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REVIEW

A review of FMRFamide- and RFamide-like peptides in metazoa

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Abstract Neuropeptides are a diverse class of signalling molecules that are widely employed as neurotransmitters and neuromodulators in animals, both invertebrate and vertebrate. However, despite their fundamental importance to animal physiology and behaviour, they are much less well understood than the small molecule neurotransmitters. The neuropeptides are classified into families according to similarities in their peptide sequence; and on this basis, the FMRFamide and RFamide-like peptides, first discovered in molluscs, are an example of a family that is conserved throughout the animal phyla. In this review, the literature on these neuropeptides has been consolidated with a particular emphasis on allowing a comparison between data sets in phyla as diverse as coelenterates and mammals. The intention is that this focus on the structure and functional aspects of FMRFamide and RFamide-like neuropeptides will inform understanding of conserved principles and distinct properties of signalling across the animal phyla.

Keywords Invertebrate · Vertebrate · Neuropeptides · FaRP · Phyla · Neuropeptide

Introduction

FMRFamide-like peptides (FLPs) are a group of neuroactive peptides that resemble the tetrapeptide FMRFamide (FMRFa), a cardioexcitatory peptide isolated from the nervous system of the clam, *Macrocallista nimbosa,* whose structure was determined by Price and Greenberg (1977). The alternative term FaRP, FMRFa-related peptide, implies a homology to FMRFa (Price and Greenberg 1989; Espinoza et al. 2000). A FLP can be defined as a peptide that ends in RFa while a FaRP is a peptide homologous to FMRFa, the latter being more difficult to demonstrate (Espinoza et al. 2000). For this reason, in the current review, the term FLP has been used in place of FaRP while for the remaining peptides the terms RFa and Fa are used. In their paper, Espinoza and colleagues present a very detailed analysis of amino acid frequency and position in FLPs, and the reader is referred to this paper for further information. In addition, for a general review on conserved patterns in bioactive peptides, the reader is referred to Liu et al. (2006). For a peptide to be designated a true FaRP, the following C-terminal sequence is required: an aromatic amino acid, viz, F (phenylalanine), Y (tyrosine) or W (tryptophan); an amino acid with a hydrophobic group, viz, F, I (isoleucine), L (leucine), M (methionine), T (threonine) or V (valine) and a C-terminal R (arginine) Famide (Wang et al. 2009). All the major phyla have examples of FLPs with the exception of the echinoderms that have Fa and Ya peptides but lack RFas. However, authentic FMRFamide has only been found in two phyla, the molluscs and annelids, and there are more examples of extended peptides that terminate in FMRFa. There are many more examples of extended peptides having a C-terminal of FLRFa or LRFa. In addition to peptides with C-terminals of MRFa or LRFa, FLPs with IRFa and GRFa (G, glycine) are also common and the following sequences also occur, QRFa (Q, glutamine), FRFa, PRFa (P, proline), YRFa, VRFa and TRFa. RYas can also be preceded by an S (serine), N (asparagine) or D (aspartate) amino acid. Interestingly, while most amino acids are represented in FLP sequences, no FLP has yet been identified with a cysteine.

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A number of enzymes are involved in the formation of a FLP. First, there is convertase cleavage at the C-terminal end of paired or single basic residues, for example, between a glycine and a lysine or arginine. Carboxypeptidases then remove the basic amino acid. Unlike the situation with some other peptide families, the FLPs are all amidated following cleavage of a peptidylglycine and in the absence of a C-terminal amide group they are inactive. Two enzymes are required for this process, peptidylglycine α-hydroxylating mono-oxygenase (PHM) and peptidyl-α-hydroxyglycine α-amidating lyase (PAL) (Grimmelikhuijzen et al. 2004). In some groups, these enzymes form part of a single bifunctional enzyme that is coded by a single gene with the PHM coding exons located at the 5′ end and exons coding for PAL at the 3′ end (Perkins et al. 1990). Initially, PHM hydroxylates peptidylglycine and then PAL cleaves peptidylhydroxyglycine into a peptidyl amide and glyoxylic acid. Indeed, all FLP receptors require an amidated ligand for activation.

The literature reviewed here has employed a number of strategies to identify neuropeptides in tissues. Initially, biochemical methods were employed, which required the use of large amounts of tissue. For example, for the identification of the insect peptide, proctolin, 125,000 adult *Periplaneta americana* were required to produce μg quantities of peptide (Brown and Starratt 1975). With the advent of more sensitive techniques, including various forms of mass spectrometry, the peptide analysis of single cells can now be achieved. Once EST (expressed sequence tags) and genome data bases for a species are available, putative neuropeptides and their genes can be more readily identified. Candidate neuropeptides identified from bioinformatic screens can then be synthesized and examined for biological activity. Using in situ hybridization, the expression patterns of genes encoding for specific peptides can be determined. Alternatively, immunohistochemical techniques have been widely used to indicate the cellular distribution of FLP-like material. A limitation of this latter approach is the difficulty of identifying the FLP due to the lack of antibody specificity. Overall, these varied approaches have generated a substantial literature on neuropeptides in metazoa. Here, we consolidate this information with respect to the FMRFamide- and RFamide-like neuropeptides.

In this review, each of the major phyla will be considered in turn and comparisons made between the FLP structures reviewed and their possible roles across the phyla considered. The volume of literature on each phylum varies and the way in which each phylum is reviewed will to some degree reflect this. There is still discussion regarding the relationships between the major phyla; however, certain conclusions can be proposed from molecular-based phylogeny (Prud'homme et al. 2002, 2003). For example, it is proposed to group the nematodes with the arthropods and the molluscs with the annelids and platyhelminths while, as deuterostomes, the echinoderms remain linked with the chordates. Using this scheme, links between FLPs in these phylum groupings and between groups will be considered.

Cnidarians

The nervous systems of metazoa probably first arose in ancestors of the present day Cnidaria and therefore their complement of neuropeptides is of interest in relation to the neuropeptides present in other metazoan phyla. FLPs in Cnidaria have been investigated by Grimmelikhuijzen and his colleagues, and the reader is referred to three comprehensive reviews (Grimmelikhuijzen et al. 1996, 2002, 2004). Cnidarians are divided into four classes, viz, Hydrozoa (*Hydra*), Cubozoa (comb jellyfish), Scyphozoa (true jellyfish) and Anthozoa (sea anemones, corals). The members of this phylum are the most primitive animals to possess a nervous system; and judging by their numbers, it would appear that neuropeptides, some of which are FLPs, play a key role in the functioning of their nervous systems although there is evidence for both amino acids and biogenic amines in cnidarian neurones and when released acting as transmitters (Anctil and Bouchard 2004; Pierobon et al. 2001; Kass-Simon and Pierobon 2007). The nervous system is composed of a nerve net that can form nerve plexuses, nerve tracks or nerve rings. No real ganglia or brain exists in Cnidaria (Grimmelikhuijzen et al. 2004). Examples of FLPs are shown in Table 1, from which it can be seen that they are all GRFa peptides. *Hydra*-RFas are synthesized by three genes each coding for a prohormone that are termed A, B and C (Mitgutsch et al. 1999). Immunostaining has revealed the presence of FLPs in neurones of all body regions and in the ectodermal (but not endodermal) nerve net of *Hydra* (Koizumi et al. 2004). It has been proposed by Espinoza et al. (2000) that some of these peptides may exist in an extended form at their N-terminal. While the three C-terminal amino acids are always GRF, Table 1, the fourth amino acid can be one of several, viz, G, K (lysine), N, Q, R or S. In addition to RFamides, there are also RIamides, RNamides, RWamides and RPamides in Cnidaria. The RW-amides are generally short, around five amino acids, for example, pQSLRWa, while the W-amides are longer, for example, PLPIGLWa (Grimmelikhuijzen et al. 2004). Some of the GLWas may play a role in metamorphosis in Cnidaria (Muller 2004). FLPs are located in dense core vesicles in neurones in Cnidaria (Koizumi et al. 1989; Westfall and Grimmelikhuijzen 1993). FLP-positive neurones may be widespread in Cnidaria, for example, around 40% of neurones in the hydrozoan planular nerve net are immunoreactive (Martin 1992).

Table 1 Summarizing RFas from Cnidaria

Name	Sequence	Species	Reference
Antho-RFamide	pQGRFa	*Anthopleura elegantissima*	Grimmelikhuijzen and Graff (1986)
Cyanea-RFa I	pQWLRGRFa	*Cyanea lamarckii*	Moosler et al. (1997)
Cyanea-RFa II	pQPLWSGRFa	*C. lamarckii*	Moosler et al. (1997)
Cyanea-RFa III	GRFa	C. lamarckii	Moosler et al. (1997)
Pol-RFa I	pQLLGGRFa	*Polyorchis penicillatus*	Grimmelikhuijzen et al. (1988)
Pol-RFa II	pQWLKGRFa	*P. penicillatus*	Grimmelikhuijzen et al. (1992)
Hydra-RFa I	pQWLGGRFa	*Hydra magnipapillata*	Moosler et al. (1996)
Hydra-RFa II	pQWFNGRFa	*H. magnipapillata*	Moosler et al. (1996)
Hydra-RFa III	KPHLRGRFa	*H. magnipapillata*	Moosler et al. (1996)
Hydra-RFa IV	HLRGRFa	*H. magnipapillata*	Moosler et al. (1996)
Hydra-RFa V	pQLMSGRFa	*H. magnipapillata*	Darmer et al. (1998)
Hydra-RFa VI	pQLMRGRFa	*H. magnipapillata*	Darmer et al. (1998)
Hydra-RFa VII	pQLLRGRFa	*H. magnipapillata*	Darmer et al. (1998)
Hydra-RFa VIII	KPHYRGRFa	*H. magnipapillata*	Darmer et al. (1998)
Hydra-RFa IX	HYRGRFa	*H. magnipapillata*	Darmer et al. (1998)

Antho-RFa immunoreactivity occurs widely in the nervous system of the sea pansy, *Renilla koellikeri*, where its distribution suggests roles associated with feeding, reproduction, neuromuscular transmission and in neuro-neuronal transmission (Pernet et al. 2004). The gametophores contain FLP immunoreactive neurones, and these FLPs may be involved in contraction and release of gametes as there is a much greater density of immunoreactive neurones during the summer spawning period. Immunohistochemical studies suggest that RFa peptides are present in interneurones associated with the transfer of visual information from the photoreceptor cells to the ring nerve in the Box jellyfish, *Tripedalia cystophora* and *Carybdea marsupialis* (Parkefelt and Ekstrom 2009). Cnidocytes or sting cells are used for a number of functions in Cnidaria, including food capture, locomotion, aggression and defense and are innervated by neurones that contain FLP immunoreactivity (Anderson et al. 2004). This peptidergic innervation may be involved in the chemosensory regulation of cnidocyte discharge.

The actions of pQGRFa on the musculature and nervous system of the sea anemone, *Calliactis parasitica,* have been investigated (McFarlane et al. 1987). Peptide of 100 nM–1 μM induced an increase in tone, contraction frequency and contraction amplitude of slow muscle but had no effect on fast muscle contraction. The peptide also increased the electrical activity of the ectodermal conducting system (SS1) when applied either internally or externally. In contrast, pQGRFa had little or no effect on the endodermal conducting system (SS2) when applied externally but did excite SS2 when applied internally. There was no clear evidence for an action of the peptide on the through conducting nerve net (TCNN), a fast conducting system that appears to innervate most endodermal muscle. The authors concluded that pQGRFa probably acted both presynaptically on neurones and postsynaptically on slow muscle as a neurotransmitter. Sporadic contractions of the peduncle (the region just above the basal disc) of *Hydra magnipapillata* are enhanced by KPHLRGRFa (HyRFaIII), but HyRFaI and II have no effect (Shimizu and Fujisawa 2003). These contractions are important for the circulation of fluid in the gastrovascular cavity, and this pumping action of the peduncle may represent a primitive heart system in Cnidaria, which is activated by an RFa in a similar manner to that seen in, for example, molluscs (Shimizu and Fujisawa 2003). Planula larvae of *Hydractinia echinata* undergo alternate periods of activity and resting behaviour during migration (Katsukura et al. 2004). pQWLGGRFa inhibits migration by reducing the initiation and length of the active period while KPPGLWa stimulates migration, mainly by increasing the length of the active periods but this peptide also increases the speed of migration during the active period.

Four cDNAs have been isolated from *H. magnipapillata* with homology to mammalian DEG/ENa channels and the resulting proteins called Hydra Na channels 1–4 (HyNaC) (Golubovic et al. 2007). These channels have two transmembrane domains with short N- and C-terminal sequences and a large extracellular loop with 12 conserved cysteines between the transmembrane domains. These Na channels, HyNaCs, are directly gated by the Hydra-RFamides. When expressed in *Xenopus* oocytes HyNaC$^{2-3}$ were activated by pQWLGGRFa and pQWFNGRFa and the resultant currents inhibited by amiloride. Replacement of the extracellular Na with N-methyl-D-glucamine abolished the inward current. Hydra-RFas were unable to induce currents in oocytes expressing ASIC or INaC

mammalian channels. The cells that express HyNaCs are located at the base of the tentacles, close to neurones, which contain Hydra-RFas.

Platyhelminths

There are four classes of platyhelminths, viz, Turbellaria (planaria), Monogenea (ectoparasitic flukes), Trematoda (endoparasitic flukes) and Cestoda (tapeworms) (Barnes et al. 1988). Many members of this phylum have evolved a parasitic lifestyle. Platyhelminths are acoelomate, without a well-defined circulatory system. This makes it difficult for neurohormones to operate and so substances released from neurones or glandular cells probably act on adjacent tissues. The nervous system can be divided into a bilobed brain with two main nerve cords that extend from the brain to the most posterior part of the animal and a peripheral nervous system. The peripheral nervous system is made up of several minor nerve cords, viz, the subepidermal, submuscular, stomatogastric and genital nerve plexi (Gustafsson et al. 2002). There have been recent reviews where FLPs in platyhelminths have been discussed, and the reader is referred to them (McVeigh et al. 2005a; Mousley et al. 2005).

Immunoreactivity to antisera raised against vertebrate neuropeptides occurs widely in the platyhelminth nervous system (Halton and Gustafsson 1996). FLP immunoreactivity occurs in nerve fibres innervating the musculature of suckers and hook-bearing organs, feeding apparatus, copulatory structures and genital tracts, indicating a role in egg production (Gustafsson et al. 2002). Evidence from several studies suggests that FLPs are involved in the reproductive system of platyhelminths (Armstrong et al. 1997; Sebelova et al. 2004; Stewart et al. 2003). For example, strong FLP ootype (egg forming apparatus) immunoreactivity is only present in the reproductive system during egg production. 5-HT is also present in axons innervating the ootype, and some of these fibres also show GYIRFa-like immunoreactivity, indicating a possible synergy between 5-HT and FLPs in contracting ootype muscles (Armstrong et al. 1997). Sensory cells are also innervated by peptide-containing fibres. There is evidence that FLPs may colocalize in platyhelminth neurones with acetylcholine (Halton and Gustafsson 1996). Four FLPs have been identified in Turbellaria and Cestoda while extended PRFas have been identified in a cestode, a turbellarian and two trematodes, Table 2. GYIRFa and YIRFa were isolated from the turbellarian, *Bdelloura candida,* by Johnston et al. (1996) and contract single *B. candida* muscle fibres. GYIRFamide was more potent and at 10 μM stimulated a higher maximum number of fibres compared with YIRFa. RYIRFa stimulated the same maximum number of fibres as GYIRFa but was less potent. Threshold values for both pentapeptides were less than 1 nM. Using an antiserum raised against GYIRFa, widespread immunoreactivity was found in the nervous system, indicating a key role for this peptide in the physiology of *B. candida.* The action of GNFFRFa, isolated from the cestode, *Moniezia expansa* (Maule et al. 1993) and the action of RYIRFa from the turbellarian, *Arthurdendyus (Artioposthia) triangulates* (Maule et al. 1994), together with FMRFa, have been examined on dispersed *Schistosoma mansoni* muscle fibres in vitro (Day et al. 1994). All three peptides contracted the muscle fibres with the order of potency of RYIRFa > FMRFa > GNFFRFa and threshold of 1, 10 and 100 nM, respectively. Using strips of body wall muscle from *Fasciola hepatica*, Graham et al. (2000) investigated the mechanism of this contraction. They provide evidence that the related peptide, GYIRFa, mediates its excitatory effect through activation of phospholipase C, which stimulates production of diacylglycerol, which then promotes protein kinase C activity. The action of platyhelminth FLPs has been tested on dispersed muscle fibres from the turbellarian, *Procerodes littoralis* (Moneypenny et al. 2001). The relative potencies were as follows: GYIRFa > YIRFa > GNFFRFa. The analogue GYIRᴅFa had no direct effect on muscle fibre contractility but reduced the excitatory effect of the FLPs on the muscle. This suggests that all three FLPs act on a common FLP receptor on turbellarian muscle. Recently using ESTs and BLAST searches, a number of putative RFas have been identified in platyhelminths from neuropeptide precursor genes, Table 2 (McVeigh et al. 2009). In addition, these authors have identified RYamides, RWamides, Wamides, Famides and two tripeptides, NYFa and NYYa (both from *Dugesia japonica*). These peptides will form the basis of important future studies in platyhelminths.

A very comprehensive structure–activity study of the excitatory action of FLPs on trematode isolated muscle fibres was undertaken by Day et al. (1997). The three turbellarian FLPs, viz, GYIRFa, YIRFa and RYIRFa were active with EC_{50}s of 1–7 nM while the cestode FLP, GNFFRFa, had an EC_{50} of 0.5 μM. The key sequence for potent activity was YIRFa, with the nature of the N-terminal aromatic amino acid important, as FIRFa and WIRFa had EC_{50}s of 100 and 0.1 nM, respectively. Interestingly, substitution of I by an amino acid more hydrophobic than I, viz, L, F or M, retained activity while substituting an amino acid less hydrophobic, viz, V or A (alanine), greatly reduced potency. Changes in the RF sequence rendered the peptide inactive or with an extremely low potency. Tripeptides, e.g., IRFa, had a very low potency or were inactive, with the exception of norLRFa, which had an EC_{50} of

Table 2 Summarizing FLP and NPF sequences in platyhelminths, together with the *L. stagnalis, D. melanogaster* and pig NPY for comparison

Sequence	Species	Reference
YIRFa	*Bdelloura candida*	Johnston et al. (1996)
YMRFa	*Macrrostomum lignano*	McVeigh et al. (2009)
GYIRFa	*Girardia tigrina*	Johnston et al. (1995)
RYIRFa	*Arthurdendyus triangulates*	Maule et al. (1994)
SVAFRFa	*Schmidtea mediterranea*	McVeigh et al. (2009)
GNFFRFa	*Moniezia expansa*	Maule et al. (1993)
SSVFRFa	*S. mediterranea*	McVeigh et al. (2009)
NHGSRFa	*Schistosoma mansoni*	McVeigh et al. (2009)
RGVAFRFa	*S. mediterranea*	McVeigh et al. (2009)
HFMPQRFa	*S. mansoni*	McVeigh et al. (2009)
AIVLTRFa	*S. mediterranea*	McVeigh et al. (2009)
NTRWPSRFa	*M. lignano*	McVeigh et al. (2009)
YTRFVPQRFa	*S. mansoni*	McVeigh et al. (2009)
WNMRWHSRFa	*M. lignano*	McVeigh et al. (2009)
WNTRWPSRFa	*M. lignano*	McVeigh et al. (2009)
NADIYESESGPRHNIGNRFa	*Schistosoma japonicum*	McVeigh et al. (2009)
NPFs		
PDKDFIVNPSDLVLDNKAALRDYLRQINEYFAIIGRPRFa	*M. expansa*	Maule et al. (1991)
KVVHLRPRSSFSSEDEYQIYLRNVSKYIQLYGRPRFa	*A. triangulates*	Curry et al. (1992)
AQALAKLMSLFYTSDAFNKYMENLDAYYMLRGRPRFa	*S. mansoni*	Humphries et al. (2004)
AQALAKLMTLFYTSDAFNKYMENLDAYYMLRGRPRFa	*S. japonica*	Humphries et al. (2004)
TEAMLTPPERPEEFKNPNELRKYLKALNEYYAIVGRPRFa	*Lymnaea stagnalis*	Tensen et al. (1998a)
SNSRPPRKNDVNTMADAYKFLQDLDTYYGDRARVRFa	*Drosophila melanogaster*	Brown et al. (1999)
YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRYa	*Sus scrofa scrofa*	Tatemoto (1982)

20 nM. It would have been interesting to test FFRFamide although judging from the potency of FIRFa and YFRFa, its EC_{50} would be <100 nM. This is lower than the EC_{50} of GNFFRFa. As expected, the YIRF free acid was inactive. Thus, for activation of this FLP receptor, the N-terminal amino acid of YIRFa should be aromatic, the second amino acid, hydrophobic, the third amino acid R and the final amino acid Fa. This receptor shows a preference for Y over F at the N-terminal while the mollusc receptor prefers F over Y (Payza 1987).

NPF, a 39 amino acid RFa with a C-terminal GRPRFa, Table 2, isolated from *M. expansa* (Maule et al. 1991), cross-reacts with a C-terminally directed pancreatic polypeptide (PP) antiserum. PP is a member of the vertebrate Neuropeptide Y superfamily. The NPF-encoding gene has been characterized in *M. expansa* (Mair et al. 2005). A similar NPF peptide with 36 amino acids has been identified in the turbellarian, *A. triangulates,* Table 2, (Curry et al. 1992), and its gene has been characterized (Dougan et al. 2002). Two NPFs have also been isolated from two species of *Schistosoma, S. mansoni* and *S. japonica* (Humphries et al. 2004). They both have 36 amino acids and only differ by one amino acid, Table 2. Recently, 8 new NPFs have been predicted with a C-terminal sequence of GRP/QRFa (McVeigh et al. 2009). NPF immunoreactivity occurs widely through the nervous system of platyhelminths, indicating a wide range of roles for this FLP (McVeigh et al. 2005a). *S. mansoni* NPF inhibits forskolin-stimulated accumulation of cAMP in *S. mansoni* tissue homogenate with an IC_{50} of 170 pM. This inhibitory effect on levels of cAMP is also seen with the vertebrate NPY family of peptides (Michel 1991) although using *S. mansoni,* pig NPY was much less potent with an IC_{50} of around 1 μM. RYIRFa, GYIRFa and YIRFa were inactive. Interestingly, a shortened *S. mansoni* NPF peptide consisting of residues 13–36 was only slightly less active than the full peptide, with an IC_{50} of 370 pM. The structure of NPY is shown for comparison in Table 2, from which the conserved feature of Y residues at positions 20 and 27 of these RFas may be noted. In the case of *M. expansa*, there are an additional 3 N-terminal amino acids, but still 6 amino acids separate the two tyrosines. There is evidence that NPF and GNFFRFa act on separate receptors in cestode larvae (*Mesocestoides vogae*) since GNFFRDFa blocks the latter's but not the former's excitatory action (Hrckova et al. 2004). These authors also found evidence for an involvement of adenylyl cyclase and Protein Kinase C in the action of NPF in cestodes.

A number of nematode and insect/crustacean FLPs have been tested on *F. hepatica* body wall muscle and *P. littoralis* isolated muscle fibres, respectively (Marks et al. 1997; Mousley et al. 2004). All the nematode FLPs tested excited *F. hepatica* muscle, including PF4 (KPNFIRFa), which is strongly inhibitory on nematode body wall muscle. Apart from PF4, with a threshold of 30 nM, all the other nematode FLPs, viz, AF1, AF2, PF1, PF2 and PF3, had thresholds in the 1–10 μM range. Interestingly, PF4 has an FIRFa C-terminal but so does AF1 (KNEFIRFa), which only activated the muscle in the μM range. Marks et al. (1997) also found that PF3 (KSAYMRFa) was 30 times less potent than the mollusc tetrapeptide, FMRFa. The myoactivity of insect/crustacean FLPs on *P. littoralis* muscle fibres ranged from an EC_{50} of 29 nM for DPSFLRFa to 1.69 μM for SDRNFLRFa, indicating the importance of the N-terminal amino acids for determining potency since both FLPs have the same four amino acids at their C-terminal. This observation is re-inforced as both HVFLRFa and VFLRFa were around ten times less potent than pQDVDHVFLRFa while PDVDHVFLRFa was around 15 times less potent than pQDVDHVFLRFa. However, none of these FLPs resembled turbellarian FLPs as they lacked an IRFa C-terminal.

The first platyhelminth neuropeptide receptor (GtNPR-1) has been identified in the turbellarian, *Girardia (Dugesia) tigrina,* as a GPCR (Omar et al. 2007).While this receptor has 33% identity with the *Caenorhabditis elegans* receptor, CeNPR-1 and 26% identity with the *Drosophila* receptor, DM-sNPFR, phylogenetic analysis evidence suggests it is more closely related to the *Drosophila* receptor than any other functionally characterized protein. When expressed in CHO cells, GYIRFamide was the most potent platyhelminth peptide to activate the receptor with an EC_{50} of 0.4 μM. However, both RYIRFa and YIRFa were only slightly less potent with EC_{50}s in the high nM range. While NPF was inactive, short FLPs from *Drosophila* had EC_{50} s greater than 10 μM. A range of FLPs from *C. elegans* had EC_{50} s in the range 0.1–0.8 μM, while FMRFa had an EC_{50} of 0.2 μM, therefore, slightly more potent than GYIRFa. These data might suggest there is another endogenous FLP in platyhelminths that is the preferred ligand for GtNPR-1, with a C-terminal of LRFa. Omar et al. (2007) also found evidence to suggest that GtNPR-1 couples to Gαi/o since pertussis toxin blocked peptide responses.

Nematodes

FLPs in nematodes have been particularly well studied in *C. elegans* (Husson et al. 2007; Kim and Li 2004; Li 2005; Li and Kim 2008) and *Ascaris suum* (Cowden and Stretton 1995; Yew et al. 2005), and there is a considerable literature on their characterization, occurrence and mode of action (Geary et al. 1995; McVeigh et al. 2006). The role of neuropeptides in *C. elegans* complex behaviours has been reviewed by de Bono and Maricq (2005). The distribution of FLPs between different species shows that many FLPs and FLP sequences probably occur widely through the phylum while others may be more restricted (McVeigh et al. 2005b). In their review of parasitic *flp* ESTs, a number of FLP sequences have been proposed, which are not recorded in *A. suum* or *C. elegans* but these have not been included in the present review. The first FLP to be isolated from *A. suum* was AF1 (Cowden and Stretton 1993). Currently, there are around 27 FLPs identified from *A. suum* (Yew et al. 2005), Table 3. A number of these have also been identified in *C. elegans* including: AF1, AF2, AF8, AF9, AF15, AF21, AF22 and AF24. A recent LC-MALDI-TOF MS study has identified 37 FLPs in *C. elegans* encoded by 19 *flp* genes, most of which were also identified in *Caenorhabditis briggsae* demonstrating that FLPs are strongly conserved between the two species (Husson et al. 2008). A total of over 70 FLPs from around 33 genes have been identified in *C. elegans* (Husson et al. 2005; Kim and Li. 2004; Li 2005; Li et al. 1999a, b; Nathoo et al. 2001; McVeigh et al. 2005b), Table 4. As can be seen from Table 4, the genes and their encoded peptides for *C. elegans* have been described in detail (Li et al. 1999a; Li 2005; McVeigh et al. 2005b) but the genes for *A. suum* are less complete. The first gene to be identified in *A. suum* was *afp-1* (Edison et al. 1997), and this gene encodes six -PGVLRFamides and is equivalent to *Ce-flp-18* of *C. elegans* and can be named *As-flp-18*. Further *flp* genes have been either characterized or identified from ESTs in *A. suum*, viz, *As-flp-6, As-flp-8, As-flp-12, As-flp-14, As-flp-16, As-flp-20* and *As-flp-24* (McVeigh et al. 2005b; Yew et al. 2005). In addition, Yew et al. have identified a gene, which they termed *afp-5* in *A. suum,* which encodes AF-17, which does not appear to have an equivalent in *C. elegans.* Recently, the gene that encodes AF-21, AF-22 and AF-23 has been cloned from *A. suum* and termed *afp-6* (Yew et al. 2007). This gene is equivalent to *flp-11* of *C. elegans* and following on from McVeigh's nomenclature should be termed *As-flp-11.* The occurrence of FLPs through the phylum has been thoroughly investigated by McVeigh et al. (2005b). In this study, the authors undertook a systematic BLAST search of nematode ESTs (Expressed Sequence Tags) from 33 species using the Genbank data base. They found that *flp-1, flp-6, flp-11, flp-14* and *flp-18* were expressed widely through the phylum, and this study increased the number of FLPs to around 90 and the number of *flp* genes to 33. Therefore, the most widely occurring FLPs have sequences or C-terminal sequences of -PNFLRFa (*flp*-1), KSAYMRFa (AF8, *flp*-6),

Table 3 Summarizing FLPs and F-amides, which have been isolated, sequenced or proposed in *Ascaris suum*

Gene	Sequence	AF number
as-flp-6 (afp-3)	KSAYMRFa	AF8
as-flp-8	KNEFIRFa	AF1
as-flp-11 (afp-6)	AMRNALVRFa	AF21
	NGAPQPFVRFa	AF22
	SGMRNALVRFa	AF23
as-flp-12	RNKFEFIRFa	AF24
as-flp-14 (afp-4)	KHEYLRFa	AF2
as-flp-16	AQTFVRFa	AF15
as-flp-18 (afp-1)	GFGDEMSMPGVLRFa	AF10
	GMPGVLRFa	AF20
	AVPGVLRFa	AF3
	GDVPGVLRFa	AF4
	SDMPGVLRFa	AF13
	SMPGVLRFa	AF14
as-flp-20	ILMRFa	AF16
as-flp-24	VPSAADMMIRFa	
Not known	SGKPTFIRFa	AF5
Not known	FIRFa	AF6
Not known	AGPRFIRFa	AF7
Not known	GLGPRPLRFa	AF9
Not known	SDIGISEPNFLRFa	AF11
Not known	NKFFLRKP	AF18
Not known	AEGLSSPLIRFa	AF19
Not known	NNFLRFa	AF25
Not known	KPNFLRFa	AF26
afp-5	DFDRDFMHFa	AF17
	EFDRDFMHFa	
	SNAFDRNFMNFa	
	ESQFSRDFLNFa	
	SDNFMNFa	
	DDAFSRDFLSFa	
	SDAFSRNFMNFa	
From *A. suum* ESTs		
CB040120	DYRPLQFa	
	DGYRPLQFa	
	KSYRPLQFa	
	SYRPLQFa	
	qDRDYRPLQFa	
CB039226	ENEKKAVPGVLRFa	

The data are mainly taken from McVeigh et al. (2005b) and Yew et al. (2005)

-RNxLVRFa (*flp*-11), KHEYLRFa (AF2, *flp-14*) and -PGVLRFa (*flp-18*). With the exception of FLP-33, all *C. elegans* FLPs have a C-terminal of IRFa, LRFa, MRFa or VRFa. McVeigh et al. (2005b) found that individual FLPs and FLP motifs were highly conserved across nematodes irrespective of whether or not they were parasitic or free-living, suggesting FLPs have a fundamental role in nematodes. For example, in the case of KHEYLRFa, this peptide has been identified in 20 species from 13 genera. It has recently been proposed (Clynen et al. 2009) that *flp-27* encodes for a NPF with a sequence of 23 amino acids, adding 15 amino acids to the sequence originally proposed (McVeigh et al. 2005b). In addition to *C. elegans* and *A. suum*, *flp* genes have also been identified in *Globodera pallida* and *Globodera rostochiensis* (Kimber et al. 2001, 2007). Five genes have been identified in *G. pallida*, viz, *Gp-flp-1, Gp-flp-6, Gp-flp-12, Gp-flp-14* and *Gp-flp-18* and a further 7 predicted from ESTs in *G. pallida* and/or *G. rostochiensis.* Interestingly, the neurones in this genus appear particularly sensitive to RNA interference. Silencing the characterized genes resulted in a disruption of motor function, particularly locomotion in the migratory behaviour assay. Knockout/down of receptor genes in *C. elegans* has provided information on the roles of specific receptors. For example, knockdown of VRFR1 gene resulted in an increase in the number of eggs laid, suggesting a role in reproduction while knockdown of FLP15R affected locomotion (Keating et al. 2003). However, many neurones in *C. elegans* are resistant to gene inactivation by RNAi due to the expression of *eri-1* (enhanced RNAi), which results in the suppression of RNA interference more intensely in neurones (and gonads) than in other tissues (Kennedy et al. 2004).

Eleven orphan GPCRs have been linked to FLPs in nematodes (McVeigh et al. 2006), and some of these have splice variants. These receptors can be named by the ligands that activate them, viz, FLP2Ra (T19F4.1a), FLP2Rb (T19F4.1b), FLP3R (C53C7.1), VRFaR1 (C26F1.6), VRFaR2 (Y59H11AL.1, FLP-7, FLP-11), FLP15R (C10C6.2), FLP18R1a (Y58G8A.4a), FLP18R1b (Y58G8A.4b), FLP18R2 (C16D6.2), FLP21R (C25G6.5) and NPR1/FLP-21-R (C39E6.6) (Kubiak et al. 2003a, b; 2008; Geary and Kubiak 2005; Mertens et al. 2004, 2005a, b, 2006; Rogers et al. 2003). These receptors and their preferred ligands are summarized in Table 5 from which it can be seen that only 8 FLPs out of over 70 so far identified in *C. elegans* have been linked to a receptor. In this table, only the most active *C. elegans* FLPs have been included, and fuller lists of peptides that possess activity on these receptors are given in the referenced papers. Interestingly, AF3, AF4 and AF20 are also potent on FLP18R1a and b (Kubiak et al. 2008). AF9 (FLP21) also activates these receptors but at high nM concentrations. However, as pointed out by McVeigh et al. (2006), only a limited number of ligands have been tested with each receptor and so the currently proposed ligand may not prove to be the ligand, which physiologically activates the receptor in vivo. Ligand-receptor analysis usually involves the expression of the receptor gene in a vertebrate system, such

Table 4 Summarizing FLPs and RFas isolated, sequenced or predicted in *C. elegans*

Gene	C-terminal sequence	Sequence
flp-1	-PNFLRFa	SADPNFLRFa
		SQPNFLRFa
		ASGNPNFLRFa
		SDPNFLRFa
		AAADPNFLRFa
		(K)PNFLRFa
		AGSDPNFLRFa
		(K)PNFMRYa
flp-2	-EPIRFa	SPREPIRFa
		LRGEPIRFa
flp-3	-GTMRFa	SPLGTMRFa
		TPLGTMRFa
		EAEEPLGTMRFa
		NPLGTMRFa
		ASEDALFGTMRFa
		EDGNAPFGTMRFa
		SAEPFGTMRFa
		SADDSAPFGTMRFa
		NPENDTPFGTMRFa
flp-4	-FIRFa	PTFIRFa
		ASPSFIRFa
flp-5	-KFIRFa	GAKFIRFa
		AGAKFIRFa
		APKPKFIRFa
flp-6	KSAYMRFa	KSAYMRFa
flp-7	-MVRFa	SPMQRSSMVRFa
		TPMQRSSMVRFa
		SPMERSAMVRFa
		SPMDRSKMVRFa
flp-8	KNEFIRFa	KNEFIRFa
flp-9	KPSFVRFa	KPSFVRFa
flp-10	-GYIRFa	QPKARSGYIRFa
flp-11	-VRFa	AMRNALVRFa
		ASGGMRNALVRFa
		NGAPQPFVRFa
flp-12	-FIRFa	RNKFEFIRFa
flp-13	-PFIRFa	AMDSPFIRFa
		AADGAPFIRFa
		APEASPFIRFa
		ASPSAPFIRFa
		SPSAVPFIRFa
		ASSAPFIRFa
		SAAAPLIRFa
flp-14	KHEYLRFa	KHEYLRFa
flp-15	-GPLRFa	GGPQGPLRFa
		RGPSGPLRFa
flp-16	-QTFVRFa	AQTFVRFa
		GQTFVRFa
flp-17	-RFa	KSAFVRFa
		KSQYIRFa
flp-18	-PGVLRFa	(DFD)GAMPGVLRFa
		EMPGVLRFa
		(SYFDEKK)SPGVLRFa
		EIPGVLRFa
		SEVPGVLRFa
		DVPGVLRFa
flp-19	-VRFa	WANQVRFa
		ASWASSVRFa
flp-20	AMMRFa	AMMRFa
flp-21	-PLRFa	GLGPRPLRFa
flp-22	-WMRFa	SPSAKWMRFa
flp-23	-DFLRFa	VVGQQDFLRFa
		(TKFQDFLRFa)
flp-24	-MVRFa	VPSAGDMMVRFa
flp-25	-RFa	DYDFVRFa
		ASYDYIRFa
flp-26	-LRFa	EFNADDLTLRFa
		GGAGEPLAFSPDMLSLRFa
flp-27	-RMRFa	EASAFGDIIGELKGKGLGGRMRFa
flp-28	-LMRFa	APNRVLMRFa
Flp-29	LSIIRFa	LSIIRFa
flp-32	-LVRFa	AMRNSLVRFa
flp-33	-KPRFa	APLEGFEDMSGFLRTIDGIQKPRFa
flp-34	-RLRFa	ALNRDSLVASLNNAERLRFa

The data were taken mainly from Li (2005), Li and Kim (2008), Husson et al. (2007) and McVeigh et al. (2005b)

as, *Xenopus* oocytes, Chinese hamster ovary (CHO) cells or human embryonic kidney (HEK) cells. The signal pathways activated following ligand-receptor interaction have been investigated although it is possible that the pathways activated in heterologously expressed receptors may not be the same as the pathways activated in *C. elegans* tissues. An example of this is shown in studies involving NPR-1, which is expressed in neurones in head ganglia, in pharyngeal neurones and terminal bulb muscle cells and in ventral cord neurones and tail sensory neurones and is a regulator of foraging behaviour and aggregation (Coates and de Bono 2002). For example, NPR-1/FLP21R when expressed in *Xenopus* oocytes activates G_i/G_o proteins with the opening of inward rectifying potassium channels and when expressed in mammalian CHO cells it also activates G_i/G_o proteins but when expressed in the pharynx of *C. elegans* it activates Gαq (Rogers et al. 2003; Kubiak

Table 5 Showing the preferred ligands, which activate nematode GPCRs

Receptor	Ligand	Gene	Reference
T19F4.1a (FLP2Ra)	SPREPIRFa	*flp-2*	Mertens et al. (2005a)
	LRGEPIRFa		
T19F4.1b (FLP2Rb)	SPREPIRFa	*flp-2*	Mertens et al. (2005a)
	LRGEPIRFa		
C53C7.1 (FLP3R)	SPLGTMRFa	*flp-3*	McVeigh et al. (2006)
	SAEPFGTMRFa[a]		
C26F1.6 (VRFaR1)	TPMQRSSMVRFa[b]	*flp-7*	Mertens et al. (2004)
	AMRNALVRFa	*flp-11*	
Y59H11AL.1 (VRFaR2)[c]	SPMERSAMVRFa	*flp-7*	Mertens et al. (2006)
	AMRNALVRFa	*flp-11*	
C10C6.2 (FLP15R)	RGPSGPLRFa	*flp-15*	Kubiak et al. (2003b)
	GGPQGPLRFa		
Y58G8A.4a (FLP18R1a)	DVPGVLRFa[d]	*flp-18*	Kubiak et al. 2008
Y58G8A.4b (FLP18R1b)	DVPGVLRFa[d]	*flp-18*	Kubiak et al. (2008)
C16D6.2 (FLP18R2)	ASPSFIRFa	*flp-4*	McVeigh et al. (2006)
	DVPGVLRFa[d]	*flp-18*	
C25G6.5 (FLP21R)	GLGPRPLRFa	*flp-21*	McVeigh et al. (2006)
C39E6.6 (NPR1/FLP21R)	GLGPRPLRFa	*flp-21*	Kubiak et al. (2003a)
	NPR-1:215F (social)		Rogers et al. (2003)
	GLGPRPLRFa	*flp-21*	Kubiak et al. (2003a)
	NPR-1:215V (solitary)		Rogers et al. (2003)
	EMPGVLRFa[e,f]	*flp-18*	Rogers et al. (2003)

It should be noted that for most receptors, there is evidence for more multiple ligands and only the most potent is listed here. Which ligands are physiologically relevant for activation of each receptor is not clear

[a] Plus 4 related *flp-3* peptides

[b] Can activate VRFaR2 but is 100 times less active than SPMERSAMVRFa

[c] Also activated by *flp-1, flp-9, flp-13* and *flp-22 FLPs*

[d] Plus 8 related *flp-18* peptides

[e] Plus 5 related *flp-18* peptides using *Xenopus* oocyte assay

[f] Using NPR-1:215F *C. elegans* pharyngeal muscle assay Rogers et al. (2003) found that FLP-18 peptides also reduced action potential frequency

et al. 2003a). It is likely that *C. elegans* ligand-receptor interactions occur best at temperatures in the 20–30°C range and need accessory proteins, which are different from those in mammalian cells (Kubiak et al. 2003a; Mertens et al. 2006) although FLP18R1a and b are both fully functional at 37°C (Kubiak et al. 2008). Recently, it has been proposed that certain FLPs, viz, AF1, AF10 and PF2, may be the putative ligands for the latrophilin-like receptor isolated from *Haemonchus contortus* (Muhlfeld et al. 2009) though these FLPs exhibited low affinities in the μM range. FLPs can also activate more than one receptor, for example, FLP-18 peptides activate NPR1/FLP21R as well as FLP18R1a, FLP18R1b and FLP18R2 and equally receptors can be activated by more than one FLP family, for example, NPR1/FLP21R can be activated by both FLP-21 and FLP-18 (Rogers et al. 2003). More than one signalling pathway can also be associated with ligand-receptor interactions, for example, FLP-18 activation of FLP181a and FLP181b involves both G_q and to a lesser extent G_s (Kubiak et al. 2008) while FLP2Ra and b are both linked to the G_q pathway (Mertens et al. 2005a).

There have been many studies on the role of FLPs in nematodes, particularly using *A. suum* (Brownlee et al. 2000; Mousley et al. 2005) but only a selection will be discussed here. The preparations used include somatic body wall muscle, pharyngeal muscle, reproductive system (vulva and vagina) muscles and neurones. Stretton and colleagues were the first to identify FLPs in *A. suum* and to test them for biological activity (Cowden et al. 1989; Cowden and Stretton 1993, 1995; Stretton et al. 1991). They used several approaches including injection of synthetic peptides, for example, AF1 (KNEFIRFa), into *A. suum* in vivo, testing the actions of FLPs on dorsal muscle strips and investigating the effects of FLPs on

motoneuron activity. Stretton and colleagues found that AF1 and AF2 (KHEYLRFa) had complex actions on muscle strips including relaxation and contraction and the induction of rhythmic activity. Some of these effects are presynaptic on motoneuron terminals since AF2 potentiated excitatory junctional potentials (ejps) recorded from the muscle cells but AF2 did not potentiate the effect of muscle depolarization due to bath-applied acetylcholine (Pang et al. 1995). Maule et al. have also conducted extensive studies on the bioactivity of FLPs (Maule et al. 1995, 1996; Mousley et al. 2005). The PF FLPs identified in *Panagrellus redivivus* are of particular interest (Geary et al. 1992; Maule et al. 1995). Four PF FLPs have been identified in *P. redivivus*, viz, PF1 (SDPNFLRFa), PF2 (SADPNFLRFa), PF3 (KSAYMRFa) and PF4 (KPNFIRFa). All four peptides relax dorsal body wall muscle but PF3 contracts ventral body wall muscle (Maule et al. 1995). It has been proposed that nitric oxide (NO) is involved in the mediation of the inhibitory effects of PF1 and PF2 on *A. suum* muscle (Bowman et al. 1995). However, while both biochemical and histochemical studies indicate that nitric oxide synthase (NOS) is present in the neuromuscular system of *A. suum* (Bascal et al. 2001), there is no evidence for the NOS gene in *C. elegans.* NOS immunoreactivity has been found in the nematode, *Trichinella britovi* (Masetti et al. 2004). The absence of NOS in *C. elegans* may not be typical of nematodes. PF4 has a very rapid relaxing effect, which has been examined in detail (Holden-Dye et al. 1997; Purcell et al. 2002). Intracellular recordings from somatic muscle cells in *A. suum* showed that PF4 and γ-aminobutyric acid (GABA) hyperpolarize the muscle cells with an increase in input conductance. The reversal potential of the rapid phase of the PF4 response indicated that it was a chloride event. The conductance increase due to PF4 was independent of the conductance increase to GABA since the latter compound was blocked by ivermectin while PF4's conductance increase was unaffected by ivermectin. The time course of the PF4 and GABA responses was similar and shorter than that of PF1. It is possible that the rapid response elicited by PF4 is associated with direct gating of the chloride channel as occurs in gastropod molluscs with FMRFa (Cottrell 1997). In contrast to the rapid inhibitory response of PF4 on *A. suum* somatic muscle cells, PF1's inhibitory effect is slow and also independent of the inhibitory action of GABA (Franks et al. 1994). In addition to their postsynaptic effect to inhibit muscle contraction, both PF1 and PF2 act presynaptically on motoneuron terminals to inhibit ejps, and this effect is independent of any effect of bath-applied acetylcholine-induced muscle cell depolarization. The postsynaptic effect of both FLPs is possibly through the inhibition of the excitation–contraction coupling of the muscle cells (Holden-Dye et al. 1995). There is evidence that FLPs are involved in the sexual turning behaviour of male *C. elegans* (Liu et al. 2007). For example, *flp-8*, *flp-10*, *flp-12* and *flp-20* mutant males increase repetitive turning behaviour compared with wild-type males. Genes, such as, *egl-3*, *egl-21*, *ida-1* and *unc-31*, which are required for neuropeptide processing and release, are also required for the inhibition of repetitive turning behaviour. Two of these genes, *egl-3* and *egl-21*, are expressed on the mechanosensitive touch receptor neurones (TRNs).

There have been relatively few studies involving the action of FLPs on either pharyngeal or reproductive system muscles. PF3 (AF8) has a biphasic action on the isolated pharyngeal pumping activity of *A. suum*, excitation followed by inhibition where the muscles are in the hypercontracted state (Brownlee et al. 1995, 1996). In contrast, the inhibition induced by AF1 leaves the pharyngeal muscle in a more relaxed state while AF2 excited the muscle. PF1, FMRFa and GNFFRFa had no observable effect on *A. suum* pharyngeal pumping. AF1, AF2, AF8 (PF3) and GAKFIRFa have been tested for activity on the pharynx of *C. elegans* (Rogers et al. 2001). These FLPs all increased pharyngeal action potential frequency while PF1 (FLP1), PF2 (FLP2), FLP3 (SAEPFGTMRFa), FLP9 (KPSVRFa), FLP13 (APEASPIRFa) and FLP16 (AQTVRFa) all inhibited the pharynx. This study was later extended to include representative FLPs from *flp* genes, *flp-1* to *flp-23* (Papaioannou et al. 2005). FLPs from genes expressed in the pharyngeal nervous system all showed clear bioactivity. In addition, some FLPs whose genes were either not expressed in the pharyngeal system or whose expression was not determined (Kim and Li 2004) were also active. Interestingly, the only inactive FLPs were FLPs whose genes had not been shown to be expressed in the pharyngeal system of *C. elegans.* FLPs have also been tested for activity on muscles associated with the reproductive system, for example, AF1 has a biphasic action on the vagina vera (ovijector), a muscular tube, which connects the uterus and vagina uteri to the gonopore of *A. suum,* while AF2 and PF3 are both inhibitory, and FLRFa is excitatory (Fellowes et al. 1998). In a second study, the actions of five FLPs were investigated using the same preparation (Fellowes et al. 2000). PF1, PF2 and PF4 all inhibited muscle activity while both AF3 and AF4 induced complex, multiphasic responses, an initial contraction with spastic paralysis followed by contractions with enhanced amplitudes. This group has also tested the effects of representative FLPs encoded by *flp1-22* from *C. elegans* on the ovijector of *A. suum* (Moffett et al. 2003). Most (16) of the FLPs tested inhibited the muscle while 4 excited it. Other effects observed were transient contraction (1 FLP), transient contraction followed by a long period of inactivity (4 FLPs) and shortening of the ovijector coupled with an increase in contraction frequency (1 FLP). An

interesting paper by Dossey et al. (2006) has attempted to relate FLP structure to activity. They used *C. elegans* NPR-1 activation by FLP-18 and NMR analysis to determine the structural features of peptides, which may determine their activity. They found that long range electrostatic interactions occurred between N-terminal aspartate (D) and the C-terminal penultimate arginine (R) together with N-terminal H-bonding interactions that form loops. These authors conclude that peptide charge and these loops may be key in determining activity.

Overall, the evidence suggests that FLPs are widely distributed in nematode tissues and play key roles in their normal physiology, including feeding (FLP-18, FLP-21), reproduction (FLP-7, FLP-11), locomotion (FLP-15) and in the modulation of neuronal circuits.

Annelids

There are two main classes of annelids, viz, Polychaeta (marine worms) and Clitellata with the latter being divided into the Oligochaeta (aquatic and earthworms) and Hirudinea (leeches) (Barnes et al. 1988). Their nervous system consists of a supra-oesophageal ganglion, a circum-oesophageal ganglionic ring and a ventral nerve cord with segmental ganglia (Barnes et al. 1988). Annelids have a closed blood system with a series of hearts, so FLPs can potentially act as neurohormones in this phylum. The neuroendocrine system of annelids has been reviewed by Salzet (2001).

Compared with other phyla relatively few FLPs have been identified in annelids but these include both FMRFa and FLRFa, Table 6 (Baratte et al. 1991; Evans et al. 1991). In addition to FMRFa, a methionyl sulphoxide derivative has been identified in both polychaetes and leeches, e.g., *Nereis diversicolor* and *Hirudo medicinalis*. Unusually, there is another tetrapeptide where T replaces M or L, FTRFamide, Table 6, (Baratte et al. 1991). There are also two tetrapeptides where tyrosine replaces phenylalanine at position 1,

Table 6 Summarizing FLPs that have been identified in Hirudinea and polychaetes

FLP	Species	Reference
FMRFa	*Nereis virens*	Krajniak and Price (1990)
FMRFa	*Hirudo medicinalis*	Evans et al. (1991)
FLRFa	*H. medicinalis*	Evans et al. (1991)
FTRFa	*Nereis diversicolor*	Baratte et al. (1991)
YMRFa	*H. medicinaslis*	Evans et al. (1991)
YLRFa	*H. medicinalis*	Evans et al. (1991)
GGKYMRFa	*H. medicinalis*	Evans et al. (1991)
GDPFLRFa	*Erpobdella octoculata*	Salzet et al. (1994)

YMRFa and YLRFa, both found in *H. medicinalis* (Evans et al. 1991). Two extended FLPs, viz, GDPFLRFa and GGKYMRFa, have been identified in leeches, e.g., *Erpobdella octoculata* and *H. medicinalis,* respectively, but not in other annelids (Evans et al. 1991; Salzet et al. 1994). Salzet et al. (1994) suggest there might be an ancestral RFamide gene common to leeches and molluscs.

The roles for FLPs in annelids have been particularly studied in leeches, possibly due to the ability to identify many neurones in the segmental ganglia (Muller et al. 1981). There is good evidence that FLPs are involved in the regulation of leech hearts. FMRFa-like immunoreactivity occurs in a defined population of central neurones in the leech, *H. medicinalis* (Kuhlman et al. 1985a) and these include three types of neurones that have a role in the regulation of the heart. The heart excitatory (HE) neurones and the heart accessory (HA) neurones directly innervate the heart while interneurone 204 has an excitatory effect on the heart central pattern generator, which is made up of 7 pairs of HN interneurones (Kuhlman et al. 1985a; Li and Calabrese 1987). FLP immunoreactivity has been identified in HE, HA and 204 neurones and in R and LPE (rostral and lateral penile erector) neurones and in the large longitudinal motoneuron (L). It is likely that a FLP colocalizes with acetylcholine in many of these neurones but acetylcholine is probably absent from HA neurones (Li and Calabrese 1987). In annelids, a FLP may act as a hormone when released into the blood but as a transmitter/modulator when released into the intercellular space adjacent to an effector organ. Application of FMRFa onto the heart produced effects, which were similar to those produced when HA was activated (Kuhlman et al. 1985b). When FMRFa was applied to the isolated central nervous system, it activated the central motor programme for heartbeat, an effect that mimics stimulation of neurone 204. The FLP immunoreactivity in the HA axon is likely to be FMRFa (Li and Calabrese 1987). These authors found evidence for the presence of FMRFa in HA cells but could not detect FMRFa-like activity in extracts of isolated HE cells even though these cells stain strongly for FMRFa-like immunoreactivity. However, HE cells exert effects on the heart when stimulated, which are similar to those when FMRFa is applied to the heart, viz, stimulates myogenic activity in the heart. Both the effect of FMRFa and the effect on the heart following HE stimulation are long lasting. Calabrese et al. (1995) and Nadim and Calabrese 1997) consider that the primary excitatory effect of FMRFa on the leech heart is through activation of a slow outward potassium current (I_{KF}) in heart interneurones, and these currents have very slow activation and deactivation kinetics. FMRFa also excites the longitudinal muscle of the leech (Norris and Calabrese 1987, 1990). FMRFa-like immunoreactivity is present in neurones associated with the pharynx of

H. medicinalis, including SW1 (swallow) neurone (O'Grady et al. 1999). When SW1 is activated the pharynx dilates and the mouth opens. However, stimulation of SW1 does not result in one to one postsynaptic potentials in pharyngeal muscle, indicating SW1 is not a motoneuron. FMRFa and other FLPs excite the pharynx, increasing both the basal tone and peak tension of the muscle. pQDPFLRFa and FMRFa were the most potent of the FLPs tested while FLRFa and GGKYMRFa were least active. The FMRFa response was reduced by an inhibitor of protein kinase C. There is also evidence that RFas play a role in the regulation of osmoregulation in the leech (Salzet et al. 1994; Salzet 2001). The extended FLP, GDPFLRFa acts as a diuretic while FMRFa acts as an antidiuretic, providing evidence they act on different receptors as occurs in other phyla. FMRFa is present in nephridial sensory nerve cells (NNCs) whose nerve terminals are located in the nephridium of *H. medicinalis* (Wenning et al. 1993; Wenning and Calabrese 1995). These neurones monitor extracellular chloride. FMRFa also modulates the excitability of NNCs, suggesting autoregulation of the receptor gain and FMRFa release. These authors propose that FMRFa has a role in control of salt output rather than volume output in leeches.

The effect of FMRFa has been tested on a number of tissues and organs in oligochaetes, e.g., FMRFa reduces the contraction amplitude of the isolated crop-gizzard of *Lumbricus terrestris* while increasing the contraction rate in the 10–100 nM range but decreases the rate at higher concentrations (Krajniak and Klohr 1999). In polychaetes, immunoreactivity for FMRFa has been found in the oesophageal muscles and gut of *Nereis virens,* and a FLP is likely to regulate gut activity (Krajniak and Greenberg 1992). There is evidence for FMRFa-like immunoreactive nerve fibres, which occur in the muscular plexus of the body wall of oligochaetes, and FMRFa directly excites the body wall muscle since its effect persists in the presence of tetrodotoxin (Csoknya et al. 2005). Interestingly, FMRFa-like immunoreactivity has been found in sensory neurones in *Lumbricus terrestris* body wall epithelium, indicating a sensory role for FLPs in oligochaetes (Reglodi et al. 1997). FMRFa-like immunoreactivity has also been found in sensory neurones of the feeding palps of the polychaetes *Dipolydora quadrilobata* and *Pygospio elegans* (Forest and Lindsay 2008). It is likely that a FLP colocalizes with serotonin in central neurones of the earthworm, *Eisenia fetida* (Lubics et al. 1997).

Although there are only seven FLPs identified in annelids evidence would suggest they play an important role in the regulation of muscle activity. Most of the physiological experiments have used FMRFa, and it would be interesting to see the effects of the other annelid FLPs on muscle and neuronal activity in this phylum.

Molluscs

The first FLP, FMRFa, was identified in a bivalve mollusc, the clam, *Macrocallista nimbosa* (Price and Greenberg 1977); and subsequently, these authors have published a number of reviews on FLPs (Greenberg and Price 1992; Price 1986; Price and Greenberg 1989, 1994). There has been a recent review on FMRFa and related peptides in molluscs (Lopez-Vera et al. 2008), and the more general review of Krajniak (2005) also considers molluscs. The number of FLPs has steadily increased and now over 20 have been proposed or identified in molluscs, Table 7. The number of amino acids making up molluscan FLPs generally varies from four to ten, and FLPs can be classified depending on the number of their amino acids. Some FLPs, such as, FMRFa, have been identified in a number of species and are likely to occur widely through the phylum. In addition to FLPs, there are also RFa and F-amides while tyrosine can replace phenylalanine to give a C-terminal ending of YLRFa. Examples of RFas are shown in Table 8. One FRa, Conorfamide-Sr1 (Cono-RF-amide, GPMGWVPVFYRFa), has only been identified in the venom of *Conus spurius* (Maillo et al. 2002). Unlike the position in some phyla, there are a number of endogenous tetrapeptides and pentapeptides, with the tetrapeptides playing a key physiological role.

FLP genes have been investigated in a number of molluscs. One group, the pulmonates, has a single FLP gene while the remaining gastropods and the other classes of molluscs have two FLP genes. FLPs in *L. stagnalis* are encoded by a multiexon gene, which undergoes alternate mRNA splicing, resulting in two different mRNAs (Worster et al. 1998). Both mRNAs share exon I while type 1 mRNA is associated with exon II and encodes for tetrapeptides while type 2 is associated with exons III-V and encodes for heptapeptides, such as, SKPYMRFa, GDPFLRFa and SDPFLRFa and the hexapeptides HDYMRFa and SSFPRYa, (Kellett et al. 1994; Santama and Benjamin 2000). The gene is differentially spliced in many neurones in a cell-specific manner with tetrapeptides occurring in around 280 neurones in all eleven ganglia of *L. stagnalis* while the heptapeptides occur mainly in neurones (around 57) in the visceral and right parietal ganglia (Bright et al. 1993). Using in situ hybridization, the paper of Bright et al. (1993) allowed precise identification of the FLP content of identified neurones and provided the basis for subsequent physiological studies on the precise roles of FLPs in gastropods. For example, heart excitatory motoneurones (E_{he}) of *L. stagnalis* contain both FMRFa and FLRFa (together with pQFLRIa, EFLRIa and pQFYRIa), and their effects on heart activity have been investigated (Buckett et al. 1990; Worster et al. 1998). Similarly, the visceral white interneurone (VWI) contains roughly equal

Table 7 Summarizing the FLPs found in molluscs

FLP	Species	Reference
FMRFa	*Macrocallister nimbosa*	Price and Greenberg (1977)
FLRFa	*Lymnaea stagnalis*	Linacre et al. (1990)
FIRFa	*Sepia officinalis*	Loi and Tublitz (1997)
AFLRFa	*Octopus vulgaris*	Martin and Voigt (1987)
NFLRFa	*Mytilus edulis*	Favrel et al. (1998)
TFLRFa	*O. vulgaris*	Martin and Voigt (1987)
SFMRFa	*L. stagnalis*	Linacre et al. (1990)
PYMRFa	*L. stagnalis*	Kellett et al. (1994)
pQFYRFa	*Helix aspersa*	Lutz et al. (1992)
HDYMRFa	*L. stagnalis*	Kellett et al. (1994)
QEYMRFa	*H. aspersa*	Lutz et al. (1992)
DPFLRFa	*Helix pomatia*	Minakata et al. (1992)
GDPFLRFa	*L. stagnalis*	Saunders et al. (1991)
NDPFLRFa	*H. aspersa*	Price et al. (1990)
pQDPFLRFa	*H. aspersa*	Price et al. (1985)
SDPFLRFa	*L. stagnalis*	Saunders et al. (1991)
SDPYLRFa	*L. stagnalis*	Saunders et al. (1991)
NDPYLRFa	*H. aspersa*	Price et al. (1990)
SEPYLRFa	*H. aspersa*	Price et al. (1990)
SKPYMRFa	*L. stagnalis*	De With and van der Schors (1992)
NGHYMRFa	*H. aspersa*	Lutz et al. (1992)
ENNNGYIRFa	*H. aspersa*	Lutz et al. (1992)
ALSGDAFLRFa	*S. officinalis*	Loi and Tublitz (1997)
ALTNDHFLRFa	*Fusinus ferrugineus*	Kanda et al. (1990)
pQGDTADNEYLRFa	*H. pomatia*	Minakata et al. (1992)

amounts of both GDPFLRFa and SDPFLRFa (together with small amounts of SDPYLRFa, SDPFFRFa and SKPYMRFa). The VWI synapses onto many follower neurones including those that regulate respiration and heart rate (Skingsley et al. 1993). The pleural interneurone PIB of *L. stagnalis* is another example of an identified neurone, which contains FMRFa (Alania et al. 2004). In *Helix aspersa,* there are two classes of FLP-encoding cDNA clones derived by alternative splicing of a single gene, one (H-1) encodes FMRFa, FLRFa and pQFYRFa while the other (H-4) encodes only N-terminally extended peptides (Lutz et al. 1992). In *H.* aspersa, tetrapeptides and extended FLPs are expressed in different neurones (Cottrell et al. 1992). Multiple copies of peptides are often encoded by the same gene where, for example, in *Aplysia californica,* around 30 copies of FMRFa but only a single copy of FLRFa are encoded (Taussig and Scheller 1986). The relative proportion of tetrapeptides and extended peptides can also vary between species.

NPY-related peptides have been found in gastropods and cephalopods and have a C-terminal sequence RPRFa in *A. californica* (Rajpara et al. 1992), *L. stagnalis* (Tensen et al. 1998a) and *Loligo vulgaris* (Smart et al. 1992) and RTRFa in *H. aspersa* (Leung et al. 1992), while the C-terminal sequence for many vertebrate NPY is RQRYa (Tensen et al. 1998a). The N-terminal sequence for vertebrate NPY also starts with a tyrosine. Vertebrate NPY peptides have around 36 amino acids, slightly fewer than found in molluscs, for example, 39 in *L. stagnalis* (see Table 2 for structure) and *H. aspersa* and 40 in *A. californica*; however, *L. vulgaris,* has only 9 amino acids. Interestingly, the NPFs identified from platyhelminths contain 36–39 amino acids with a C-terminal of GRPRFa (Curry et al. 1992; Maule et al. 1991). All the NPY-related peptides have a proline at position 5 and a tyrosine at position 27 of the vertebrate NPY. Immunocytochemical studies have demonstrated the colocalization of NPY- and FMRFa-like immunoreactivity in the optic lobe and peduncular complex of *Octopus valgaris*, suggesting a role for both peptides in the modulation of visual information (Suzuki et al. 2002). In vertebrates, NPY plays a key role in the regulation of food intake and energy flow (Frankish et al. 1995). In contrast, LyNPY has no short-term effects on food consumption but only affects energy flow, that is, it can stop reproduction and growth (De Jong-Brink et al. 2001). However, there is evidence for a leptin-like compound in *L. stagnalis,* which inhibits food consumption. In vertebrates, there are up to six NPY receptors, Y-1 to Y-6, and Y-5 activation may stimulate feeding. It is

Table 8 Summarizing the RFas found in molluscs

RFa	Species	Reference
TIFRFa	*Loligo opalescens*	Loi and Tublitz (1997)[a]
GNLFRFa	*L. opalescens*	Loi and Tublitz (1997)[a]
GSLFRFa	*Fusinus ferrugineus*	Kanda et al. (1990)
NSLFRFa	*L. opalescens*	Loi and Tublitz (1997)[a]
SSLFRFa	*F. ferrugineus*	Kanda et al. (1990)
STLFRFa	*Aplysia californica*	Greenberg and Price (1992)
GGALFRFa	*A. californica*	Greenberg and Price (1992)
SDPFFRFa	*Lymnaea stagnalis*	Kellett et al. (1994)
GGAALFRFa	*A. californica*	Greenberg and Price (1992)
pQGGSLFRFa	*Helix pomatia*	Minakata et al. (1992)
pQGGTLFRFa	*H. pomatia*	Minakata et al. (1992)
AdLAGDHFFRFa	*Mytilus edulis*	Fujisawa et al. (1992)
SGQSWRPQGRFa	*Achatina fulica*	Fujimoto et al. (1990)
SAPSWRPQGRFa	*A. californica*	Aloyz and DesGroseillers (1995)
TPHWRPQGRFa	*L. stagnalis*	Tensen et al. (1998b)
GGTLLRFa	*Acanthopleura granulata*	Greenberg and Price (1992)
GSLLRFa	*A. granulata*	Greenberg and Price (1992)
YAIVARPRFa	*Loligo vulgaris*	Smart et al. (1992)
GPMGWVPVFYRFa	*Conus spurius*	Maillo et al. (2002)
STQMLSPPER-19-YYAIMGRTRFa	*Helix aspersa*	Leung et al. (1992)
TEAMLTPPER-19-YYAIVGRPRFa	*L. stagnalis*	Tensen et al. (1998a)
DNSEMLAPPP-20-YYSIMGRPRFa	*A. californica*	Rajpara et al. (1992)

[a] Unpublished work of D. A. Price quoted by Loi and Tublitz (1997)

proposed by De Jong-Brink et al. (2001) that the LsNPY receptor more closely resembles the vertebrate Y-1 receptor and that LsNPY exerts its action through a Y-1-like receptor in *L. stagnalis*. In molluscs and arthropods, NPY is synthesized from a prohormone similar to vertebrate NPY precursors while platyhelminth NPYs are synthesized in a different way, making De Jong-Brink et al. question whether or not they should be considered as real homologues of vertebrate NPY.

RFas that occur in molluscs include FRFas, GRFas, LRFas and PRFas Table 8. The FRFas are not alternatively spliced products of the FMRFa gene and are therefore less closely related to FLPs than are heptapeptides (Price and Greenberg 1994). The leucine of *Mytilus*FFRFa, A_DLAG-DHFFRFa, is a D isomer but the synthetic peptide with an L isomer is equiactive on the anterior byssus retractor muscle, both peptides potentiate phasic contractions of the muscle but at high concentrations cause tonic contraction (Fujisawa et al. 1992). Two LLRFas, viz, GSLLRFa and GGTLLRFa have been reported in a chiton, *Acanthopleura granulata* (Price and Greenberg 1994). A YRFa, pQFYRFa, has been identified in the FMRFa gene of *H. aspersa* (Lutz et al. 1992); and an extended form, GPMGWVPVFYRFa, has been identified from the venom of *Conus spurious* and when injected into mice this peptide induced hyperactivity (Maillo et al. 2002). However, this hyperactivity can only be elicited in mice older than 16 days. Interestingly, the naturally occurring pQFYRFa antagonizes the effect of FMRFa on the ligand-gated channel expressed in *Xenopus* oocytes (Cottrell 1997). This raises the possibility that this peptide might act as an endogenous antagonist to reduce or terminate the action of FMRFa in *H. aspersa*.

There have been extensive immunohistochemical studies in molluscs, particularly in gastropods and cephalopods but these will not be considered in detail. The recent review of Lopez-Vera et al. (2008) provides a useful overview and references to these studies. There have also been many investigations regarding the effects of FLPs on molluscan excitable tissues. For example, FLRFa can excite the heart, rectum and pedal retractor muscle of the clam, *Meretrix lusoria* (Ohtani et al. 1995) while FMRFa, 10–100 nM, relaxes acetylcholine-induced catch tension of *Mytilus edulis* anterior byssus retractor muscle but at 1 μM causes contraction of the muscle (Muneoka and Saitoh 1986). Tetrapeptides and heptapeptides vary in terms of their relative potencies depending on the tissue. For example, the *H. aspersa* FLP, pQDPFLRFa, is 100 times more potent than FMRFa on the heart and occurs in the blood at concentrations, which can excite the heart, suggesting a physiological role as a cardioexcitant (Price et al. 1985). In

contrast, pQDPFLRFa is less potent than FMRFa on the radula protractor muscle of *H. aspersa.* It is likely that extended FLPs act as neurohormones as they are more resistant to enzymatic breakdown compared to tetrapeptides (Price 1986). In addition, the methionine is replaced by leucine in the extended FLPs making them less prone to oxidation. FMRFa can modulate the activity of *A. californica* buccal motoneurones, suggesting a role in the regulation of feeding in gastropods (Sossin et al. 1987). On the heart of *Achatina fulica,* FMRFa, FLRFa and the nematode FLP, SDPNFLRFa, excited the heart, (threshold 1 nM) while the avian FLP, LPLRFa, the nematode FLP, KHEYLRFa and the synthetic RFa, GSFFRFa, were weak excitants, suggesting the importance of the FM/LRFa C-terminal for potent excitation (Koch et al. 1993). Interestingly, the synthetic FLP, DNFLRFa, was a potent inhibitor of the heart. The *Fuscinus ferruginius* RFa, SSLFRFa, was also a potent inhibitor of activity while LFRFa and GSLFRFa were weak inhibitors of the heart. The nematode FLP, KNEFIRFa, was also a weak inhibitor of activity. MRFa, LRFa and RFa were inactive, indicating the importance of the tetrapeptide structure with F as the first amino acid for activation of the heart FLP receptor. FMRFa itself was the subject of a study in which it was shown to directly gate amiloride-sensitive sodium channels in the C2 neurone of *H. aspersa* (Cottrell et al. 1990). This work has been reviewed in depth (Cottrell 1997) and will be discussed later in this section. Apart from this fast excitatory response, FLPs can have other effects on gastropod neurones, suggesting multiple receptors (Cottrell and Davis 1987; Cottrell et al. 1984). For example, FLPs can excite some neurones through an increase in permeability to sodium but inhibit others through an increase in permeability to potassium. YGGFMRFa was found to be more active in producing the excitatory response. Some neurones showed a biphasic response (Cottrell et al. 1984). These experiments were extended to show that FLPs could have up to four actions on neurones in the *H. aspersa* suboesophageal ganglia, viz, a slow increase in potassium conductance, a fast increase in potassium conductance, an increase in sodium conductance and a decrease in potassium conductance (Cottrell and Davis 1987). Clear differential actions of the FLPs tested provided evidence for more than one FLP receptor on snail neurones. In addition to having a direct action on gastropod central neurones, FLPs can also reduce the excitatory response to acetylcholine (ACh). For example, FMRFa, SKPYMRFa and its acetylated derivative can all reduce the ACh-induced inward current of identified neurones of *Helix lucorum* (Pivovarov and Walker 1996).

The occurrence and actions of FLPs have been examined on several cephalopods, and a total of ten FLP/RFas have been described, including tetrapeptides, pentapeptides, hexapeptides and decapeptides, Tables 7 and 8. The first FLPs identified in this class were FMRFa, FLRFa, AFLRFa and TFLRFa in the optic lobe and vena cava of *O. vulgaris* (Martin and Voigt 1987). Their presence in these areas suggests roles in both visual and cardiovascular physiology. Subsequently, partial sequences for two cDNAs were isolated, one coding FMRFa, FLRFa and ALSGDAFLRFa and one coding TIFRFa, GNLFRFa, GSLFRFa and NSLFRFa from *Loligo opalescens* (D.A.Price, quoted by Loi and Tublitz 1997). A full length FLP cDNA has been isolated from the brain of the cuttlefish, *Sepia officinalis,* which encodes a precursor protein for FMRFa, FLRFa, FIRFa and ALSGDAFLRFa (Loi and Tublitz 1997). This precursor protein encodes eleven copies of FMRFa and one copy of each of the other FLPs. All four FLPs induce chromatophore expansion in vitro. A MALDI (matrix-assisted laser desorption/ionization) mass spectrometric investigation into tissues from three species of cephalopod, viz, *S. officinalis, L. opalescens* and *Dosidicus gigas,* was undertaken by Sweedler et al. (2000). These authors found molecular masses, which corresponded to FMRFa and FLRFa and went onto sequence a FLP gene in *L. opalescens*. This gene encodes a prohormone that can be processed into four FLPs, viz, FMRFa, FLRFa, FIRFa and ALSGDAFLRFa. As with the gene from *S. officinalis*, this gene also contained eleven copies of FMRFa and one each of the other FLPs. It is likely that FMRFa from the subpeduncular area of the brain influences the secretory activity of the optic glands in *O. vulgaris* (Di Cosmo and Di Cristo 1998). These glands control the maturation of the reproductive system in the octopus. FMRFa and FLRFa stimulate contraction of the oviduct of *S. officinalis* while FIRFa and ALGSDAFLRFa reduce the tone, frequency and amplitude of contraction (Henry et al. 1999). In *O. vulgaris* a combination of FMRFa and dopamine reduce the protein content of both previtellogenic and early vitellogenic oviduct glands (Di Cristo and Di Cosmo 2007). NPY has also been proposed to regulate endocrine function in *O. vulgaris* (Suzuki et al. 2002). Both FLRFa and SDPFLRFa potentiated transmission across the giant synapse of the squid, *Loligo peallei* but no effect could be recorded from either the presynaptic or postsynaptic terminals (Cottrell et al. 1992). They concluded that a change in ion permeability did not underlie the potentiation and suggested a possible effect on transmitter mobilization. Under high frequency of activity, transmission at this synapse can fail and they suggest that a FLP might reactivate transmission. This might occur when the squid was under predator attack. These authors also detected FMRFa and FLRFa in the stellate ganglion of *L. peallei*.The action of FMRFa has been investigated on spontaneously excitatory and inhibitory postsynaptic currents (sE/IPSCs)

recorded from neurones in the central optic lobe of *S. officinalis* (Chrachri and Williamson 2003). FMRFa reduced both the frequency and amplitude of spontaneous and evoked EPSCs but increased the frequency and amplitude of spontaneous and evoked IPSCs. Since FMRFa had no effect on the interval or amplitude distributions of sE/IPSCs and its effect was blocked by tetrodotoxin, these authors concluded FMRFa was acting presynaptically in the optic lobe, possibly affecting calcium entry. This suggestion agrees with an earlier study on the action of FMRFa on L- and T-type calcium currents recorded from type I and type II dissociated heart muscle cells from *Loligo forbseii* (Chrachri et al. 2000). FMRFa blocked the L-type calcium current of type I cells while potentiating the L-type current in type II cells but had no effect on T-type calcium currents. The effect on type II heart muscle cells was not mediated via a pertussis toxin-sensitive pathway. While YGGFMRFa excites the octopus heart, it is not clear whether this FLP occurs naturally in cephalopods (Voigt et al. 1987).

Relatively, few FLP and RFa receptors have been characterized in molluscs but there are examples of both GPCRs and FLP-gated ion channel receptors. Using ^{125}I-desaminoYFnorLRFa (^{125}I-daYFnLRFa), a radioligand-binding assay was developed to identify and characterize an FMRFa receptor in the brain and heart of *H. aspersa* (Payza 1987). Binding of ^{125}I-daYFnLRFa to brain membranes was reversible, saturable and specific, with a K_d of 14 nM and a B_{max} of 85fmol/mg brain, designated a high affinity site and a K_d of 245 nM and B_{max} of 575 fmol/mg, designated a low affinity site. Payza performed an extensive structure–activity study using both a heart bioassay and competitive displacement of ^{125}I-daYFnLRFa from heart and brain membranes. Extended heptapeptides, viz, pQDPFLRFa, NDPFLRFa and SDPFLRFa, had a low potency in displacing the ligand while YGGFMRFa was a potent displacer. Payza found that the structure–activity profile for the heart bioassay and for ligand displacement from heart and brain membranes was similar, suggesting that the high-affinity-binding sites in the heart and brain membranes were FMRFa receptors. The optic lobe of *L. peallei* contains a FLP receptor, which binds to [^{125}I]-daYFnLRFa and is coupled to G_s (Chin et al. 1994). This receptor shows high affinity to FMRFa and FLRFa (IC_{50} 0.4 nM for both FLPs). Interestingly, the amide is essential for binding activity. An RFa GPCR from *L. stagnalis* has been cloned (GRL106) and its ligand, LyCEP, characterized (Tensen et al. 1998b). This receptor is expressed in the brain and heart and when activated by LyCEP, TPHWRPQGRFa, the heart is excited. Using the isolated *L. stagnalis* auricle preparation LyCEP excited the auricles, threshold around 10 nM and EC_{50} around 200 nM. When the receptor is expressed in *Xenopus* oocytes LyCEP activates a current with a threshold around 1 nM. LyCEP also inhibits activity of *L. stagnalis* caudodorsal cells. Tensen et al. (1998b) concluded that LyCEP is primarily a cardioactive neuropeptide with a structure that is closely related to ACEP-1 (SGQSWRPGRFa) from *A. fulica* and LUQIN (SAPSWRPQGRFa) from *A. californica* (Aloyz and DesGroseillers 1995; Fujimoto et al. 1990). LyCEP-containing fibres innervate the cells that produce egg-laying hormone, and these cells express GRL105. When applied to egg-laying hormone cells, LyCEP hyperpolarized the cell membrane potential (Tensen et al. 1998b). The cDNA of the FMRFa-gated receptor/channel protein (FaNaC) from *H. aspersa* has been cloned and sequenced and its properties analysed (Lingueglia et al. 1995). The channel has two membrane spanning regions and a large extracellular loop and has been expressed in *Xenopus* oocytes where it is activated by FMRFa and by a number of FLPs, which act as full agonists, viz, WMRFa, FnLRFa (nL, norleucine), FMRWa, FMRYa and WnLRFa. YMRFa was about 200 times less active than FMRFa while FLRFa was about ten times less active and was a partial agonist (Cottrell 1997). For optimal FMRFa-like activity, a non-polar aromatic amino acid, for example, phenylalanine or tryptophan, is required at position 1 and 4. If methionine is replaced by leucine, there is a considerable loss of activity but when replaced by norleucine, there is no loss of activity. When leucine is replaced by lysine, the tetrapeptide becomes an antagonist. When arginine is replaced by norleucine or D-arginine, there is always a considerable loss of activity, and even when replaced by lysine. A similar situation was observed by Payza (1987) in his study. FLRFa heptapeptides are inactive on FaNaC while YGGFMRFa is around 300 times less active than FMRFa, which contrasts with the receptor described by Payza (1987) where YGGFMRFa was more potent than FMRFa. These data are informative as they provide an insight regarding when other aromatic amino acids can replace phenylalanine and the neuropeptide can still be considered a FLP in terms of FLP-like activity. HaFaNaC has been expressed in CA3 pyramidal neurones from rat hippocampal slice cultures (Schanuel et al. 2008). FMRFa depolarizes and excites these neurones inducing bursts of activity, and this effect is blocked by amiloride. In contrast, mammalian NPFF and RFa-related peptide-1 have no effect on these channels and neither do NPFF antagonists. The FaNaCh protein forms covalent multimers in stably transfected human embryonic kidney cells (HEK-293) that associate as tetrameric complexes (Coscoy et al. 1998). A FMRFa-gated sodium channel (LsFaNaC) has also been cloned from *L. stagnalis* with 93% sequence identity to HaFaNaC and found to possess acid sensitivity, suggesting that FaNaCs share a common ancestry with mammalian acid-sensing ion channels (ASICs) (Perry et al.

2001). While not generating a current itself, FMRFa and FRRFa can potentiate the duration of H^+-gated currents from ASICs (Askwith et al. 2000). The mammalian NPFF also modulated ASICs but has a greater effect on DRASICs (dorsal root acid-sensing ion channels or ASIC3 s). The direct modulation of ASICs by FMRFa has been reviewed recently (Lingueglia et al. 2006).

It is clear that FLPs, together with other neuropeptides, play a key role in the physiology of molluscs. They occur widely in the central and peripheral nervous systems and occur in nerves that probably innervate all organs. At both central and peripheral levels, FLPs regulate respiration, feeding and the cardiovascular system. FLPs are also involved in the sensory and reproductive systems and probably play a role in development. FLPs can act as transmitters or are colocalized with classical transmitters and can modulate their postsynaptic actions. More than one FLP can be expressed in a neurone and so can modulate each other's mode of action. FLPs can also function as neurohormones, acting at sites distant from their site of action. This is particularly true for the extended FLPs.

Arthropods

The arthropods make up more than 80% of animal species, and the majority of the work on FLPs has been carried out on the two major classes in this phylum, crustacea and insecta. The stomatogastric and cardiac ganglia of crustaceans have been extensively used in the study of neuropeptides including the action of FLPs on neurones in the circuits of these two ganglia (Skiebe 2001; Cooke 2002). The ganglia contain neurones, which can be identified from preparation to preparation (Skiebe 2003). The crustacean stomatogastric system is made up of four ganglia, viz, a pair of commissural ganglia, an oesophageal ganglion and the stomatogastric ganglion. The major neurosecretory/neurohaemal structures in crustacean are the pericardial and postcommissural organs, the Y-gland and X-organ/sinus gland complex (Hartenstein 2006). In insects, most of the neurosecretory cells are in the intercerebralis and pars lateralis of the protocerebrum (Hartenstein 2006). Their axons project to the corpora cardiaca and corpora allata endocrine glands. Neurosecretory cells also occur in the tritocerebrum, ventral nerve cord and stomatogastric nervous system of insects. The prothoracic gland secretes a steroid prohormone.

Only extended FLPs have been identified in arthropods, Tables 9 and 10. The main FLP structures in crustacean are the N-terminal extended FLRFamides, which are often preceded by an asparagine (N), and these FLPs have been identified in a number of species including *Homarus americanus, Penaeus monodon, Macrobrachium rosenbergii, Cancer borealis, Cancer productus* and *Procambarus clarkii* (Trimmer et al. 1987; Sithigorngul et al. 2001, 2002; Huybrechts et al. 2003; Cruz-Bermudez et al. 2006; Fu et al. 2005; Ma et al. 2009b; Mercier et al. 2003). The first extended FLRFas to be identified were SDRNFLRFa (F2, FLI 3) and TNRNFLRFa (F1, FLI 4), which were identified in the lobster, *H. americanus* (Trimmer et al. 1987). Later studies identified the related peptides NRNFLRFa and DRNFLRFamide (D_2) in *P. clarkii* (Mercier et al. 1993). More recently, four extended YLRFas have been found in *P. monodon, C. borealis*, *Carcinus maenas* and *C. productus,* viz, AYSNLNYLRFa, GAHKNYLRFa, GLSRNYLRFa and GYSKNYLRFa, respectively (Sithigorngul et al. 2002; Cruz-Bermudez et al. 2006; Ma et al. 2009b; Fu et al. 2005). Sithigorngul et al. (2002) identified 7 extended Famides in the eyestalk of *P. monodon* of which only one had a FLRFa C-terminal, while four had RLRFa C-terminals, one had a YLRFa C–terminal, and one had a GSIFa C-terminal. The eyestalk contained a large number of FLP immunoreactive cells, indicating a role for FLPs in the crustacean visual system. Lobster gastropyloric receptor neurones also contain FLP-like immunoreactivity, indicating a sensory role for FLPs in the crustacean stomatogastric system (Kilman et al. 1999). Extended N-terminal peptides with an RLRFa C-terminal (short NPF) have also been found in *M. rosenbergii* (Sithigorngul et al. 2001). RYamide peptides have been identified in crustacean, for example, in *C. borealis*, viz, FVNSRYa, FYANRYamide, FYSQRYa, SGFYANRYa and PAFYSQRYa, but these peptides lack activity on the stomatogastric system (Li et al. 2003). However, following application of high potassium saline to the pericardial organs, RYas were released, indicating a possible hormonal role for them in crustacean. These authors also found that NRNFLRFa had a similar physiological action to TNRNFLRFa on the pyloric and gastric mill rhythms, suggesting that the heptapeptide sequence is all that is required for receptor recognition and binding. RYas also occur in *C. productus*, including SSRFVGGSRYa, RFVGGSRYa, FVGGSRYa and L/IFVGGSRY (Fu et al. 2005). Neuropeptides have been characterized in *Carcinus maenas* using mass spectrometry and functional genomics (Ma et al. 2009b). Twenty-five FLPs, 9 RYas and 3 SIFamides were identified. Two FLPs, QDLDHVFLRFa and pQDLDHVFLRFa, having the myosuppressin C-terminal sequence, were found in the brain, thoracic ganglia and pericardial organs of *C. maenas*. Six FLPs had a C-terminal sequence of LRLRFa, indicating they were members of the sNPF group. SIFamides have been identified or predicted in a number of crustacean and are widely distributed in the CNS and may play a role in olfaction (Verleyen et al. 2009; Yasuda et al. 2004). In contrast to the situation in insects, relatively few studies have been conducted for sulfakinins

Table 9 Summarizing FLPs identified or proposed to occur in crustaceans

FLPa	Species	Reference
RNFLRFa	*Cancer productus*	Fu et al. (2005)
RSFLRFa	*C. productus*	Fu et al. (2005)
DRNFLRFa	*Procambarus clarkii*	Mercier et al. (1993)
NRNFLRFa	*P. clarkii*	Mercier et al. (1993)
SRNYLRFa	*Carcinus maenas*	Ma et al. (2009b)
LNPFLRFa	*Cancer borealis*	Huybrechts et al. (2003)
NRSFLRFa	*C. maenas*	Ma et al. (2009b)
pQGNFLRFa	*C. maenas*	Ma et al. (2009b)
GDRNFLRFa	*Penaeus monodon*	Sithigorngul et al. (2002)
RDRNFLRFa	*C. productus*	Fu et al. (2005)
SDRNFLRFa	*Homarus americanus*	Trimmer et al. (1987)
GNRNFLRFa	*C. productus*	Fu et al. (2005)
TNRNFLRFa	*H. americanus*	Trimmer et al. (1987)
APRNFLRFa	*C. productus*	Fu et al. (2005)
ADKNFLRFa	*Macrobrachium rosenbergii*	Sithigorngul et al. (1998)
APQGNFLRFa	*C. maenas*	Ma et al. (2009b)
NYDKNFLRFa	*M. rosenbergii*	Sithigorngul et al. (1998)
GAHKNYLRFa	*C. borealis, C. productus, Cancer magister*	Cruz-Bermudez et al. (2006)
GYSKNYLRFa	*C. productus*	Fu et al. (2005)
VSHNNFLRFa	*M. rosenbergii*	Sithigorngul et al. (2001)
DGGRNFLRFa	*M. rosenbergii*	Sithigorngul et al. (2001)
DGNRNFLRFa	*C. maenas*	Ma et al. (2009b)
SENRNFLRFa	*C. productus*	Fu et al. (2005)
APQRNFLRFa	*C. borealis*	Huybrechts et al. (2003)
GLSRNYLRFa	*C. maenas*	Ma et al. (2009b)
AYNRSFLRFa	*C. borealis*	Huybrechts et al. (2003)
GYNRSFLRFa	*Callinectes sapidus*	Krajniak (1991)
YGNRSFLRFa	*C maenas*	Ma et al. (2009b)
GYGDRNFLRFa	*M. rosenbergii*	Sithigorngul et al. (2001)
AYSNLNYLRFa	*P. monodon*	Sithigorngul et al. (2002)

in crustacea. Sulfakinins have been identified in *H. americanus, P. monodon* and *Litopenaeus vannamei*, and as with insects sulfakinins occur both as short and long isoforms, Table 11 (Dickinson et al. 2007; Johnsen et al. 2000; Torfs et al. 2002). In the case of *H. americanus,* the study was the first molecular characterization of a sulfakinin-encoding cDNA in crustacean (Dickinson et al. 2007). These authors found that both Hoa-SK-I and II increased the frequency and amplitude of the heart beat of *H. americanus.* Immunocytochemical studies indicate that very few neurones in the CNS of *P. monodon* exhibit sulfakinin-like immunoreactivity (Johnsen et al. 2000). Sulfakinins from *L. vannamei* stimulated the hindgut of the locust, *Leucophaea maderae* (Torfs et al. 2002). It has been proposed that sulfakinins and gastrin/CCK originate from a common ancestral peptide (Torfs et al. 2002). In an interesting comparative study, pQDLDHVFLRFa was identified in 32 species from seven decapod infraorders while GYRKPPFNGSIFa was found in all species apart from two species of Homarus, indicating the broad conservation of some peptides in crustacean (Stemmler et al. 2007). In the case of *H. americanus* and *H. gammarus,* the N-terminal G was replaced by V. B-type allatostatins, which have a C-terminal W occur in crustacean (Fu et al. 2005; Ma et al. 2009a). For example, VPNDWAHFRGSWa is present in the pericardial organs of *C. borealis* and *C. productia* where it modulates the pyloric rhythm (Fu et al. 2007). A C-type allatostatin with a C-terminal CFa (Table 11) has been identified in *Daphnia pulex, C. borealis* and *H. americanus* (Gard et al. 2009; Ma et al. 2009c). This peptide inhibits the pyloric rhythm of the stomatogastric ganglion in *C. borealis* when its frequencies are < 0.7 Hz. Using *in silico* analyses of ESTs, two NPFs have been identified in *Marsupenaeus japonicus* and *Daphnia magna,* Table 11 (Christie et al. 2008). Although FMRFa-like immunoreactivity has been demonstrated in cirripeds, for

Table 10 Summarizing extended FLPs identified or proposed in insects

Extended FLP	Species	Reference
AFIRFa	*Locusta migratoria*	Lange et al. (1994)
GNSFLRFa	*Manduca sexta*	Kingan et al. (1996)
DPSFLRFa	*M. sexta*	Kingan et al. (1996)
GDNFMRFa	*Lucilia cuprina*	Rahman et al. (2009)
PDNFMRFa	*Drosophila melanogaster*	Schneider and Taghert (1988)
SDNFMRFa	*D. melanogaster*	Schneider and Taghert (1988)
MDSNFIRFa	*D. melanogaster*	Schneider and Taghert (1988)
GKQDFIRFa	*Periplaneta americana*	Predel et al. (2004)
ARPDNFIRFa	*P. americana*	Predel et al. (2004)
SVQDNFIRFa	*L. cuprina*	Rahman et al. (2009)
GGKQDNFIRFa	*P. americana*	Predel et al. (2004)
GKSDNFIRFa	*P. americana*	Predel et al. (2004)
DRSDNFIRFa	*P. americana*	Predel et al. (2004)
GRSDNFIRFa	*P. americana*	Predel et al. (2004)
GKTDNFIRFa	*P. americana*	Predel et al. (2004)
GGKNDNFIRFa	*P. americana*	Predel et al. (2004)
GGRSNDNFIRFa	*P. americana*	Predel et al. (2004)
GGKSGSNFIRFa	*P. americana*	Predel et al. (2004)
APPQPSDNFIRFa	*Neobellieria bullata*	Meeusen et al. (2002)
GGNDFMRFa	*L. cuprina*	Rahman et al. (2009)
MDSNFMRFa	*Drosophila virilis*	Taghert and Schneider (1990)
AKDNFLRFa	*Rhodnius prolixus*	Ons et al. (2009)
PDRNFLRFa	*Anopheles gambiae*	Scholler et al. (2005)
GANDFMRFa	*Calliphora vomitoria*	Duve et al. (1992)
APSDFMRFa	*D. virilis*	Taghert and Schneider (1990)
GQERNFLRFa	*L. migratoria*	Lange et al. (1994)
PDVDHFLRFa	*L. migratoria*	Schoofs et al. (1993)
TPAEDFMRFa	*D. melanogaster*	Schneider and Taghert (1988)
APGQDFMRFa	*C. vomitoria*	Duve et al. (1992)
AAGQDFMRFa	*L. cuprina*	Rahman et al. (2009)
ASGQDFMRFa	*C. vomitoria*	Duve et al. (1992)
DPKQDFMRFa	*D. melanogaster*	Nambu et al. (1988)
SPKQDFMRFa	*D. melanogaster*	Schneider and Taghert (1990)
QANQDFMRFa	*L. cuprina*	Rahman et al. (2009)
KPNQDFMRFa	*C. vomitoria*	Duve et al. (1992)
NPQQDFMRFa	*L. cuprina*	Rahman et al. (2009)
TPQQDFMRFa	*C. vomitoria*	Duve et al. (1992)
DPSQDFMRFa	*D. virilis*	Taghert and Schneider (1990)
pQPSQDFMRFa	*N. bullata*	Meeusen et al. (2002)
GPSQDFMRFa	*L. cuprina*	Rahman et al. (2009)
SPSQDFMRFa	*C. vomitoria*	Duve et al. (1992)
TPSQDFMRFa	*C. vomitoria*	Duve et al. (1992)
SPTQDFMRFa	*L. cuprina*	Rahman et al. (2009)
A/S/TPNRDFMRFa	*C. vomitoria*	Duve et al. (1992)
TPNRDFMRFa	*C. vomitoria*	Duve et al. (1992)
APPSDFMRFa	*D. virilis*	Taghert and Schneider (1990)
AGQDGFMRFa	*C. vomitoria*	Duve et al. (1992)
AGQDNFMRFa	*L. cuprina*	Rahman et al. (2009)

Table 10 continued

Extended FLP	Species	Reference
pQDVVHSFLRFa	*M. sexta*	Kingan et al. (1990)
AAGQDNFMRFa	*C. vomitoria*	Duve et al. (1992)
AAASDNFMRFa	*L. cuprina*	Rahman et al. (2009)
SANAKNDFMRFa	*L. cuprina*	Rahman et al. (2009)
SANTKNDFMRFa	*L. cuprina*	Rahman et al. (2009)
SVNTKNDFMRFa	*C. vomitoria*	Duve et al. (1992)
TPPQPADNFIRFa	*L. cuprina*	Rahman et al. (2009)

example, *Balanus amphitrite,* no FLPs have yet been identified (Gallus et al. 2009).

The actions of FLPs have been examined on a range of crustacean tissues in addition to the stomatogastric and cardiac ganglion circuits; and in general, they appear to be excitatory (Mercier et al. 2003). For example, both DRNFLRFa and NRNFLRFa are cardioexcitatory and enhance transmission at the nerve-muscle junction of *P. clarkii* (Mercier et al. 1993). DRNFLRFa excites isolated longitudinal muscle of the hindgut of this crayfish, threshold 1 nM, but has no effect on circular muscle (Mercier and Lee 2002). Interestingly, *P. clarkii,* hindgut contains very little FLP-like immunoreactivity and so it is likely that if these peptides affect the gut physiologically then they do so as neurohormones. However, the haemolymph content of FLP-like peptide immunoreactivity is $<$1 nM. Both SDRNFLRFa and TNRNFLRFa, 100 nM, activate pyloric and gastric rhythms in stomatogastric quiescent preparations of *C. borealis* (Weimann et al. 1993). Both peptides are present in the stomatogastric nervous system and activate neurones in the stomatogastric circuit. SDRNFLRFa can excite both cardiac ganglion neurones and heart muscle fibres of *H. americanus,* indicating the presence of neuronal and myocyte FLP receptors in this species (Wilkens et al. 2005). These authors concluded that the high affinity inotropic effects of SDRNFLRFa are due to modulation of voltage-gated calcium channels while low affinity effects are mediated by activation of ligand-gated calcium transporters in the sarcolemma. When TNRNFLRFa and SDRNFLRFa are applied to isolated hearts of *Cancer magister,* they excite the heart but when injected into intact animals these peptides are inhibitory, inducing a fall in heart rate, stroke volume and cardiac output (McGaw et al. 1995). This difference is probably due to the peptides activating neuronal circuits, which release inhibitory compounds onto the heart. The interactions between peripheral and central effects of FLPs on crab (*Callinectes sapidus)* heartbeat have been studied in detail by Fort et al. (2007), who conclude their effects include both feedback and feedforward interactions involving a central pattern generator effector system. Using four preparations from *H. americanus*, viz, the intact animal, an in vitro heart preparation, a stimulated heart muscle preparation and an isolated cardiac ganglion preparation for intracellular recordings Stevens et al. (2009) have found evidence that the myosuppressin, pQDLDHVFLRFa, acts at several sites to modulate the cardiac neuromuscular system. TNRNFLRFa and SDRNFLRFa excite most of the muscles in the stomach musculature of *C. borealis* (Jorge-Rivera and Marder 1996) with a threshold of 0.1–1 nM. Since these muscles do not receive a direct peptidergic innervation but have FLP receptors, it is assumed the peptides reach the muscles via the blood, which is supported by the finding that FLPs are present in the pericardial organs. TNRNFLRFa also excites neurones in the stomatogastric system through activation of a non-selective cation voltage-dependent inward current, which is also activated by four other peptides and pilocarpine (Swensen and Marder 2000). There is also evidence that FLPs like TNRNFLRFa and DRNFLRFa can act both directly to induce tonic contractions and indirectly to release transmitter onto somatic muscle of crustacean (Mercier et al. 2003; Worden et al. 1995). These and other FLPs can induce long-lasting desensitization of muscle contractures (Worden et al. 1995). These actions of extended FLPs occur at concentrations 1–10,000 times lower than the concentration of FMRFa required to produce similar intensity responses. An YLRFa peptide, GAHKNYLRFa has been identified in three species of *Cancer,* including *C. borealis,* where it excites cardiac ganglion neurones and pyloric and gastric mill rhythms, mimicking the actions of TNRNFLRFa and SDRNFLRFa (Cruz-Bermudez et al. 2006). These authors found that GAHKNYLRFa is degraded by extracellular peptidase at a slower rate than TN- or SDRNFLRFa, suggesting a longer-lasting physiological action.

There are over 50 deduced or isolated insect FLPs, examples are shown in Table 10, and from this it can be seen that FLPs are particularly diverse in flies (diptera) (Rahman et al. 2009). Unlike the situation in crustacean, the main C-terminal FLP in insects is MRFa, followed by IRFa and then LRFa. In addition, there are RFamides, e.g.,

Table 11 Summarizing RFas, RYas and F-amides in crustaceans

Group	Sequence	Species	Reference
Myosuppressins	pQDLDHVFLRFa	*Carcinus maenas*	Ma et al. (2009b)
-HVFLRFa	QDLDHVFLRFa	*C. maenas*	Ma et al. (2009b)
	PELDHVFLRFa	*Cancer borealis*	Huybrechts et al. (2003)
NPF	-YYSQVSRPRFa[a]	*Marsupenaeus japonicus*	Christie et al. (2008)
	-VYGQAARPRFa[b]	*Daphnia magna*	Christie et al. (2008)
sNPF (LRLRFa)	PSLRLRFa	*C. maenas*	Ma et al. (2009b)
	PSMRLRFa	*C. maenas*	Ma et al. (2009b)
	APALRLRFa	*Macrobrachium rosenbergii*	Sithigorngul et al. (1998)
	EMPSLRLRFa	*C. maenas*	Ma et al. (2009b)
	SMPSLRLRFa	*C. maenas*	Ma et al. (2009b)
	DRTPALRLRFa	*M. rosenbergii*	Sithigorngul et al. (1998)
	DARTPALRLRFa	*C. maenas*	Ma et al. (2009b)
	DVRTPALRLRFa	*C. borealis*	Huybrechts et al. (2003)
	SDRSPSLRLRFa	*Daphnia pulex*	Gard et al. (2009)
Sulfakinins	pEFDEY$_S$GHMRFa	*Homarus americanus*	Dickinson et al. (2007)
(Y$_S$GHL/MRFa)	pQFDEY$_S$GHMRFa	*Penaeus monodon*	Johnsen et al. (2000)
	VGGEY$_S$DDY$_S$GHLRFa	*P. monodon*	Johnsen et al. (2000)
	GGGEY$_S$DDY$_S$GHLRFa	*H. americanus*	Dickinson et al. (2007)
	-GEY$_S$DDY$_S$GHL/IRFa[c]	*P. monodon*	Johnsen et al. (2000)
RYa	FYANRYa	*C. borealis*	Li et al. (2003)
	FYSQRYa	*C. borealis*	Li et al. (2003)
	FVNSRYa	*C. borealis*	Li et al. (2003)
	FVGGSRYa	*Cancer productus*	Fu et al. (2005)
	SGFYADRYa	*C. maenas*	Ma et al. (2009b)
	SGFYANRYa	*C. borealis*	Li et al. (2003)
	SGFYAPRYa	*C. maenas*	Ma et al. (2009b)
	PAFYSQRYa	*C. borealis*	Li et al. (2003)
	L/IFVGGSRYa	*C. productus*	Fu et al. (2005)
	RFVGGSRYa	*C. productus*	Fu et al. (2005)
	PEGFYSQRYa	*C. maenas*	Ma et al. (2009b)
	SSRFVGGSRYa	*C. productus*	Fu et al. (2005)
SIFa	RKPPFNGSIFa	*C. maenas*	Ma et al. (2009b)
	TRKLPFNGSIFa	*D. pulex*	Verleyen et al. (2009)
	GYRKPPFNGSIFa[d]	*Procambarus clarkii*	Yasuda et al. (2004)
	VYRKPPFNGSIFa	*Homarus gammarus*	Stemmler et al. (2007)
C-Allatostatin	SYWKQCAFNAVSCFa	*D. pulex*	Gard et al. (2009)
Orcokinin	NFDEIDRSGFa	*C. maenas*	Ma et al. (2009b)
	NFDEIDRSSFa	*C. maenas*	Ma et al. (2009b)
Others	LRNLRFa	*C. borealis*	Huybrechts et al. (2003)

[a] The N-terminal sequence of this NPF contains another 22 amino acids (KPDPSQLANMAEALKYLQELDK)

[b] The N-terminal sequence of this NPF contains another 28 amino acids (DGDVMGGGEGGEMTAMADAIKYLQGLDK)

[c] The N-terminal sequence of this sulfakinin contains another 8 amino acids (AGGSGGVG)

[d] This SIFa has also been found in *Procambarus clarkia, C. borealis*, *Cancer magister*, *C. productus* and *C. maenas*

D. melanogaster Neuropeptide F with 36 amino acids (NPF, SNSRPPRKNDVNTMADAYKFLQDLDTYYGD RARVRFa) (Brown et al. 1999), which is related to mammalian NPY, Table 12 and F-amides, e.g., NQKT MSFa, Allatotropin (*Manduca sexta*) (GFKNVEMM-TARGFa) (Kataoka et al. 1989; Utz et al. 2008) but the allotropins will not be considered in this review. NPF is the only member of the family in *D. melanogaster* and plays a

Table 12 Summarizing examples of myosuppressins, short and long NPFs, sulfakinins and SIFamides identified or proposed in insects

Peptide group	Sequence	Species	Reference
Myosuppressins	pEDVVHSFLRFa	*Manduca sexta*	Kingan et al. (1990)
-HVFLRFa	PDVDHVFLRFa	*Schistocerca gregaria*	Robb et al. (1989)
	QDLDHVFLRFa	*Rhodnius prolixus*	Ons et al. (2009)
	pQDVDHVFLRFa	*Leucophaea maderae*	Holman et al. (1986)
	pQDVDHVFLRFa	*Apis mellifera*	Hummon et al. (2006)
	TDVDHVFLRFa	*Drosophila melanogaster*	Nichols (1992)
	ADVGHVFLRFa	*Locusta migratoria*	Lange et al. (1994)
Short NPFs	APSLRLRFa	*Leptinotarsus decemlineata*	Spittaels et al. (1996)
-PxxRLRFa	SPSLRLRFa	*A. mellifera*	Hummon et al. (2006)
	APTQRLRWa	*Anopheles gambiae*	Riehle et al. (2002)
	ARGPQLRLRFa	*L. decemlineata*	Spittaels et al. (1996)
	ANRSPSLRLRFa	*Periplaneta americana*	Veenstra and Lambrou 1995
	SGRSPSLRLRFa	*Tribolium castaneum*	Hauser et al. 2008
	ANRSPSLRLRFa	*P. americana*	Veenstra and Lambrou (1995)
	AQRSPSLRLRFa	*D. melanogaster*	Vanden Broeck (2001)
	AVRSPSLRLRFa	*A. gambiae*	Riehle et al. (2002)
	FAPRSPQLRLRFa	*Acrosternum hilare*	Predel et al. (2008)
Long NPFs	VAAGRPRFa	*R. prolixus*	Ons et al. (2009)
	-KHAQHARPRFa[a]	*Aedes aegypti*	Veenstra and Costes (1999)
	-KHAQHARPRFa[b]	*A. gambiae*	Riehle et al. (2002)
	-YYGDRARVRFa[a]	*D. melanogaster*	Brown et al. (1999)
Sulfakinins	FDDY$_S$GHMRFa	*D. melanogaster*	Nichols (1988)
-YGHM/LRFa	pQSDDY$_S$GHMRFa	*Leucophaea maderae*	Nachman et al. (1986a)
	pQFNEY$_S$GHMRFa	*R. prolixus*	Ons et al. (2009)
	EQFDDY$_S$GHMRFa	*P. americana*	Veenstra (1989)
	pQQFDDY$_S$GHLRFa	*A. mellifera*	Hummon et al. (2006)
	pQTSDDY$_S$GHLRFa	*T. castaneum*	Hauser et al. (2008)
	GEEPFDDY$_S$GHMRFa	*T. castaneum*	Hauser et al. (2008)
	pQLASDDY$_S$GHMRFa	*L. migratoria*	Schoofs et al. (1997)
	GGDDQFDDY$_S$GHMRFa	*D. melanogaster*	Nichols et al. (1988)
	GGEEQFDDY$_S$GHMRFa	*Calliphora vomitoria*	Duve et al. (1995)
	GGEGDQFDDY$_S$GHMRFa	*A. gambiae*	Riehle et al. (2002)
SIFas	FRKPPFNGSIFa	*Acrythosiphon pisum*	Verleyen et al. (2009)
	TYKKPPFNGSIFa	*R. prolixus*	Ons et al. (2009)
	AYRKPPFNGSIFa	*D. melanogaster*	Verleyen et al. (2004)
	GYRKPPFNGSIFa	*A. gambiae*	Riehle et al. (2002)
	TYRKPPFNGSIFa	*T. castaneum*	Hauser et al. (2008)
	AYRKPPFNGSLFa	*Neobellieria bullata*	Janssen et al. (1996)
-FVRFa	GNSNFVRFa	*P.americana*	Predel et al. (2004)
	GRPSNNFVRFa	*P. americana*	Predel et al. (2004)

[a] An additional 36 amino acids at N-terminal

[b] An additional 32 amino acids at N-terminal. Only a selection of peptides are given for each group

role in the link between chemosensory inputs and behaviour and stimulates the feeding response in the larvae (Wu et al. 2003). Interestingly, disruption of the NPF/NPFR1 system also reduces the sensitivity of *D. melanogaster* to alcohol (Wen et al. 2005). NPFs in insects have been reviewed by Roller et al. (2008). Another group of Fa peptides has a C-terminal SIFa or SLFa, and these peptides are highly conserved in insects (Verleyen et al. 2004, 2009). A CFa, an example of a C-type allatostatin, SYWKQCAFNAVSCFa, has been identified in *A. mellifera*

(Hummon et al. 2006; Boerjan et al. 2010). There are also FMHRas, for example, SVKQDFMHFa (Taghert and Schneider 1990), which is reminiscent of AF17 in *A. suum*, Table 3. While very few peptides end in tyrosine (Y), for example, *Tribolium castaneum,* allatotropin (Hauser et al. 2008) and GRNDLNFIRYa from *A. mellifera* (Hummon et al. 2006), there are several which end in tryptophan (W), for example, adipokinetic hormone (Drm-AKH, pQLTF SPDWa), cricket-type allatostatins (Drostatin-B1-5, e.g., AWQSLQSSWa), Drm-sNPF-3 (PQRLRWa), Drm-sNPF-4 (PMRLRWa). The latter two can be included with the short NPFs, see later. There are also examples of FLPs with C-terminals of YLRFa, for example in *T. casteneum* (Hauser et al. 2008). Insect FLPs can be divided into five types, FMRFas, FLRFas, GHMRFas, RVRFas and RLRFas based on their presence on five *D. melanogaster* genes (Nassel 2002). FLPs with a sequence, xDVxHxFLRFa, Table 12, are called myosuppressins (Nassel 2002) while the extended GHMRFas are the sulfakinins (Nachman et al. 1986a, b), and the RLRFas are short NPFs (Spittaels et al. 1996). The first N-terminally extended RFa to be sequenced in insects was leucomyosuppressin (LMS, pQDVDHVFLRFa) from the cockroach, *Leucophaea maderae* (Holman et al. 1986). This peptide inhibited spontaneous contractions of the locust hindgut. Since then a number of myosuppressins have been sequenced, Table 12, including one from D. melanogaster, TDVDHVFLRFa (Nichols 1992; Nichols et al. 1997). Other extended FLRFas have been identified, which are not myosuppressins, together with extended FIRFas, KTRFas and RLRFas (Orchard et al. 2001). Extended FMRFas were first identified in diptera (Schneider and Taghert 1988; Nambu et al. 1988), particularly from *Calliphora, Lucilia* and *Drosophila* (Duve et al. 1992) and generally have a total of 9–10 amino acids, Table 10. Subsequently, using tandem mass spectrometry, extended FLPs have been identified from a single gene in *P. americana* although none contained an FMRFa C-terminal (Predel et al. 2004; Neupert and Predel 2005; Neupert and Gundel 2007). The 24 peptides included 10 FIRFas, 3 FIRLas, 2 FVRFas and one LMRFa while 4 were non-amidated. All 24 peptides were expressed in postero-lateral cells in the thoracic ganglia that project to the perisympathetic organs. Certain of these peptides, viz, DRSDNFIRFa, GKQDFIRFa and ARPSSNFIRLa inhibited DUM (dorsal medium unpaired) cell activity by decreasing intracellular calcium but excited the antenna-heart preparation. The extended HMRFas, the sulfakinins, with a common GHMRFa C-terminal, mainly contain a sulphated tyrosine. The first sulfakinin was isolated from *L. maderae* (Nachman et al. 1986a,b) but a number of peptides have since been identified from other insect species, Table 12. The sulfakinins play a role in feeding but it is likely they exert their effects centrally as they are localized in the brain (Audsley and Weaver 2009). A number of short NPFs have been identified in insects with a C-terminal RLRFa or RLRWa, and their occurrence is fully covered in invertebrates in the paper of Clynen et al. (2009). sNPFs normally have a C-terminal sequence of RxPxxRLRFa where the N-terminal arginine in this sequence is important for binding and normally have <12 amino acids. These authors also propose a second group of sNPFs where the N-terminal amino acid or the ninth amino acid from the C-terminal is tyrosine. sNPF immunoreactivity occurs widely in interneurones in the brain of *D. melanogaster,* suggesting this group of peptides is involved in a number of functions, including olfaction, central control of locomotion and a role in the regulation of the hormonal system (Johard et al. 2008; Nassel et al. 2008). In addition, they play a role in reproduction, feeding and digestion (Clynen et al. 2009) and are possibly involved in the regulation of adult diapause in *Leptinotarsa decemlineata* (Huybrechts et al. 2004). Interestingly, the majority of sNPF immunoreactive interneurones do not appear to colocalize with classical transmitters. To activate the receptor for these peptides, an arginine at position N-4 is required since replacing it with phenylalanine renders the peptide inactive on the short NPFa receptor (Mertens et al. 2002). Using bioinformatics and mass spectrometry, a number of RFa-like peptides have been predicted/identified in *D. melanogaster*, *T. castaneum, A. mellifera Tenebrio molitor* and *Rhodnius prolixus* (Baggerman et al. 2002; Hauser et al. 2006, 2008; Hummon et al. 2006; Ons et al. 2009; Weaver and Audsley 2008). FLP immunoreactivity has been identified throughout the insect central and peripheral nervous systems and in the midgut and this has been reviewed in depth (Orchard et al. 2001; Nassel 2002). This immunoreactivity has been found in all types of neurones and immunoreactive axons extend to the corpora cardiaca and to tissues including the heart and somatic muscle. Extended FMRFas are expressed throughout the nervous system and are the most abundant neuropeptides in thoracic neuroendocrine cells (Predel and Neupert 2007; Rahman et al. 2009).

There have been many studies in which FLPs have been tested on various insect preparations involving visceral, cardiac and somatic muscles but only a few examples will be given. FLPs with a common C-terminal sequence of amino acids tend to have similar actions on insect tissues. For example, both extended FLRFas and FMRFas tend to inhibit movement of the crop, heart, midgut and oviducts, but facilitate somatic muscle contractions (Krajniak 2005) and excite the ileum of *M. sexta* (Kingan et al. 1996). An excellent summary of the earlier work is provided in the review of Orchard et al. (2001). Duve et al. (1992) carried out an analysis of FLPs present in the blowfly, *Calliphora vomitoria.* They characterized 13 extended FLPs, which

included seven QDFMRFas with N-terminal sequences of TPQ, TPS, SPS, KPN, APG, ASG and AxG and two NDFMRFas with N-terminal sequences of GA and SVNTK. Six QDFMRFas were tested for activity on the *C. vomitoria* heart but only two were active (Duve et al. 1993). APGQDFMRFa, 10 nM–1 μM, increased both the frequency and amplitude of the heart while TPQQDFMRFa at the same concentration range only increased the frequency of heart beat. Non-amidated analogues were inactive. Four other QDFMRFas, viz, TPS, SPS, KPN and ASG were inactive. This shows the high degree of selectivity for the activation of the FLP receptor on the heart of *C. vomitoria* since changing a proline to a serine resulted in loss of activity. SchistoFLRFa (PDVDHVFLRFa) was also inactive while leucomyosuppressin (pQDVDHVFLRFa), 1 μM, inhibited the heart. The interaction of FLPs and 5-hydroxytryptamine (5-HT) has been investigated on the pupal heart of *D. melanogaster* (Nichols 2006). FLPs and 5-HT appear not to colocalize in central neurones or in fibres innervating the anterior dorsal vessel. FLPs, particularly dromyosuppressin (TDVDHVFLRFa) inhibited the heart while 5-HT enhanced contractions. The effect of dromyosuppressin was non-additive with 5-HT, suggesting an interaction between the two compounds. The role of the histidine in the myosuppressins has been investigated by Starrat et al. (2000) who found it to be key for the inhibitory effect on locust oviduct. TPAEDFMRFa decreases contraction of the *D. melanogaster* crop although there is no evidence for this FLP in the crop, suggesting it acts as a neurohormone (Duttlinger et al. 2003). In contrast, two other peptides encoded by the dFMRFa gene, DPKQDFMRFa and SDNFLRFa had no effect on the crop. The myosuppressin peptide, TDVDHVFLRFa completely stopped crop contraction. The minimum sequence required to inhibit cockroach hind gut activity is VFLRFa (Nachman et al. 1993). The minimum required for full activity is DHVFLRFa. These authors concluded that hydrophobic valine in position 6 and leucine in position 8 are important in the inhibitory actions of myosuppressins while valine and leucine at these positions are absent in the stimulatory sulfakinins. Interestingly, FMRFa, which is not an endogenous peptide in *D. melanogaster,* had a very similar inhibitory effect to TPAEDFMRFa when applied at a similar concentration. This would suggest that the N-terminal sequence TPAED does not enhance activity on the crop while an N-terminal sequence of DPKQD prevents the action of FMRFamide. It would be interesting to know whether DPKQDFMRFa blocked the action of FMRFa on the crop. Histidine in the HVFLRFa myosuppressins has been found to be key for its inhibitory effect, for example, on the locust oviduct, since removal of H, leaving VFLRFa, removes biological activity although the peptide can still bind to its receptor (Nachman et al. 1986a; Wang et al. 1995). Interestingly, although VFLRFa is devoid of biological activity, it is a strong antagonist of PDVDHVFLRFa activity on the oviduct. Immunological studies indicate that SIFamides are only found in 4 neurones in the CNS and have a role in the modulation of sexual behaviour (Terhzaz et al. 2007; Verleyen et al. 2009). Short NPFs stimulate food intake and increase body size in insects (Lee et al. 2004). The role of FLPs in feeding behaviour is complex and has recently been reviewed in depth by Audsley and Weaver (2009). These authors concluded there are many issues yet to be resolved regarding the roles of neuropeptides in the regulation of feeding behaviour. FLPs, including DRSDNFIRFa, have been implicated in the regulation of the circadian clock in the cockroach, *Leucophaea maderae* (Soehler et al. 2008).

Genome projects have been completed for several insects including *D. melanogaster, D. pseudoobscura, A. gambiae, Aedes aegypti, Culex quinquefasciatus, Bombyx mori, Acyrthosiphon piscum, Nasonia vitripennis, Pediculuc humanus corporis* and *Apis mellifera* (Adams et al. 2000; Ashburner and Bergman 2005; Holt et al. 2002; International Silkworm Genome Consortium 2008; Mita et al. 2004; Pittendrigh et al. 2006; Weinstock et al. 2006; Sabater-Munoz et al. 2006; Nene et al. 2007). In addition, the genomes of ten further species of *Drosophila* have been analysed and their phylogeny compared (Clark et al. 2007). The first insect FMRFa GPCR, CG2114 (DrmFMRFa), was cloned from *D. melanogaster* by two groups (Cazzamali and Grimmelikhuijzen 2002; Meeusen et al. 2002). This receptor was sensitive at around 1 nM to a number of FMRFas, including DPKQDFMRFa and PDNFMRFa. The following receptors have been cloned from *D. melanogaster*, long NPF receptor (Li et al. 1992), short neuropeptide F receptor (CG7395) (Mertens et al. 2002), SIFa receptor (CG10823) (Jorgensen et al. 2006), sulfakinin receptor, DSK-R1 (CG6881) (Kubiak et al. 2002) and myosuppressin receptors (CG8985, CG13803) (Egerod et al. 2003). Orthologues of these receptors have been cloned from genes from both *Tribolium castaneum* and *Apis mellifera* (Hauser et al. 2006, 2008) while a myosuppressin receptor has been cloned from *Anopheles gambiae* (Scholler et al. 2005). This receptor does not respond to PDRNFLRFa and is not related to insect FMRFa receptors, the two systems having evolved independently, myosuppressins should not be termed FLPs according to Scholler et al. (2005). A short NPF receptor has been characterized from *A. gambiae* and expressed in CHO cells (Garczynski et al. 2007). *A. gambiae* sNPF, AVRSPSLRLRFa, displaced a radiolabeled peptide with an IC_{50} of 3 nM. Removal of the N-terminal AV and AVR amino acids reduced the IC_{50} 10- and 100-fold, respectively. AVRSPSLRLRFa inhibited forskolin-stimulated cAMP accumulation.

By comparison with crustacean and insects, there have been relatively few studies regarding the presence and role of neuropeptides in arachnids. FMRFa-like immunoreactivity has been found in the nervous system of the horseshoe crab, *Limulus polyphemus* (Watson et al. 1984). Four FLPs and one LRLRFa have been identified in *L. polyphemus* with sequences of DEGHKMLYFa (LP-1), GHSLLHFa (LP-2), PDHHMMYFa (LP-3), DHGNM LYFa (LP-4) and a sNPF, GGRSPSLRLRFa (LP-5) (Gaus et al. 1993). This sNPF sequence has been proposed for the tick, *Ixodes scapularis,* based on an *in silico* investigation Christie (2008). Christie also proposed the presence in the tick of two sulfakinins, *viz,* pQDDDY$_S$GHMRFa and SDDY$_S$GHMRFa, and three SIFamides, *viz,* AYR-KPPFNGSIFa, ASRKPPFNSSIFa and RKPPFNSSIFa. Two of the peptides isolated from *L. polyphemus* were also tested on the *L. polyphemus* heart (Gaus et al. 1993). While GGRSPSLRLRFa slightly reduced the amplitude of the heartbeat at 5 μM, GHSLLHFa produced a concentration-dependent decrease in the heartbeat amplitude, threshold 4 nM. Crustacean FLPs were subsequently tested on the heart of *L. polyphemus* and excited the heart (Groome et al. 1994). For example, TNRNFLRFa, SDRNFLRFa, GY-NRSFLRFa and pQDPFLRFa, increased both the frequency and amplitude of the heartbeat, with a threshold around 1 nM. FMRFa and N-terminally extended analogues of FLRFa relax the gut of *L. polyphemus* (Groome et al. 1992). These FLPs also reversed the contractions induced by proctolin.

Immunocytochemical and physiological studies suggest that RFas play key roles in the modulation of activity throughout the insect digestive, reproduction and cardiovascular systems and somatic muscle (Orchard et al. 2001). The FLPs and related peptides are involved in feeding, reproduction, vision and the regulation of muscle activity. The myosuppressins are mainly inhibitory, blocking muscle contraction and nervous system activity and inhibiting the release of ecdysone, which is required for growth, moulting and metamorphosis. The sulfakinins, NPFs and short NPFs play a role in feeding while SIFamides modulate sexual behaviour.

Echinoderms

The echinoderms can be divided into five main classes, viz, crinoids (feather-stars and sea-lilies), asteroids (starfish), ophiuroids (brittle-stars), echinoids (sea-urchins) and holothurians (sea-cucumbers) (Barnes et al. 1988). FMRFa-like immunoreactivity was first described in the starfish, *Asterias rubens*, by Elphick et al. (1989) but subsequent studies identified this immunoreactivity as being due to a new family of neuropeptides with a C-terminal Fa (Elphick et al. 1991). These peptides were designated S1 and S2 and had the following structures, GFNSALMFa and SGPYS FNSGLTFa, respectively, Table 13. Both peptides occur in the radial nerve cords of the nervous system and in organs, including the tube feet, apical muscle and cardiac stomach. In initial experiments, neither peptide had any effect on the tone of the tube feet and apical muscle but S2 relaxed the cardiac stomach following its contraction with acetylcholine (Elphick et al. 1995). S2 was detected in the coelomic fluid, suggesting a neurohormonal role in starfish. In a radioimmunoassay using antisera selective for either S1 or S2, FMRFa, FLRFa and LPLRFa showed very little immunoreactivity. In contrast, a SALMFa from the sea-cucumber, *Holothuria glaberimma*, GFSKLYFa (Diaz-Miranda et al. 1992) had a similar binding profile to S2 with the antisera used. GFSKLYFa relaxed the intestine and longitudinal body wall muscle of *H. glaberimma* (Diaz-Miranda et al. 1992). The nitric oxide (NO) donor, SNAP, also relaxed the cardiac muscle of *A. rubens,* and this relaxation was reduced in the presence of ODG, a soluble guanylyl cyclase inhibitor (Elphick and Melarange 1998, 2001). It would be very interesting to know the interactive roles of these two relaxing molecules in the physiology of echinoderms. There is evidence that NO is produced endogenously in the starfish stomach since L-arginine can also induce relaxation. In a later paper, Melarange and Elphick (2003) showed that the NO donor SNAP, S1 and S2 relaxed tube feet and apical body wall muscle of *A. rubens*, with the following relative potencies, SNAP > S2 > S1. The phosphodiesterase inhibitor, IBMX, increased the basal levels of cGMP and cAMP in cardiac stomach muscle but co-treatment with IBMX and either S1, S2 or SNAP failed to increase the levels further. It is therefore not clear which signalling mechanism the SALMFas use to induce relaxation. Unless a tissue can be found with a high level of cells expressing SALMFa receptors then it may be necessary to clone and express the gene encoding the receptor in a suitable system (Melarange and Elphick 2003). In a subsequent paper, Elphick and Thorndyke (2005) identified additional SALMFas in a sea-urchin, *Strongylocentrotus purpuratus* by sequencing its genome. A total of seven putative SALMFas were identified, Table 13, and named SpurS1-7. This paper is the first description of a neuropeptide precursor gene in an echinoderm that is made up of two exons with the first exon encoding an N-terminal signal sequence and the second exon encoding the seven peptides. From Table 13, it can be seen that SALMFas have been identified in starfish, holothurians and sea-urchins, and it is likely they occur throughout the phylum with a relaxing/inhibitory effect on muscle. While none of the SALMFas appear to excite echinoderm tissues, S1 has been tested on identified *Helix aspersa* neurones where GFNSALMF-1 excited cell F2

Table 13 Summarizing the SALMFa peptides identified or proposed to occur in echinoderms

Peptide	Species	Reference
GFSKLYFa	*Holothuria glaberrima*	Diaz-Miranda et al. (1992)
GYSPFMFa	*Stichopus japanicus*	Muneoka et al. (2000)
FKSPFMFa	*S. japonicus*	Muneoka et al. (2000)
GMSAFSFa	*Strongylocentrotus purpuratus*	Elphick and Thorndyke (2005)
AQPSFAFa	*S. purpuratus*	Elphick and Thorndyke (2005)
GDLAFAFa	*S. purpuratus*	Elphick and Thorndyke (2005)
SGYSVLYFa	*H. glaberrima*	Diaz-Miranda et al. (1992)
GFNSALMFa	*Asterias rubens*	Elphick et al. (1991)
DAYSAFSFa	*S. purpuratus*	Elphick and Thorndyke (2005)
GLMPSFAFa	*S. purpuratus*	Elphick and Thorndyke (2005)
PHGGSAFVFa	*S. purpuratus*	Elphick and Thorndyke (2005)
PPVTTRSKFTFa	*S. purpuratus*	Elphick and Thorndyke (2005)
SGPYSFNSGLTFa	*A. rubens*	Elphick et al. (1991)

with a low pM or even lower threshold (Pedder et al. 1992). There is also a Ya in the sea-cucumber, *Stichopus japonicus*, NGIWYa, which contracts both the radial longitudinal muscle and the intestine of the sea-cucumber (Muneoka et al. 2000). NGIWYa also stiffens the body wall connective tissue of *S. japonicus* (Birenheide et al. 1998; Inoue et al. 1999). Recently, a gene has been identified in *S. purpuratus* that encodes two copies of an NGIWYa-like peptide, viz, NGFFFa (Elphick and Rowe 2009). NGFFFa contracted the tube foot and oesophagus from *Echinus esculentus.*

It has been proposed that the C-terminal sequence of the SALMFas is made up of SxL/FxFa (Elphick and Melarange 2001) and out of the 13 peptides listed in Table 13, ten have this sequence while the remaining three have FxFa. A structure–activity study was undertaken to try and determine whether the N-terminal extension of S2 conveyed its increased sensitivity over S1 as a relaxant of starfish cardiac stomach (Otara et al. 2004). Removal of the sequence SGPY from S2 and its addition to S1 did not alter the relative potency of the two peptides. The difference in potency would appear to reside in the C-terminal amino acid sequence rather than in the extended N-terminal amino acid sequence, which is an unexpected and therefore, interesting finding.

Vertebrate RFamides

The RFas in vertebrates present a complex story, which may be unfamiliar to many who work primarily on invertebrates and so this section will be reviewed in more detail. There have been several recent reviews covering different vertebrate RFas, and the reader is recommended to consult these for further information (Chartrel et al. 2006b; Fukusumi et al. 2006; Tsutsui and Ukena 2006). The entire issue 5 of volume 27 of the journal Peptides was devoted to articles on RFa peptides. Until very recently, no peptides had been identified in vertebrates with their C-terminal amino acid sequence consisting of either FMRFa or FLRFa, Table 14. Apart from two FLPs, which will be considered at the end of this section the nearest C-terminal sequence is LRFamide, found in the avian brain (Dockray et al. 1983; Tsutsui et al. 2000), bovine hypothalamus (Fukusumi et al. 2001) and human placenta (Ohtaki et al. 2001). It is likely that the mammalian hypothalamic RFRPs (RF-related peptides) are orthologues of avian LPLRFa, which was the first vertebrate RFa to be identified (Dockray et al. 1983). The first mammalian RFas to be identified were the NPFF and NPAF peptides isolated from the bovine hypothalamus with a C-terminal sequence of PQRFa (Yang et al. 1985) and later identified in humans (Perry et al. 1997). This sequence is shared with RFRP-3 and one of the GnIHs, GnIH-RP-2 (gonadotropin-inhibiting-hormone-related peptide-2), isolated from the quail (*Coturnix japonica*), Table 14 (Yoshida et al. 2003; Satake et al. 2001). The PrRPs (Prolactin-releasing peptide) have a RGIRPVGRFa C-terminal sequence while the carp (*Carassius auratus*) C-RFa has an RGVRPIGRFa C-terminal (Hinuma et al. 1998; Fujimoto et al. 1998). Interestingly, I and V have been exchanged in the C-terminal sequences of these two peptides. As can be seen in Table 14, there are other amino acid similarities between these peptides. The human metastin peptide has a C-terminal sequence of GLRFa while QRFP and 26RFa both have a C-terminal sequence of KGGFSFRFa, Table 14, (Ohtaki et al. 2001; Chartrel et al. 2003). The only RFa C-terminal, which is similar between vertebrate and *C. elegans* is PLRFa, which is shared between the avian RFa peptides, the mammalian RFRP-1 and FLP15, FLP21 (AF9 of *A. suum*) and FLP30 of *C. elegans.* However, the C-terminal sequence of carp C-RFa has similarities with the gastropod cardioexcitatory

Table 14 Showing examples of vertebrate RFamides. For longer neuropeptides, intermediate residues have been omitted. The number of intervening amino acids is given in the sequence

Name of peptide	Species	Structure	Reference
Avian brain peptide	*Gallus domesticus*	LPLRFa	Dockray et al. (1983)
GnIH	*Coturnix japonica*	SIKPSAYLPLRFa	Tsutsui et al. (2000)
GnIH-RP-1	*C. japonica*	VPNSVANLPLRFa	Satake et al. (2001)
GnIH-RP-2	*C. japonica*	SSIQSLLNLPQRFa	Satake et al. (2001)
RFRP-1	*Bos taurus*	SLTFEEVKDW-15-PSAANLPLRFa	Fukusumi et al. (2001)
RFRP-1	*Homo sapiens*	SLNFEELKDW-17-HSFANLPLRFa	Liu et al. (2000)
RFRP-3	*B. taurus*	AMAHLPLRLG- 9-WVPNLPQRFa	Yoshida et al. (2003)
RFRP-3	*H. sapiens*	ATANLPLRSG-8-RRVPNLPQRFa	Hinuma et al. (2000)
RFRP-3	*Rattus norvegicus*	ANMEAGTMSHFPSLPQRFa	Ukena et al. (2002)
GRP/R-RFa	*Rana esculenta*	SLKPAANLPLRFa	Chartrel et al. (2002)
GRP-RP-1/NRP	*Rana catesbeiana*	SIPNLPQRFa	Ukena et al. (2003)
GRP-RP-2	*R. catesbeiana*	YLSGKTKVQSMANLPQRFa	Ukena et al. (2003)
GRP-RP-3	*R. catesbeiana*	AQYTNHFVHSLDTLPLRFa	Ukena et al. (2003)
LPXRFa	*Carassius auratus*	SGTGLSATLPQRFa	Sawada et al. (2002)
NPFF	*B. taurus*	FLFQPQRFa	Yang et al. (1985)
NPFF	*H. sapiens*	SQAFLFQPQRFa	Perry et al. (1997)
NPAF	*B. taurus*	AGEGLSSPFWSLAAPQRFa	Yang et al. (1985)
NPSF	*R. norvegicus*	SLAAPQRFa	Egido et al. (2006)
PrRP20	*B. taurus*	TPDINPAWYAGRGIRPVGRFa	Hinuma et al. (1998)
PrRP20	*R. norvegicus*	TPDINPAWYTGRGIRPVGRFa	Hinuma et al. (1998)
PrRP20	*H. sapiens*	TPDINPAWYASRGIRPVGRFa	Hinuma et al. (1998)
PrRP31	*B. taurus*	SRAHQHSMEI-11-GRGIRPVGRFa	Hinuma et al. (1998)
PrRP31	*R. norvegicus*	SRAHQHSMET-11-GRGIRPVGRFa	Hinuma et al. (1998)
PrRP31	*H. sapiens*	SRTHRHSMEI-11-SRGIRPVGRFa	Hinuma et al. (1998)
C-RFa	*C. auratus langsdorfi*	SPEIDPFWYVGRGVRPIGRFa	Fujimoto et al. (1998)
Metastin/Kiss-pectin-54	*H. sapiens*	GTSLSPPPES-34-YNWNSFGLRFa	Otaki et al. (2001)
26RFa	*H. sapiens*	TSGPLGNLAE-6-RKKGGFSFRFa	Jiang et al. (2003)
26RFa	*R. norvegicus*	ASGPLGTLAE-6-RRKGGFSFRFa	Chartrel et al. (2003)
26RFa	*R. esculenta*	VGTALGSLAE-6-RKKGGFSFRFa	Chartrel et al. (2003)
QRFP	*H. sapiens*	pQDEGSEATGF-23-RKKGGFSFRFa	Fukusumi et al. (2003)
	Kassina maculata	IPPQFMRFa	Wang et al. (2009)
	Phylictimantis verrucosus	EGDEDEFLRFa	Wang et al. (2009)

peptide, ACEP-1, both having RPxGRFa, where x is Q in ACEP-1 but I in C-RFa (Fujimoto et al. 1990; Fujimoto et al. 1998). Cnidarian RFas, such as Antho-RFa, also have a C-terminal GRFa (Grimmelikhuijzen and Graff 1986).

Related RFas from different vertebrates vary in both length and the identity of their amino acids. For example, the human NPFF (Neuropeptide FF) is 3 amino acids longer than bovine NPFF, having SQA at its N-terminal (Perry et al. 1997). The human NPAF is the same length as the bovine peptide but differs with respect to 2 amino acids, having an N in place of an S at position 6 and a Q in place of a P at position 8, Table 14 (Chartrel et al. 2003). The human RFRP-1 has 37 amino acids with its last 7 C-terminal amino acids the same as the bovine RFRP-1 with 35 amino acids while the human and bovine RFRP-3 both have 2 with the C-terminal 8 amino acids in common (Chartrel et al. 2003; Fukusumi et al. 2001; Yoshida et al. 2003). However, the human RFRP-1 may also occur as a shorter 12 amino acid sequence, MPHSFANLPLRFa (Hinuma et al. 2000). The last 8 C-terminal amino acids of frog, rat and human 26RFa are the same, viz, KGGFSFRFa (Chartrel et al. 2003). Human 26RFa has also been identified as P518 (Jiang et al. 2003).

The five vertebrate peptide genes have been named *farp-1 to 5,* together with the receptor genes, *rfr-1* to *5*, for their peptides (Dockray 2004), Table 15. For example, *farp-1* is the gene for both NPAF and NPFF (Perry et al. 1997) while its receptor gene is *rfr-3*. The naming of the RFa receptors is confusing, each having more than one name. It is further complicated since vertebrate RFas can have high affinity

Table 15 A summary of mammalian and *R. esculenta* genes for RFa peptides and their receptors (after Dockray (2004) and Fukusumi et al. (2006)

Peptide gene	Peptide name	Species	Receptor gene	Receptor name
farp-1	NPAF	*Homo sapiens*	*rfr-3*	GPR74 (or NPGPR; HLWAR77; NPFF-2)
	NPFF	*H. sapiens*		GPR147; (or NPFF-1; OT7T022)
farp-2	PrRP	*H. sapiens*	*rfr-1*	GPR10 (or hGR3; UHR-1)
farp-3	RFRP-1 (or NPSF)	*Bos taurus* (hypothalamus)	*rfr-2*	GPR147 (or NPFF-1; OT7T022)
	RFRP-3 (or NPVF)	*B. taurus*		GPR74 (or NPGPR; HLWAR77; NPFF-2)
farp-4	Metastin (Kisspeptins)	*H. sapiens* (placenta)	*rfr-4*	GPR54 (or OT7T175; AXOR12; KiSS-IR)
farp-5	26RFa (or P513)	*Rana esculenta*; *Rattus norvegicus*; *H. sapiens* (hypothalamus)	*rfr-5*	GPR103 (or AQ27; SP9155)
	QRFP	*H. sapiens*		GPR103

for more than one receptor type. The receptor for PrRP was the first one to be identified and called hGR_3 with subsequent names of GPR10 or UHR-1 (rat) and its gene designated as *rfr-1*, Table 15 (Hinuma et al. 1998). In compiling this table, the classification relating peptides and their receptors put forward by Dockray (2004) has been followed. PrRP is encoded by *farp-2*. NPFF, hRFRP-1 and hRFRP-3 all have low affinity for hGR_3 (Engstrom et al. 2003). The receptor genes *rfr-2* and *rfr-3* both encode receptors that have been termed NPFF, viz, NPFF-1 (or OT7T022) and NPFF-2 (or HLWAR77), respectively, Table 15 (Fukusumi et al. 2006). NPFF specifically binds to both receptors and both receptors are activated by NPFF (Bonini et al. 2000). Elshourbagy et al. (2000) have shown that both NPFF and NPAF have high affinity for NPFF-2, and these authors conclude that these peptides are the cognate ligands for this receptor. However, Engstrom et al. (2003) noted that hPrRP31 has a significantly higher efficacy compared with NPFF at the hNPFF-2 receptor, which indicates the promiscuity of some of these RFas. It is not clear whether NPFF-1 has a physiological role in mediating the action of NPFF although NPFF is a potent agonist of the NPFF-1 receptor (Bonini et al. 2000). Bonini et al. (2000) consider that NPFF-1 and NPFF-2 may belong to the same evolutionary lineage as NPY receptors, having arisen by the gene duplication of ancestral NPY receptor genes. *farp-3* encodes RFRP-1 and RFRP-3 while its receptor gene, *rfr-2,* encodes NPFF-1 (OT7T022 or GPR147) (Hinuma et al. 2000). RFRP-3 binds with a high affinity to OT7T022 but with a low affinity to HLWAR77 (Yoshida et al. 2003). Structure–activity studies indicate that the C-terminal four amino acids, PQRFa, are core for binding to both receptors while the addition of three further amino acids to give PNLPQRFa and LFQPQRFa confers selectivity for OT7T022 and HLWAR77, respectively. *farp-4* encodes metastin (alternatively called KiSS-1 peptide) and its receptor gene, *rfr-4*, encodes GPR54 (the human orthologue of rat GPR54 is called AXOR12 or hOT7T175) (Lee et al. 1999; Muir et al. 2001; Ohtaki et al. 2001). *farp-5* encodes QRFP and 26RFa (P518, Jiang et al. 2003) while *rfr-5* encodes for their receptor, GPR103 (or AQ27 or SP9155) (Fukusumi et al. 2003; Jiang et al. 2003; Lee et al. 2001).

OT7T022 has been identified as the receptor for RFRP-1 and RFRP-3 (Hinuma et al. 2000). CHO cells transfected with a rat cDNA-encoding OT7T022 responded to both RFRP-1 and 3 but not to RFRP-2. This is not surprising since the C-terminal sequence for this peptide is LPLRSa rather than LPLRFa or LPQRFa in the case of RFRP-1 and 3, respectively. Both RFRP-1 and 3 potently inhibited forskolin-induced production of cAMP with IC_{50} values in the sub nM range. Both chicken LPLRFa and bovine NPFF also inhibited cAMP production but with IC_{50} values around ten times greater. PQRFa was relatively inactive with an IC_{50} of 300 nM while PrRP was inactive. The highest levels of OT7T022 expression were in the hypothalamus with lower levels in the thalamus, midbrain, hindbrain and eye while RFRP mRNA was selectively expressed in the rat hypothalamus and eye. Some neurones in the periventricular nucleus exhibited expression for both OT7T022 and tyrosine hydroxylase mRNA, suggesting that RFRPs may modulate the reduction in prolactin secretion by dopamine. Dopamine secretion is induced by a rise in cAMP and so it is possible that RFRPs inhibit dopamine release. Interestingly, Fujimoto et al. (1998) found that human RFRP-1 increased prolactin plasma levels in rats. Hypothalamic GnIH peptides inhibit release of gonadotropins in birds (Tsutsui et al. 2000). OT7T022 shows homology to the NPY receptor and NPY, which has a C-terminal RYamide, might interact with OT7T022. A pancreatic polypeptide has been identified in the frog, *Rana temporaria*, with an RFamide C-terminal (McKay et al. 1990) and also one in an alligator (Glover et al. 1984). A number of RFa peptides have been identified in the frog

central nervous system and their structures and functions recently reviewed by Ukena et al. (2006). These are either RFRP-1-, RFRP-3- or QRFP-like in structure, Table 14, (Chartrel et al. 2002, 2006a, b). Evidence suggests frog Growth Hormone-Releasing Peptide, fGRP or Rana-RFa, R-RFa, has a clear effect on Growth Hormone-releasing activity. fGRP immunoreactivity has been localized in fibres in the median eminence and dorsal horn of the spinal cord and in neurones in the hypothalamus, suggesting this peptide may act as a neurohormone and play a role in the transmission of pain (Chartrel et al. 2002). A number of RFa peptides have been identified in the bird brain with a C-terminal of LPXRFa where X is either L or Q, Table 14, (Tsutsui and Ukena 2006). For example, GnIH, gonadotropin-inhibiting hormone is localized in the hypothalamo-hypophysial system and decreases the release of gonadotropin from cultured anterior pituitary. Two further related peptides, GnIH-RP-1 and GnIH-RP-2, have been identified in the quail brain, Table 14, (Satake et al. 2001). As seen from the table, these are related in structure to both RFRP-1 and RFRP-2 and the frog GRP peptides described earlier. There is an LPXRFa-3 in goldfish, Table 14, which occurs in the hypothalamus and may act to regulate pituitary hormone release (Sawada et al. 2002).

hGR_3 is a G-protein-coupled receptor encoded by the gene *rfr-1,* which is specifically expressed in the human pituitary (Hinuma et al. 1998). The ligand for this receptor is PrRP, which occurs in the hypothalamus, possibly in noradrenaline-containing neurones (Chen et al. 1999). PrRP has at least two isoforms, one consisting of 31 amino acids and one of 20 amino acids, Table 14. PrRP has been found to release prolactin from isolated anterior pituitary cells (Hinuma et al. 1998). PrRP can induce intracellular calcium influx and reduce forskolin-stimulated cAMP production in CHO cells expressing hGR_3 and can stimulate extracellular signal-regulated protein kinase (ERK) in rat pituitary tumour cells. This stimulation is blocked by pertussis toxin, suggesting PrRP activates GR_3 which is linked to Gαq or Gαi/o (Fukusumi et al. 2006). However, there is evidence that certain PrRP effects occur through the activation of NPFF receptors, possibly NPFF2 rather than GPR10 (Ma et al. 2009a). The role of PrRP as a physiologically important hormone for the release of prolactin in mammals has been challenged but it may have a physiological role in the regulation of stress responses and feeding behaviour (Fukusumi et al. 2006). An RFamide, which is structurally related to PrRP20 and 31 has been isolated from the brain of the carp and salmon, Table 14, (Fujimoto et al. 1998; Moriyama et al. 2002). This peptide is present in cell bodies in the hypothalamus and in fibres running to the ventral telencephalon. Immunoreactive fibres also run to the pituitary and terminate near prolactin cells in the rostral pars distalis and to somatolactin cells in the pars intermedia. In the trout, plasma levels of prolactin and somatolactin were increased while levels of growth hormone were decreased following intraperitoneal injection of C-RFa. Perifusion of trout pituitary with C-RFa, 10 pM–100 nM, induced maximum prolactin release at 100 pM and maximum somatolactin release at 10 and 100 nM. Levels of growth hormone were not affected. Therefore, C-RFa may have a physiological role in prolactin release in teleost fish. Evidence in support of this comes from the recent work of Fujimoto et al. (2006). These authors injected C-RFa and anti-C-RFa antiserum intraperitoneally into goldfish and trout and found that while C-RFa increased pituitary mRNA level of prolactin and decreased water inflow in the gills, anti-C-RFa had the opposite actions. Thus, both C-RFa and prolactin act to restrict branchial water permeability. These results indicate that C-RFa is required to maintain prolactin levels and osmotic balance in freshwater teleosts. In a structure–activity study using a fish stomach assay, Fujimoto et al. (1998) found that the minimum C-RFa sequence necessary for activity was IGRFamide while PIGRFamide was almost equipotent with C-RFa.

NPFF has been implicated in a range of physiological processes including insulin release, cardiovascular responses, food intake and electrolyte balance (Allard et al. 1995; Panula et al. 1996). In addition, NPFF would appear to have a key role in the regulation of nociception (Panula et al. 1999). NPFF precursor mRNA in the mammalian spinal cord can be up-regulated following carrageenan injection, a model for inflammatory pain (Vilim et al. 1999). However, there is conflicting evidence as to whether NPFF-like peptides exert pro- or anti-opioid actions (Liu et al. 2001; Panula et al. 1999). For example, intraventricular NPFF exerts anti-opioid effects while intrathecal NPFF potentiates the analgesic effect of morphine. Interestingly, NPY has also been found to both enhance and reduce pain (Brumovsky et al. 2007). High levels of both NPFF-1 and NPFF-2 mRNA are expressed in the CNS of both humans and rats although the relative distribution between the two receptors and the two species varies (Bonini et al. 2000). For example, while there is a high level of NPFF-1 in the human spinal cord, the level of NPFF-2 in the cord is very low. In contrast, in the rat spinal cord, there are high levels of NPFF-2 but only moderate levels of NPRR-1. These differences would suggest that NPFF-1 and NPFF-2 not only have different roles in the same species but their roles vary between species. Interestingly, there are high levels of NPFF-2 in the human placenta but only trace levels of NPFF-1. Bonini et al. (2000) consider that the distribution of NPFF-like immunoreactivity, NPFF-2 mRNA and NPFF-2 receptor-binding sites in the dorsal root ganglia and spinal cord, spinal trigeminal, parafascicular and raphe nuclei and the lateral

hypothalamus, indicates a role for NPFF-2 receptors in nociception. Extensive NPFF structure–activity-binding studies, mainly using rat spinal cord, have been reviewed by Vyas et al. (2006). The first 3 amino acids at the N-terminal of FLFQPQRFa do not appear to be important for receptor affinity. The last 4 C-terminal amino acids, together with amidation of the C-terminal F, are key for affinity but the extended N-terminal is probably important in determining efficacy. Interestingly, the rat NPSF, an octapeptide, has the same amino acid sequence as the last eight amino acids of the C-terminal of bovine and human NPAF, Table 14 (Vilim et al. 1999). HLWAR77, the receptor proposed for NPFF and NPAF, Table 15, is expressed in brain areas associated with opiate activity (Elshourbagy et al. 2000). This provides further support for a pain regulating role for these peptides. Their mode of action appears to be through activation of $G_{i/o}\alpha$-G protein subunits with the inhibition of adenylyl cyclase (Elshourbagy et al. 2000). A role for NPFF has been proposed in the release of vasopressin from the paraventricular nucleus of the rat hypothalamus (Jhamandas et al. 2006). These authors found that NPFF stimulates GABA inhibitory interneurones which synapse onto magnocellular neurosecretory cells, thus inhibiting release of vasopressin. Currently, there are no high affinity antagonists for NPFF receptors (Vyas et al. 2006). Recently, the distribution of NPFF receptors has been investigated in the human brain (Goncharuk and Jhamandas 2008). Neurones expressing hFF2 receptors were located in the forebrain and medulla oblongata, particularly in the anterior amygdala and the dorsal motor nucleus of the vagus.

Metastin or Kisspectin-54 is an RFa peptide, encoded by *KiSS-1* gene, a human metastasis suppressor gene and is the ligand for a human orphan GPCR, OT7T175 (GPR54) (Kotani et al. 2001; Muir et al. 2001; Ohtaki et al. 2001). Three peptides are derived from the *KiSS-1* gene, viz, Kisspectin-54 (Metastin), Kisspectin-14 and Kisspectin-13. Kisspectin-14 and Kisspectin-13 have the same amino acid sequences as the C-terminal of Kisspectin-54, Table 14. KiSS-1 peptide has 145 amino acids, of which Kisspectin-54 corresponds to residues 68–121 (Muir et al. 2001). Metastin was isolated from the placenta and found to inhibit the chemotaxis, invasion and metastasis of cells expressing human OT7T125 (GPR54) receptors (Ohtaki et al. 2001). Structure–activity studies showed that the C-terminal decapeptide was a potent agonist at AXOR12 receptor but removal of the two terminal amino acids, resulted in an octapeptide with a significant drop in activity (Muir et al. 2001). This showed that N^{113} and Y^{112} are key amino acids for potent efficacy of the peptide (numbers refer to amino acid sequence of KiSS-1 peptide). Low level expression of KiSS-1 mRNA was identified in the hypothalamus and basal ganglia with high levels in the placenta. These peptides bind to rat and human GPR54 expressed in CHO K1 cells and stimulates PIP_2 hydrolysis, calcium mobilization, arachidonic release, ERK1/2 and p38 MAP kinase phosphorylation (Kotani et al. 2001). The Kisspectins and their receptor have also been implicated in the control of gonadotropin secretion, viz, follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Gottsch et al. 2004; Matsui et al. 2004; Navarro et al. 2004, 2005).

A 26 amino acid RFa peptide has been identified in the frog, together with related 26 amino acid peptides in humans, rats and ox, Table 14, (Chartrel et al. 2003, 2006a). This RFa has a C-terminal sequence containing three phenylalanines, viz, KGGFSFRFamide, a sequence, which is different from all other vertebrate RFa peptides so far identified. It is suggested that this octapeptide structure is required for biological activity. There is also a common LAEEL sequence in the central region portion of the peptide, Table 14. 26RFa is primarily expressed in the hypothalamus of the rat. Interestingly, prepro-26RFa mRNA is strongly expressed in the human cerebellum and medulla oblongata but at much lower levels in the hypothalamus, suggesting 26RFa has a different role in human compared with rats. This peptide is the ligand for the human orphan receptor GPR103, Table 15, (Chartrel et al. 2006a). In CHO cells expressing mammalian GPR103, mammalian 26RFa induces a rise in intracellular calcium with a fall in cAMP, suggesting it is coupled with $G_{i/o}$ and G_q signalling. However, 26RFa induced a dose-dependent stimulation of cAMP in rat pituitary cells (Chartrel et al. 2003). While the binding sites for 26RFa occur widely in the brain, the expression of GPR103 mRNA is primarily restricted to the mid and hind brain and the superficial layers of the dorsal horn of the cord (Bruzzone et al. 2007). Interestingly, a high density of binding sites for 26RFa also occurs in this area of the cord. However, there is evidence that 26RFa may act at both spinal and super spinal levels to modulate nociception (Yamamoto et al. 2008, 2009). An extended form of 26RFa with 43 amino acids has also been identified in the human brain. This extended peptide also inhibits cAMP and is slightly more potent than the 26 amino acid analogue. The C-terminal sequence GGFSFRFa has very weak affinity for GPR103 while the non-amidated 26RF does not bind to the receptor (Fukusumi et al. 2003; Jiang et al. 2003). Intracerebral injection of 26RFa induces a dose-dependent stimulation of food consumption in semi-fasted mice and stimulates locomotor activities (Chartrel et al. 2003; do Rego et al. 2006). The extended form, QRFP43, also has a role in regulating appetite and energy expenditure (Moriya et al. 2006). The expression of GPR103 in the pituitary, adrenal gland and testis suggests a neuroendocrine role for 26RFa. 26RFa may also play a role in the inhibition of insulin secretion (Egido et al. 2007) and the regulation of dietary intake (Primeaux et al. 2008).

Recently, two very interesting FLPs have been identified in the secretions of the skin of two species of frog, *Kassina* (*Hylambetes*) *maculate* and *Phylictimantis verrucosus* (Wang et al. 2009). These FLPs are IPPQFMRFa and EGDEDEFLRFa and are the first FLPs identified in vertebrates with a C-terminal FMRFa and FLRFa, respectively. Interestingly, while these frogs are hosts to endoparasites, they have relatively few ectoparasites, suggesting that these FLPs may act as natural antiparasitics. It would be very relevant to investigate the actions of both peptides on nematodes preparations, EGDEDEFLRFa in particular since it has five acidic amino acids. It is also of note that both peptides are amides, possibly suggesting an interaction with a FLP receptor in the parasite whose natural receptor ligand is a parasite FLP.

It is clear from this section of the review that vertebrate RFamides likely play a key role in normal function and in pathophysiology. They are found in a number of areas in the CNS, particularly in the hypothalamus of all classes of vertebrate where they have been investigated. Their functions include the modulation of several neuroendocrine systems, of behaviour, pain perception and the autonomic system (Tsutsui and Ukena 2006). Their proposed roles in satiety and pain provide areas for future research into novel compounds to modulate these roles.

Conclusions

It is clear from this review that the volume of research into FLPs and RFas varies between phyla. For example, there is considerable interest in these peptides in nematodes, arthropods and in vertebrates. The depth of the research is enhanced where the genome has been sequenced, which is particularly the situation with nematodes, insects and mammals. A bioinformatic analysis can be used to predict peptide sequences from genes-encoding preprohormone precursors, which can then be tested for bioactivity. Furthermore, this also allows the identification of putative neuropeptide receptors that can be expressed and investigated for their preferred ligand. In the case of insects and nematodes, there is added interest in the development of new insecticides and anthelmintics with the synthesis of compounds, which will disrupt the physiology of the animal associated with the activation of peptide receptors and other systems in these groups (Grimmelikhuijzen et al. 2007). In the case of mammals, it is probable that RFas play key roles in both their physiology and pathophysiology.

If we now compare the structures of FLPs and RFas across the groups, we can see certain trends. In the cnidarians, there are no FMRFa-related peptides, all of the RFas end in GRFa, with a range of amino acids as the fourth amino acid, Table 1. This C-terminal is also found in molluscs, and some molluscan peptides have a C-terminal sequence of QGRFa, as found in cnidarians. C-terminal GRFas, for example, PrRPs, also occur in vertebrates. Cnidaria is of interest since tryptophan (W) is the C-terminal amino acid in place of phenylalanine in a number of peptides and in an insect peptide, Table 12. Tryptophan rarely replaces phenylalanine to give WxRFa but occurs in SPSAKWMRFa in *C. elegans*, Table 4. FLP tetrapeptides have only been found in Platyhelminths and other members of the lophotrochozoa, viz annelids and molluscs. Platyhelminths have a tetrapeptide sequence YMRFa (Table 2) while annelids and molluscs have FMRFa (Tables 6, 7, respectively). In addition, platyhelminths also have NPFs, which terminate in GRPRFa, a grouping shared with crustacean and insects, Tables 11 and 12. Nematodes have a vast range of FLPs and RFas. For example, their FLPs include Y as the first C-terminal amino acid but not W. The second C-terminal amino acid can be one of M, I, L or V but no examples of FRFa or TRFa have been found. In addition, there are examples of RFas and F-amides. Annelids have short FLPs and like molluscs contain examples of FMRFa and FLRFa but unlike molluscs also have the tetrapeptide, FTRFa. Molluscs have a number of FRFas, two of which, SDPFFRFa and AdLAGDHFFRFa have a C-terminal sequence of FFRFa. The remaining peptides in Table 8 are RFas. The arthropods provide a large number of FLPs, Tables 9 and 10, together with extended myosuppressins, which have a C-terminal, HVFLRFa, Tables 11 and 12. In addition, there are a large number of RFas and related peptides. The echinoderms have no FLPs or RFas but a range of F-amides, Table 13. While there have been relatively few structure–activity studies considered in this review, some conclusions can be drawn on the requirements to activate FLP receptors. All FLP receptor ligands must have a C-terminal amide and in its absence the ligand is inactive. For FLP-like activity at least four amino acids are required with a sequence F/YxRFa. In the case of myosuppressins, a minimum C-terminal sequence of VFLRFa is required to activate receptors on some insect tissues. The role of the N-terminal amino acids varies between receptors, however, in general a reduction in the number of amino acids leads to a reduction in potency. The occurrence of NPY-like peptides in platyhelminths, Table 2, molluscs, Table 8, arthropods and in vertebrates with a C-terminal of RP/TRFa or RQ/PRYa and a Y at position 10 from the C-terminal amide is intriguing together with the suggestion that *L. stagnalis* receptor GRL105 is a molluscan homologue of a vertebrate NPY-like receptor. Is there evidence from these data in support of the link between nematodes and arthropods, or molluscs with annelids and platyhelminths, or echinoderms with vertebrates (chordates)? Both the nematodes and arthropods have extended FLPs and

Table 16 A summary of the possible roles of FLPs and RFas in the major metazoan phyla including vertebrates

System	Cnidarians	Platyhelminths	Nematodes	Annelids	Molluscs	Arthropods	Echinoderms	Vertebrates
Metamorphosis	x							
Feeding	x		x	x	x	x		x
Reproduction	x	x	x		x	x		x
Neuromuscular transmission	x	x	x	x	x	x	x	x
Sensory	x	x		x	x	x		x
Cardiovascular	x			x	x	x		x
Larval movement	x							
Osmoregulation				x				x
Respiration					x			
Development					x			
Diapause						x		
Hormonal release						x		x

both lack FMRFa or FLRFa tetrapeptides, although *A. suum* has a FIRFa and *L. migratoria,* an AFIRFa. Among the C-terminals of extended FLPs, both nematodes and arthropods have FLRFas and FMRFas. Comparing molluscs, annelids and platyhelminths, all have FLP tetrapeptides and both molluscs and annelids have FMRFa and FLRFa while platyhelminths have a YMRFa. Both molluscs and annelids also share a C-terminal of YMRFa. Finally, there is little in common between RFas in echinoderms and vertebrates since the former have only F-amides. Apart from two recent FLPs from the skin of two amphibians, vertebrates have a range of LRFas and RFas, including C-terminal FRFas and GRFas.

From work, particularly in nematodes, it is likely that certain FLPs are widely distributed in a phylum and perhaps others are more restricted to related families. However, no generalization can be made until more research has been undertaken using other phyla. It is interesting that some phyla have a number of short FLPs while others have only extended FLPs. This may be related to their use as neurohormones or as transmitters, the former having to resist enzyme breakdown. From Table 16, it is clear that FLPs play a key role in the physiology of all metazoan animals. Many research groups are interested in the occurrence and function of RFas in vertebrates and their results can be taken as a guide to the roles of these peptides in other groups. For example, there is evidence that RFas modulate the activity of a number of vertebrate hormones that have roles in feeding and reproduction. RFas also modulate the vertebrate cardiovascular system and play a role in the modulation of sensory systems, including nociception. In parallel, there is clear evidence that FLPs play a key role in feeding and reproduction in invertebrate phyla, both systems which could be disrupted through the development of anthelmintics and pesticides which act through the FLP system. Interestingly, FLPs also play a role in the transfer of sensory information into the nervous system, particularly being associated with vision. It is likely that FLPs either initiate or modulate neurone-muscle and neurone-neurone transmission in all groups, often being colocalized with, for example, acetylcholine. However, the literature indicates that FLPs are colocalized with many different classes of small molecule 'classical' transmitters. FLPs can also modulate metamorphosis, growth, development and larval movement, and these actions may be widespread through the phyla. By comparing the current state of FLP research across the metazoa, an insight can be obtained for future research. While significant early research was undertaken in molluscs, currently the major areas for FLP research involve nematodes, insects, mammals and amphibians.

In conclusion, a review of the literature covering the FMRFamide and RFamide-like neuropeptides in the metazoan phyla reveals a diverse array of neuropeptides within this family of signalling molecules. These neuropeptides are intimately involved in the regulation of vital physiological processes and, as such, are of considerable interest with respect to the development of novel pharmaceuticals, antiparasitics and insecticides. As the peptide sequences are often phylum, if not species specific, the potential for targeting the pathways with selective agents is promising and is likely to provide the impetus for future research in this rapidly developing field.

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