residues located in the N-
109 terminal region of these peptides (Fig. 1). Thus, there are a variety of structural characteristics
110 shared between echinoderm SALMFamide neuropeptides, chordate GnIH/NPFF-type neuropeptides
111 and protostome SIFamide-type neuropeptides that lend support to the notion that these peptides may
112 all be derived from a common ancestral peptide signaling system. Furthermore, these findings
113 provide a basis to investigate NPFF/SIFamide-type receptors in echinoderms as mediators of the
114 effects of SALMFamide neuropeptides.
115 It is noteworthy that by comparison with just four GnIH/NPFF-type neuropeptides in
116 humans and a single SIFamide in *Drosophila*, there are fourteen GnIH/NPFF-type neuropeptides
117 derived from two precursor proteins in the invertebrate chordate *Branchiostoma floridae* (Fig. 1;
118 [26, 33]). This may be associated with the occurrence of a remarkably expanded family of thirty-six
119 NPFF1/NPFF2-type receptors in *Branchiostoma floridae* [26]. Interestingly, a similar expansion of
120 NPFF1/NPFF2-type receptors (twenty-seven) has recently been reported in the hemichordate
121 *Saccoglossus kowalevskii* [21]. Putative ligands for these receptors have as yet not been identified
122 in hemichordates [26] but the occurrence of sixteen SALMFamide neuropeptides derived from two
123 precursors in the starfish *Patiria miniata* (Fig. 1; [7]) may reflect a similar expansion of

NPFF1/NPFF2-type receptors in echinoderms. Thus, it appears that the existence of expanded families of GnIH/NPFF/SALMFamide-type neuropeptides and an apparently correlated expansion of the gene repertoire encoding NPFF1/NPFF2-type receptors may be a general feature of deuterostomian invertebrates.

Identification of a putative relationship between echinoderm SALMFamide neuropeptides, protostomian SIFamide-type neuropeptides and chordate GnIH/NPFF-type neuropeptides based on sequence similarities provided a basis to investigate similarities in the physiological roles of these neuropeptides. As highlighted above, GnIH has an important role in reproductive physiology, inhibiting release of gonadotropic hormones from the pituitary and inhibiting hypothalamic release of gonadotropin-releasing hormone (GnRH) [39]. Is there evidence that SIFamide-type neuropeptides and/or SALMFamide neuropeptides are similarly involved in regulation of reproductive physiology/behaviour in protostomes and echinoderms, respectively?

In *Drosophila* the SIFamide precursor gene is expressed in four neurons located in the pars intercerebralis, a neuroendocrine gland in insects that is functionally, and possibly evolutionarily, homologous to the hypothalamus [15]. Thus, here there are parallels with hypothalamic expression of GnIH in vertebrates [39]. Interestingly, ablation of the four SIFamide-expressing cells or RNAi-mediated knockdown of SIFamide expression in these cells results in flies that are promiscuous: "males perform vigorous and indiscriminant courtship directed at either sex, while females appear sexually hyper-receptive" [36]. Thus, it is proposed that SIFamide acts physiologically to inhibit sexual behaviour [36]. This striking similarity with the physiological role of GnIH in birds and mammals provides powerful supporting evidence that GnIH and SIFamide are orthologous peptides with evolutionarily conserved physiological roles that may date back to the common ancestor of bilaterians.

Further evidence of a conserved role for SIFamide-type neuropeptides in regulation of reproductive processes can be found in experimental studies on a molluscan species, the pond snail *Lymnaea stagnalis*. SIFamide-type neuropeptides were first reported in insects in 1996 [18] but

prior to this a neuropeptide with the amino-acid sequence GLTPNMNSLFFamide was identified in *Lymnaea* and named neuropeptide FF on account of the C-terminal pair of phenylalanine residues (FF) [22]. With the identification of precursors of SIFamide-type precursors in molluscan species [41] it became apparent that *Lymnaea* neuropeptide FF is in fact a member of the protostomian SIFamide-type neuropeptide family. Furthermore, it is interesting that *Lymnaea* neuropeptide FF was originally isolated on account of its *in vitro* pharmacological effect in causing an enhancement in the contraction frequency and contraction amplitude of the vas deferens in this species. Thus, again we see evidence of a conserved physiological role for SIFamide-type neuropeptides in regulation of reproductive processes. In this case the effect is stimulatory, which contrasts with the inhibitory effects of GnIH in birds and mammals [39] and the inhibitory of effect of SIFamide on *Drosophila* [36]. However, comparison of physiological actions here is complicated by the fact that *Lymnaea* is a hermaphrodite species and therefore neuropeptides may have counteracting effects of male and female reproductive systems. For example, like neuropeptide FF, the vasopressin-type neuropeptide "conopressin" induces muscular contractions of the vas deferens in *Lymnaea*, but it also inhibits central neurons that control female reproductive behavior [40]. Accordingly, perhaps SIFamide-type neuropeptides also have inhibitory effects on female reproductive behaviour in *Lymnaea*.

What about SALMFamide neuropeptides? Is there any evidence that SALMFamides regulate reproductive processes in echinoderms? The first SALMFamides to be identified were the starfish neuropeptides SALMFamide-1 (S1) and SALMFamide-2 (S2), which were both isolated from the starfish species *Asterias rubens* and *Asterias forbesi* [10, 11]. Immunocytochemical analysis of the expression S1 and S2 in *Asterias rubens* revealed a widespread pattern of expression in nerve fibres associated with a variety of neuromuscular organs, including the cardiac stomach, tube feet and apical muscle [9, 30, 31]. Accordingly, *in vitro* pharmacological studies have revealed that both S1 and S2 cause dose-dependent relaxation of cardiac stomach, tube foot and apical muscle preparations *in vitro* [8, 9, 24, 25]. Furthermore, there is evidence that SALMFamides have

176 a general role as muscle relaxants throughout the echinoderms [4, 8, 12]. However, in addition to
177 inhibitory effects on muscle, there is also evidence that SALMFamides have a physiological role in
178 suppression of reproductive activity. Gamete release in starfish is triggered by a neuropeptide
179 hormone that is known as gonad-stimulating substance (GSS), which was recently identified as
180 dimeric peptide related to the mammalian hormone relaxin [28]. Furthermore, *in vitro* experiments
181 have revealed that the starfish SALMFamide neuropeptide S1 inhibits potassium-induced release of
182 GSS from radial nerve cords in the starfish *Asterina pectinifera* [27]. Thus, as with GnIH in
183 vertebrates and SIFamide-type neuropeptides in protostomes, these findings are supportive of the
184 notion that SALMFamides inhibit reproductive processes in echinoderms.
185 In conclusion, the findings presented here support the notion that GnIH, SALMFamides and
186 SIFamides belong to a bilaterian family of neuropeptides that have evolutionarily ancient and
187 conserved physiological roles in regulation of reproductive activity. It should be noted, however,
188 that these neuropeptides are not only involved in regulation of reproductive physiology, as is
189 evident in the effects of PxRFamides in attenuating morphine-induced anti-nociception in mammals
190 [44] and the muscle-relaxing effects of SALMFamides in echinoderms [8]. Nevertheless, it is
191 effects on reproductive processes that provide a unifying functional perspective on this family of
192 neuropeptides as well as a basis for further investigation of roles in regulation of reproductive
193 physiology throughout the bilateria.

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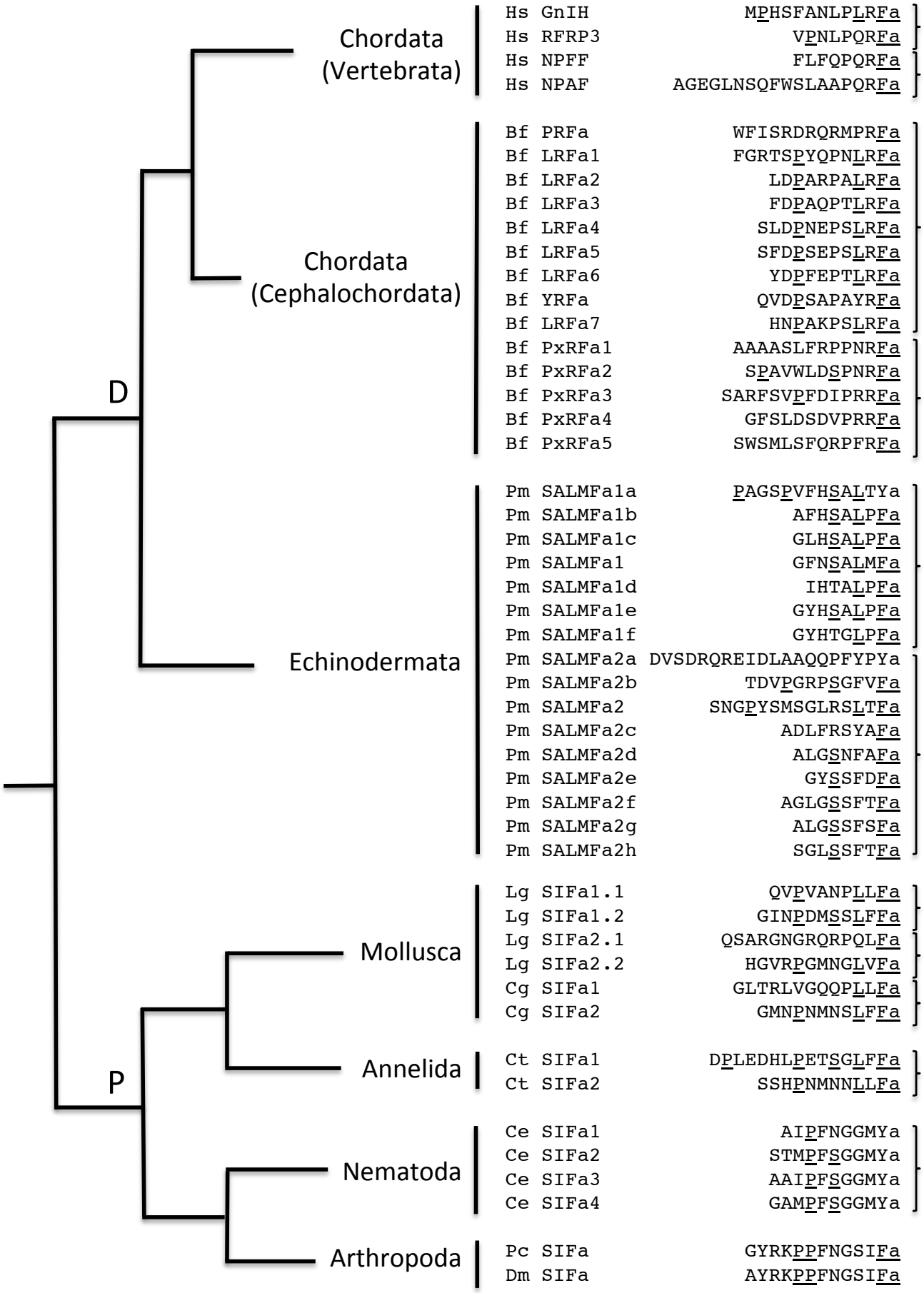
Figure Legend

Fig. 1. Phylogenetic C-terminal alignment of vertebrate PxRFamide-type neuropeptides and protostomian SIFamide-type neuropeptides with cephalochordate PxRFamides/LRFamides and echinoderm SALMFamides reveals structural similarities (underlined) indicative of a common evolutionary ancestry. A key feature is the C-terminal SxLxFamide motif that characterises L-type SALMFamides in echinoderms. This motif, or elements of it, are apparent in several chordate PxRFamides/LRFamides and protostomian SIFamide-type neuropeptides. Another recurring feature is the presence of a proline (P) residue in the N-terminal region of the peptides. The D and P denote deuterostome and protostome clades, respectively. The brackets on the right group peptides derived from the same precursor protein. Abbreviations: Hs, *Homo sapiens*; Bf, *Branchiostoma floridae* (amphioxus or lancelet); Pm, *Patiria miniata* (starfish); Lg, *Lottia gigantea* (limpet); Cg, *Crassostrea gigas* (oyster); Ct, *Capitella teleta* (polychaete); Ce, *Caenorhabditis elegans*; Pc, *Procambarus clarkii* (crayfish); Dm, *Drosophila melanogaster*. References: Hs, [17, 32]; Bf, [26, 33]; Pm, [7]; Lg, [26, 41]; Cg, [26, 46]; Ct, [26, 42]; Ce, [26]; Pc, [45]; Dm, [3, 16].

Figures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Lg SIFa1.1 QVPVANPLLFa
Lg SIFa1.2 GINPDMSSLFFa
Lg SIFa2.1 QSARGNGRQRPQLFa
Lg SIFa2.2 HGVRPGMNGLVFa
Cg SIFa1 GLTRLVGQQPLLFa
Cg SIFa2 GMNPNMNSLFFa
Ct SIFa1 DPLEDHLPETSGLFFa
Ct SIFa2 SSHPNMNNLLFa
Ce SIFa1 AIPFNGGMYa
Ce SIFa2 STMPFSGGMYa
Ce SIFa3 AAIPFSGGMYa
Ce SIFa4 GAMPFSGGMYa
Pc SIFa GYRKPPFNGSIFa
Dm SIFa AYRKPPFNGSIFa