A COMPARATIVE SURVEY OF THE RF-AMIDE PEPTIDE SUPERFAMILY

EDITED BY: Karine Rousseau, Sylvie Dufour and Hubert Vaudry

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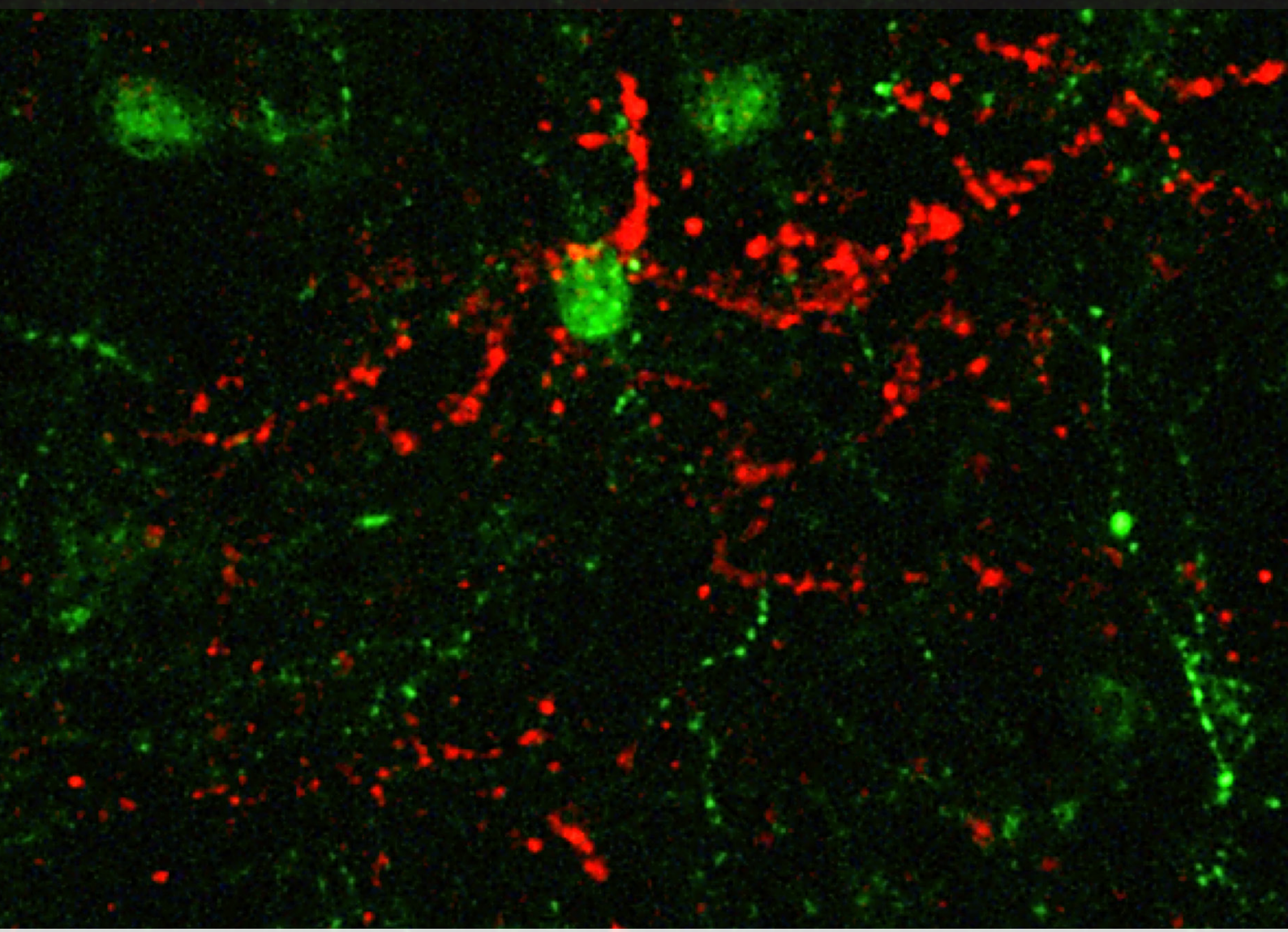

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A COMPARATIVE SURVEY OF THE RF-AMIDE PEPTIDE SUPERFAMILY

Topic Editors:
Karine Rousseau, Museum National d'Histoire Naturelle, France
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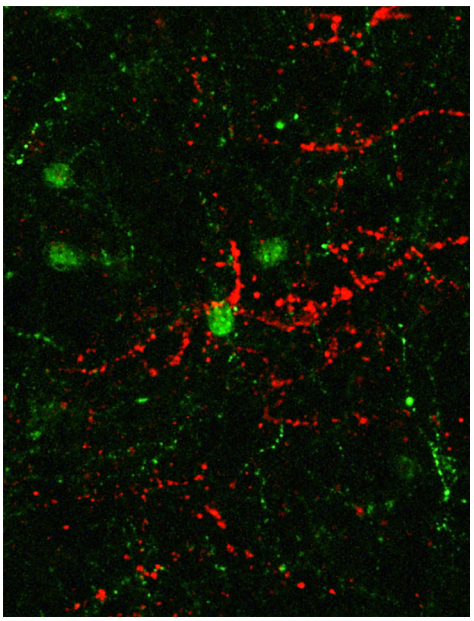
The discovery, twelve years ago, that the RF-amide peptide kisspeptin, acting via GPR54, is essential for the onset of puberty and reproduction, has been a major breakthrough in reproductive physiology. It has also put in front of the spotlights RF-amide peptides and allowed to revive research on this superfamily.

The first member of this family to be characterized, in 1977, was the cardioexcitatory peptide, FMRFamide, isolated from the ganglia of the clam *Macrocallista nimbosa*. Since then, a large number of these peptides, designated after their C-terminal arginine (R) and amidated phenylalaline (F) residues, have been identified in representative species of all major phyla. By means of phylogenetic analyses, the superfamily of RFamide peptides has been divided into five families in vertebrates: kisspeptin, QFRP (including 26RFa), LPXRFa (including GnIH and RFRP), PQRFa (including NPFF) and PrRP. Recent data reveal that SIFamide-type neuropeptides in protostomian invertebrates and SALMFamide-type neuropeptides in deuterostomian invertebrates share a common evolutionary origin with vertebrate LPXRFa and PQRFa. Interestingly, in invertebrates as in vertebrates, multiple genes, as well as multiple mature peptides, are often present in a single species, questioning the need for such diversity in term of function. Comparative studies on non-mammalian vertebrates and invertebrates allow major advances in the knowledge of the evolutionary history of the RF-amide peptide superfamily. Such phylogenetical studies also contribute to improve classification and nomenclature of both peptides and receptors.

RF-amide peptides from different families have major evolutionary conserved roles in the control of reproduction, but also of food intake, metabolism, energy expenditure, cardiovascular function, nociception and stress. They are also involved in the integration of environmental signals, notably the photoperiod, to regulate reproduction. For instance, in most vertebrate species and especially in seasonal mammals, kisspeptin and GnIH/RFRP have complementary but opposite effects in the control of reproductive function. In addition, recent data show cross-activities between the members of the RF-amide peptide superfamily and



Double-immunofluorescent staining for immunoreactivities of the RF-amide peptide, gonadotropin-inhibitory hormone (GnIH), and the norepinephrine (NE) neuronal marker, dopamine β-hydroxylase (DBH) in the paraventricular nucleus (PVN) of the hypothalamus of male quail.

Representative fluorescent photomicrographs showing GnIH (green) and DBH (red). Fluorescence microscopy indicates the anatomical relationship between DBH-IR fibers and GnIH-IR neurons. DBH-IR fibers exist in close proximity to GnIH-IR cell bodies in the PVN of male quail.

Image by K Tsutsui based on Tobari. Y., Son, Y.L., Ubuka, T., Hasegawa, Y., Tsutsui, K. (2014). A new pathway mediating social effects on the endocrine system: female presence acting via norepinephrine release stimulates gonadotropin-inhibitory hormone in the paraventricular nucleus and suppresses luteinizing hormone in quail. J. Neurosci. 34, 9803-9811.

their receptors. For example, PrRP, kisspeptin and 26RFa are able to modulate nociception via NPFF receptors. Comparative studies have the potential to reveal novel regulatory mechanisms that could give a better comprehension of physiological functions and lead to new therapeutic treatments for related human pathologies. Thus, kisspeptin antagonists have been developed as novel tools for treatment of hormone-dependent disorders of reproduction such as precocious puberty and endometriosis or kisspeptin agonists for treatment of infertility, in humans. Studies on lower vertebrate models can also contribute to the discovery of new roles of these peptides, as seen recently with kisspeptin being involved in the early development of the medaka.

This research topic will aim at gathering major advances achieved through comparative studies in (mammalian and non-mammalian) vertebrates and invertebrates, in the knowledge of RF-amide peptides in term of evolutionary history and physiological roles.

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EDITORIAL
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Editorial: A comparative survey of the RF-amide peptide superfamily

Karine Rousseau[1], Sylvie Dufour[1] and Hubert Vaudry[2]*

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Keywords: RF-amide peptides, receptors, evolution, functions, deuterostomes, protostomes

The first member of the RF-amide peptide superfamily to be characterized, in 1977, was the cardioexcitatory peptide, FMRFamide, isolated from the ganglia of the clam *Macrocallista nimbosa* (1). Since then, a large number of such peptides, designated after their C-terminal arginine (R) and amidated phenylalanine (F) residues, have been identified in representative species of all major phyla. The discovery, 12 years ago, that the RF-amide peptide kisspeptin, acting via GPR54, was essential for the onset of puberty and reproduction, has been a major breakthrough in reproductive physiology (2–4). It has also put in front of the spotlights RF-amide peptides and has invigorated research on this superfamily of regulatory neuropeptides. The present Research Topic aims at illustrating major advances achieved, through comparative studies in (mammalian and non-mammalian) vertebrates and invertebrates, in the knowledge of RF-amide peptides in terms of evolutionary history and physiological significance.

Since 2006, by means of phylogenetic analyses, the superfamily of RFamide peptides has been divided into five families/groups in vertebrates (5, 6): kisspeptin, 26RFa/QRFP, GnIH (including LPXRFa and RFRP), NPFF, and PrRP. Recent data reveal that SIFamide-type neuropeptides in protostomian invertebrates and SALMFamide-type neuropeptides in deuterostomian invertebrates share a common evolutionary origin with vertebrate LPXRFa and PQRFa (7). Comparative studies on non-mammalian vertebrates and invertebrates allow major advances in the knowledge of the evolutionary history of the RF-amide peptide superfamily. Such phylogenetical studies also contribute to refine classification and nomenclature of both peptides and receptors. In this issue, Yun et al. (8) show that the concept of coevolution of peptide ligands and their cognate receptors helps to re-examine not only the classification of receptors but also their peptides. They thus report that kisspeptin should be classified in the galanin/spexin family rather than in the RF-amide peptide family. Another example is given by Tachibana and Sakamoto (9) who propose non-mammalian PrRP (C)-RFa to be renamed PrRP2. With the identification of the QRFPR genes in coelacanth and spotted gar, Larhammar et al. (10) demonstrate that the QRFP system is complex in the early stages of vertebrate evolution and secondarily becomes restricted in mammals.

In their review, Elphick and Mirabeau (11) recount the occurrence of the RFamide motif in bilaterian neuropeptide families. They report that peptides, such as NPY/NPF, have acquired modified C-terminal characteristics in vertebrates, while RFamide-type peptides like luqins have been lost in the vertebrate lineage. They also underline some neuropeptide families (e.g., CCK/sulfakinins) in which the RFamide motif is unique to protostomian members. Osugi et al. (12) show that identification of GnIH in agnathans (lamprey) and amphioxus reveals that the C-terminal amide motif of GnIH can differ, being QPQRF or RPQRF, in addition to previously observed LPXRF in birds, mammals and most of fish, and MPQRF in grass puffer and medaka.

As indicated above, the characterization of the first RF-amide peptide was carried out in a mollusk. Since then, many different genes have been identified in invertebrates, and the reviews by Zatylny-Gaudin and Favrel (13) in mollusks, and by Li and Kim (14) and Peymen et al. (15) in nematodes, emphasize the need of identifying receptors for these peptides in invertebrates and characterizing their signaling pathways.

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RF-amide peptides from different families have major evolutionary conserved roles in the control of reproduction, food intake, metabolism, energy expenditure, cardiovascular function, nociception, and stress (16–20). The review by Ayachi and Simonin (21) presents the emerging evidences in rodents that all RF-amide peptides and their receptors are involved in the modulation of nociception in basal and chronic pain conditions, as well as of opioid-induced hyperalgesia. The reviews on GnIH by Osugi et al. (12) and by Ogawa and Parhar (22) report that even if the inhibitory role of GnIH is well established in later-evolved vertebrates, such as birds and mammals, the situation is less clear in teleosts and may vary according to the maturational stage. Comparative studies have the potential to reveal novel regulatory mechanisms that could give a better comprehension of physiological functions. Interestingly, in invertebrates, as in vertebrates, multiple genes as well as multiple mature peptides are often present in a single species, questioning the need for such diversity in term of function. In this Research Topic, Tachibana and Sakamoto (9) report that physiological actions of PrRP and PrRP2 seem to overlap in non-mammalian vertebrates, while converging into those of PrRP in mammals. Studies on lower vertebrate models can also contribute to the discovery of new roles of these peptides. For example, Sandvik et al. (23) review the role of RF-amide peptides in development, first established in medaka (24). In addition, Bouteau et al. (25) provide the first evidence that FMRFamide like peptides (FLPs) may be involved in physiological processes related to hyperosmotic stress responses in plants, widening the scope of RFamide peptides far beyond bilaterian perspectives.

We are particularly indebted to all the researchers who have enthusiastically answered to our invitation to contribute articles to this Research Topic, and to the reviewers who helped us reach the highest standards. It is our hope that this Research Topic will become a major set of references for those working on the phylogenetic history of RFamide-related peptides, and will raise interest of others who are not (yet) involved in this research area.

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Does kisspeptin belong to the proposed RF-amide peptide family?

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Kisspeptin (KISS) plays a key role in regulating reproduction by binding to its receptor, GPR54. Because of the Arg-Phe (RF) sequence at its carboxyl terminus, KISS has been proposed to be a member of the RF-amide peptide family consisting of neuropeptide FF (NPFF), neuropeptide VF (NPVF), pyroglutamylated RF-amide peptide (QRFP), and prolactin-releasing hormone (PRLH). Evolutionary relationships of protein families can be determined through phylogenetic analysis. However, phylogenetic analysis among related peptide families often fails to provide sufficient information because only short mature peptide sequences from full preprohormone sequences are conserved. Considering the concept of the coevolution of peptide ligands and their cognate receptors, evolutionary relationships among related receptor families provide clues to explore relationships between their peptides. Although receptors for NPFF, NPVF, and QRFP are phylogenetically clustered together, receptors for PRLH and KISS are on different branches of the phylogenetic tree. In particular, KISS has been proposed to be a member of the KISS/galanin/spexin family based on synteny analysis and the phylogenetic relationship between their receptors. This article discusses the evolutionary history of the receptors for the proposed RF-amide peptide family and proposes that, from an evolutionary aspect, KISS has emerged from an ancestor, which is distinct from those of the other RF-amide peptides, and so should be classed separately.

Keywords: kisspeptin, RF-amide, spexin, galanin, coevolution, gene duplication, evolutionary history

INTRODUCTION

The RF-amide peptides that harbor the Arg-Phe-amide sequence in their carboxyl (C)-termini were first discovered in a species of mollusk, *Macrocallista nimbosa* (1). Following this discovery, various RF-amide peptides have been identified in other invertebrate species (2, 3). In vertebrates, neuropeptide FF (NPFF) along with its receptor GPR74 (NPFFR2) was the first to be identified in the central nervous system of mammals (4). Fifteen years later, paralogous peptides, gonadotropin-inhibitory hormone (GnIH) in quail brain (5), and neuropeptide VF (NPVF) in mammals (6, 7) were identified. Currently, GnIH and NPVF are found to be orthologous to each other and activate the receptor GPR147 (NPFFR1) (6–10). Another paralogous peptide, pyroglutamylated RF-amide peptide (QRFP or 43RF-amide peptide), and its receptor GPR103 (QRFPR) have been identified in the hypothalamus and spinal cord of mammals (11–13). The receptors for these three vertebrate RF-amide peptides, NPFFR1, NPFFR2, and QRFPR, are phylogenetically very close to each other (**Figure 1**), indicating an evolutionarily common origin for these receptors. The other RF-amide-related peptides in vertebrates are prolactin-releasing hormone (PRLH or PrRP) and kisspeptin (KISS). Interestingly, the receptors for PRLH (PRLHR) and KISS (KISSR) are phylogenetically distant from NPFFR1, NPFFR2, and QRFPR. They are more closely related to neuropeptide Y (NPY) receptors (14, 15) and galanin (GAL) receptors (16), respectively (**Figure 1**).

The RF-amide peptides do not exhibit any sequence similarity to each other, other than the presence of the common RF-amide at their C-termini (17). As most neuropeptides have coevolved with their cognate receptors (18, 19), phylogenetic analysis of the related receptor families may mirror the evolutionary history of the peptide families (20, 21). Phylogeny of the RF-amide peptide receptor family, however, revealed distant relationships between PRLHR, KISSR, and the other RF-amide receptors (15, 16). This suggests that PRLH and KISS, at least, are likely to have originated from ancestors different from that of NPFF, NPVF, and QRFP. Therefore, it is timely to redefine the RF-amide family group members according to the evolutionary histories of individual RF-amide peptides. This article reviews the evolutionary relationships between KISS, and the other RF-amide peptides along with their paired receptors, and proposes that KISS is independent from the RF-amide peptide family.

GENERAL MECHANISM FOR THE EVOLUTION OF NEUROPEPTIDES AND THEIR RECEPTORS

Neuropeptide and receptor families have expanded through whole-genome duplications (WGD) and local tandem gene duplications before and after WGD during vertebrate evolution (14, 16, 20, 22–26). To date, synteny analyses of vertebrate genome fragments and comparison of entire chromosomes of evolutionarily distinct taxa support two rounds (2R) of WGD during early vertebrate evolution. These events produced four paralogous

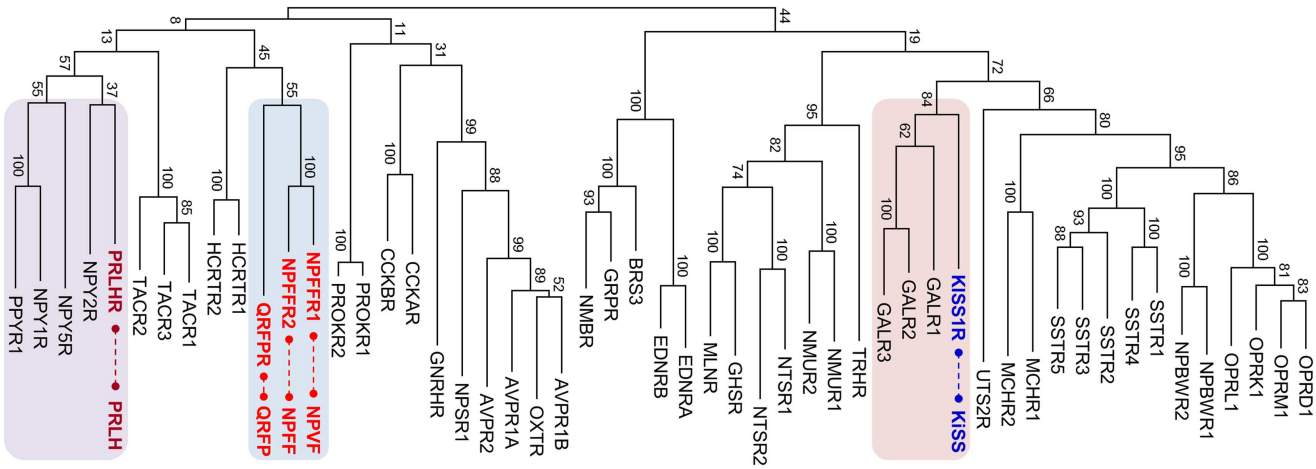

FIGURE 1 | Maximum likelihood phylogenetic tree for human rhodopsin-like neuropeptide GPCRs. The amino acid sequences of the receptors were aligned using MUSCLE, and the tree was constructed by MEGA 5.1. The RF-amide peptide receptor groups are indicated with colored boxes and corresponding peptides are connected by dashed lines. Bootstrap values represent 100 replicates. MLNR, motilin receptor; GHSR, ghrelin receptor; NTSR, neurotensin receptor; NMUR, neuromedin U repceptor; TRHR, thyrotropin-releasing hormone receptor; NMBR, neuromedin B receptor; GRPR, gastrin-releasing peptide receptor; BRS, bombesin receptor; EDNR, endothelin receptor; PRLHR, prolactin-releasing hormone receptor; NPYR, neuropeptide Y receptor; PROKR, prokineticin receptor; TACR, tachykinin receptor; CCKR, cholecystokinin receptor; HCRTR, hypocretin (orexin) receptor; QRFPR, pyroglutamylated RFamide peptide receptor; NPFFR, neuropeptide FF receptor; AVPR, arginine vasopressin receptor; OXTR, oxytocin receptor; NPSR, neuropeptide S receptor; GnRHR, gonadotropin-releasing hormone receptor; UTSR, urotensin receptor; GALR, galanin receptor; KISSR, kisspeptin receptor; MCHR, melanin-concentrating hormone receptor; SSTR, somatostatin receptor; NPBWR, neuropeptides B/W receptor; OPR, opioid receptor.

chromosomal regions (paralogons) sharing similar sets of genes (27–29). A third round (3R) of WGD during an early phase of bony fish emergence resulted in octupled paralogons in teleosts (30).

The evolutionary history of a gene family can be traced by phylogenetic analysis. However, in the case of peptide gene families, phylogenetic analysis often does not provide sufficient information to conclude the evolutionary relationship among related peptide gene families (20, 31). In general, signal peptide sequences are not conserved and propeptide sequences, other than the mature peptide, are highly variable because they are free from evolutionary selective pressure (32). Sequence comparison of only a short, conserved mature peptide is not enough to extrapolate correct relational information. Furthermore, paralogous peptide genes that arose by local gene duplication before 2R exhibit considerable variation even in the mature peptide sequences (20, 31). For instance, the mature peptide sequences of the ligand genes for class B (secretin-like) G-protein-coupled receptors (GPCR) are highly variable (20, 31) while maintaining conserved three-dimensional structure to a general extent (33). Thus, alternative methods need to be applied to explore the evolutionary relationships among peptide gene families.

In contrast to peptide genes, evolutionary relationships among related receptor families can be readily assessed by phylogenetic analysis since the transmembrane domains of the receptors are relatively conserved across vertebrate species and the amino acid sequences of receptors are long enough to generate a convincing phylogenetic tree. Thus, tracing relationships among related receptor families can provide clues to understand the evolutionary history of their corresponding peptide genes. For instance, the evolutionary histories of receptors for secretin family peptides, including corticotropin-releasing hormone, calcitonin, parathyroid hormone, glucagon, and secretin subfamilies may allow us to speculate about possible evolutionary histories for the cognate peptide genes, for which the sequence similarities are not well preserved (20, 25, 31). In addition, locating related peptide genes on the reconstructed pre-2R ancestral chromosomes (or linkage group) is an alternative tool to explore the relationships among related gene families (20, 34). Paralogous genes that emerged through local duplications before 2R reside in the same vicinity on the pre-2R linkage group. For instance, many secretin family peptide genes are located on the same pre-2R linkage group, indicating close evolutionary relationships among the peptide genes (20). The gene families of the neuropeptides, KISS, GAL, and spexin (SPX), are in the vicinity of the same pre-2R linkage group (16). This possibility is further supported by phylogenetic relationships among the receptors of these peptides (**Figure 1**) (16). The neuropeptide Y (*NPY*) gene and its paralogous genes, including peptide YY (*PYY*) and pancreatic polypeptide (*PPY*), reside on the same chromosome or on paralogons (35, 36). Likewise, the NPY receptor (NPYR) family seems to have been generated by local gene duplications followed by 2R WGD (14, 15, 37).

EVOLUTIONARY HISTORY OF NPFF/NPVF, QRFP, AND PRLH

Neuropeptide NPFF was the first RF-amide peptide characterized in the central nervous system of mammals (4, 8, 38, 39). NPFF is known to be involved in morphine tolerance, adipogenesis, and anorectic activity (40–45). In 2000, Tsutsui et al. found a new RF-amide peptide, GnIH, in quail brain (5, 46). This turned out to be an ortholog of NPVF in humans (9, 47). Albeit with some

cross-reactivity, NPFF has a high affinity to NPFFR2 (4) while NPVF more selectively activates NPFFR1 (6, 48, 49).

The neuropeptides NPFF and NPVF seem to be 2R-generated paralogs (ohnologs) as their genes are located on two 2R-generated paralogons of human chromosomes. *NPFF* is located in the vicinity of the neuropeptide *tachykinin 3* (*TAC3*) gene on human chromosome 12 while *NPVF* is on human chromosome 7, which also contains the *TAC1* gene. Likewise, the receptors *NPFFR1* and *NPFFR2* are ohnologous to each other since *NPFFR1/TACR2* and *NPFFR2/TACR3* pairs are on paralogons of human chromosomes 10 and 4, respectively (6, 15, 50). It is of interest to note that human chromosome 10 also harbors *PRLHR* and *PPYR* (a receptor for NPY family peptides) and that human chromosome 4 has *QRFPR* and three *NPYRs*, *NPY1R*, *NPY2R*, and *NPY5R* (14, 15, 51). The phylogenetic tree in **Figure 1** also shows that these receptors are clustered together. These observations suggest that *NPFFRs*, *QRFPR*, *TACRs*, *NPYRs*, and *PRLHR* may have emerged through local duplications from a common ancestor before 2R and expanded their members via 2R. Although the hypocretin (orexin) receptor (HCRTR) is shown in that branch, the *HCRTR* gene is not shown in the paralogons. This may be due to translocation of the gene to other chromosomal regions during evolution (20). Similarly, many peptide genes for these receptors are clustered on 2R-generated paralogons. For instance, the *HCRT*, *PYY*, *PPY*, and *TAC4* genes are closely located on a region of human chromosome 17, which is likely another paralogon of *NPFF/TAC*-containing regions of human chromosomes 7 and 12 (15). Thus, it seems likely that these peptide genes arose during vertebrate evolution in a manner similar to that of receptor genes. It is noteworthy that the chicken C-RF-amide peptide (*PRLH2*), an ohnolog of *PRLH* resides near *NPY*, *TAC1*, and *NPVF* on the chromosomes of chickens, medaka, and tetraodon (52, 53). These findings suggest the presence of evolutionary relationships between the NPFF family and the other RF-amide peptides QRFP and PRLH, along with non-RF-amide peptides such as NPY, TAC, and HCRT.

Pyroglutamylated RF-amide peptide (43RF-amide), a long form of 26RF-amide, has been discovered in the hypothalamus and spinal cord of humans (11–13, 54). Intravenous or intracerebroventricular administration of QRFP increased plasma aldosterone levels and food intake in rats (11, 12, 55). While humans have only one QRFP receptor (12, 13), rodents have two receptors QRFPR1 and QRFPR2 (56, 57). QRFPR2 appears to be an ohnolog of QRFPR1. A region of mouse chromosome 6 has *QRFPR2* and *TACR1*, indicating that this region is another paralogon for the *QRFPR/NPFFR/TACR*-containing chromosomal regions as described previously. As the phylogenetic tree reveals a close relationship between QRFPR and NPFFRs, an evolutionarily close relationship between QRFR and NPFF/NPVF can be postulated.

Prolactin-releasing hormone was first identified by a reverse pharmacological approach in 1998 (58). Physiological roles of PRLH include regulation of stress response (59–61), reduced appetite (62–64), and stimulation of luteinizing hormone and follicle stimulating hormone (65). The orphan receptor GPR10 was identified as the receptor for PRLH (58, 66, 67). PRLHR exhibits a close relationship in its sequence identity with the NPYR family (14, 15). *NPY4R*, a member of the NPYR family is positioned together with *PRLHR1* on the same chromosome of humans and chickens (15). There are also some structural similarities between PRLH and NPY family peptides. Both *PRLH* and *NPY* family genes have two coding exons. The first coding exon contains a signal peptide sequence followed by the N-terminal region of the mature peptide sequence. The second coding exon contains the C-terminal region of the mature peptide with conserved Arg-Phe-Gly (RFG) or Arg-Tyr-Gly (RYG) residues followed by a dibasic cleavage site. Like PRLH, some NPY peptide family members such as bovine PYY and PPY have RF-amide sequences, while NPY, PPY, and PYY from most vertebrates contain the Arg-Tyr-amide (RY-amide) sequence (15, 68, 69). It is also interesting to note that the NPY peptide is able to activate PRLHR1 at micromolar levels (15). These observations together with phylogenetic analysis of neuropeptide receptors suggest that the *PRLH/NPY* family genes and their receptor genes emerged by local gene duplications during early vertebrate evolution (14, 15). These local duplication events are likely to have occurred after the genes split from their common ancestors into the PRLH/NPY and NPFF/QRFP systems.

COEVOLUTION OF THE SPEXIN/GALANIN/KISSPEPTIN FAMILY

The *KISS* gene was first identified as a tumor suppressor gene expressed in human melanoma and breast cancer cells (70, 71). Later, the *KISS* gene was found to produce a functional peptide with an RF-amide sequence (designated as kisspeptin or metastin) that activates an orphan GPCR, GPR54 (KISSR) (72–74). KISS and KISSR are involved in the onset of puberty and the control of reproduction and food consumption (75–80). After the discoveries of mammalian *KISS* and *KISSR*, two paralogs of the *KISS* gene (*KISS2* and *KISS3*) and three paralogs of the *KISSR* gene (*KISSR2*, *KISSR3*, and *KISSR4*) have been identified in a variety of vertebrate species (32, 81–83). All these paralogs seem to be ohnologs as each of them is located on 2R-generated paralogons (32, 82, 83).

Although KISS has been acknowledged as a member of the RF-amide peptide family, no supporting evidence, other than the presence of the RF-amide sequence in its C-terminus, has been provided. KISSR was originally reported to have considerably high degree of sequence similarity with galanin receptors (GALR) (84). This result raises the possibility that KISS/KISSR and GAL/GALR pairs have diverged from a common ancestor and not from the RF-amide peptide/receptor ancestors. Recently, it was shown that the novel neuropeptides spexin1 (SPX1) and spexin2 (SPX2) are functional ligands for GALR2 and GALR3 but not GALR1 (16, 85). In particular, SPXs are more potent than GAL in activation of GALR3, while they show potencies to GALR2 similar to that of GAL (16). Synteny analysis and relocation of the gene families on the reconstructed vertebrate ancestral linkage groups show that *SPX*, *GAL*, and *KISS* family genes are distributed among 2R-generated 4 paralogons (4 linkage groups). Three linkage groups contain *SPX1* and *KISS2*, *SPX2* and *GAL*, and *KISS3* and galanin-like peptide (*GALP*), respectively. The fourth linkage group has *KISS1* alone (16, 86). This study proposed that ancestral forms of *KISS*, *GAL*, and *SPX* arose by tandem local duplications before 2R and expanded their family members through 2R (16). Likewise, KISSRs and GALRs are likely to have emerged through local duplications before 2R, producing three subgroups in vertebrates:

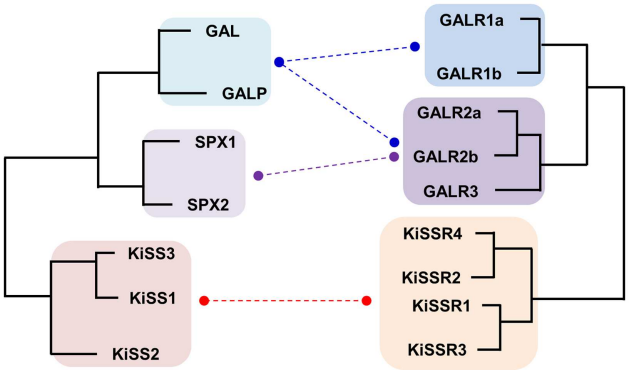

FIGURE 2 | Schematic diagram for the evolutionary relationship between KISS/GAL/SPX family peptides and their corresponding receptors in vertebrates. Paralogs for each peptide and receptor found in vertebrates are included. Functional interaction between the ligands and receptors are indicated by dashed lines. The KISS peptides are able to activate all types of KISSRs with some differences in their ligand potencies but they do not activate any type of GALRs. GALs exhibit high potencies toward GALR1 and GALR2 family receptors but low potencies toward GALR3. SPXs activate GALR2 and GALR3 with high affinities but do not activate GALR1.

KISSRs (4 KISSRs), GALR1 (GALR1a and GALR1b), and GALR2/3 (GALR2a, GALR2b, and GALR3) (**Figure 2**). Altogether, these results suggest that the evolutionary origin of the KISS/SPX/GAL family is far distant from those of the NPFF/QRFP and PRLH/NPY families.

CONCLUSION

Rapid accumulation of genomic sequence information from various invertebrate and vertebrate species and the development of bioinformatic tools, including phylogenetic analysis, small scale genome comparison to identify orthologous and paralogous relationships of genes, and reconstruction of ancient chromosomes, have facilitated exploration of the relationships and origins of peptide and receptor gene families (20, 28, 32, 34, 87, 88). Based on the phylogenetic analysis of neuropeptide receptors and the syntenic relationships of neuropeptide genes, we suggest that the proposed RF-amid peptide family arose from three different ancestors. NPFF and NPVF are likely 2R-generated paralogs and have a close evolutionary relationship with QRFP. PRLH is evolutionarily closer to the NPY family than the NPFF/NPVF/QRFP group. KISS is likely a member of the KISS/GAL/SPX peptide family and their evolutionary origin is far distant from those of the other RF-amide peptides. This study may provide an insight into the mechanism for coevolution of neuropeptides and their receptors.

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Functions of two distinct "prolactin-releasing peptides" evolved from a common ancestral gene

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Prolactin-releasing peptide (PrRP) is one of the RF-amide peptides and was originally identified in the bovine hypothalamus as a stimulator of prolactin (PRL) release. Independently, another RF-amide peptide was found in Japanese crucian carp and named *Carassius*-RFa (C-RFa), which shows high homology to PrRP and stimulates PRL secretion in teleost fish. Therefore, C-RFa has been recognized as fish PrRP. However, recent work has revealed that PrRP and C-RFa in non-mammalian vertebrates are encoded by separate genes originated through duplication of an ancestral gene. Indeed, both PrRP and C-RFa are suggested to exist in teleost, amphibian, reptile, and avian species. Therefore, we propose that non-mammalian PrRP (C-RFa) be renamed PrRP2. Despite a common evolutionary origin, PrRP2 appears to be a physiological regulator of PRL, whereas this is not a consistent role for PrRP itself. Further work revealed that the biological functions of PrRP and PrRP2 are not limited solely to PRL release, because they are also neuromodulators of several hypothalamus–pituitary axes and are involved in some brain circuits related to the regulation of food intake, stress, and cardiovascular functions. However, these actions appear to be different among vertebrates. For example, central injection of PrRP inhibits feeding behavior in rodents and teleosts, while it stimulates it in chicks. Therefore, both PrRP and PrRP2 have acquired diverse actions through evolution. In this review, we integrate the burgeoning information of structures, expression profiles, and multiple biological actions of PrRP in higher vertebrates, as well as those of PrRP2 in non-mammals.

Keywords: prolactin-releasing peptide, prolactin-releasing peptide-2, C-RFa, vertebrates, prolactin, feeding, stress

DISCOVERY OF PROLACTIN-RELEASING PEPTIDES

Hinuma et al. (1) first identified a peptide, which is a ligand of the orphan 7-transmembrane receptor hGR3, by using a reverse-pharmacological technique. The peptide was named "prolactin-releasing peptide (PrRP)" because it was found to specifically promote prolactin (PRL) release from rat anterior pituitary cells *in vitro*.

Independent of the discovery of mammalian PrRP, a novel RF-amide peptide, which has a similar amino acid sequence, was isolated from the brain of the Japanese crucian carp using an intestine contracting assay (2) and named *Carassius*-RFa (C-RFa). In addition to the sequence homology, C-RFa is a strong candidate as the stimulator of PRL synthesis and release, and this peptide has been regarded as a teleost PrRP. However, some studies reported that PrRP did not show a PRL-releasing effect in mammals (3). These differences implied that PrRP and C-RFa might not be orthologous. Finally, with a synteny analysis, Lagerström et al. (4) and Wang et al. (5) found that PrRP and C-RFa originated in gene duplication from a common ancestral gene, and we propose that the widely used name of PrRP in lower vertebrate species be renamed "PrRP2."

BIOCHEMISTRY AND MOLECULAR BIOLOGY OF PrRP AND PrRP2

PrRP

Prolactin-releasing peptide was first identified in the bovine hypothalamus (1). There are two types of PrRP in mammals: one consists of 20 amino acids (PrRP-20) and the other consists of 31 amino acids (PrRP-31) (**Figure 1**), with PrRP-20 being a C-terminal fragment of PrRP-31. As well as in mammals, PrRP-20 is also predicted from the cDNA sequences in some non-mammalian vertebrates, such as chicken, *Xenopus tropicalis*, and zebrafish (5), while the existence of a longer form of PrRP such as PrRP-31 has been unclear (**Figure 1**). These amino acid sequences of PrRP-20 are well conserved. Among mammals, rat and mouse PrRP-20 are identical and their amino acid sequence shows high homology to bovine and human counterparts. Chicken and *X. tropicalis* have the same PrRP-20, whose sequence is different from murine PrRP-20 at only three amino acids. The amino acid sequence of zebrafish PrRP-20 shows moderate homology to those of mammals, and of chicken or *X. tropicalis*. All PrRPs have the Arg-Phe-NH_2 (RF-amide) motif at the C-terminus. The C-terminus amidation is necessary for interaction with the receptors because PrRP-20 with a non-amidated C-terminus cannot efficiently interact (1). Rat and

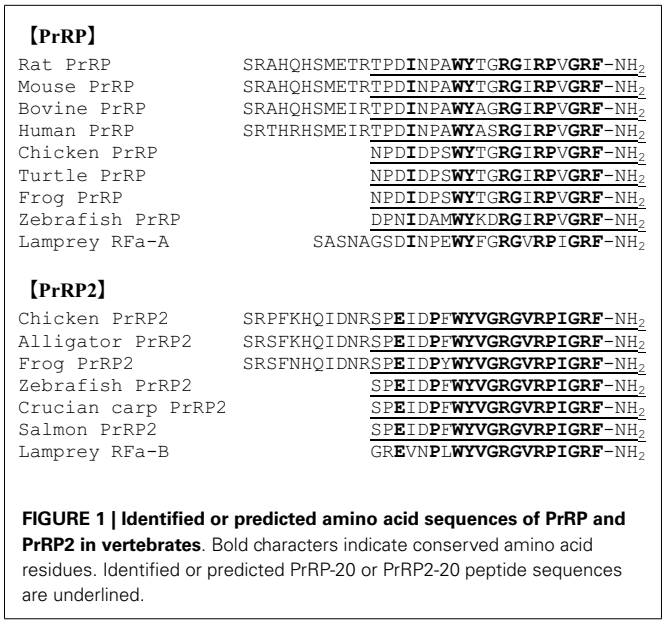

FIGURE 1 | Identified or predicted amino acid sequences of PrRP and PrRP2 in vertebrates. Bold characters indicate conserved amino acid residues. Identified or predicted PrRP-20 or PrRP2-20 peptide sequences are underlined.

human PrRP genes are located on chromosomes 9q36 and 2q37.3, respectively. The genomic organization and promoter function of the rat PrRP gene have been examined by Yamada et al. (6).

In non-mammalian vertebrates, the structure of the PrRP gene has not been well investigated. Since non-mammalian vertebrates have two types of PrRP (namely, PrRP and PrRP2), which is orthologous to mammalian PrRP was a problem. This problem was solved by the synteny analysis done by Wang et al. (5). Chicken, *X. tropicalis*, and zebrafish PrRP genes are located on chromosome 7, scaffold 15, and chromosome 9, respectively. These PrRP genes are surrounded by several genes such as *leucine-rich repeat interacting protein 1*, *ras-related protein Rab-17*, *melanophilin*, and *collagen type VI, alpha 3* (5). These neighboring genes (surrounding the PrRP gene) are also observed on human chromosome 2q37.3, where the PrRP gene is located. Based on PrRP genes in non-mammalian vertebrates, the amino acid sequence of their preproPrRPs was predicted and its sequence showed low-to-moderate homology to mammalian PrRP.

PrRP2

Fujimoto et al. (2) first isolated a novel bioactive peptide from the Japanese crucian carp. Since the peptide contains an RF-amide sequence at the C-terminus, it was named *Carassius* RFamide (C-RFa). Later, C-RFa homologs were isolated from several teleosts, such as chum salmon and tilapia, and their amino acid sequences are identical to the crucian carp (7–9). This amino acid sequence shows similarity to those of mammalian PrRP-20 (**Figure 1**). Together with their stimulation of PRL release in teleosts (10, 11), they have been considered the teleost orthologs of PrRP-20 (12). However, a recent phylogenetic study revealed that they do not originate from the same gene as mammalian PrRP (5). It is, therefore, natural that the nomenclature should be reviewed and it would be better renamed PrRP2 (5). Sea bream and zebrafish PrRP2-20s predicted from the cDNA also show the identical sequence [**Figure 1**; (5, 13, 14)]. Additionally, two putative PrRP2 consisting of 37 or 36 amino acids, like PrRP-31, were predicted from the analyses of cleavage sites, but such a longer form of PrRP2 has not been identified in teleosts.

PrRP2 was also identified in chicken: both PrRP2-31, which consists of 31 amino acids and the C-terminal PrRP2-20 are present in the brain (15). In several non-mammalian vertebrates, such as *Xenopus laevis* and *X. tropicalis*, PrRP2s have been predicted from cDNA (5, 16). All the PrRP2s are expected to have RF-amide motifs at the end of their C-terminuses and their PrRP2-20s show high homology to teleost PrRP2. The amino acid sequences of teleost PrRP2 and chicken PrRP2-20 are identical (**Figure 1**), and the sequence of *Xenopus* shows high homology (**Figure 1**). In one animal species, the sequence of PrRP2 is moderately similar to that of the respective PrRP. PrRP-like RF-amide peptides are also found in sea lamprey, and named RFa-A and RFa-B (17) (**Figure 1**). These peptides show relatively high homology to teleost, *Xenopus*, and chicken PrRP2 but not to PrRP, suggesting that they are homologous to PrRP2. RFa-B, in particular, has an amino acid sequence similar to teleost, *Xenopus*, and chicken PrRP2s compared with RFa-A. If lamprey RFa-A and RFa-B are, respectively, orthologous to PrRP and PrRP2, these peptides originated at least from the stage of primitive vertebrates.

Chicken and *X. laevis* preproPrRP2 consist of 108 amino acids (15, 16). Chicken and *X. tropicalis* preproPrRP2, respectively, have low homology with their preproPrRP (5, 15). Their amino acid sequences suggest the occurrence of a longer form of PrRP2 such as PrRP2-31 in *X. laevis* (16) as shown in chickens. The deduced amino acid sequence of *X. laevis* PrRP2-31 shows high homology with chicken PrRP2-31 and moderate homology with mammalian PrRP-31. Although PrRP2-20 can activate PrRP-R1, PrRP-R2, and PrRP2-R in chickens (5), N-terminal 11 residue sequence of PrRP2-31 also seem to be important for activity because central injection of PrRP2-20 did not affect feeding behavior and blood constituents while PrRP2-31 stimulates feeding behavior and modified blood constituents (15, 18).

In contrast to non-mammalian vertebrates, PrRP2 has not been identified in mammals. PrRP2 genes are located on chromosome 3, scaffold 359, and chromosome 24 in chicken, *X. tropicalis*, and zebrafish, respectively, and genes of *myosin VIIA* and *Rab interacting protein* (*MYRIP*) exist on each chromosome or scaffold (5). The *MYRIP* gene is located on human chromosome 3, but the PrRP2 gene has not been identified. Based on a phylogenetic relationship among PrRPs and PrRP2s (**Figure 2**), divergence of PrRP and PrRP2 from the common ancestral gene occurred faster than their specialization in each vertebrate. It is, therefore, possible that PrRP2 might have been lost during mammalian evolution. Lagerström et al. (4) could not find PrRP or PrRP2 in any invertebrate genome, suggesting that these peptides most likely arose during the tetraploidizations.

RECEPTORS FOR PrRP AND PrRP2

Hinuma et al. (1) first identified the human PrRP receptor (hGR3, PrRP-R), which is virtually identical to the orphan receptor GPR10 (19). The hGR3 receptor was expected to be a counterpart of the rat orphan receptor UHR-1 (20), sharing 89% amino acid homology. These PrRP-Rs share a slight homology with the neuropeptide Y (NPY) receptor in amino acid sequences, and a micromolar level

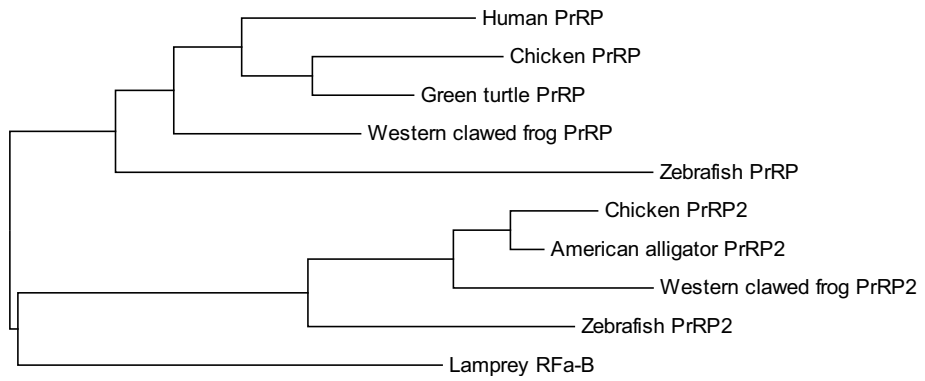

FIGURE 2 | Phylogenetic relationship between proPrRP and proPrRP2 in vertebrates. The tree was constructed using the maximum likelihood method, plotted in MEGA6.

of NPY was able to bind and inhibit completely the PrRP-induced response in cells, which express PrRP-R, suggesting that PrRP-R shares a common ancestor with NPY receptors (13). On the other hand, it is likely that PrRP binds to not only the PrRP receptor but also other receptors such as neuropeptide FF receptor-2 (21).

In non-mammalian vertebrates, Lagerström et al. (13) first identified PrRP-R. They found three PrRP-Rs (PRLHR1, PRLHR1B, and PRLHR2) in chicken, two receptors (PRLHR1 and PRLHR2) in pufferfish, and one receptor (PRLHR2) in zebrafish. Thereafter, Wang et al. (5) suggested that PRLHR1 and PRLHR1B are receptors for both PrRP and PrRP2 in chicken while PRLHR2 is a specific receptor for PrRP2 in chicken. Based on these features, the names were changed from PRLHR1 and PRLHR1B to PrRPR1 and PrRPR2, respectively, and from PRLHR2 to C-RFaR (5). As noted above, we suggest that C-RFa be renamed PrRP2, so C-RFa-R should be renamed PrRP2-R. In this review, we use the PrRP receptor terms PrRP-R1, PrRP-R2, and PrRP2-R for chicken and other non-mammalian vertebrates.

PrRP-R

Mammalian PrRP-R belongs to the 7-transmembrane receptor (7TMR) superfamily. Radioiodinated PrRP-20 and -31 bind equally and with high affinity to these orphan 7TMRs expressed in CHO cells and HEK293 cells (1, 22, 23). Except for non-mammalian PrRP2, none of the known native ligands including RF-amide neuropeptide FF demonstrate any affinity for GPR10 (23). These studies indicate that PrRPs are specific, high affinity ligands for PrRP-R. In rats, Satoh et al. (24) suggested that there may be different subtypes of PrRP-R, which may be different from UHR-1.

The human PrRP-R gene is located on chromosome 10q26.13 and consists of two exons and one intron (25), although the entire coding region is intronless. In GH3 pituitary tumor cells or primary cultures of anterior pituitary cells from rat, the PrRP receptor appears to signal via multiple kinase pathways including mitogen-activated protein kinase (MAPK), Jun N-terminal kinase (JNK), and serine/threonine kinase (Akt/protein kinase B) to the PRL promoter; these pathways require an Ets transcription factor (26, 27). Since the Akt pathway is associated with cell survival and growth, PrRP may function not only to control PRL expression, but also to maintain PRL cell number (28).

In non-mammalian vertebrates, two subtypes of the PrRP receptor, PrRP-R1 and PrRP-R2, have been predicted (5, 13). In *X. tropicalis* and chicken, the amino acid sequences of PrRP-R1 and PrRP-R2 are moderately conserved and show moderate homology with mammalian PrRP-R. The PrRP-R1 and PrRP-R2 genes are located on scaffold 605 and 40, and on chromosome 6 and 22 in chickens (5), respectively. Their surrounding genes are well conserved between human beings, chickens, and *Xenopus*. Based on a synteny analysis, PrRP-R1 but not PrRP-R2 is thought to be orthologous to mammalian PrRP-R [**Figure 3**; (13)]. Although the PrRP-R2 gene is expected to exist on chromosome 8 in human beings based on the surrounding genes, it has not yet been identified. Like mammalian PrRP-R, the entire coding region of cPrRPR1 is intronless while the coding region of cPrRPR2 is interrupted by an intron (5, 13). In chickens, both PrRP and PrRP2 show affinity to PrRP-R1 and PrRP-R2 to a similar extent (5). Both receptors possess conserved structural motifs, which are also well conserved within the rhodopsin family of G-protein coupled receptors. In chickens, PrRP-R1 and PrRP-R2 appear to be functionally coupled with the intracellular protein kinase A signaling pathway (5). The activation of these receptors is also expected to trigger Ca^{2+} release from intracellular stores (5).

Teleost PrRP-Rs are also predicted from their genome. For example, Watanabe and Kaneko (29) characterized PrRP-R of tilapia. This receptor may be categorized as PrRP-R2 because its amino acid sequence shows higher homology to *X. tropicalis* and chicken PrRP-R2 rather than other receptors.

PrRP2-R

PrRP2-Rs have been identified in several non-mammalian vertebrates, such as zebrafish, *X. tropicalis*, and chickens (5, 13). Among these animals, the structures of *Xenopus* and chicken PrRP2-R have been well studied. The entire coding region of the chicken PrRP2-R gene is intronless (5), while *X. tropicalis* PrRP2-R consists of two exons and one intron as does mammalian PrRP-R. *Xenopus* and chicken PrRP2-R, respectively, consists of 364 and 361 amino acids, and show moderate homology between them. In contrast,

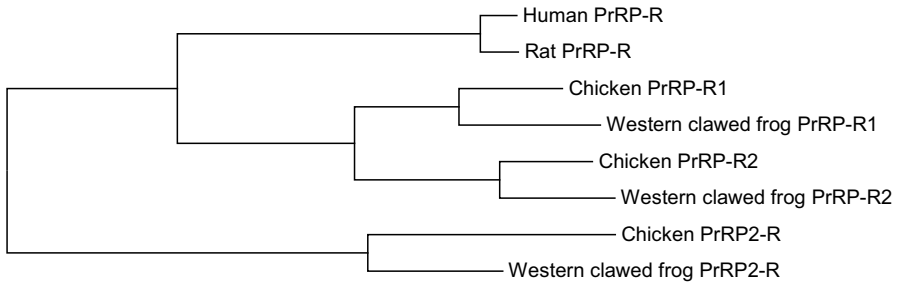

FIGURE 3 | Phylogenetic relationship between PrRP-R, PrRP-R1, PrRP-R2, and PrRP2-R in vertebrates. The tree was constructed using the maximum likelihood method, plotted in MEGA6.

Xenopus and chicken PrRP2-R also show lower homology to their PrRP-R1 and PrRP-R2, respectively.

Like PrRP-Rs, PrRP2-R belongs to the neuropeptide receptor family of the 7TMR superfamily. In chickens, stimulation of PrRP2-R also activates the intracellular protein kinase A signaling pathway. Chicken PrRP2-R is activated by both PrRP and PrRP2 *in vitro*, but PrRP2 is 150 times more potent than PrRP (5). On the other hand, chicken PrRP-Rs are similarly activated by PrRP and PrRP2. Therefore, PrRP2-R is a specific receptor for PrRP2 in chickens. This idea was supported by studies using teleost showing that Japanese crucian carp PrRP2 can also interact with human PrRP-R (30). In addition, teleost PrRP2, not mammalian PrRP, stimulates PRL release from tilapia pituitary, suggesting that PrRP2-R might be related to PRL secretion and mammalian PrRP would not be able to bind to teleost PrRP2-R (8).

Chicken, *X. tropicalis*, and zebrafish PrRP2-R genes are located on chromosome 5, scaffold 627, and chromosome 17, respectively (5). Genes surrounding the PrRP2-R gene are well conserved between vertebrates including mammals. The human PrRP2-R gene is expected to exist on chromosome 15, but it has not yet been identified as well as PrRP2 gene (5). Phylogenetic study implies that the PrRP2 and PrRP-R2 genes disappeared through the evolution of mammals.

DISTRIBUTION OF PrRP, PrRP2, AND THEIR RECEPTORS

PrRP AND PrRP-R

Prolactin-releasing peptide is widely distributed in central and peripheral organs in mammals (**Table 1**). In the rat brain, PrRP nerve cell bodies are distributed in the hypothalamic dorsomedial nucleus (DMN), the nucleus of the solitary tract (NTS), and ventral and lateral reticular nuclei (VLRN) in the medulla oblongata (31–33). PrRP neurons in the VLRN and NTS are considered to be A1 and A2 noradrenergic neurons, respectively (34, 35). PrRP nerve fibers project to several brain regions, such as the bed nucleus of the stria terminalis, hypothalamic paraventricular nucleus (PVN), hypothalamic periventricular nucleus (PerVN), and basolateral amygdaloid complex (31, 36). PrRP nerve fibers in the hypothalamus appear to contact catecholaminergic, oxytocin, corticotrophin-releasing hormone (CRH), and somatostatin neurons (36–39). On the other hand, PrRP neurons do not project to the external lamina of the median eminence (33, 40), where hypophysiotropic factors of hypothalamic origin gain access to the hypophysial portal vasculature and the anterior lobe. An axonal transport study (35) also revealed that the hypothalamic PrRP neurons are not directly connected to the anterior pituitary. The distribution of PrRP in the brain was also investigated in human beings and sheep, and their distributions are basically similar to

Table 1 | Comparison of the distribution of PrRP and PrRP2 mRNA among mammals, avians, and teleosts.

	Mammal[a]	Avian[b]		Teleost
	PrRP	PrRP	PrRP2	PrRP2
Whole brain	+	+	+	+[c]
Telencephalon	–	+	+	–[d], +[e]
Diencephalon	+	+	+	+[d,e]
Midbrain	–	+	+	+[d,e]
Cerebellum	–	+	+	–[d]
Hindbrain	+	+	+	+[d,e]
Spinal cord	+	–	–	+[d]
Pituitary	–	+	+	+[c,d], –[e]
Thyroid gland	+			
Heart	–	–	+	–[d]
Lung	+	+	+	
Liver	–	–	+	+[c]
Spleen	–	–	–	–[c]
Pancreas	+	–	+	
Kidney	+	–	+	–[c,d]
Gut	+	–	+	+[c,d]
Adipose tissue	–			
Skeletal muscle	–	+	+	–[c]
Testis	+	+	+	
Ovary	–	+	+	+[c]

[a] *Rat (41).*
[b] *Chicken (5).*
[c] *Mudskipper (12).*
[d] *Sea bream (14).*
[e] *Goldfish (45).*
+: Definite expression; –: little or nothing.
Blank columns indicate "not examined."
Distribution of PrRP has not yet been investigated in teleost.
In rats, PrRP mRNA expression is also observed in thymus, trachea, submandibular gland, adrenal gland, and uterus (see PrRP and PrRP-R).

those of rats (41–43). PrRP and its mRNA have also been found in a number of peripheral tissues, including the pituitary gland, adrenal gland, lung, pancreas, and testis (41, 44) (**Table 1**).

As with PrRP itself, PrRP-R-expressing neurons are widely distributed throughout the brain, including the PVN, hypothalamic preoptic area (POA), PerVN, ventrolateral hypothalamus, DMN, reticular thalamus, and area postrema (22, 33, 38). In contrast to the central nervous system, the distribution of PrRP-R seems to be sparse in the peripheral tissues. PrRP receptor mRNA has been found in the pituitary gland, adrenal gland, stomach, and femur (41).

In contrast to mammals, the distribution of PrRP in non-mammalian vertebrates has not been well investigated. Wang et al. (5) found that PrRP mRNA is expressed in both central and peripheral tissues in chickens. In the central nervous system, PrRP mRNA is present in various brain regions including the hypothalamus, but not in the spinal cord (**Table 1**). In the peripheral tissues, PrRP mRNA is present in the pituitary, muscle, lung, testis, and ovary. These distributions in chickens are almost similar to those of mammals.

In chickens, PrRP-R1 and PrRP-R2 mRNA are widely and similarly distributed throughout the brain as they are in mammals (5). On the other hand, their distribution in the peripheral tissues is different (5). PrRP-R1 mRNA is present in the pituitary, heart, small intestine, kidney, liver, lung, muscle, testis, ovary, and spleen. PrRP-R2 mRNA is distributed in similar tissues to PrRP-R1, but it is not present in the pituitary, liver, and spleen. The difference in distribution indicates that they are functionally differentiated in chickens. In tilapia, PrRP-R (probably PrRP-R2) mRNA is expressed in the brain, pituitary, heart, spleen, kidney, and rectum but not liver (29). The mRNA expressions of PrRP-R in the peripheral tissues are different between vertebrates, suggesting that the action of PrRP is different between chickens and mammals.

PrRP2 AND PrRP2-R

PrRP2 mRNA is distributed in the central nervous system in non-mammalian vertebrates (5, 12, 14, 45, 46) (**Table 1**). Further, a histological survey has been performed only in teleosts using the antisera against teleost PrRP2 whose cross-reactions with PrRP are unclear. Based on these studies, PrRP and/or PrRP2 perikarya and nerve fibers are thought to be distributed in the hypothalamus and pituitary of teleosts (7, 47). Further studies using specific antiserum for PrRP2 will clarify the actual distribution of PrRP2 in the brain of teleosts.

PrRP2 mRNA is abundantly expressed not only in the brain but also in the peripheral organs of teleosts (**Table 1**). In mudskippers, PrRP2 mRNA is expressed in the liver, gut, and ovary, while significant levels of expression were also detected in the skin and kidney (48). Within the cyprinid retina, PrRP2 mRNA is also abundantly expressed (46). Corresponding to the distribution of PrRP2 mRNA in the mudskipper, relatively high expression of extrapituitary PRL was observed in the liver, gut, and ovary (48). PrRP2 may stimulate PRL expression in an autocrine/paracrine manner as observed in human decidua PrRP-PRL (49).

In chickens, PrRP2 mRNA and mature peptides (PrRP2-20 and PrRP2-31) are expressed in the telencephalon, midbrain, cerebellum, hindbrain, and hypothalamus of the brain (5, 15). Messenger RNA is also expressed in the peripheral organs such as the heart, small intestine, kidney, liver, lung, muscle, ovary, testis, pituitary, and pancreas (5). PrRP2-R mRNA is also observed in similar tissues to PrRP2, but it is not expressed in the heart and pancreas.

BIOLOGICAL ACTIONS OF PrRP AND PrRP2

In vertebrates, it has been reported that both PrRP and PrRP2 are related to many physiological roles such as the regulation of pituitary hormone release, feeding/energy metabolism, stress response, cardiovascular regulation (50), retinal information processing (51), regulation of sleep (52), and stimulation of visceral muscle contraction (2) (**Table 2**). Brain PrRP is also thought to

Table 2 | Comparison of representative actions of PrRP and PrRP2 between mammals, avians, and teleosts.

Action	Mammal	Avian[a]		Teleost
	PrRP	PrRP	PrRP2	PrRP2
PRL release				
In vitro	↑[b,c], →[b,c]			↑[d,e]
In vivo				
Peripheral injection	↑[b], →[f,g]		↑	↑[d,e]
Central injection	↑[b,f], →[g]	↓	↓	
GH release				
In vitro	↑[c]			↓[e], →[d]
In vivo				
Peripheral injection			↓	↓[d], →[e]
Central injection	↓[b]		↓	
Activation of HPA axis				
Peripheral injection				→[e,h]
Central injection	↑[b,f], →[g]	↑	→	
Food intake				
Peripheral injection		→	→	↓[h]
Central injection	↓[b,i], →[f]	↑	↑	

[a] *Chicken (15, 18, 57).*

[b] *Rat (1, 3, 37, 38, 52, 58, 67).*

[c] *Human being (59).*

[d] *Rainbow trout (7).*

[e] *Tilapia [PRL-releasing effect induced by peripheral injection was observed only in females and suppression of GH release in vitro was observed only in males, (8)].*

[f] *Cattle (60, 61).*

[g] *Sheep (43, 62).*

[h] *Goldfish (11, 45).*

[i] *Mouse (63).*

↑: Increase; ↓: Decrease; →: No change.

Blank columns indicate "not examined."

Actions of PrRP have not yet been investigated in teleost.

In rats, PrRP is related to the releases of oxytocin, vasopressin, FSH, and LH, and the regulation of stress response, sleeping, blood pressure, and so on (see Biological Actions of PrRP and PrRP2).

In Japanese crucian carp, PrRP alters somatolactin secretion and contracts the intestine (see Discovery of Prolactin-Releasing Peptides and Biological Actions of PrRP and PrRP2).

be related to the regulation of reproduction because brain PrRP mRNA expression increases during proestrus of the gonadal cycle and mid-period of pregnancy (53). In *Xenopus*, PrRP2 might be related to the regulation of metamorphosis because PrRP2 mRNA expression peaks at premetamorphosis (16). These functions have been well summarized in several reviews [for example, see Ref. (12, 54–56)]. Among them, the regulations of pituitary hormone release, feeding behavior, and stress have been well studied in both PrRP and PrRP2. In this section, we describe the current knowledge of the actions of PrRP and PrRP2 in PRL release, hypothalamus–pituitary axes, feeding/energy metabolism, and stress.

EFFECT ON PRL SECRETION

PrRP

Hinuma et al. (1) first found that PrRP stimulates PRL release from the rat pituitary adenoma-derived cell line and from primary cultures of anterior pituitary cells harvested from lactating female rats, the most sensitive model of PRL-releasing factor activity (64). This PRL-releasing activity appeared to be specific since PrRP did not affect the release of other anterior pituitary hormones such as luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyroid-stimulating hormone, growth hormone (GH), and adrenocorticotropic hormone (ACTH). However, further studies revealed that the effect of PrRP on PRL secretion was inconsistent. For example, PrRP stimulated PRL release only when a higher dose was applied (65, 66) or had no effect (3). *In vivo*, intravenous (IV) injection of PrRP-31 induces PRL release dependent on the estrus cycle and estrogen in rats (67, 68). On the other hand, IV injection of PrRP has no effect on PRL secretion in cattle (69), while intracerebroventricular (ICV) injection of PrRP temporarily increases PRL secretion (60). In addition to this, neither IV nor ICV injections induce PRL secretion in female sheep (43), suggesting that PrRP is not important in stimulating PRL secretion in ruminants.

As noted above, PrRP neuronal terminals are not observed in the external layer of the median eminence, suggesting that PrRP is expected to stimulate PRL release with different mechanism(s) from ordinary hypophysiotropic hormones. The small number of PrRP nerve fibers in the posterior pituitary (36) suggests that PrRP in the posterior pituitary might be transported to the anterior pituitary through a short hypophyseal portal system. PrRP-R expressed not only in the anterior pituitary but also in the rostral and medio-basal hypothalamus and in the hypothalamic PVN suggests possible indirect pathways, which modulate PRL release, but the effects of PrRP on PRL release are not consistent among studies in rats (3, 52, 70–73). In the hypothalamic explants, PrRP-31 increases the release of vasoactive intestinal peptide (VIP) and galanin, which are known to stimulate PRL release (70). ICV injection of PrRP stimulates activity of tuberoinfundibular dopaminergic neurons, which are involved in regulation of PRL secretion (39). It is, therefore, possible that PrRP modifies the activity of dopaminergic, VIP, and galanin neurons in the hypothalamus and then stimulates PRL release. In human beings, PrRP and PrRP-R are expressed in the uterine decidua and PrRP increases PRL release from the primary cultures of decidual stromal cells *in vitro* (49), suggesting that PrRP is also a local modulator of decidual PRL release.

The effect of PrRP on PRL release is controversial in mammals, and such a role has not yet been well investigated in non-mammalian vertebrates. Incubation of the tilapia pituitary with mammalian PrRP had no effect on the release of two PRL isoforms, PRL177 or PRL188, although PrRP2 stimulated the release. Interestingly, ICV injection of mammalian PrRP decreases plasma PRL level in chicks (18). Further studies using homologous systems of fish, amphibians, reptiles, and birds are needed.

PrRP2

In contrast to PrRP, PrRP2 is recognized as a strong candidate for the specific stimulator of PRL secretion in teleosts. For example, PrRP2 stimulates PRL release from primary cultures of rainbow trout pituitaries (7). In tilapia, PrRP2 also stimulates PRL release *in vitro* from the rostral pars distalis of the pituitary (8) equipotently with GnRH, another candidate PRL-releasing factor. The PRL-releasing effect of PrRP2 is also observed *in vivo*: plasma PRL concentration increases with intra-arterial (10) and intraperitoneal (IP) injection of PrRP2 (7). In tilapia, injection of PrRP2 elevates plasma PRL levels only in females but not in males (8), in accordance with the gender-biased effects in rats. Additionally, intra-arterial injection of PrRP2 increases the PRL mRNA level in the pituitary of trout *in vivo* (10, 48). Moreover, PrRP2 antiserum decreases mRNA expression of PRL in goldfish, suggesting that endogenous PrRP2 is essential for PRL expression (11).

PrRP2 is thought to be involved in adapting to new osmotic conditions in euryhaline teleosts. This idea is also supported by PRL having an important role in osmotic regulation in teleosts. PRL mRNA expression in the pituitary increases during adaptation to low osmotic conditions. Similarly, PrRP2 mRNA expression increases under freshwater and terrestrial conditions in the brain of euryhaline mudskippers and under ion-poor freshwater in the hypothalamus of goldfish (45). As brain PrRP2, pituitary and gut PrRP2 are also related to acclimation to low osmotic conditions because PrRP2 mRNA in the pituitary and gut increases when euryhaline silver sea bream and mudskipper, respectively, adapt to low salinities (14, 74, 75). These data suggest that PRL expression is regulated by not only brain PrRP but also peripheral PrRP2 in teleosts. In fact, the changes in PRL mRNA in the gut parallel those in PrRP2 mRNA in the gut of mudskipper (74). In addition, the changes in PrRP2 mRNA in the hypothalamus do not parallel that of PRL mRNA in the pituitary (14, 75). These PrRP2 mRNA expressions during osmotic acclimation might be regulated by the olfactory system because removing the olfactory rosette lowers the increase in PrRP2 mRNA expression under hypo-osmotic conditions in sea bream (75).

On the other hand, from bullfrog pituitary cells, PrRP2-20 and PrRP2-31 increased PRL release (to 130–160% of control) *in vitro*, but their effects were much less potent than thyrotropin-releasing hormone-induced PRL release (16). In chicks, similarly, IP injection of PrRP2-31 increased plasma PRL concentration, but the effect was not marked [to 140% of control, (15)]. In contrast, ICV injection of PrRP2-31 decreases plasma PRL level in chicks (15), but the mechanism of this inhibition of PRL release is unclear.

EFFECT ON HYPOTHALAMUS–PITUITARY AXES

CRH–ACTH axis

In the rat brain, PrRP is produced in some A1/A2 noradrenergic neurons in the medulla oblongata, which mediate stress signals in the central nervous system (76–80). In addition, it has been demonstrated that PrRP neurons innervate the CRH neurons by synaptic connection in rats (37). Stress information sent by A1/A2 noradrenergic neurons activates the hypothalamic CRH neurons, and then stimulates ACTH release from the anterior pituitary (81–83), suggesting that PrRP stimulates ACTH secretion via activating CRH neurons in rodents. Indeed, ICV injection of PrRP increases c-Fos expression in the CRH neurons and plasma ACTH level while systemic injection had no effect (37, 84). Additionally, PrRP-induced ACTH release is blocked by peripheral pretreatment with the CRH antagonist, alpha-helical CRH (37), demonstrating that PrRP activates the CRH neurons for the hypothalamic–pituitary–adrenal (HPA) axis in the stress response. A similar effect of PrRP was also observed in castrated bulls because ICV injection of PrRP increases cortisol secretion (60). In sheep, on the other hand, ICV injection of PrRP does not alter cortisol secretion (62), suggesting that the effect of PrRP is different between animal species. It has been reported that vasopressin rather than CRH might be a major secretagogue of ACTH in sheep, which is different from rats and pigs (85–87). Therefore, the difference in the hypothalamic regulation of the HPA axis might contribute to the unique effect of PrRP in sheep (60).

Intracerebroventricular injection of mammalian PrRP increases the plasma corticosterone level in chicks (18), suggesting that brain PrRP-induced activation of the HPA axis is conserved. On the other hand, the plasma corticosterone level is not changed by ICV injection of PrRP2-20 in chicks (18). In addition, plasma cortisol levels are not increased by IP injection of PrRP2 in tilapia and goldfish (8, 11). These results suggest that PrRP2 might not activate the HPA axis in chicks and teleosts.

Somatostatin–GH axis

In rats, somatostatin neurons in the hypothalamic PerVN are contacted by PrRP nerve terminals and express the PrRP receptor. These facts suggest that PrRP alters the activity of somatostatin neurons. Indeed, ICV injection of PrRP-31 induces expression of the immediate early gene (NGFI-A) in these somatostatin neurons in rats (38). The injection of PrRP-31 decreases the plasma GH level (38, 52), and the effect is abolished by depletion or neutralization of somatostatin in rats (38). Therefore, PrRP directly activates somatostatin neurons to stimulate somatostatin release and then inhibits GH release from the anterior pituitary in rodents.

Although the relationship between PrRP and somatostatin has not been well investigated in non-mammalian vertebrates, ICV injection of mammalian PrRP was reported to reduce the plasma GH level in chicks (18), suggesting that their relationship is conserved in other vertebrates. The decrease in GH level was also observed in chicks after PrRP2-31 was centrally injected (15). IP injection of PrRP2 also decreases the GH level in rainbow trout (7). It is, therefore, possible that PrRP2 is also related to the somatostatin–GH axis in non-mammalian vertebrates. PrRP2 does not affect GH secretion in cultured pituitaries of rainbow trout *in vitro* (7), implying that the inhibitory effect of PrRP2 may also be mediated by the release of somatostatin. Thus, it is likely that the inhibitory effect on GH release is well conserved in vertebrates and between PrRP and PrRP2.

Other

In mammals, PrRP is thought to regulate oxytocin release because PrRP neurons have synaptic contact with oxytocin neurons in the PVN (36) and PrRP receptors are present in the oxytocin neurons in rats (33). Indeed, central injection of PrRP (10 nmol/300 g rat) elevates the plasma oxytocin level in conscious rats (88). Yamashita et al. (89) also indicated that oxytocin neurons in the hypothalamus and bed nucleus of the stria terminalis show immunoreactivity of PrRP receptors, and that application of PrRP to isolated supraoptic nuclei facilitates the release of oxytocin. They also demonstrated that vasopressin neurons in the hypothalamus show immunoreactivity of PrRP receptors and that PrRP stimulates vasopressin release from the supraoptic nuclei in rats. Similarly, central injection of PrRP also increases the plasma vasopressin level in rats, although the effect is observed only in females (88). PrRP stimulates release of LH and FSH via a hypothalamic mechanism, such as VIP and galanin in rats (70). PrRP is also related to the ovarian steroid-induced LH surge because it is reduced by ICV injection of PrRP antiserum (90). PrRP gene expression is directly regulated by gonadal steroid hormones because PrRP neurons in the medulla oblongata are co-localized with receptors for estrogen or progesterone (53). In addition, administering estrogen or progesterone to ovariectomized rats induces PrRP mRNA expression in the medulla oblongata, as well as release of PrRP in the medial POA (53, 68, 91).

PrRP2 decreases plasma somatolactin levels in rainbow trout at 30 min after intra-arterial injection of PrRP2 (48). On the other hand, Moriyama et al. (7) demonstrated that PrRP2 increases the plasma somatolactin level at 9 h after IP injection, and stimulates somatolactin release only by pharmacological doses of PrRP *in vitro*. The effect of PrRP on somatolactin release might be mediated by an indirect endocrine mechanism as suggested by Moriyama et al. (7), although the distribution of PrRP neuronal terminals are also located near somatolactin cells in the trout pituitary.

REGULATION OF FOOD INTAKE AND ENERGY HOMEOSTASIS

Central injection of PrRP, especially into the DMN, reduces food intake in rats without inducing conditioned taste aversion (58, 92–94). The anorexigenic effect is also observed in mice (63). In addition, PrRP mRNA expression decreases with fasting during states of negative energy balance, like other anorexigens, in rats (58). Furthermore, immuno-neutralization of endogenous PrRP using monoclonal antibody has been demonstrated to induce hyperphagia in mice (95). Moreover, hyperphagia is also observed in PrRP-deficient mice (95) and PrRP-R-deficient mice (100). These results show that endogenous PrRP is one of the anorexigenic peptides in mammals. Takayanagi et al. (95) also reveal that PrRP regulates meal size rather than meal frequency in mice. It has been demonstrated that meal size is regulated by several satiety signals including cholecystokin (CCK). CCK is released from the intestine and then activates afferent vagal nerves that project to the medulla oblongata. CCK is also thought to activate PrRP neurons because injection of CCK induces c-Fos expression in PrRP-containing

neurons distributed in the NTS (93). In addition, the anorexigenic effect of CCK disappears in both PrRP-deficient mice (95) and PrRP-R-deficient mice (63). Thus, PrRP also appears to mediate the anorexigenic effect of CCK. Leptin is also considered an upstream regulator of the anorexigenic effect of PrRP because the leptin receptor is expressed in PrRP neurons in the brainstem and hypothalamus (94, 96). In addition, PrRP mRNA expression is low in Zucker rats, which are obese due to the lack of a leptin receptor (94). Furthermore, leptin induces the expression of the phosphorylated signal transducer and activator of transcriptional protein 3 in PrRP neurons (95). Moreover, leptin-induced anorexia is not observed in PrRP-deficient mice. Therefore, PrRP also mediates the anorexigenic signal of leptin (95).

The downstream mechanisms underlying the anorexigenic effect of PrRP remain to be clarified. Seal et al. (92) suggested that the anorexigenic effect of PrRP is also mediated by alpha-melanocyte-stimulating hormone (MSH) and neurotensin, both of which are well-known anorexigenic peptides. CRH and oxytocin are also thought to be related to the anorexigenic mechanism of PrRP. As noted above, central injection of PrRP activates CRH or oxytocin neurons in the hypothalamus, and the anorexigenic effect of PrRP is attenuated by a CRH receptor antagonist or oxytocin receptor antagonist (63, 97), showing that they mediate PrRP-induced anorexia. Furthermore, an oxytocin receptor antagonist attenuates the anorexigenic effect of CCK (98, 99). Re-feeding and CCK-induced Fos expression in several brain regions including the PVN was impaired in PrRP-deficient mice. CCK-induced oxytocin release was also impaired in PrRP-deficient mice. Collectively, it is expected that the CCK-PrRP-oxytocin system is an important anorexigenic pathway of PrRP.

Endogenous PrRP is also related to energy metabolism in mammals because PrRP-deficient or PrRP-R-deficient mice show late-onset obesity (95, 100–102). Furthermore, the Otsuka Long-Evans Tokushima Fatty (OLETF) rat strain, which exhibits obesity and diabetes, has a mutated GPR10 gene (103). Streptozotocin-induced diabetic rats also show lower mRNA expression of PrRP and it is reversed by insulin (96). In rats, ICV injection of PrRP increases body temperature and oxygen consumption, also suggesting that brain PrRP is related to the control of energy metabolism (58, 97). In addition to obesity, PrRP-deficient mice show increased food intake, body fat mass, glucose tolerance, and increased levels of blood insulin, leptin, cholesterol, and triacylglycerol (95, 100, 102). Similar changes were also observed in PrRP-R-deficient mice (100, 101). However, PrRP-deficient mice do not show any change in body temperature, oxygen consumption, or locomotion activity (95). PrRP-R-deficient male mice also show no difference in oxygen consumption while consumption is slightly low in PrRP-R-deficient females (101). The PrRP-R-deficient mice become obese but the extent is pronounced in females (101). Thus, it is likely that the role of PrRP in energy metabolism is different between the sexes. The difference is explained by estrogen because PrRP-containing neurons in the brainstem express the estrogen receptor (53). Obesity observed in PrRP-deficient mice is thought to be related to hyperphagia because pair-feeding abolishes the induction of obesity in PrRP-deficient mice (95).

PrRP2 is also recognized as an anorexigenic peptide in teleosts because ICV injection of PrRP2 significantly inhibits feeding behavior in goldfish (45). In mudskipper, a euryhaline fish, brain PrRP2 mRNA expression is induced when they are kept in fresh water (48) where their food intake decreases compared with rearing in sea water (our unpublished observation). Indeed, several euryhaline fish grow slower in fresh water than in seawater (104), suggesting the involvement of brain PrRP2.

In contrast to rodents and teleosts, PrRP shows a unique effect on feeding behavior in steers and chicks. In steers, ICV injection of PrRP has no effect on food intake (61). Furthermore, ICV injection of mammalian PrRP increases food intake in chicks (57). The orexigenic effect is also observed when chicken PrRP is ICV injected (our unpublished data). Similarly, ICV injection of PrRP2-31 also stimulates feeding behavior in chicks, although PrRP2-20 has no effect (15). Thus, the effects of both PrRP and PrRP2 on feeding behavior are completely opposite to those in rodents and teleosts, and seem to have changed during the process of evolution. The reason why PrRP and PrRP2 stimulate feeding behavior in chicks has not yet been well clarified. Interestingly, ICV injection of leptin (105) has no effect on feeding behavior in chicks (105), although CCK, CRH, MSH, and mesotocin (avian homolog of oxytocin) have an anorexigenic effect in chicks (106–109) as well as rodents. The difference in the feeding inhibitory mechanism may contribute to the reason why PrRP does not suppress feeding behavior in chicks. It is possible that NPY, an orexigenic factor, is related to the orexigenic effect of PrRP because ICV injection of PrRP2-31 significantly increases the NPY mRNA level in the diencephalon in chicks (110). As well as in feeding regulation, PrRP2 might also be related to energy metabolism in non-mammalian vertebrates because ICV injection of PrRP2-31 decreases blood insulin, glucose, and non-esterified fatty acid in chicks (110).

STRESS RESPONSE

As noted in Section "CRH–ACTH Axis," PrRP neurons project directly to CRH neurons and oxytocin neurons in the hypothalamus (33), and ICV injection of PrRP increases ACTH (84) and oxytocin release in rats (88). These facts suggest that PrRP is a mediator of the HPA axis for stress response. In fact, water immersion-restraint stress dramatically induces c-Fos expression in the A1/A2 neurons containing PrRP (84). Other stress stimuli, such as restraint, conditioned fear, foot shocks, hemorrhage, exercise, and inflammatory stress, also activate PrRP neurons in the medulla oblongata and/or DMN (111–114). Additionally, Seal et al. (92) found that ICV injection of PrRP-31 increases grooming associated with stress response. Immuno-neutralization of PrRP decreases the activation of the neurons in the hypothalamic PVN after noxious stimuli (113) or oxytocin release in response to conditioned fear (111). In addition, contextual conditioned fear-induced oxytocin and ACTH releases are impaired in PrRP-deficient mice (115). It is, therefore, likely that brain PrRP has a facilitative role in stress responses.

In contrast, the injection of PrRP antibodies facilitates ACTH release in response to exercise, suggesting that PrRP inhibits ACTH release in response to exercise (116). Restraint stress-induced corticosterone release is enhanced in PrRP-deficient mice (102). These data suggest that PrRP also inhibits neuroendocrine responses to stress. Additionally, contextual conditioned fear-induced

neuroendocrine responses disappear in PrRP-deficient mice while the freezing behavior is augmented (115). The mechanisms underlying these contradictions have not been clarified to date. Takayanagi and Onaka (55) suggest that the role of PrRP in stress responses may depend on the nature of the stressful stimuli used. Thus, PrRP is involved in integration in the control of stress responses, whereas the underlying detailed mechanisms need further investigation.

Intracerebroventricular injection of mammalian PrRP significantly increases the plasma corticosterone level in chicks, implying that brain PrRP is also related to stress response (18). On the other hand, ICV injection of chicken PrRP2-20 has no effect on the plasma corticosterone level, suggesting that PrRP2 might not be involved in stress (18). It is also reported that IP injection of PrRP2 does not alter the plasma cortisol level in tilapia (8). It is, therefore, possible that PrRP2 is not related to activating the HPA axis and stress in non-mammalian vertebrates.

CONCLUDING REMARKS

Prolactin-releasing peptide was originally identified as a stimulator of PRL release in mammals. Although further work revealed that PrRP had less effect on PRL release, PrRP has been shown to be involved in many physiological actions, such as reproduction, endocrine functions, feeding behavior and metabolism, and stress response in mammals, and possibly in other vertebrates (**Table 2**). This PrRP may be the less appropriate nomenclature, at least for mammals. At the same time, as the discovery of PrRP, PrRP2, whose amino acid sequence is similar to PrRP, was identified from Japanese crucian carp. PrRP and PrRP2 are thought to have originated from a common ancestral gene by the second round of whole genome duplication. In contrast to PrRP, PrRP2 is thought to exist only in non-mammalian vertebrates, and to be essential for PRL secretion in teleosts and important for non-mammalian vertebrates. The physiological actions of PrRP and PrRP2 seem to overlap in non-mammalian vertebrates (**Table 2**), and may have converged into those of PrRP in mammals. Consequently, the PrRP2 gene might have diminished in mammals during evolution.

The origin of PrRP and PRL might provide a key to knowing how PrRP acquired its diverse functions in vertebrates. Moriyama et al. (17) found two RF-amide peptides (RFa-A and RFa-B), homologous to PrRP, from sea lamprey. The amino acid sequence is similar to PrRP2 rather than PrRP (**Figures 1** and **2**), especially in RFa-B. If lamprey RFa-A and RFa-B are, respectively, orthologous to PrRP and PrRP2, these peptides originated at least from the stage of primitive vertebrates. Interestingly, lamprey is thought not to possess PRL (117), suggesting that PrRP is generated prior to the occurrence of PRL. Especially in teleosts, PRL is widely expressed in extrapituitary organs, and PrRP2 and its receptor system might concomitantly obtain to regulate these PRL expressions. Conversely, the lack of this extrapituitary PRL might be associated with the fact that the PrRP2-PrRP2 receptor system was not necessary for regulating PRL and disappeared in mammals.

On the other hand, the effect of PrRP on GH secretion is commonly observed among vertebrates. Moriyama et al. (17) found that both RFa-A and RFa-B decrease GH mRNA expression *in vitro* in lamprey. As noted above, PrRP or PrRP2 inhibits GH release from the pituitary in mammals, chicks, and teleosts, suggesting that the roles of PrRP and PrRP2, at least the suppression of GH release, occur with the appearance of primitive vertebrates. Since PRL is a member of the GH family and originated from a common ancestor, the diversity of the physiological roles of PRL and PrRP are thought to have developed with the evolution of GH rather than PRL.

The conserved effect of PrRP on feeding behavior may also be generated in ancestral PrRP. Thus, the original role of PrRP and PrRP2 would be the regulation of GH release and feeding rather than PRL release. However, the physiological roles of PrRP on the releases of PRL and GH, feeding behavior, and stress response in non-mammalian vertebrates have not been clarified (**Table 2**) because their PrRPs were recently predicted. Information regarding the effect of PrRP2 on the endocrine system and stress response, which have been investigated in rodents, is also insufficient. To acquire a clearer understanding of the biological and physiological roles of PrRP and PrRP2, future investigations should focus on the physiological roles of PrRP and PrRP2 in non-mammalian vertebrates. Such studies should reveal the evolution of the PrRP family in vertebrates.

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Unexpected multiplicity of QRFP receptors in early vertebrate evolution

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The neuropeptide QRFP, also called 26RFa, and its G protein-coupled receptor GPR103 have been identified in all vertebrates investigated. In mammals, this peptide-receptor pair has been found to have several effects including stimulation of appetite. Recently, we reported that a QRFP peptide is present in amphioxus, *Branchiostoma floridae*, and we also identified a QRFP receptor (QRFPR) that mediates a functional response to sub-nanomolar concentrations of the amphioxus peptide as well as short and long human QRFP (Xu et al., submitted). Because the ancestral vertebrate underwent two tetraploidizations, it might be expected that duplicates of the QRFP gene and its receptor gene may exist. Indeed, we report here the identification of multiple vertebrate QRFPR genes. Three QRFPR genes are present in the coelacanth *Latimeria chalumnae*, representing an early diverging sarcopterygian lineage. Three QRFPR genes are present in the basal actinopterygian fish, the spotted gar. Phylogenetic and chromosomal analyses show that only two of these receptor genes are orthologous between the two species, thus demonstrating a total of four distinct vertebrate genes. Three of the QRFPR genes resulted from the early vertebrate tetraploidizations and were copied along with syntenic neuropeptide Y receptor genes. The fourth QRFPR gene may be an even older and distinct lineage. Because mammals and birds have only a single QRFPR gene, this means that three genes have been lost in these lineages, and at least one of these was lost independently in mammals and birds because it is still present in a turtle. In conclusion, these results show that the QRFP system gained considerable complexity in the early stages of vertebrate evolution and still maintains much of this in some lineages, and that it has been secondarily reduced in mammals.

Keywords: QRFP, QRFP receptor, RFamide peptide, 26RFamide, G protein-coupled receptor, coelacanth, Latimeria chalumnae, spotted gar

INTRODUCTION

Many of the biologically active peptides that end with the sequence RFamide, i.e., arginine-phenylalanine-amide, bind to receptors that are more closely related to each other than to other G protein-coupled receptors. These peptides include NPFF, GnIH, PRLH, and QRFP/26RFa, henceforth called QRFP (Fukusumi et al., 2006; Osugi et al., 2006, 2011; Elphick and Mirabeau, 2014). Furthermore, peptides ending with RYamide, namely the neuropeptide Y (NPY) family including peptide YY (PYY) and pancreatic polypeptide (PP), bind to a large family of receptors closely related to those responding to the peptides listed above (Sundstrom et al., 2008). Thus, many RFamide/RYamide peptides have receptors that form an evolutionary clade. On the other hand, some peptides with carboxyterminal RFamides bind to more distantly receptors, indicating that some peptides have found their receptor partners in independent evolutionary events, such as kisspeptin.

The NPY receptor family is probably the largest peptide GPCR family in vertebrate genomes with seven ancestral members (Larsson et al., 2008, 2009; Larhammar and Bergqvist, 2013) and with five members present in many presently living mammals. This family consisted of three ancestral linked genes before the origin of vertebrates, then this triplet was quadrupled (Larhammar and Salaneck, 2004) in the two ancestral vertebrate tetraploidizations called 1R and 2R (Nakatani et al., 2007; Putnam et al., 2008). After 2R, gene losses reduced the number to a total of 7 NPY-family receptors in the ancestor of gnathosomes, i.e., the jawed vertebrates (Larsson et al., 2009; Larhammar and Bergqvist, 2013). The RFamide peptides, in contrast, have quite modest receptor repertoires in mammals with just a single receptor each for the four peptides NPFF, GnIH (called RFRP-3 in mammals), PRLH and QRFP, albeit NPFF and GnIH can activate also one another's receptors (Liu et al., 2001; Mollereau et al., 2002). This might be taken as evidence that these four receptors arose as a result of the two tetraploidizations. Recently, however, the PRLH receptor family alone was found to consist of as many as four members in a broader vertebrate perspective, probably as a result of the two basal vertebrate tetraploidizations (Kuraku and Kuratani, 2011). Furthermore, we have recently identified and characterized a QRFP receptor and its peptide ligand from amphioxus (Xu et al., submitted), implying that all four RFamide peptides were already established before the

vertebrate tetraploidizations. The origin of the four RFamide peptides and their receptors before the emergence of vertebrates is also supported by global analyses of genomes for the presence of peptide and receptor genes (Jekely, 2013; Mirabeau and Joly, 2013; Elphick and Mirabeau, 2014).

Our searches in the ENSEMBL genome databases retrieved multiple QRFPR-like sequences from many vertebrate species and our phylogenetic analyses showed that these sequences represent clades with deep roots in the vertebrate tree. We therefore proceeded to complement the sequence-based phylogenetic analyses with information about the chromosomal locations of the QRFPR genes in the genomes with most reliable assemblies in order to obtain clues to the mechanisms of gene duplication. This would allow more precise determination of the time points for the gene duplications. Our studies build upon our previous extensive analyses of the NPY receptor family genes, some of which are located on the same chromosome as the QRFPR gene in the human genome. In addition, we have incorporated information from the recently completed genome of a basal ray-finned fish, the spotted gar, *Lepisosteus oculatus*, which in many instances has been found to have retained a large proportion of the ancestrally duplicated vertebrate genes, and to have undergone comparatively few chromosomal rearrangements (Amores et al., 2011). Furthermore, this species diverged before the teleost ancestor underwent its third tetraploidization, 3R (Jaillon et al., 2004), making it simpler to analyze than many teleost fishes. Another useful species for this type of comparison thanks to its slow evolution is the African coelacanth, *Latimeria chalumnae*, which has many examples of retained ancestral genes, albeit its genome assembly does not have contigs that are sufficiently large to allow comparisons of conserved synteny except in a few cases (Amemiya et al., 2013).

In contrast to a recent study that proposed three ancestral vertebrate QRFPR subtypes (Ukena et al., 2014), our analyses identified four ancestral vertebrate QRFP receptor genes. We present here the evidence for this conclusion and propose a likely scenario for the gene duplications in early vertebrate evolution in relation to the vertebrate tetraploidizations. These findings imply that mammals have lost much of the QRFP system, possibly explaining why the single QRFP peptide found in mammalian genomes seems to have diverged from other vertebrates which still retain more extensive QRFP receptor repertoires.

MATERIALS AND METHODS

The Ensemble sequence database was searched with Blast/Blat using as query sequence the human QRFP receptor. As new receptor sequences were found and confirmed to be QRFPR-related, they were used as queries in additional searches. A list of the genes whose predicted receptor sequences were analyzed phylogenetically is shown in **Supplementary Table 1** which contains their accession numbers. Amino acid alignments were made in Jalview 2.8 version 14.0 (Waterhouse et al., 2009) using the MUSCLE web tool with standard settings. During the course of the work, phylogenetic Neighbor-Joining (NJ) (Saitou and Nei, 1987) trees were made by using Clustal X version 2.0.12 (Larkin et al., 2007), with standard settings and 1000 bootstrap replicates. The tree shown in **Figure 1** was made with the Maximum Likelihood (ML) method using the PhyML3.0 algorithm (Guindon et al., 2010) with the following settings: amino acid frequencies (equilibrium frequencies), proportion of invariable sites (with optimized p-invar) and gamma shape parameters were estimated from the alignments, the number of substitution rate categories was set to 8, BIONJ was chosen to create the starting tree, both NNI and SPR tree optimization methods were considered and both tree topology and branch length optimization were chosen.

RESULTS

PHYLOGENETIC ANALYSES OF QRFPR SEQUENCES

Extensive searches in vertebrate genomes identified three QRFPR genes in three species, namely in spotted gar, coelacanth, and zebrafish. Several other genomes were found to contain two genes, these include the western clawed frog *Xenopus tropicalis*, the Chinese soft-shelled turtle, the Japanese eel, and the cave fish. Two genes were also found in the anole lizard and the American alligator, but in these species one of the genes was incomplete and could not be included in the phylogenetic analysis. Finally, it has been known since before that mouse as well as rat has two receptor genes as a result of a recent gene duplication (Takayasu et al., 2006), probably in the rodent lineage.

As the QRFP gene has five introns in the vertebrate genes, several annotations contained obviously erroneous predictions of splice junctions. Also, the beginning and end of some genes were missing. After manual curation of the sequences to comply with consensus rules, the sequences were aligned with MUSCLE in Jalview (see Materials and Methods) and analyzed phylogenetically, first with the NJ method and finally with the maximum likelihood (ML) method. The tree thus obtained (**Figure 1**) shows clearly that the sequences cluster in four major clades, each one supported by high significance. The sequence used as out-group to root the tree was the amphioxus QRFP receptor (Xu et al., submitted) which in our previous calculations, using more distantly related sequences as out-group, would always branch off basally to all of the vertebrate QRFPR sequences.

No single species contains all four genes. Nevertheless, by combining information from the 20 species included in the tree, the taxonomic distribution implies that four genes existed in early vertebrate evolution. The largest clade is the one containing the human QRFP receptor, henceforce called QRFPR1 to distinguish it from the other subtypes. Orthologs of this gene exist in the mammals included in the analysis, as well as in chicken and in representatives from three other major amniotes lineages, i.e., an alligator, a lizard, and a turtle (**Figure 1**). This subtype is also found in the frog and the coelacanth.

The smallest clade is the one given the subtype name QRFPR2 which is present in gar, coelacanth, frog, and turtle. The two former species also possess QRFPR4 which is also found in three of the teleost fish species. Also the single receptor sequence of the sea lamprey, *Petromyzon marinus*, belongs to this clade with high statistical support. The final clade, QRFPR3, is present in all ray-finned fish species investigated, both the spotted gar and all of the teleosts. Finally, it should be added that both the alligator and the lizard have a second receptor gene, which however could not be assigned to a specific subtype due to incomplete information in the sequence databases. Two of the zebrafish sequences cluster

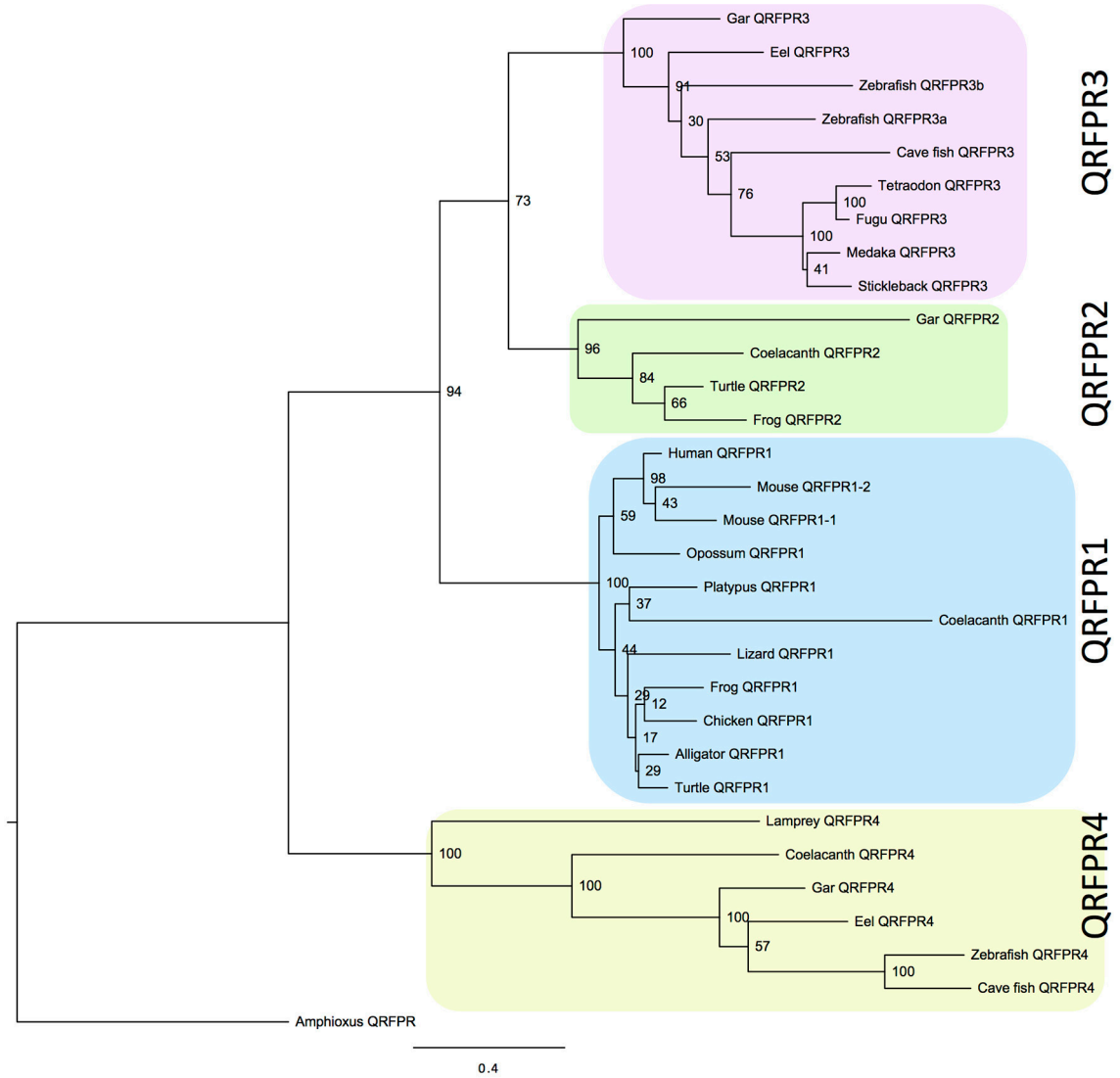

FIGURE 1 | Phylogenetic tree calculated with the maximum likelihood (ML) method showing the relationships of the QRFPR receptors for human (*Homo sapiens*), mouse (*Mus musculus*), gray short-tailed opossum (*Monodelphis domestica*), platypus (*Ornithorhynchus anatinus*), chicken (*Gallus gallus*), American alligator (*Alligator mississippiensis*), anole lizard (*Anolis carolinensis*), Chinese soft-shelled turtle (*Pelodiscus sinensis*), western clawed frog (*Xenopus tropicalis*), African coelacanth (*Latimeria chalumnae*), spotted gar (*Lepisosteus oculatus*), Japanese eel (*Anguilla japonica*), zebrafish (*Danio rerio*), cave fish (*Astyanax mexicanus*), medaka (*Oryzias latipes*), three-spined stickleback (*Gasterosteus aculeatus*), fugu (*Takifugu rubripes*), green spotted pufferfish (*Tetraodon nigroviridis*), sea lamprey (*Petromyzon marinus*). The amphioxus QRFPR (*Branchiostoma floridae*) was used as out-group to root the tree (Xu et al., submitted). The tree was calculated using PhyML3.0 (Guindon et al., 2010) and the complete sequences aligned with MUSCLE. For alignment see **Supplementary Figure 1**.

close to each other in the QRFPR3 clade, supporting duplication in conjunction with the teleost 3R event.

SYNTENY AND PARALOGON ANALYSES OF QRFPR SEQUENCES

The presence of four QRFPR clades naturally suggests origin by duplications resulting from the two basal tetraploidizations, 1R and 2R, thus forming a paralogon (a set of chromosome regions containing members of the same gene families as a result of duplication of a large block or an entire chromosome). However, due to the many losses of QRFPR genes in the different species or lineages, sequence-based analyses may be skewed due to uneven selection pressures. Therefore, some additional type of information should be considered. The human QRFPR1 gene is located on chromosome 4, the same chromosome that contains no less than three NPY-family receptors, namely subtypes 1, 2, and 5 (**Figure 2**). This synteny suggests that QRFPR1 arose as a duplicate of the ancestral NPYR (or the other way around). As the NPYR triplet was clearly quadrupled in the 1R-2R events (Larhammar and Salaneck, 2004; Larhammar and Bergqvist, 2013), this suggests that QRFPR1 could have been duplicated simultaneously. Therefore, we have analyzed the chromosomal locations of the other QRFPR genes, especially in the species

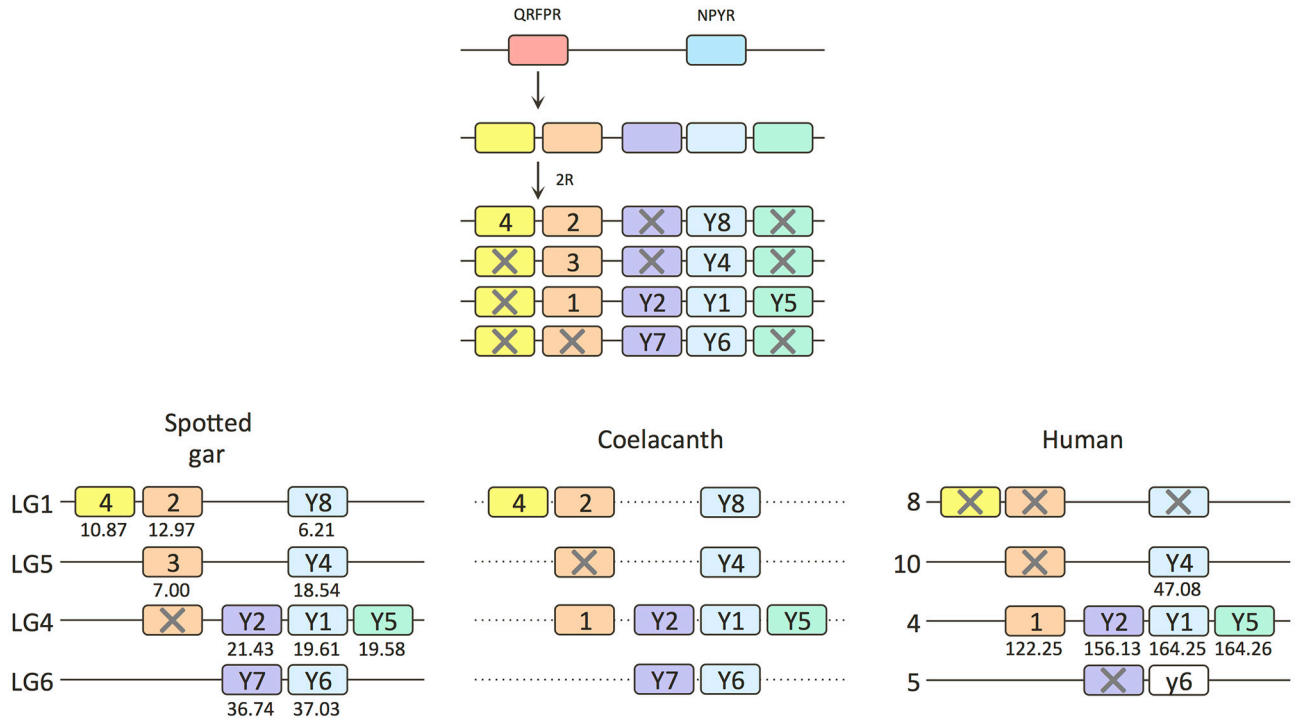

FIGURE 2 | Gene duplication events for the QRFP receptor family in early vertebrate evolution including local duplications and the two basal vertebrate tetraploidizations. NPYR stands for neuropeptide Y receptors. Crosses mark gene losses. The human Y6 gene is a pseudogene. The dashed lines for the coelacanth indicate that it is not known if the genes are syntenic. Numbers under gene boxes show map positions along the chromosomes in megabase pairs.

which is known to have a genome with very few chromosomal rearrangements, the spotted gar (Amores et al., 2011).

Two of the receptors in the spotted gar, QRFPR2 and 4, are located on linkage group 1 (LG1) which also contains NPY8R (**Figure 2**). All three of these genes are missing in the human genome, but NPY8R is known to be in a chromosome region in teleost fishes (Larsson et al., 2008) that displays conserved synteny with human chromosome 8 (abbreviated Hsa8 for *Homo sapiens* chromosome 8). The coelacanth has all three of these genes, NPY8R, and QRFPR2 and 4, but they are located on separate scaffolds.

The gar QRFPR3 gene is located on LG5 together with NPY4R which in human is on Hsa10, also a member of this paralogon (**Figure 2**). The QRFPR1 subtype found in mammals is unfortunately missing in the gar, but as this gene is located on Hsa4 together with the NPY receptor cluster, which is present in gar on LG4, the missing QRFPR1 gene probably once resided there too.

The chromosome harboring NPY receptors Y6 and Y7 in gar (**Figure 2**) as well as chicken (Larsson et al., 2009) corresponds to Hsa5 where the NPY6R pseudogene is located. NPY7R was lost in the mammalian ancestor. No QRFPR subtype has been identified in any species that might correspond to a gene located on this chromosome.

The two zebrafish QRFPR genes belonging to the QRFPR3 clade are located on zebrafish chromosomes 12 and 13 (**Supplementary Table 1**). These are known to be 3R duplicates of the chromosome corresponding to Hsa10 containing NPY4R (Larsson et al., 2008) and to gar LG5, hence the zebrafish genes have been named QRFPR3a and b.

DISCUSSION

The high statistical support for the four clades in **Figure 1**, and the species distribution of the receptors, argues strongly for an ancestral quartet of QRFP receptors although no single extant species or lineages is in possession of all four subtypes. The two crucial vertebrate lineages are represented by the spotted gar, an early branching ray-finned fish, and the African coelacanth, or latimeria, an early branch in the clade of lobe-finned fishes (Sarcopterygii) including also lungfishes and tetrapods. Both the gar and the coelacanth have been reported to evolve more slow than several other lineages, the gar by having fewer chromosomal rearrangements and the coelacanth by having fewer amino acid changes in many proteins. Therefore, it is of great importance that we found three QRFPR genes in both of these species, two of which are orthologs, namely QRFPR2 and QRFPR4. The remaining subtypes are found in each of these two species, QRFPR1 in coelacanth and QRFPR3 in gar. Taken together, this information points to four distinct receptor subtypes before the divergence of ray-finned and lobe-finned fishes.

As the QRFPR1 gene in human (and other mammals) and the QRFPR2 and QRFPR3 genes in gar are located in chromosome regions that belong to the well-characterized paralogon encoding the seven NPY receptor subtypes, which is known to have undergone chromosome duplications in 2R (Larhammar and Salaneck,

2004; Larsson et al., 2008, 2009; Larhammar and Bergqvist, 2013), this argues strongly for origin of these three QRFPR subtypes as a result of chromosome duplications in 1R and 2R, and by extension the origin of zebrafish QRFPR3a and 3b in the teleost 3R tetraploidization.

The origin of the QRFPR4 gene is more difficult to deduce. It has broad taxonomic distribution, being present in both coelacanth, gar, and teleost fishes, as well as the sea lamprey. In the phylogenetic tree it branches off with high statistical support prior to the radiation of the other three subtypes. As the QRFPR4 gene is located in the same linkage group as QRFPR2 in the gar, this indicates origin of the two by duplication from a common ancestor. If so, the phylogenetic tree implies that this duplication took place prior to the chromosome duplications in 1R and 2R. This would mean that any 1R/2R duplicates of QRFPR4 have been lost from the chromosomes corresponding to gar LG4, 5, and 6.

Another possibility is that QRFPR4 is actually the fourth and missing member of the QRFP1,2,3 quartet resulting from 2R, but has been translocated from the chromosome corresponding to LG6 (which presently lacks a QRFP receptor) onto LG1 near the QRFPR2 gene. Such translocations by unequal crossing over were probably more likely to happen soon after 2R while the quadrupled chromosomes were still highly identical to each other. This scenario would require that QRFPR4 has had a higher evolutionary rate, to explain its basal position in the three. Unfortunately the coelacanth scaffolds are not large enough to say whether QRFPR2 and 4 belong on the same chromosome. More detailed analyses of the synteny groups of all of the QRFP receptor genes may resolve these alternative scenarios. Unfortunately our searches in the genome of a cartilaginous fish, the elephant shark *Callorhinchus milii*, has so far yielded no certain QRFP receptor sequences, nor a QRFP peptide precursor gene. More extensive studies are required before conclusions can be drawn about possible loss of the entire QRFP system in chimeras or the entire class of Chondrichthyes.

Analyses of teleost fish chromosomes is more complicated due to the high frequency of rearrangements, probably as a result of the teleost 3R tetraploidization. However, it is obvious that very few 3R duplicates of the QRFPR genes have survived. In fact, the zebrafish QRFPR3a and 3b are the only ones in the entire QRFPR tree. Also some other GPCR families show this remarkable degree of gene loss after 3R, for instance the NPY receptor family where only a singly 3R duplicate has survived, namely for NPY8R (Larsson et al., 2008). For the distantly related families of kisspeptin receptors and visual opsins, not a single one of the four and five ancestral genes, respectively, have survived 3R (Pasquier et al., 2012; Lagman et al., 2013). In contrast, some GPCR families gained multiple members in 3R: in the somatostatin receptor 2-3-5 family, all three members were duplicated (Ocampo Daza et al., 2012), for the endothelin receptors two out of three members have 3R duplicates (Braasch and Schartl, 2014), and for the opioid receptor family two of the four ancestral receptors have 3R duplicates (Dreborg et al., 2008).

Although the coelacanth has been found to have a slow pace of evolution (Amemiya et al., 2013), some genes seem to have evolved more rapidly in this lineage. Interestingly, this seems to concern QRFPR1 as indicated by its long branch, as seems to be the case also for gar QRFPR2 (**Figure 1**). More detailed scrutiny of the receptor sequences may give clues as to what aspects of receptor function might be affected by the increased evolutionary rate.

Despite the presence of four QRFPR subtypes in the ancestral vertebrate after 2R, there are no reports about additional copies of the QRFP peptide gene. This implies that this peptide alone served as ligand on the four ancestral receptors. On the other hand, amphioxus seems to have three QRFP-related peptides (Mirabeau and Joly, 2013) and a single receptor (Xu et al., submitted). However, it remains to be investigated if all three peptides act on this receptor.

In conclusion, the QRFP receptor family shows considerable complexity with four members in the ancestral vertebrate and three members still existing in spotted gar and coelacanth. This invites to further studies of this peptide-receptor system in these species, as well as many other species with more than just the single receptor found in mammals.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fnins.2014.00337/abstract

Supplementary Figure 1 | Alignment of the QRFP receptor sequences used for calculating the phylogenetic tree in Figure 1.

Supplementary Table 1 | Ensemble gene ID or NCBI accession numbers for vertebrate QRFP receptors. As indicated in the right hand column, the annotation of many of the entries had to be curated by manual editing.

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The evolution and variety of RFamide-type neuropeptides: insights from deuterostomian invertebrates

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Five families of neuropeptides that have a C-terminal RFamide motif have been identified in vertebrates: (1) gonadotropin-inhibitory hormone (GnIH), (2) neuropeptide FF (NPFF), (3) pyroglutamylated RFamide peptide (QRFP), (4) prolactin-releasing peptide (PrRP), and (5) Kisspeptin. Experimental demonstration of neuropeptide–receptor pairings combined with comprehensive analysis of genomic and/or transcriptomic sequence data indicate that, with the exception of the deuterostomian PrRP system, the evolutionary origins of these neuropeptides can be traced back to the common ancestor of bilaterians. Here, we review the occurrence of homologs of vertebrate RFamide-type neuropeptides and their receptors in deuterostomian invertebrates – urochordates, cephalochordates, hemichordates, and echinoderms. Extending analysis of the occurrence of the RFamide motif in other bilaterian neuropeptide families reveals RFamide-type peptides that have acquired modified C-terminal characteristics in the vertebrate lineage (e.g., NPY/NPF), neuropeptide families where the RFamide motif is unique to protostomian members (e.g., CCK/sulfakinins), and RFamide-type peptides that have been lost in the vertebrate lineage (e.g., luqins). Furthermore, the RFamide motif is also a feature of neuropeptide families with a more restricted phylogenetic distribution (e.g., the prototypical FMRFamide-related neuropeptides in protostomes). Thus, the RFamide motif is both an ancient and a convergent feature of neuropeptides, with conservation, acquisition, or loss of this motif occurring in different branches of the animal kingdom.

Keywords: RFamide, neuropeptide, receptor, evolution, deuterostome, echinoderm, hemichordate

INTRODUCTION

Neuropeptides are evolutionarily ancient mediators of neuronal signaling in nervous systems that have fundamental roles in regulation of physiological processes and animal behavior. They are remarkably diverse, ranging from 3 to >40 residues, but all are derived from larger precursor proteins. Some neuropeptide precursors are simple, giving rise to a single neuropeptide, while others yield multiple related or unrelated neuropeptides (1–3).

A major challenge in neurobiology is to understand the evolutionary and functional significance of neuropeptide diversity in animals. Determining evolutionary relationships between neuropeptides in different phyla has proven to be difficult because they comprise relatively short stretches of amino acids, often with only a few conserved residues. However, recent advances in comparative genomics/transcriptomics are transforming our understanding of neuropeptide signaling systems. Thus, a picture is emerging of a core set of neuropeptide–receptor signaling pathways that can be traced back to the common ancestor of the Bilateria, with neuropeptide orthologs being identified in an increasingly wide range of animal phyla (4–8).

In this review, our attention is focused on neuropeptides that are unified by a common structural characteristic – the presence of a C-terminal Arg–Phe–NH_2 (RFamide) motif. In vertebrates, five types of RFamides have been identified, which are derived from different precursor proteins: (1) Gonadotropin-inhibitory hormone (GnIH)-type, (2) Neuropeptide FF (NPFF)-type, (3) Pyroglutamated RFamide peptide (QRFP)-type, (4) Prolactin-releasing peptide (PrRP)-type, and (5) Kisspeptin-type (9, 10). Here, we briefly review how these diverse RFamides were discovered and then we proceed to our main objective – to explore the phylogenetic distribution and evolutionary origins of these neuropeptides. Our primary focus will be on deuterostomian invertebrates (urochordates, cephalochordates, hemichordates, and echinoderms) because these animals share a more recent common ancestor with vertebrates than the protostomian invertebrates (e.g., arthropods, nematodes, annelids, and mollusks) (11). However, in reviewing research on RFamides, the story begins (and ends) with what at first sight might be considered an inauspicious protostomian invertebrate – the sunray venus clam *Macrocallista nimbosa*, a bivalve mollusk.

FMRFamide – THE PROTOTYPICAL RFamide-TYPE NEUROPEPTIDE

The tetrapeptide Phe–Met–Arg–Phe–NH_2 (FMRFamide) was purified from extracts of ganglia from the clam *Macrocallista nimbosa* on account of its cardioexcitatory activity. A chromatographically distinct component of molluscan ganglia that stimulates beating of quiescent molluscan hearts, known as peak C, was first reported in 1967 (12). Ten years later Price and Greenberg identified peak C as FMRFamide, reporting their finding in the

journal *Science* (13). FMRFamide might have remained an obscurity of molluscan pharmacology were it not for the discovery that FMRFamide-like peptides occur throughout the animal kingdom. For example, using antibodies to FMRFamide, cross-reacting peptides were detected immunocytochemically in the nervous systems of an insect, a fish, and a mammal (14). And so began an era in which neuropeptides that share a C-terminal RFamide motif with FMRFamide were identified in a wide range of taxa, including cnidarians, nematodes, insects, and vertebrates (15, 16). A comprehensive survey of the diverse set of RFamide-type neuropeptides that have been identified in the animal kingdom is beyond the scope of this review, where the focus is on RFamides that were first discovered in vertebrates. However, we will take on a broader phylogenetic perspective in the last section of this review, highlighting, for example, RFamide-type neuropeptides that have ancient bilaterian origins but which have been lost in the vertebrate lineage.

FROM LPLRFamide TO GnIH: THE BIRD BRAIN'S CONTRIBUTION TO RFamide DISCOVERY IN VERTEBRATES

The first RFamide-type neuropeptide to be identified in a vertebrate was purified from chicken brain extracts on account of its cross-reactivity with antibodies to FMRFamide. Reporting in the journal *Nature* in 1983, the purified peptide was identified as the pentapeptide Leu–Pro–Leu–Arg–Phe–NH_2 (LPLRFamide) (17). Seventeen years later, using antibodies to RFamide, an RFamide-type neuropeptide was purified from quail brain extracts and identified as the dodecapeptide SIKPSAYLPLRFamide (18). Therefore, it is likely that the LPLRFamide peptide originally isolated from chicken brain was a fragment of a homolog of this dodecapeptide. Investigation of the physiological roles of SIKPSAYLPLRFamide has revealed that it inhibits pituitary release of gonadotropic hormones – hence this peptide was named GnIH. The GnIH precursor contains two related peptides known as GnIH-RP1 and GnIH-RP2, which have the C-terminal motif LPxRFamide (where x is L or Q) (19). Importantly, GnIH-like neuropeptides that suppress reproductive activity have also been identified in mammals (20, 21). Furthermore, the orphan receptor GPR147 has been identified as the G-protein coupled receptor that mediates effects of GnIH-type neuropeptides. GPR147 is also now known as NPFFR1 or NPFF1 (22–24).

Analysis of the phylogenetic distribution of GnIH-type neuropeptides has revealed that they occur throughout the vertebrates, from primitive agnathan vertebrates through bony fish and amphibians to reptiles, birds, and mammals (25, 26). A comprehensive survey of the properties and functions of GnIH-type neuropeptides in each of the vertebrate classes is beyond the scope of this review and has been discussed elsewhere. Therefore, we will focus here on studies in the most primitive extant vertebrates, the agnathans (lamprey, hagfish). A cDNA encoding a precursor of two GnIH-type peptides has been identified in the sea lamprey *Petromyzon marinus*, with expression revealed in the hypothalamus and gonads (26). Interestingly, injection of the GnIH-type peptides stimulates expression of GnRH and gonadotropins in lamprey, which contrasts with the inhibitory effects of GnIH in birds and mammals. Thus, the physiological role of GnIH as a regulator of reproductive processes can be traced back to the common ancestor of vertebrates, but inhibitory or stimulatory effects are observed in different vertebrate lineages (26).

F8Fa (NPFF) AND A18Fa (NPAF): BOVINE FMRFamide-LIKE IMMUNOREACTIVE PEPTIDES AND PROTOTYPES FOR THE NEUROPEPTIDE FF FAMILY

The first RFamides to be identified in mammals were purified from extracts of bovine brain, employing antibodies to FMRFamide in a radioimmunoassay. Two neuropeptides were identified: FLFQPQRFamide (F8Fa or NPFF) and AGEGLSSPFWSLAAPQRFamide (A18Fa or NPAF), which have a common C-terminal motif – PQRFamide (27). Subsequently, it has been found that these peptides are derived from the same precursor protein (28, 29) and exert effects by binding to the G-protein coupled receptor GPR74, which is also referred to as NPFFR2 or NPFF2 (22, 24, 30).

Early on it was found that NPFF and NPAF attenuate morphine-induced antinociception and cause hyperalgesia (27); consistent with these effects, NPFF2 receptors are expressed in the dorsal horn of the spinal cord (31). However, evidence of roles in regulation of other physiological processes has been obtained subsequently; for example, increasing blood pressure and slowing heart rate (32–34).

Analysis of the phylogenetic distribution of NPFF/NPAF-type neuropeptides has revealed that they occur throughout the vertebrates, from primitive agnathan vertebrates through bony fish and amphibians to reptiles, birds, and mammals (35). For example, in the agnathan *P. marinus* (sea lamprey) a cDNA encoding a precursor protein that gives rise to three neuropeptides with a C-terminal PQRFamide motif has been identified (36). The PQRFamide-type precursor is expressed in several regions of the lamprey brain, including the hypothalamus, mesencephalon, and medulla oblongata (36). Interestingly, *in vivo* pharmacological tests have revealed that one of the PQRFamide-type peptides triggers increased expression of GnRH-II in the lamprey (37). In hagfish, there are two PQRFamide-type precursors and *in vitro* tests with one of the derived PQRFamide-type peptides revealed stimulation of gonadotropin expression in the hagfish pituitary (35).

RFamides AS LIGANDS FOR ORPHAN G-PROTEIN COUPLED RECEPTORS: DISCOVERY OF QRFP, PrRP, AND KISSPEPTIN

By the late 1990s, a growing number of orphan G-protein coupled receptors had been cloned and sequenced on account of their similarity with known receptors. And so began an era in which the search for endogenous ligands for these receptors became a priority and between 1998 and 2003, three novel types of RFamide neuropeptides were discovered in this period of receptor de-orphanization or "reverse pharmacology" (38, 39).

QRFP

In 2003, the endogenous ligand for the orphan receptor GPR103 was identified as a 26-residue peptide with a C-terminal RFamide motif, which is known pyroglutamylated arginine–phenylalanine-amide peptide (QRFP) or 26RFa (40–42). Subsequent studies have revealed that QRFP is expressed in regions of the hypothalamus involved in control of feeding behavior and accordingly pharmacological studies have revealed that this peptide increases intake of a high fat diet (43–46).

Analysis of the phylogenetic distribution of QRFP-type neuropeptides has revealed that they occur throughout the vertebrates, from bony fish and amphibians to reptiles, birds, and mammals (47, 48). The occurrence of QRFP-type neuropeptides in agnathans has, as yet, not been reported; however, notwithstanding secondary loss, it is expected because the phylogenetic distribution of QRFP-type neuropeptides extends to invertebrates [see **Figure 2B**; (8)] and because a QRFP-type receptor is present in lamprey (48). Investigation of the physiological roles of the QRFP-type neuropeptide in a teleost species, the goldfish *Carassius auratus*, revealed that injection of synthetic QRFP caused an increase in serum gonadotropins, but only at the highest dose of QRFP tested (1 μg/g body weight). Interestingly, upregulation of hypothalamic QRFP precursor mRNA expression was observed following 4 days of food deprivation in goldfish, indicating that QRFP-type neuropeptides may have an evolutionarily ancient role in regulation of food intake in vertebrates (47). Further studies are now needed to investigate more widely the physiological roles of QRFP-type neuropeptides in non-mammalian vertebrates.

PrRP

In 1998, endogenous ligands for the orphan receptor GPR10 were isolated from hypothalamic extracts and identified as a 31-residue peptide with a C-terminal RFamide motif and a N-terminally truncated 20-residue isoform of the 31-residue peptide. Investigation of the physiological roles of these peptides revealed that they stimulate release of prolactin from anterior pituitary cells and hence, they were named PrRPs (49). However, the physiological relevance of this *in vitro* effect of PrRPs has been questioned and alternative roles in regulation of feeding and stress hormone release have been proposed (50, 51).

Analysis of the phylogenetic distribution of PrRP-type neuropeptides has revealed that they occur throughout the vertebrates from primitive agnathans (52) to bony fish and tetrapods [(53); **Figure 2C**]. Interestingly, phylogenomic analysis has led to the proposal that PrRP and its cognate receptor originated from the neuropeptide Y (NPY) peptide-receptor system following the genome duplications that occurred during the early evolution of vertebrates (53). Thus, the evolution and physiological roles of PrRP-type peptides needs to be considered in the context of a bilaterian family of neuropeptides that includes NPY in vertebrates (C-terminal RYamide motif) and invertebrate neuropeptide F (NPF)-type peptides, which have a C-terminal RFamide motif [(54); see also below for further discussion of this topic].

Comparative analysis of the physiological roles of PrRP-type peptides has revealed that PrRP-type peptides stimulate prolactin release in bony fish (55), indicating that this is an ancient role in vertebrates. Furthermore, evidence of roles for PrRP-type peptides in regulation of feeding behavior and growth hormone release in non-mammalian vertebrates has been obtained (52, 56).

KISSPEPTIN

In 2001, the endogenous ligands for the orphan receptor GPR54 were identified as a 54-residue peptide with a C-terminal RFamide motif and N-terminally truncated isoforms of the 54-residue peptide comprising 13 or 14 residues. It was discovered that these peptides are derived from metastasis-suppressor protein KiSS-1 and hence they were named kisspeptins (57). Subsequently, evidence that kisspeptins are important physiological regulators of reproductive development was obtained. Thus, loss-of-function mutations in the kisspeptin receptor (GPR54) cause a failure to progress through puberty due to hypogonadotropic hypogonadism (58).

Analysis of the phylogenetic distribution of kisspeptins has revealed homologs throughout the vertebrates, from agnathans to mammals (59). Furthermore, in some vertebrates up to three genes encoding precursors of kisspeptins have been found; for example, in the coelacanth, a sarcopterygian fish, and the elephant shark, a chondrichthyan fish. Accordingly, multiple copies of candidate kisspeptin receptors are found in some vertebrates; for example, in the coelacanth and the spotted gar (an actinopterygian fish), there are four genes encoding kisspeptin-type receptors and phylogenomic analysis indicates this reflects the two rounds of whole genome duplication that are thought to have occurred in a basal ancestral vertebrate (60). However, in the majority of vertebrates there has been loss of one or more kisspeptin precursor and kisspeptin receptor paralogs. For example, in the zebrafish *Danio rerio* there are two kisspeptin precursors (61), while the most extreme loss is seen in some bird species where no genes encoding a kisspeptin precursor or kisspeptin receptor have been found (62). Investigation of the physiological roles of kisspeptins in non-mammalian vertebrates indicates that the kisspeptin signaling system has an ancient role in regulation of reproductive development (63).

THE PHYLOGENETIC DISTRIBUTION AND EVOLUTIONARY ORIGINS OF VERTEBRATE RFamide-TYPE NEUROPEPTIDES: INSIGHTS FROM DEUTEROSTOMIAN INVERTEBRATES

Orthologs of receptors for vertebrate RFamide-type neuropeptides occur in deuterostomian and protostomian invertebrates (8), as illustrated in **Figure 1**. Thus, based on this phylogenetic distribution, the evolutionary ancestry of NPFF/GnIH-type, QRFP-type, and kisspeptin-type receptors can be traced back to the common ancestor of the bilaterians. The PrRP receptor is an exception in as much as orthologs of this receptor are only found in deuterostomes.

The discovery of invertebrate orthologs of vertebrate receptors for RFamide-type neuropeptides is fascinating because it provides a basis for discovery of ligands for these receptors and investigation of their physiological roles in invertebrates. An example of where this has been accomplished is the discovery that SIFamide-type neuropeptides are ligands for protostomian orthologs of NPFF/GnIH-type receptors (64). SIFamides share limited sequence similarity with vertebrate NPFF/GnIH-type neuropeptides (**Figure 2A**); however, analysis of the physiological roles of SIFamide in *Drosophila* indicates that it acts to suppress reproductive behavior (65). This is intriguing because it is consistent with the role of GnIH as an inhibitory regulator of reproductive processes in vertebrates (20). Thus, it appears that the evolutionary origin of GnIH/SIFamide-type neuropeptides as reproductive inhibitors may trace back to the common ancestor of the Bilateria (66).

Little is known about the molecular identity of peptide ligands for invertebrate orthologs of other vertebrate RFamide-type receptors. Therefore, here we have addressed this issue, focusing on

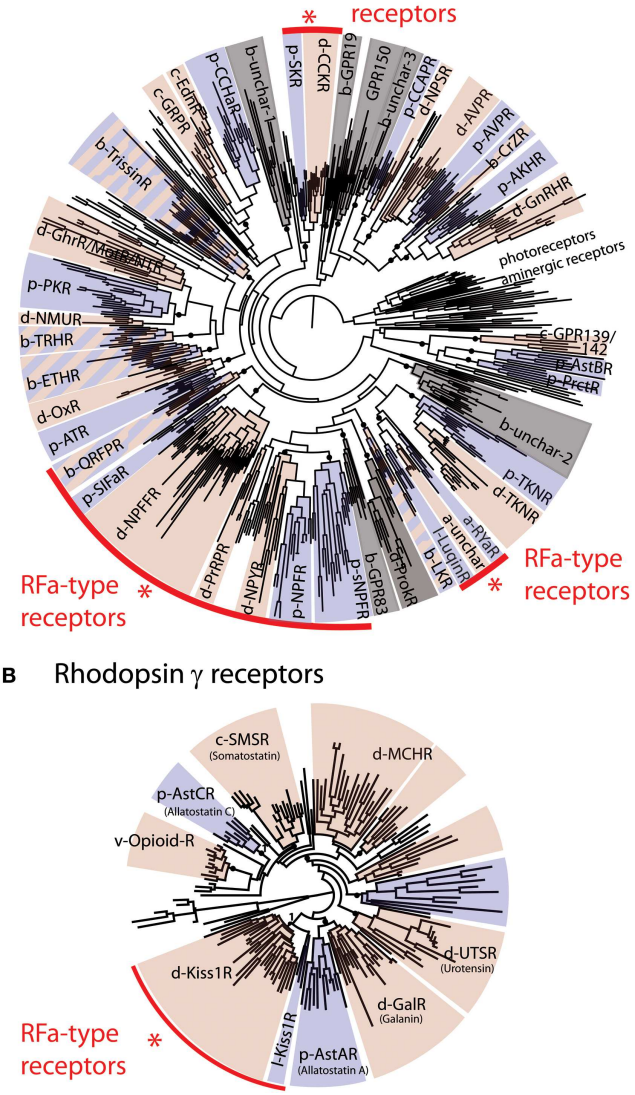

FIGURE 1 | Phylogenetic analysis of bilaterian rhodopsin β-type (A) and rhodopsin γ-type (B) receptors. The red arcs highlight four groups that include receptors that are known to be activated by RFamide-type peptides. These include a large group of receptors for QRFP (pyroglutamylated RFamide peptide), SIFa (SIFamide), NPFF (Neuropeptide FF and Gonadotropin-inhibitory hormone), PrRP (Prolactin-releasing peptide), NPY/NPF (Neuropeptide Y/F), sNPF (short Neuropeptide F), and Luqin, and two isolated groups of RFamide-type receptors, CCK/SK (Cholecystokinin/Sulfakinin), and Kiss1 (Kisspeptin) receptors. In **(A)** rhodopsin α-type receptors (photoreceptors and aminergic receptors) are included as an outgroup. The prefixes b-, d-, p- designate subgroups of, respectively, bilaterian, deuterostomian, and protostomian receptors. Deuterostomian and protostomian clades have been colored in pink and blue, respectively. Gray sections of the trees correspond to groups of receptors for which either only deuterostomian or protostomian ligands are known. The fact that most of the rhodopsin β-type RFamide receptors fall in the same region of the tree suggests that these probably originated from a common ancestral RFamide-type neuropeptide signaling system. However, the occurrence of other groups of receptors that are activated by RFamides (CCK, Kiss1) indicates that RFamide-type neuropeptides have evolved independently at least three times in bilaterian history. Figure adapted from Ref. (8).

deuterostomian invertebrates. These include two sub-phyla of the phylum Chordata – the urochordates [e.g., the sea squirt *Ciona intestinalis*; (67)] and the cephalochordates [e.g., *Branchiostoma floridae*; (68)]. Other deuterostomian phyla are the hemichordates [e.g., acorn worm *Saccoglossus kowalevskii*; (69)] and the echinoderms [e.g., the sea urchin *Strongylocentrotus purpuratus* (70)], which are sister phyla in a clade of deuterostomian invertebrates known as the ambulacraria (11).

GnIH/NPFF-TYPE NEUROPEPTIDES IN DEUTEROSTOMIAN INVERTEBRATES

Orthologs of GnIH/NPFF-type receptors are not present in the sea squirt *C. intestinalis* and therefore, it appears that this neuropeptidergic system has been lost in the urochordate lineage. In the cephalochordate *B. floridae*, there is an expanded family of GnIH/NPFF-type receptors (8) and accordingly at least two precursors of putative peptide ligands for these receptors have been identified, with the predicted neuropeptide products sharing a common C-terminal PxRFamide motif (where *x* is variable) with vertebrate GnIH/NPFF-type neuropeptides [(8); **Figure 2A**].

GnIH/NPFF-type neuropeptides in vertebrates can be divided into two functional groups – peptides that are PQRFamides, which modulate nociception (28) and LRFamides, which inhibit the release of gonadotropins (19). Peptides derived from both of the GnIH/NPFF-type precursors in *B. floridae* are LRFamides, suggesting that the ancestral GnIH/NPFFamide-type neuropeptides in chordates were LRFamides that may have had a role in regulation of reproduction. Furthermore, the LxFamide motif is conserved in lophotrochozoan (e.g., *Capitella* and *Lottia*) SIFamide-type peptides, suggesting that a leucine at position-2 relative to a C-terminal amidated phenylalanine and a role in reproduction are ancient characteristics of this family of orthologous peptides.

As highlighted above, SIFamide-type neuropeptides have been identified as ligands for GnIH/NPFF-type receptors in protostomian invertebrates. However, SIFamides share very limited sequence similarity with chordate GnIH/NPFF-type peptides; in fact the only universally shared feature is a C-terminal Phe–NH_2 motif (**Figure 2A**), although this does extend to Leu–x–Phe–NH_2 in some cases. Thus, when comparing chordate GnIH/NPFF-type peptides and protostomian SIFamides, sequence divergence has rendered the orthologous relationship between these peptides almost completely unrecognizable. In this context, it would be interesting to identify putative ligands for GnIH/NPFF/SIFamide-type receptors in non-chordate deuterostomes.

Recently, it was proposed that SALMFamide-type neuropeptides may be echinoderm homologs of GnIH/NPFF/SIFamide-type neuropeptides (66). The rationale for this hypothesis was that L-type SALMFamides share sequence similarity with some SIFamides – in particular, the C-terminal SxLxFamide motif. However, we have not obtained support for this hypothesis from analysis of the occurrence of GnIH/NPFF/SIFamide-type receptors in echinoderms. Thus, orthologs of GnIH/NPFF/SIFamide-type receptors do not appear to be present in the sea urchin *S. purpuratus* (8). Therefore, the sequence similarity shared by SALMFamides and some SIFamides may reflect convergence and it appears, based on the data currently available, that GnIH/NPFF/SIFamide-type neuropeptide signaling may have been lost in the echinoderm lineage.

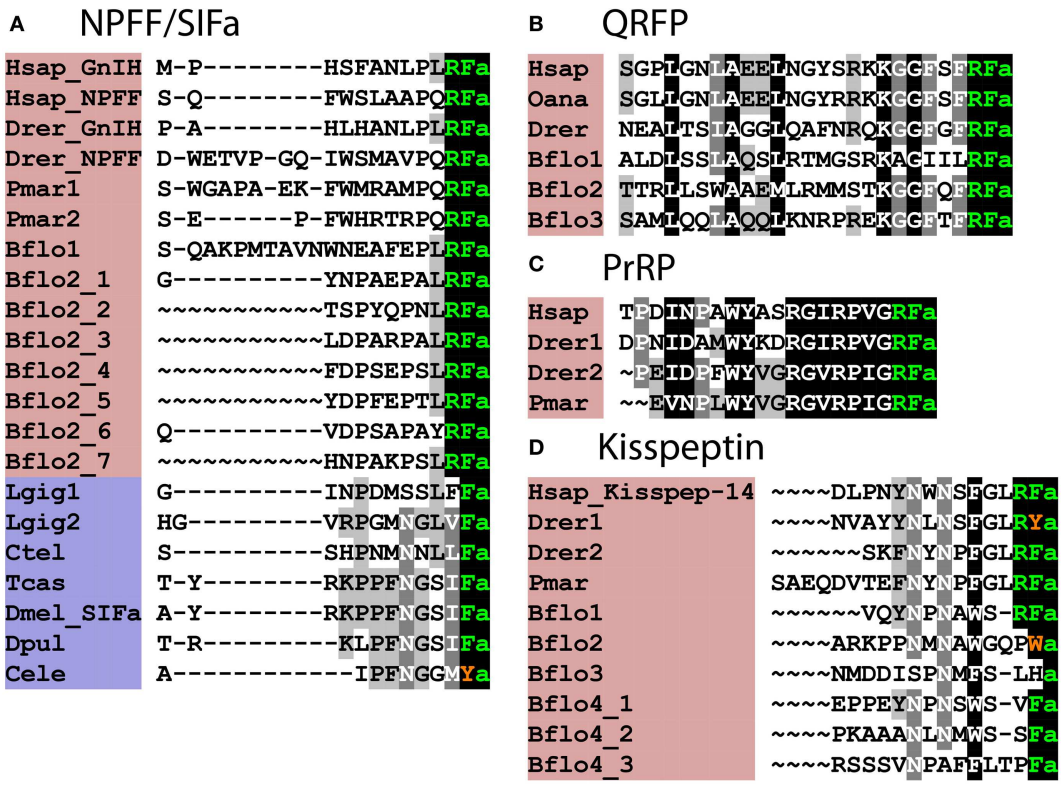

FIGURE 2 | Alignment of known orthologous peptides from (A) bilaterian NPFF/SIFa (B), chordate QRFP, (C) vertebrate PrRP, and (D) chordate kisspeptin families. The names of deuterostomian and protostomian peptides are shaded in pink and blue, respectively. The C-terminal amide groups are represented by an "a" at the end of aligned sequences. The lamprey sequences are from Ref. (26), other vertebrate and *B. floridae* sequences are from Ref. (8), the *Lottia gigantea* sequences are from Ref. (4), and the *Capitella teleta* sequence is from Ref. (5). For vertebrate sequences, only one peptide from each precursor is shown in the alignment. Genbank (GI) or JGI IDs of all precursor sequences are listed below: **(A)** alignment of chordate NPFF/SIFa peptides: Hsap_GnIH, *Homo sapiens* GnIH (GI:11125707); Hsap_NPFF, *Homo sapiens* NPFF precursor (GI:219878493); Drer_GnIH, *Danio rerio* GnIH precursor (GI:283825363), *Danio rerio* NPFF precursor (GI:116078056); Pmar1, *Petromyzon marinus* NPFF/GnIH precursor 1 (GI:88595366); Pmar2, *Petromyzon marinus* NPFF/GnIH precursor 2 (GI:374849285); Bflo1, *Branchiostoma floridae* NPFF/GnIH precursor 1 (GI:260808912); Bflo2_1–7, *Branchiostoma floridae* NPFF/GnIH precursor 2 (GI:260829186); Lgig1, *Lottia gigantea* SIFamide precursor 1 (JGI: 176362); *Lottia gigantea* SIFamide precursor 2 (JGI: 175046); Ctel, *Capitella teleta* SIFamide precursor (GI:161198869); Tcas, *Tribolium castaneum* SIFamide precursor (GI:189239683); Dmel_SIFa, *Drosophila melanogaster* SIFamide precursor (GI:386768581); Dpul, *Daphnia pulex* SIFamide precursor (JGI: 260818); Cele, *Caenorhabditis elegans* SIFamide precursor (GI:392894563). **(B)** Alignment of chordate QRFP peptides: Hsap, *Homo sapiens* pyroglutamylated RFamide peptide precursor (GI:38016139); Oana *Ornithorhynchus anatinus* QRFP precursor (GI:620943939); Drer, *Danio rerio* QRFP precursor (GI:528509692); Bflo1–3, *Branchiostoma floridae* QRFP precursors (GI:260828082, GI:260828080, and JGI:107075). **(C)** Alignment of vertebrate PrRP peptides: Hsap, *Homo sapiens* prolactin-releasing peptide/hormone (GI:7705679); Drer1-2, *Danio rerio* prolactin-releasing peptide precursors (GI:350539516, GI:528519441); Pmar, *Petromyzon marinus* prolactin-releasing peptide precursor (Ensembl scaffold: GL490889). **(D)** Alignment of Kisspeptins: Hsap_Kisspep-14, *Homo sapiens* KiSS-1 metastasis-suppressor precursor (GI:116829963); Drer1-2, *Danio rerio* KiSS-1 precursors (GI:157061759, GI:217272819); Pmar, *Petromyzon marinus* KiSS-1 precursors (GENSCAN00000116455); Bflo1-4, *Branchiostoma floridae* KiSS-1 precursors (GI:260826607, GI:260793233, GI:260826605, and GI:260827077).

The hemichordates are a sister phylum to the echinoderms and a GnIH/NPFF-type receptor has been identified in the acorn worm *S. kowalevskii* (8). Therefore, the presence of a gene encoding a GnIH/NPFF-type neuropeptide precursor is anticipated, but it remains to be discovered. If the endogenous ligand for the GnIH/NPFF/SIFamide-type receptor in *S. kowalevskii* is identified, it would be fascinating to investigate the physiological roles of this peptide. Does it, for example, act as an inhibitor of reproductive processes in *S. kowalevskii*? – a role that would be consistent with the physiological roles of GnIH and SIFamide in vertebrates and *Drosophila*, respectively.

QRFP-TYPE NEUROPEPTIDES IN DEUTEROSTOMIAN INVERTEBRATES

Orthologs of vertebrate QRFP-type receptors are present in the cephalochordate *B. floridae*, the hemichordate *S. kowalevskii*, the echinoderm *S. purpuratus*, and in lophotrochozoan protostomes but not in urochordates or ecdysozoan protostomes (8). Thus, the evolutionary origin of the QRFP-type peptide signaling system can be traced back to the common ancestor of the Bilateria but with subsequent loss in ecdysozoan protostomes and urochordates.

Three precursors of candidate ligands for QRFP-type receptor(s) have been identified in *B. floridae*. Like most QRFP-type

neuropeptides in vertebrates, all three peptides are predicted to comprise 25 residues with a C-terminal RFamide motif. Other features in common with vertebrate QRFP-type neuropeptides include a conserved leucine residue at position four, a conserved alanine residue at position eight, a conserved lysine residue at position 18, and a conserved glycine residue at position 20 [(8); **Figure 2B**].

QRFP-type receptors are present in echinoderms (*S. purpuratus*) and hemichordates (*S. kowalevskii*) (8); Table S1 in Supplementary Material) but precursors of QRFP-type peptides have as yet not been identified in these phyla. It has been noted though that F-type SALMFamide neuropeptides in echinoderms are similar to vertebrate QRFP-type peptides in having a C-terminal FxFamide motif (where x is variable) (71). However, the organization of F-type SALMFamide precursors is different from that of QRFP-type precursors because they comprise multiple copies of shorter peptides, ranging in length from seven to twenty residues (72). This contrasts with QRFP-type precursors that have been identified in chordates, which comprise a single copy of a 25-residue peptide (**Figure 2B**). More extensive analysis of echinoderm and hemichordate genome/transcriptome sequence data is now required to investigate the existence of precursors of QRFP-type peptides in these non-chordate deuterostomian invertebrates. Likewise, QRFP-type receptors are present in lophotrochozoan protostomes (8) but candidate peptide ligands for these receptors have yet to be identified. If this can be accomplished, then comparative functional analysis of QRFP-type peptides in a variety of invertebrates would provide fascinating insights into the origins and evolution of the physiological roles of this ancient bilaterian neuropeptidergic signaling system.

PrRP-TYPE NEUROPEPTIDES IN DEUTEROSTOMIAN INVERTEBRATES

Orthologs of vertebrate PrRP-type receptors are present in the cephalochordate *B. floridae* and the hemichordate *S. kowalevskii* but not in urochordates, echinoderms, or protostomes (8). We have identified one and three PrRP-type receptors in the hemichordate *S. kowalevskii* and the cephalochordate *B. floridae*, respectively, but analysis of genome/transcriptome data for these species has thus far not yielded candidate precursors of peptide ligands for these receptors [(8); Table S1 in Supplementary Material]. Nevertheless, the existence of PrRP-type receptors in *B. floridae* and *S. kowalevskii* indicates that the duplication of a NPY-type receptor gene and a NPY-type precursor gene that is proposed to have given rise to the PrRP peptide-receptor system occurred earlier in animal evolution than has been proposed previously (53). Our data indicate that these gene duplications occurred in a common ancestor of deuterostomes, with subsequent loss in urochordates and echinoderms.

KISSPEPTIN-TYPE NEUROPEPTIDES IN DEUTEROSTOMIAN INVERTEBRATES

Orthologs of vertebrate kisspeptin-type receptors are present in the cephalochordate *B. floridae*, the hemichordate *S. kowalevskii*, the echinoderm *S. purpuratus*, and in lophotrochozoan protostomes but not in urochordates or ecdysozoan protostomes [(8); Table S1 in Supplementary Material]. Based on these findings, the evolutionary origin of kisspeptin-type peptide signaling can be traced back to a common ancestor of the Bilateria but with subsequent loss in urochordates and ecdysozoan protostomes.

In *B. floridae*, an expanded family of 16 kisspeptin-type receptors and 4 precursors of kisspeptin-like peptides have been identified [(8); **Figure 2D**; Table S1 in Supplementary Material]. One of the precursors (Bflo1; GI:260826607) contains a peptide with a putative C-terminal RFamide motif, in common with vertebrate kisspeptins (**Figure 2D**). Another kisspeptin-type precursor (Bflo4; GI:260827077) gives rise to peptides with putative C-terminal VFamide, SFamide, and PFamide motifs and two paralogous precursors (Bflo2; GI:260793233 and Bflo3; GI:260826605) yield peptides with a C-terminal Wamide and Hamide, respectively. However, features that are conserved between all chordate kisspeptins are two conserved asparagine residues located seven or eight, and five or six residues from the C-terminal amidated residue (**Figure 2D**) and a conserved phenylalanine or tryptophan located three or four residues from the C-terminal amidated residue (**Figure 2D**).

Hitherto, kisspeptin-type peptides have only been identified in chordates (8). However, the occurrence of kisspeptin-type receptors in *S. kowalevskii* and *S. purpuratus* (8) indicates that kisspeptin-type peptides may exist in hemichordates and echinoderms. Likewise, the presence of kisspeptin-type receptors in lophotrochozoan invertebrates indicates that kisspeptin-type peptides may exist in these animals, but they remain to be discovered. If kisspeptin-type peptides can be identified in ambulacrarians (hemichordates and echinoderms) and lophotrochozoans (e.g., mollusks and annelids) then investigation of their functions in these animals may provide insights into the ancestral physiological roles of kisspeptins in the common ancestor of bilaterians.

OTHER RFamide-TYPE NEUROPEPTIDES IN BILATERIANS

The primary focus of this review has been on neuropeptide families where vertebrate representatives have a C-terminal RFamide motif. However, there are also other bilaterian neuropeptide families that include RFamide-type peptides but where vertebrate representatives have either lost this feature or have been lost altogether. Furthermore, some RFamide-type neuropeptides appear to have evolved only within the protostomian lineage. Below, we briefly discuss some examples and in so doing illustrate that in characterizing neuropeptides as "RFamides," the representatives of different neuropeptides families that are assembled may vary depending on the phylogenetic perspective taken.

NPY/NPF-TYPE NEUROPEPTIDES

NPY-type neuropeptides include the mammalian peptides neuropeptide Y (NPY), pancreatic polypeptide (PP), and peptide YY (PYY), which have a C-terminal RYamide motif (73). However, some representatives of this family in non-mammalian vertebrates have a C-terminal RFamide motif – e.g., alligator PP (74). An NPY-type neuropeptide in the cephalochordate *B. floridae* also has a C-terminal RYamide motif but the NPY-type neuropeptide in the hemichordate *S. kowalevskii* has a C-terminal RFamide motif (8). Protostomian orthologs of the NPY family are characterized by a C-terminal RFamide motif and hence are named NPF (54) (**Figure 3A**). Thus, it appears that in the NPY/NPF-type

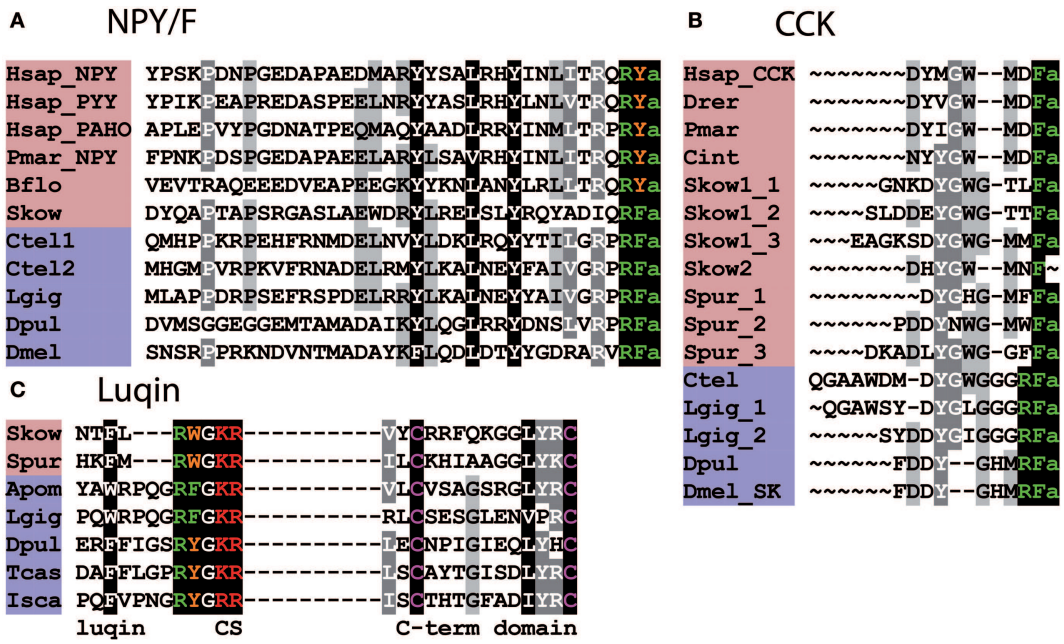

FIGURE 3 | Alignment of known orthologous peptides from (A) bilaterian NPY/NPF, (B) bilaterian Cholecystokinin/Gastrin (CCK), and (C) bilaterian Luqin/RYamide families. The names of deuterostomian and protostomian peptides are shaded in pink and blue, respectively. The C-terminal amide groups are represented by an "a" at the end of aligned sequences. Skow Luqin is from Ref. (7), Spur Luqin is from Ref. (75), and Tcas Luqin/RYamide is from Ref. (76). The *Lottia gigantea* sequences are from Ref. (4), the *Capitella teleta* sequences are from Ref. (5), and other sequences are from Ref. (8). For vertebrate sequences, only one peptide from each precursor is shown in the alignment. Genbank (GI) or JGI IDs of all precursor sequences are listed below: **(A)** Alignment of bilaterian NPY/F-type peptides: Hsap_NPY, Hsap_PYY, and Hsap_PAHO; *Homo sapiens* Neuropeptide Y, Peptide Tyrosine tyrosine, and Pancreatic polypeptide precursors (GI:189273, GI:300068955, and GI:35589); Pmar_NPY, *Petromyzon marinus* Neuropeptide Y precursor (GI:57231270); Bflo, *Branchiostoma floridae* NPY-like precursor (GI:260829184); Skow, *Saccoglossus kowalevskii* NPY-like precursor (GI:585716458); Ctel1-2, *Capitella teleta* NPF precursors (GI:161315271, JGI:204022); Lgig, *Lottia gigantea* NPF precursor (GI:163562021); Dpul, *Daphnia pulex* NPF precursor (GI:168841503); Dmel, *Drosophila melanogaster* NPF precursor (GI:442619467). **(B)** Alignment of bilaterian Cholecystokinin/Sulfakinin-type peptides: Hsap, *Homo sapiens* CCK precursor (GI:84040232); Drer, *Danio rerio* CCK-like precursor (GI:42490839); Pmar, *Petromyzon marinus* CCK-like precursor (GI:299891581); Cint, *Ciona intestinalis* CCK-like (Cionin) precursor (GI:296983); Skow1-2, *Saccoglossus kowalevskii* CCK-like precursors (GI:585688033, GI:187061456); Spur, *Strongylocentrotus purpuratus* CCK-like precursor (GI:390355380); Ctel, *Capitella teleta* Sulfakinin (SK)-type precursor (GI:161296032); Lgig, *Lottia gigantean* SK-type precursor (GI:163526260); Dpul, *Daphnia pulex* SK-type precursor (JGI:242979); Dmel, *Drosophila melanogaster* SK-type precursor (GI:386765036). **(C)** Alignment of bilaterian Luqin-type peptides: in addition to the luqin sequences, which are followed by a dibasic cleavage site (CS), the sequences of a conserved cysteine-containing C-terminal domain of the precursor proteins are also shown. The residues connecting the two domains are not represented. Skow, *Saccoglossus kowalevskii* Luqin-like precursor (GI:187205184); Spur, *Strongylocentrotus purpuratus* Luqin-type precursor (GI:390331827); Apom, *Alvinella pompejana* Luqin-type precursor (GI:223786475), Lgig, *Lottia gigantea*, Luqin-type precursor (GI:163510328); Dpul, *Daphnia pulex*, RYamide-type precursor (JGI:251691); Tcas, *Tribolium castaneum*, RYamide-type precursor (GI:347807413); Isca, *Ixodes scapularis* RYamide-type precursor (GI:156462907).

neuropeptide family the C-terminal RYamide motif is a derived characteristic of the chordates and the ancestral motif in the common ancestor of bilaterians was probably RFamide. Also of relevance here are the PrRP-type neuropeptides in mammals and other vertebrates, one of five families of RFamide-type peptides discussed above, which are thought to have arisen by duplication of a gene encoding an NPY/NPF-type precursor in a common ancestor of the deuterostomes (see above). Thus, when taking a bilaterian phylogenetic perspective, the NPY/NPF-type family of neuropeptides can be considered as members of the heterogeneous assemblage of neuropeptides that are categorized as RFamides.

CCK/GASTRIN-TYPE NEUROPEPTIDES

Cholecystokinin (CCK) and gastrin are gut hormones/neuropeptides in mammals and other vertebrates that share a common C-terminal motif – GWMDFamide (77, 78) (**Figure 3B**). Furthermore, a CCK/gastrin-type neuropeptide identified in the urochordate *C. intestinalis* also has this motif (79). However, the first invertebrate representatives of the CCK/gastrin family to be identified were the sulfakinins (SKs), which have been isolated from several insect species, and these neuropeptides have a C-terminal RFamide motif (80, 81). In the nematode *C. elegans*, CCK/gastrin-type peptides have a C-terminal QFamide motif (82); however, this is probably a derived feature because CCK/gastrin-type peptides in lophotrochozoan protostomes (e.g., annelids, mollusks) are like the insect SKs in having a C-terminal RFamide motif (4, 5, 8) (**Figure 3B**). Thus, from a protostomian perspective CCK/gastrin-type neuropeptides are RFamides.

CCK/gastrin-type neuropeptide precursors have recently been identified in non-chordate deuterostomian invertebrates, providing important new insights on the evolution of this neuropeptide

family. In the hemichordate *S. kowalevskii*, there is a CCK/gastrin-type precursor, which gives rise to three putative peptides that have the C-terminal motifs GTLFamide, GTTFamide and GMMFamide [(8); **Figure 3B**], while in the echinoderm *S. purpuratus*, there is a CCK/gastrin-type precursor that gives rise to three putative peptides that have the C-terminal motifs GMFFamide, GMWFamide and GGFFamide [(8); **Figure 3B**]. Thus, these ambulacrarian CCK/gastrin-type peptides do not have a C-terminal RFamide motif and therefore, it appears that only the C-terminal Famide motif is a common feature that is found in both protostomian and deuterostomian CCK/gastrin-type peptides (**Figures 3B** and **4**).

LUQINS

Luqin is an RFamide-type neuropeptide (APSWRPQGRFamide) that was originally isolated from the mollusk *Aplysia californica* and named luqin because it is expressed in the dorsal left upper quadrant (LUQ) cells of the abdominal ganglion in this species (83). Subsequently, luqin-type neuropeptides with a C-terminal RFamide motif have been identified in other molluscan species and in annelids (4, 5), while members of the luqin family in arthropods and nematodes are characterized by a C-terminal RYamide motif and hence are referred to as RYamides (8, 76) (**Figure 3C**). Luqin-type neuropeptides have also been identified in hemichordates and echinoderms and these peptides have a C-terminal RWamide motif (7, 75) (**Figure 3C**). However, luqin-type neuropeptide signaling has been lost in the chordate lineage (8).

Thus, in conclusion, the luqins are a bilaterian neuropeptide family but only from a lophotrochozoan perspective are they RFamide-type neuropeptides.

FMRFamide-RELATED NEUROPEPTIDES IN PROTOSTOMES

Last but not least, we return to where the RFamide story began – with the tetrapeptide FMRFamide that was isolated from molluscan ganglia in 1977 (13). FMRFamide-related peptides that share with FMRFamide a C-terminal FxRFamide motif have been identified throughout the protostomes (15, 16) but not in deuterostomes. Thus, it appears that neuropeptides with the C-terminal FMRFamide-type motif FxRFamide are a uniquely protostomian invention.

THE RFamide MOTIF: AN ANCIENT AND A CONVERGENT FEATURE OF NEUROPEPTIDE EVOLUTION

A primary objective here was to review evidence of the occurrence of RFamide-type peptides and their receptors in deuterostomian invertebrates and a summary of our findings is shown in **Figure 4** and in a complementary supplementary table. With an anthropocentric perspective, the main focus of this review has been on five types of RFamide neuropeptides that are found in vertebrates: (1) Gonadotropin-inhibitory hormone (GnIH)-type, (2) NPFF-type, (3) Pyroglutamated RFamide peptide (QRFP)-type, (4) Prolactin-releasing peptide (PrRP)-type, and (5) Kisspeptin-type. Four of these neuropeptide types, GnIH, NPFF, QRFP, and PrRP, exert their effects via receptors that belong to distinct clade of the rhodopsin-β-type G protein-coupled neuropeptide–receptors, as illustrated in **Figure 1A**. Not all of the receptors in this clade are activated by RFamide-type neuropeptides (e.g., tachykinin receptors and leucokinin receptors). But some of the invertebrate

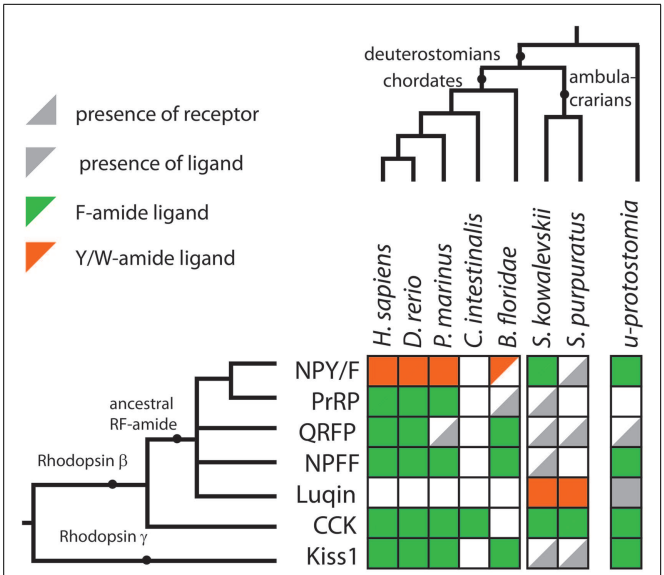

FIGURE 4 | Summary of RFamide-type peptides and receptors that have been identified in representative deuterostomian taxa. A gray triangle in the bottom right indicates that a receptor has been identified but a candidate ligand has not yet been identified. A gray triangle in the top left indicates that a peptide(s) has been identified but a candidate cognate receptor has not yet been identified. An empty white square indicates that neither a receptor nor a candidate peptide has been identified. The color of the triangle indicates that the peptide is either a Phe-amide (Fa, green), a Tyr-amide, or Trp-amide (Y/Wa, orange) or unknown (gray) in a given species or group of species. The last column of squares to the right describes the expected situation in the last common ancestral protostomian (*u-protostomia*). A supplementary table provides further details of IDs for the sequence data used to compile this figure.

members of this receptor clade are activated by RFamide-type peptides (e.g., luqin, NPF), while their vertebrate counterparts have either been lost or are activated by peptides with C-terminal motifs that are structurally similar to RFamide (e.g., RYamide in NPY-type peptides). Therefore, we speculate that the common ancestor of this clade of receptors may have been activated by a neuropeptide with a C-terminal RFamide motif. If this is correct, then we have a scenario where multiple gene duplications followed by diversification has given rise to neuropeptides where the C-terminal RFamide motif has either been retained, slightly modified (e.g., RYamide or RWamide) or lost, but with different patterns of retention, modification, or loss occurring in different branches of the animal kingdom.

But the RFamide motif is not unique to neuropeptide ligands of the clade of rhodopsin-β-type G protein-coupled receptors that includes GnIH-, NPFF-, QRFP-, and PrRP-type receptors. As highlighted above, the ligands for CCK/gastrin-type receptors in protostomian invertebrates also have an RFamide motif (**Figures 1A** and **3B**). This may be a consequence of convergent evolution, with CCK/gastrin-type neuropeptides having acquired an RFamide motif in the protostomian lineage. However, an alternative, and more provocative, hypothesis would be that the RFamide motif in protostomian CCK/gastrin-type neuropeptides reflects conservation of an ancient motif that was a characteristic

of the neuropeptide ligand that activated the receptor that is the common ancestor of all rhodopsin-β-type G protein-coupled neuropeptide–receptors in bilaterians.

In this scenario, neuropeptide diversification following multiple gene duplications would have resulted in loss of the RFamide motif in many of the neuropeptides that act as ligands for rhodopsin-β-type G protein-coupled neuropeptide–receptors in bilaterians. Support for the notion of an ancient RFamide neuropeptide signaling system can be found in the discovery that neuropeptides with an RFamide-type motif are present not only in bilaterians but also in basal animal groups such as the cnidarians (7, 84). However, the receptors that mediate the effects of RFamide-type neuropeptides in cnidarians have, as yet, not been identified and it is possible, of course, that receptors for RFamides in cnidarians are not rhodopsin-β-type receptors. Furthermore, the RFamide motif does appear to be a convergent feature of neuropeptide systems and this can be seen in kisspeptins, which exert effects via rhodopsin-γ-type G protein-coupled receptors. Thus, neuropeptides with a C-terminal RFamide motif act as ligands for both rhodopsin-β-type G protein-coupled receptors (GnIH-, NPFF-, QRFP-, and PrRP-type receptors) and rhodopsin-γ-type G protein-coupled receptors (kisspeptin-type receptors).

Interestingly, the abundance of neuropeptide types that share a C-terminal RFamide-type or RFamide-like motif (e.g., RYamide) could, in principle, give rise to cross-talk between neuropeptide signaling systems. Thus, a promiscuous RFamide receptor could potentially bind multiple RFamide-type neuropeptides derived from a variety of different precursor proteins. However, evidence that this occurs physiologically has, to the best of our knowledge, not been obtained. Demonstrating it will require evidence of not only peptide-receptor interaction *in vitro*, but also *in vivo*.

In conclusion, the RFamide motif appears to be both an ancient and a convergent feature of neuropeptide evolution, which then poses the ultimate question as to why this is. It has been proposed that from an enzymatic perspective, the occurrence of an arginine residue in the penultimate position may be a preferred characteristic for cleavage at neighboring monobasic or dibasic cleavage sites. Furthermore, non-polar and aromatic (e.g., phenylalanine and tyrosine) amino acids may be favored substrates for C-terminal amidation (15). Another observation that could explain the occurrence of C-terminal aromatic residues (Phe, Tyr, and Trp) in many neuropeptides, including RFamides, is the fact that rhodopsin α, a class of receptors phylogenetically related to neuropeptide–receptors (85) (see also **Figure 1A**), bind monoamine neurotransmitters (dopamine, norepinephrine, tyramine, and serotonin), which all have an aromatic functional group that is similar to the aromatic amino acids. This observation raises the possibility that ancestral neuropeptide ligands for rhodopsin-type receptors could have been peptides with a C-terminal aromatic amino acid (Phe/Tyr/Trp-amides).

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fendo.2014.00093/abstract

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Review: evolution of GnIH and related peptides structure and function in the chordates

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Discovery of gonadotropin-inhibitory hormone (GnIH) in the Japanese quail in 2000 was the first to demonstrate the existence of a hypothalamic neuropeptide inhibiting gonadotropin release. We now know that GnIH regulates reproduction by inhibiting gonadotropin synthesis and release *via* action on the gonadotropin-releasing hormone (GnRH) system and the gonadotrope in various vertebrates. GnIH peptides identified in birds and mammals have a common LPXRF-amide (X = L or Q) motif at the C-terminus and inhibit pituitary gonadotropin secretion. However, the function and structure of GnIH peptides are diverse in fish. Goldfish GnIHs possessing a C-terminal LPXRF-amide motif have both stimulatory and inhibitory effects on gonadotropin synthesis or release. The C-terminal sequence of grass puffer and medaka GnIHs are MPQRF-amide. To investigate the evolutionary origin of GnIH and its ancestral structure and function, we searched for GnIH in agnathans, the most ancient lineage of vertebrates. We identified GnIH precursor gene and mature GnIH peptides with C-terminal QPQRF-amide or RPQRF-amide from the brain of sea lamprey. Lamprey GnIH fibers were in close proximity to GnRH-III neurons. Further, one of lamprey GnIHs stimulated the expression of lamprey GnRH-III peptide in the hypothalamus and gonadotropic hormone β mRNA expression in the pituitary. We further identified the ancestral form of GnIH, which had a C-terminal RPQRF-amide, and its receptors in amphioxus, the most basal chordate species. The amphioxus GnIH inhibited cAMP signaling *in vitro*. In sum, the original forms of GnIH may date back to the time of the emergence of early chordates. GnIH peptides may have had various C-terminal structures slightly different from LPXRF-amide in basal chordates, which had stimulatory and/or inhibitory functions on reproduction. The C-terminal LPXRF-amide structure and its inhibitory function on reproduction may be selected in later-evolved vertebrates, such as birds and mammals.

Keywords: gonadotropin-inhibitory hormone (GnIH), RF-amide peptides, reproduction, evolution, chordates, lamprey, amphioxus

INTRODUCTION

Reproduction is one of the essential mechanisms for life. In vertebrates, the hypothalamic-pituitary-gonadal (HPG) axis is known as the core mechanism regulating reproduction. Gonadotropin-releasing hormone (GnRH) is the key hypothalamic neuropeptide that regulates the HPG axis by stimulating secretion of gonadotropins, i.e., luteinizing hormone (LH) and follicle-stimulating hormone (FSH), from the anterior pituitary. GnRH was first discovered in the brain of mammals at the beginning of 1970s (Matsuo et al., 1971; Burgus et al., 1972), and it was subsequently identified in the brain of non-mammalian vertebrates (King and Millar, 1982; Miyamoto et al., 1982, 1984; Sherwood et al., 1986). On the other hand, until recently no hypothalamic neuropeptide that inhibits gonadotropin release has been identified, although gonadal sex steroids and inhibin can inhibit gonadotropin release.

It became clear that the regulatory mechanism of reproduction is not as simple as it was once considered, since gonadotropin-inhibitory hormone (GnIH), a novel hypothalamic neuropeptide, was found to be involved in the regulation of the HPG axis (Tsutsui et al., 2000). GnIH was originally identified in birds (Tsutsui et al., 2000) and it was subsequently identified in other vertebrates from fish to humans (for reviews, see Ukena and Tsutsui, 2005; Tsutsui and Ukena, 2006; Tsutsui et al., 2006, 2007, 2010a,b, 2012, 2013; Tsutsui, 2009; Tsutsui and Ubuka, 2012). The discovery of GnIH has now changed our understanding about regulation of the reproductive axis fundamentally (for reviews, see Tsutsui et al., 2006, 2007, 2010a,b, 2012, 2013; Tsutsui, 2009; Tsutsui and Ubuka, 2012; Ubuka et al., 2013a).

To investigate the evolutionary origin of GnIH, we identified the orthologous gene of GnIH and mature GnIH peptides in the brain of lamprey, one of the oldest lineage of vertebrates, Agnatha (Osugi et al., 2012). Recently we further identified the ancestral form of GnIH in amphioxus, the most basal chordates (Osugi et al., 2014). These studies suggest that the origin of GnIH-like peptides may date back to the time of the emergence of early chordates. Based on these new findings, this review highlights the evolution of GnIH peptide structure and its function.

DISCOVERY OF GnIH IN THE BRAIN AS A NOVEL KEY FACTOR REGULATING REPRODUCTION

A neuropeptide possessing C-terminal Arg-Phe-NH_2 motif (RF-amide peptide) was first identified in the ganglia of venus clam *Macrocallista nimbosa* (Price and Greenberg, 1977). Since important functions of RF-amide peptides as neurotransmitters, neuromodulators or peripheral hormones were revealed in invertebrates (Greenberg and Price, 1992), there have been attempts to identify RF-amide peptides in the central nervous system of vertebrates. Tsutsui et al. (2000) discovered a novel RF-amide peptide from brains of the Japanese quail *Coturnix japonica* (Tsutsui et al., 2000). An immunohistochemical study showed that the GnIH-immunoreactive cell bodies exist in the paraventricular nucleus (PVN) and their fibers project to the median eminence where neurochemicals that regulate the anterior pituitary are released (Tsutsui et al., 2000). Therefore, this RF-amide peptide was considered to regulate the function of anterior pituitary in quail (Tsutsui et al., 2000). Indeed this novel RF-amide peptide inhibited gonadotropin release from the cultured quail pituitary and thus the RF-amide peptide was termed GnIH (Tsutsui et al., 2000).

Quail GnIH is a dodecapeptide having a C-terminal RF-amide motif, SIKPSAYLPLRF-amide (**Table 1**). The sequence of the five amino acids at the C-terminal of quail GnIH was identical to chicken LPLRF-amide that was isolated as a first RF-amide peptide in vertebrates (Dockray et al., 1983). This chicken LPLRF-amide may be a fragment of chicken GnIH (for reviews, see Tsutsui, 2009; Tsutsui et al., 2010a,b). In 2001, a cDNA encoding GnIH precursor polypeptides was identified in quail (Satake et al., 2001). Now GnIH cDNAs have been identified in several avian species, such as chickens, sparrows, starlings and zebra finches (for reviews, see Tsutsui, 2009; Tsutsui et al., 2010a,b). The GnIH precursor encodes one GnIH and two GnIH-related peptides (GnIH-RP-1 and GnIH-RP-2) that possess a conserved Leu-Pro-Xaa-Arg-Phe-NH_2 (LPXRF-amide; X = L or Q) motif at their C-termini in all birds studied (**Table 1**). Thus, GnIH and related peptides are called LPXRF-amide peptides from a structural point of view (for reviews, see Tsutsui, 2009; Tsutsui et al., 2010a,b). GnIH was further isolated as an endogenous ligand in European starling *Sturnus vulgaris* (Ubuka et al., 2008) and zebra finch *Taeniopygia guttata* (Tobari et al., 2010) and endogenous GnIH-RP-2 was also identified in quail (**Table 1**; Satake et al., 2001).

UNITY AND DIVERSITY OF GnIH STRUCTURE IN CHORDATES

A mammalian GnIH, also known as RFamide-related peptide (RFRP), orthologous gene has been identified by using *in silico* analysis (Hinuma et al., 2000). The mammalian GnIH cDNAs encoded two GnIH peptides (RFRP-1 and -3) (**Table 1** and **Figure 1**). Human, macaque, bovine and ovine precursor cDNAs also encoded a putative GnIH-like peptide that possesses a C-terminal LPLRSamide or LPLRLamide motif, which was named RFRP-2. However, rodent GnIH precursors lost RFRP-2 (**Figure 1**) (for reviews, see Tsutsui, 2009; Tsutsui et al., 2010a,b). Interestingly, the putative horse RFRP-2 possesses a C-terminal LPLRFamide motif (**Figure 1**) (Thorson et al., 2014). The mammalian GnIHs, RFRP-1 and/or RFRP-3, were identified as mature peptides in the brains of bovine *Bos taurus* (Fukusumi et al., 2001; Yoshida et al., 2003), rat *Rattus norvegicus* (Ukena et al., 2002), Siberian hamster *Phodopus sungorus* (Ubuka et al., 2012a), monkey *Macaca mulatta* (Ubuka et al., 2009a), and human *Homo sapiens* (**Table 1**; Ubuka et al., 2009b). GnIH and related peptides identified in birds and mammals have a conserved LPXRF-amide motif at the C-terminus (**Table 1**).

In reptiles, a putative GnIH gene was found in the Ensembl genome database of anole lizard, Chinese softshell turtle and painted turtle (**Table 1**; **Figure 1**). Recently, the crocodilian genome project was completed and the genome data of American alligator *Alligator mississippiensis* and saltwater crocodile *Crocodylus porosus* are available on the website of Crocodilian Genome Project (St. John et al., 2012; http://crocgenomes.org/). The putative GnIH gene was found in the genome data of crocodilians by using a tblastn program and exon-intron calculation based on the GT-AG rule (**Figure 1**; Mount, 1982). The putative reptilian GnIH peptides possess a C-terminal LPXRF-amide (X = L or Q) motif and showed a high sequence similarity with avian GnIH peptides that reflects a close phylogenetic position between birds and reptiles (**Table 1**; **Figure 1**).

In amphibians, a GnIH peptide was identified in the hypothalamus of bullfrog *Rana catesbeiana* and named frog growth hormone-releasing peptide (fGRP) (**Table 1**; Koda et al., 2002). cDNA cloning revealed that the precursor polypeptide encodes four GnIH peptides (fGRP, fGRP-RP-1, -RP-2, and RP-3) (Sawada et al., 2002a). fGRP-RP-1, -RP-2, and RP-3 were also identified as mature peptides (**Table 1**; Ukena et al., 2003). fGRP was independently isolated from the European green frog *Rana esculenta* and named *Rana* RFamide (R-RFa) (Chartrel et al., 2002). A GnIH cDNA was also cloned from the Japanese red-bellied newt, an urodele amphibian (Chowdhury et al., 2011). The deduced precursor encoded four GnIH peptides (nLPXRFa-1, -2, -3, -4), and these peptides were identified as mature peptides from the brain extracts (**Table 1**; Chowdhury et al., 2011). The rate of amino acid substitution or deletion may have been lower in the lineage of amphibians compared with other vertebrates, resulting in the conservation of four LPXRF-amide (X = L or Q) peptides encoded in the precursor (**Figure 1**).

In teleost fish, a GnIH cDNA encoding three peptides (gfLPXRFa-1, -2, and -3) which have C-terminal LPXRF-amide (X = L or Q) sequences was cloned from the brain of goldfish *Carassius auratus*, and one peptide (gfLPXRFa-3) was identified as a mature peptide (**Table 1**; Sawada et al., 2002b). A GnIH gene was also identified in the grass puffer *Takifugu niphobles*. The grass puffer GnIH precursor contained two putative GnIH peptides which have C-terminal MPMRF-amide or MPQRF-amide sequences and one possible RY-amide peptide (**Table 1**; Shahjahan et al., 2011). The medaka GnIH precursor contained two putative GnIH peptides which have C-terminal MPLRF-amide or MPQRF-amide sequences and one LPQRF-amide peptide (**Table 1**; XM_004073848). Therefore, Leu, Met, and Glu are substituted by each other in some fish species. The CUG codon encoding Leu can be mutated to AUG encoding Met by a single nucleotide substitution. Similarly, a single nucleotide substitution in the codon encoding Glu (CAA and CAG) can produce CUA and CUG encoding Leu. Thus, nucleotide substitutions in

Table 1 | Amino acid sequences of GnIHs in chordates.

	Animal	Name	Sequence	References
Mammals	Human	RFRP-1	MPHSFANLPLRFa	Ubuka et al., 2009b
		RFRP-3	VPNLPQRFa	Ubuka et al., 2009b
	Macaque	RFRP-1*	MPHSVTNLPLRFa	Ubuka et al., 2009a
		RFRP-3	SGRNMEVSLVRQVLNLPQRFa	Ubuka et al., 2009a
	Bovine	RFRP-1	SLTFEEVKDWAPKIKMNKPVVNKM PPSAANLPLRFa	Fukusumi et al., 2001
		RFRP-3	AMAHLPLRLGKNREDSLSRWVPNLPQRFa	Yoshida et al., 2003
	Horse	RFRP-3*	IPNLPQRFa	Thorson et al., 2014
	Rat	RFRP-1*	SWFQELKDWGAKKDIKMSPAPANKVPHS AANLPLRFa	Ukena et al., 2002
		RFRP-3	ANMEAGTMSHFPSLPQRFa	Ukena et al., 2002
	Siberian hamster	RFRP-1	SPAPANKVPHSAANIiPLRFa	Ubuka et al., 2012a
		RFRP-3	TLSRVPSLPQRFa	Ubuka et al., 2012a
Birds	Quail	GnIH	SIKPSAYLPLRFa	Tsutsui et al., 2000
		GnIH-RP-1*	SLNFEEMKDWGSKNFMKVNTPTVN KVPNSVANLPLRFa	Satake et al., 2001
		GnIH-RP-2	SSIQSLLNLPQRFa	Satake et al., 2001
	Chicken	GnIH*	SIRPSAYLPLRFa	Ikemoto and Park, 2005
		GnIH-RP-1*	SLNFEEMKDWGSKNFLKVNTPTVNKV PNSVANLPLRFa	Ikemoto and Park, 2005
		GnIH-RP-2*	SSIQSLLNLPQRFa	Ikemoto and Park, 2005
	White-crowned sparrow	GnIH*	SIKPFSNLPLRFa	Osugi et al., 2004
		GnIH-RP-1*	SLNFEEMEDWGSKDIIKMNPFTASKMPNS VANLPLRFa	Osugi et al., 2004
		GnIH-RP-2*	SPLVKGSSQSLLNLPQRFa	Osugi et al., 2004
	European starling	GnIH	SIKPFANLPLRFa	Ubuka et al., 2008
		GnIH-RP-1*	SLNFDEMEDWGS KD IIKMNPFTVS KMPNS VANL PLRFa	Ubuka et al., 2008
		GnIH-RP-2*	GSSQSLLNLPQRFa	Ubuka et al., 2008
	Zebra finch	GnIH	SIKPFSNLPLRFa	Tobari et al., 2010
		GnIH-RP-1*	SLNFEEMEDWRSKDIIKMNPFAASKMPN SVANLPLRFa	Tobari et al., 2010
		GnIH-RP-2*	SPLVKGSSQSLLNLPQRFa	Tobari et al., 2010
Reptiles	Anole lizard	GnIH*	SIKPAANLPLRFa	EN SACAG00000013069
		GnIH-RP-1*	SMDLESMNDWELNKIIRRTTPEMKKMA HAAVNLPLRFa	EN SACAG00000013069
		GnIH-RP-2*	APDVQSLSRSLANLPQRFa	EN SACAG00000013069
	Chinese softshell turtle	GnIH*	IIKPVANLPLRFa	EN SPSIG00000017952
		GnIH-RP-1*	SLNFEELKDWGSKNIIKMSTPTVNKM PNSVANLPLRFa	EN SPSIG00000017952
		GnIH-RP-2*	TPFVKTSSQLFPNLPQRFa	EN SPSIG00000017952
Amphibians	Bullfrog	fGRP/R-RFa	SLKPAANLPLRFa	Chartrel et al., 2002; Koda et al., 2002
		fGRP-RP-1	SIPNLPQRFa	Ukena et al., 2003
		fGRP-RP-2	LSGKTKVQSMANLPQRFa	Ukena et al., 2003
		fGRP-RP-3	QYTNHFVHSLDTLPLRFa	Ukena et al., 2003
	Red-bellied newt	nLPXRFa-1	SVPNLPQRFa	Chowdhury et al., 2011
		nLPXRFa-2	MPHASANLPLRFa	Chowdhury et al., 2011
		nLPXRFa-3	SIQPLANLPQRFa	Chowdhury et al., 2011
		nLPXRFa-4	APSAGQFIQTLANLPQRFa	Chowdhury et al., 2011
Teleost fish	Goldfish	gfLPXRFa-1*	PTHLHANLPLRFa	Sawada et al., 2002b
		gfLPXRFa-2*	AKSNINLPQRFa	Sawada et al., 2002b
		gfLPXRFa-3	SGTGLSATLPQRFa	Sawada et al., 2002b

(Continued)

Table 1 | Continued

	Animal	Name	Sequence	References
	Medaka	mdLPXRFa-1*	**PLHMHANMPLRFa**	XM_004073848
		mdLPXRFa-2*	**VSNSSPNMPQRFa**	XM_004073848
		mdLPXRFa-3*	**EAPSPVLPQRFa**	XM_004073848
	Grass puffer	gpLPXRFa-1*	**SLDMERINIQVSPTSGKVSLPTIVRLYPT**	Shahjahan et al., 2011
			LQPHHQHVN	
			-MPMRFa	
		gpLPXRFa-2*	**DGVQGGDHVPNLNPNMPQRFa**	Shahjahan et al., 2011
		gpRYa*	**SWKVIRLCEDCSKVQGVLKHQVRYa**	Shahjahan et al., 2011
Agnathans	Sea lamprey	ILPXRFa-Ia	**SGVGQGRSSKTLFQPQRFa**	Osugi et al., 2012
		ILPXRFa-Ib	**AALRSGVGQGRSSKTLFQPQRFa**	Osugi et al., 2012
		ILPXRFa-2	**SEPFWHRTRPQRFa**	Osugi et al., 2012
Protochordates	Amphioxus	PQRFa-1	**WDEAWRPQRFa**	Osugi et al., 2014
		PQRFa-2	**GDHTKDGWRPQRFa**	Osugi et al., 2014
		PQRFa-3	**GRDQGWRPQRFa**	Osugi et al., 2014

Ensembl accession numbers or Genbank accession numbers are referred to for reptile GnIHs or medaka GnIHs.

**Indicates putative peptides.*

the codon encoding the third and the fifth amino acids from the C-terminal may have occurred in some fish species, such as medaka and grass puffer (**Figure 2**). We further searched for GnIH-like sequences in the genome database of phylogenetically important fish, such as the elephant shark, skate, and spotted gar. A partial GnIH-like sequence was found in the Ensembl genome database of the spotted gar (chromosome LG11, nt 40715843 to nt 40716142, reverse strand). The C-terminal motifs of spotted gar GnIH-like peptides were LPLRF or LPQRF and their codons were similar to those of other fish (**Figure 2**). On the other hand, we could not find any GnIH-like sequence in the elephant shark genome database (http://esharkgenome.imcb.a-star.edu.sg/) and the skate genome database (http://skatebase.org/). Further researches are needed to clarify the presence of GnIH in cartilaginous fish.

Recently, we have identified a GnIH orthologous gene by using synteny analysis and cDNA cloning in lamprey, one of the most basal vertebrates (**Figure 1**; Osugi et al., 2012). Mature lamprey GnIH peptides were also identified by using immunoaffinity purification and mass spectrometry (**Table 1**; Osugi et al., 2012). The lamprey GnIH peptides possessed a C-terminal QPQRF-amide or RPQRF-amide motif and the third or the fifth Leu from the C-terminal was not conserved (**Table 1**; Osugi et al., 2012). The codon encoding Glu in QPQRF-amide and RPQRF-amide were all CAG, and the codon encoding the first Arg in RPQRF-amide was CGG (**Figure 2**). The codon CAG or CGG can be mutated to CUG encoding Leu by a single nucleotide substitution. Therefore, the fifth and the third Leu from the C-terminal may have appeared and conserved after the emergence of gnathostomes.

We further searched for GnIH in amphioxus, the most basal chordate, to investigate the evolutionary origin of GnIH. A novel gene encoding RF-amide peptides and mature peptides were identified in the amphioxus *Branchiostoma japonicum* by using genome database search in *Branchiostoma floridae*, cDNA cloning and immunoaffinity purification (**Table 1**; **Figure 1**; Osugi et al., 2014). The identified amphioxus RF-amide peptides possessed a C-terminal RPQRF-amide motif that was identical to the C-terminal of lamprey LPXRFa-2 (**Table 1**). The codon encoding the first Arg in RPQRF-amide was CGC, CGA, or CGG, which can be mutated to CUC, CUA, or CUG encoding Leu by a single nucleotide substitution, respectively (**Figure 2**). The codon encoding the third Glu in RPQRF-amide was CAG or CAA, which can be mutated to CUG or CUA encoding Leu by a single nucleotide substitution, respectively (**Figure 2**). Accordingly, nucleotide mutations may have occurred at the codon encoding the first Arg and the third Glu in RPQRF-amide during the course of vertebrate evolution, resulting in the C-terminal LPQRF-amide or LPLRF-amide motif of GnIH peptides in gnathostomes.

UNITY AND DIVERSITY OF GnIH FUNCTION IN VERTEBRATE REPRODUCTION

The function of GnIH and related peptides are summarized in **Table 2**. As described above, the gonadotropin inhibiting effect of GnIH was first demonstrated in the quail pituitary *in vitro* (Tsutsui et al., 2000). An *in vivo* study further revealed that GnIH inhibits the release and expression of gonadotropins in quail (Ubuka et al., 2006). In addition to the direct effect of GnIH on the pituitary, GnIH also inhibited GnRH-induced elevation in plasma LH in song sparrow (Osugi et al., 2004). The close proximity of GnIH immunoreactive fibers to GnRH neurons and the expression of GnIH receptor in GnRH neurons support the effect of GnIH on GnRH neurons in birds (Ubuka et al., 2008). To investigate the mode of action of GnIH in birds, the receptor for GnIH was identified in quail brain (Yin et al., 2005). GnIH receptor (GnIH-R) is a G-protein-coupled receptor, also known as GPR147, and it was expressed in the pituitary and several brain regions including diencephalon (Yin et al., 2005). GnIH-R showed high affinities to GnIH, GnIH-RPs, and RFRPs, which have LPXRF-amide (X = L or Q) motif

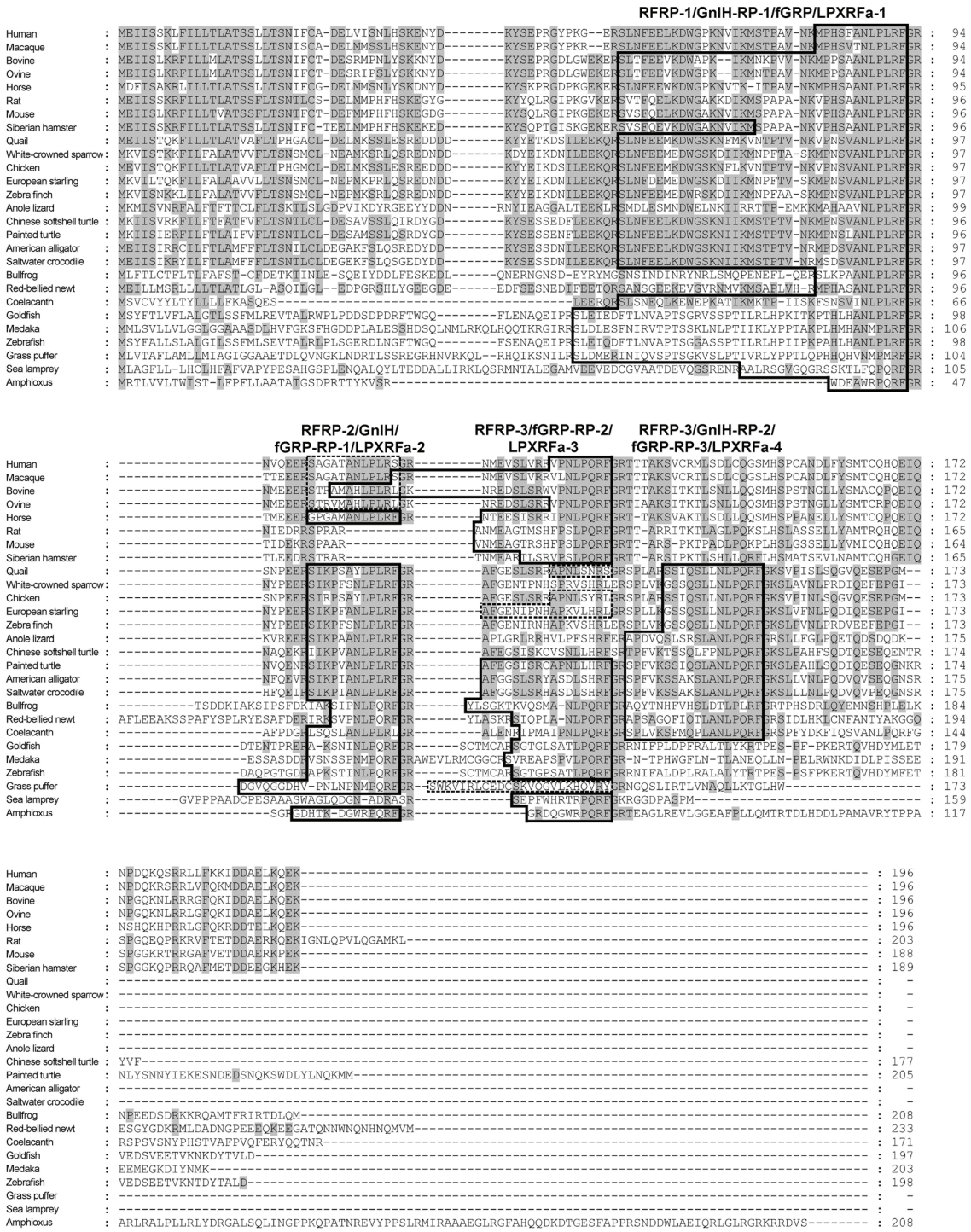

FIGURE 1 | Comparison of GnIH precursor amino acid sequences in representative species of chordates. The identical amino acids are shaded. The peptide coding regions are boxed. The precursors of human, macaque, bovine, ovine, quail, chicken, starling, and grass puffer encode putative peptides with C-terminal RS-amide, RL-amide, or RY-amide motifs that are boxed by broken lines.

at their C-termini (Yin et al., 2005). Non-amidated GnIH failed to bind the receptor, suggesting that the C-terminal LPXRF-amide (X = L or Q) motif is responsible for its binding to GnIH-R (Yin et al., 2005). It was further demonstrated that GnIH-R couples to $G_{\alpha i}$ and GnIH inhibits GnRH-induced cAMP responsive element (CRE) activation in the chicken, suggesting that GnIH regulates GnRH signaling by inhibiting cAMP signaling pathway (Bédécarrats et al., 2009; Shimizu and Bédécarrats,

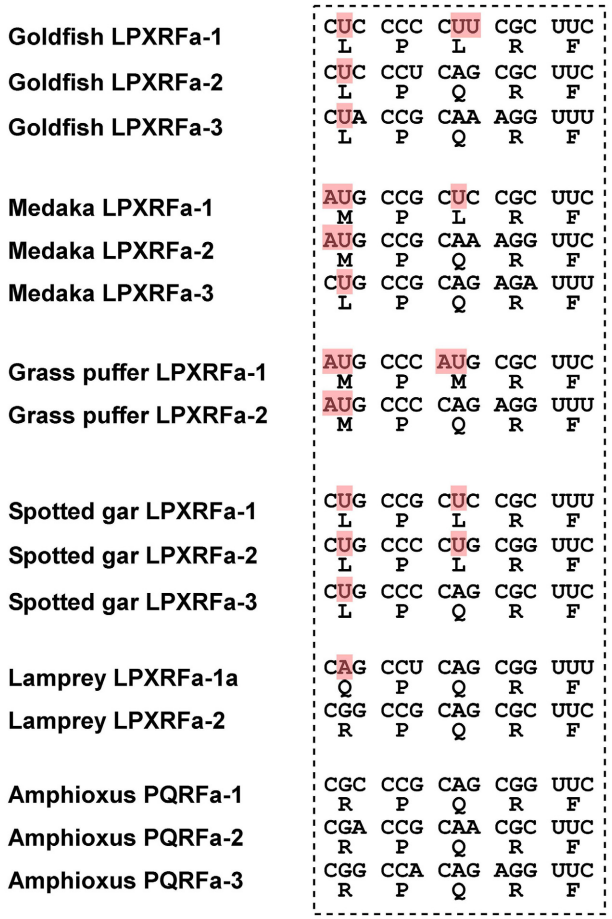

FIGURE 2 | Comparison of the C-terminal five amino acid sequences of GnIH peptides and their corresponding codons in teleost fish (goldfish, medaka, grass puffer, spotted gar), agnathan (lamprey), and protochordate (amphioxus). The nucleotides that have been modified during evolution are shaded in red.

2010). From the viewpoint of the behavioral regulation, intracerebroventricularly (ICV) administered GnIH inhibited reproductive behavior of female white-crowned sparrows (Bentley et al., 2006). By using the RNAi technique, it was shown that GnIH regulates aggressive and sexual behaviors in male white-crowned sparrow or quail (Ubuka et al., 2012b, 2013b). Recently, it was further demonstrated that GnIH inhibits socio-sexual behavior of male quail by increasing neuroestrogen synthesis in the hypothalamus (Ubuka et al., 2014). At the peripheral level, GnIH decreased plasma testosterone concentration, induced testicular apoptosis and decreased spermatogenic activity in adult male quail, suggesting a direct action of GnIH at the testis or an action *via* reduced gonadotropin secretion (Ubuka et al., 2006). In addition, GnIH also reduced the testicular weight in immature birds, suggesting that GnIH is involved in gonadal development and maintenance (Ubuka et al., 2006). Taken together, GnIH acts as an inhibitory neuropeptide and exerts multiple effects on the reproductive systems in the brain as well as peripheral organs.

In mammals, two GnIH peptides (RFRP-1 and RFRP-3) are encoded in the precursor. As in birds, RFRP-3 inhibited gonadotropin synthesis and/or release in various mammalian species (Kriegsfeld et al., 2006; Johnson et al., 2007; Clarke et al., 2008; Murakami et al., 2008; Kadokawa et al., 2009; Sari et al., 2009; Ubuka et al., 2012a). In addition, immunoreactive GnIH fibers were in close proximity to GnRH neurons in the hypothalamus of human, monkey, sheep, rat and hamster (Kriegsfeld et al., 2006; Johnson et al., 2007; Smith et al., 2008; Ubuka et al., 2009a,b, 2012a). Expression of GnIH-R was also observed in GnRH neurons (Rizwan et al., 2012; Ubuka et al., 2012a). Consistent with histochemical studies, RFRP-3 was shown to inhibit the firing rate of GnRH neurons in mice (Ducret et al., 2009). In pig, RFRP-3 also inhibited the synthesis and release of GnRH (Li et al., 2013). Recently, it was found that ICV administration of RFRP-1 also inhibits gonadotropin release in hamsters (Ubuka et al., 2012a). Therefore, both RFRP-1 and RFRP-3 may act as GnIH in mammals. Regarding the signal transduction mechanisms in mammals, it was demonstrated that RFRP-3 reduces GnRH-stimulated cytoplasmic calcium response and extracellular signal-regulated kinase (ERK) phosphorylation in sheep pituitary (Clarke et al., 2008; Sari et al., 2009). The detailed mechanisms were further investigated using mouse gonadotrope cell line (LβT2 cells). It was revealed that the inhibitory action of mouse GnIHs (RFRPs) on gonadotropin gene expression is mediated by an inhibition of adenylate cyclase (AC)/cAMP/cAMP-dependent protein kinase A (PKA)-dependent ERK pathway (Son et al., 2012). These studies in mammals suggest that the inhibitory mechanism of GnIH on the reproductive system is conserved among mammalian and avian animals. In Siberian hamsters, the expression of GnIH decreased in short day conditions by the action of pineal melatonin (Ubuka et al., 2012a). Siberian hamster GnIHs (RFRP-1 and RFRP-3) stimulated LH release in short day conditions and inhibited LH release in long day conditions (Ubuka et al., 2012a). Because Siberian hamsters are long day breeders and short day conditions represent an inhibitory photoperiod, GnIH may sustain appropriate concentration of LH in short day condition. Taken together, GnIH may have acquired a stimulatory function in the lineage of photoperiodic mammals to optimize their reproductive activities according to the season.

In teleost fish, functional diversity was observed compared to mammals and birds. Goldfish GnIHs (gfLPXRFa peptides) stimulated release of gonadotropins and growth hormone (GH) in Sockeye salmon *Oncorhynchus nerka* (Amano et al., 2006). In grass puffer, the expression of GnIH and GnIH-R mRNA were increased during the spawning season in the brain and pituitary (Shahjahan et al., 2011). In addition, goldfish GnIH (gfLPXRFa-1) stimulated the expression of gonadotropin mRNAs in the pituitary in grass puffer, suggesting that GnIH may be involved in the stimulation of reproductive axis in grass puffer (Shahjahan et al., 2011). In contrast to the stimulatory effects of fish GnIH, zebrafish GnIH decreased serum LH level in goldfish *in vivo* (Zhang et al., 2010). Similarly, goldfish GnIHs (gfLPXRFa-2 and -3) decreased salmon GnRH and FSHβ mRNA levels and gfLPXRFa-2 decreased LHβ mRNA levels in goldfish *in vivo* (Qi et al., 2013). Although single administrations of goldfish GnIHs

Table 2 | Functions of GnIH and related peptides in vertebrates.

Animal	Name	Function	References
Syrian hamster	GnIH	Inhibition of LH release	Kriegsfeld et al., 2006
Siberian hamster	RFRP-1 and-3	Inhibition or stimulation of LH release	Ubuka et al., 2012a
Rat	RFRP-3	Inhibition of LH secretion	Johnson et al., 2007
		Inhibition of GnRH-elicited LH release	Murakami et al., 2008
Mouse	RFRP-3	Inhibition of the firing rate of GnRH neurons	Ducret et al., 2009
Ovine	RFRP-3	Inhibition of GnRH-elicited gonadotropin release	Clarke et al., 2008
		Inhibition of gonadotropin secretion	Sari et al., 2009
Bovine	RFRP-3	Inhibition of LH release	Kadokawa et al., 2009
Pig	RFRP-3	Inhibition of GnRH secretion	Li et al., 2013
Quail	GnIH	Inhibition of LH release	Tsutsui et al., 2000
		Inhibition of gonadotropin secretion	Ubuka et al., 2006
		Inhibition of socio-sexual behavior	Ubuka et al., 2014
		Inhibition of plasma testosterone concentration	Ubuka et al., 2014
		Reduction of testicular weight	Ubuka et al., 2006
Sparrow	GnIH	Inhibition of GnRH-induced elevation in plasma LH	Osugi et al., 2004
		Inhibition of reproductive behavior	Bentley et al., 2006
		Inhibition of aggressive and sexual behaviors	Ubuka et al., 2012b, 2013b
		Inhibition of socio-sexual behavior	Ubuka et al., 2014
Chicken	GnIH	Inhibition of GnRH-induced CRE activation	Bédécarrats et al., 2009; Shimizu and Bédécarrats, 2010
Sockeye salmon	gfLPXRFa-1	Stimulation of gonadotropin and GH release	Amano et al., 2006
	gfLPXRFa-2	Stimulation of gonadotropin and GH release	Amano et al., 2006
	gfLPXRFa-3	Stimulation of gonadotropin and GH release	Amano et al., 2006
Grass puffer	gfLPXRFa-1	Stimulation of gonadotropin expression	Shahjahan et al., 2011
Goldfish	zfLPXRFa	Inhibition of plasma LH concentration	Zhang et al., 2010
Goldfish	gfLPXRFa-2	Inhibition of sGnRH and FSH expression	Qi et al., 2013
Goldfish	gfLPXRFa-2	Inhibition of LH expression	Qi et al., 2013
Goldfish	gfLPXRFa-3	Inhibition of sGnRH and FSH expression	Qi et al., 2013
Goldfish	gfLPXRFa-3	Inhibition of GnRH-stimulated gonadotropin synthesis	Qi et al., 2013
Goldfish	GnIH	Inhibition or stimulation of gonadotropin secretion	Moussavi et al., 2012, 2013
Lamprey	LPXRFa-2	Stimulation of gonadotropin expression	Osugi et al., 2012

(gfLPXRFa-2 and -3) showed no effect on gonadotropin synthesis in the primary culture of goldfish pituitary cells, gfLPXRFa-3 inhibited GnRH-stimulated LHβ and FSHβ synthesis (Qi et al., 2013). According to the maturational status of goldfish, goldfish GnIH exerted both stimulatory and inhibitory effects on the expression of gonadotropin mRNAs and the serum LH level (Moussavi et al., 2012, 2013). The studies in teleost fish suggest that the functions of GnIH are diverse even within the teleost fish lineage. The action of GnIH also changes depending on the physiological conditions of the fish.

In agnathans, lamprey GnIH-immunoreactive fibers were in close proximity to GnRH-III neurons (Osugi et al., 2012). One of the GnIH peptides (lamprey LPXRFa-2) administered *in vivo* increased GnRH-III concentration in the brain and mRNA expression of gonadotropin β subunit in the pituitary (Osugi et al., 2012). These effects of GnIH are similar to that of some of teleost fish, suggesting that the stimulatory action of GnIH may have been conserved in several lineages of basal vertebrates.

EVOLUTIONARY ORIGIN OF GnIH AND ITS ANCESTRAL STRUCTURE AND FUNCTION

Neuropeptide FF (NPFF), also known as PQRF-amide peptide is a pain-modulatory neuropeptide and is considered to be a paralogous gene of GnIH. The C-terminal motifs of GnIH and NPFF that are important for the interaction with their receptors showed high sequence similarity (Osugi et al., 2006, 2011, 2012). The receptors for GnIH (NPFFR1; GPR147) and NPFF (NPFFR2; GPR74) also showed a high sequence similarity (Yin et al., 2005). In addition, the GnIH gene locates near the *HOXA* clusters and NPFF gene locates near the *HOXC* clusters on the chromosome (**Figure 3**; Ikemoto and Park, 2005; Osugi et al., 2012). The sequence similarity of GnIH and NPFF and a common belief that the *HOX* clusters have duplicated from a common ancestral gene during whole genome duplication events through vertebrate evolution (Venkatesh et al., 2007) led to the strong hypothesis that GnIH gene and NPFF gene have diverged from a common ancestral gene through chromosome duplication (Ikemoto and

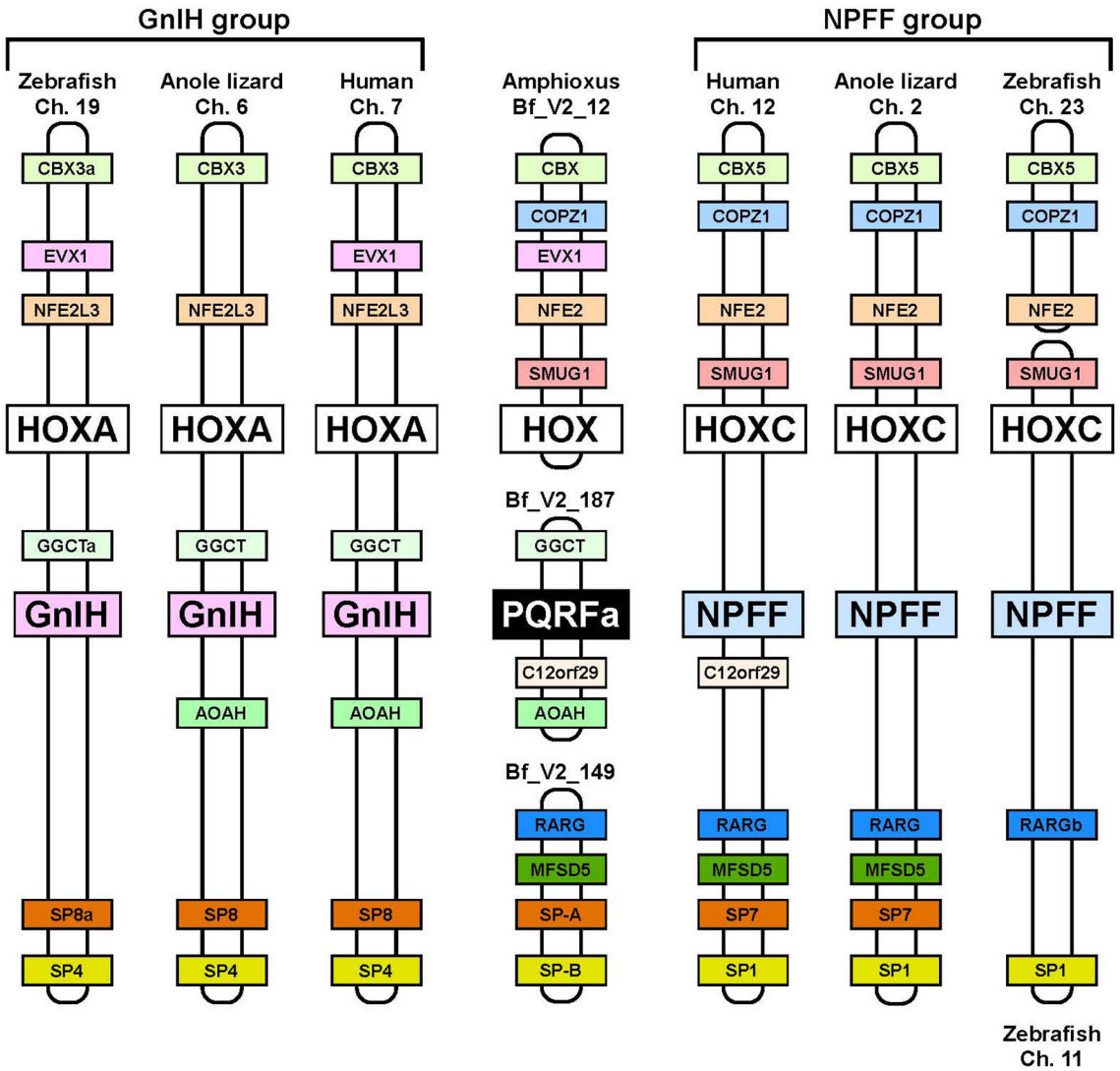

FIGURE 3 | Synteny analysis of the loci of amphioxus PQRF-amide peptide gene, GnIH gene, and NPFF gene. The names of animals and chromosome or scaffold numbers are shown on the top or bottom of each chromosome or scaffold region. Orthologous genes are shown in a same color. The amphioxus PQRF-amide peptide gene, vertebrate GnIH genes and vertebrate NPFF genes are shown in black, pink, or blue boxes. HOX clusters are shown in white boxes. The conserved synteny region exists around the loci of amphioxus PQRF-amide peptide gene, GnIH gene and NPFF gene. The distance of the genome fragments analyzed were as follows: 15.08 Mbp (human Ch. 7), 17.24 Mbp (anole lizard Ch. 6), 18.46 Mbp (zebrafish Ch. 19), 1.09 Mbp (human Ch. 12), 3.91 Mbp (anole lizard Ch. 2), 40 Kbp (zebrafish Ch. 23), and 259.76 Mbp (zebrafish Ch. 11). Note that anole lizard HOXA and SP8 are on the scaffolds GL343275.1 and GL343212.1. The homology between these genome regions was identified based on the gene annotation in the Ensembl genome database. The homologous genes of amphioxus were searched by the blast program in the Joint Genome Institute.

Park, 2005; Osugi et al., 2012, 2014). The presence of both GnIH and NPFF in agnathans, the most ancient vertebrates, further suggests that GnIH and NPFF genes have diverged before the emergence of vertebrates (Osugi et al., 2006, 2011, 2012). As described above, we identified a novel gene encoding RF-amide peptides and mature peptides in the amphioxus *Branchiostoma japonicum* (**Table 1**; **Figure 1**; Osugi et al., 2014). We further identified putative receptors for the identified amphioxus RF-amide peptides. Molecular phylogenetic analysis and synteny analysis indicated that these genes are closely related to GnIH and NPFF genes and their receptors of vertebrates (**Figure 3**; Osugi et al., 2014). The identified amphioxus RF-amide peptides inhibited forskolin induced cAMP signaling in the COS-7 cells transfected with one of the identified amphioxus RF-amide peptide receptors (Osugi et al., 2014). The study of amphioxus thus indicates that the identified protochordate RF-amide peptide gene is likely to be an ancestral form of both GnIH and NPFF. We could not find any GnIH-like peptide sequence in other early deuterostome genomes, such as *Ciona intestinalis*, sea urchin and acorn worm. Therefore, the origin of GnIH may date back to the time of the emergence of early chordates (**Figure 4**). The role of the ancestral GnIH may have been an inhibitory peptide and its functions

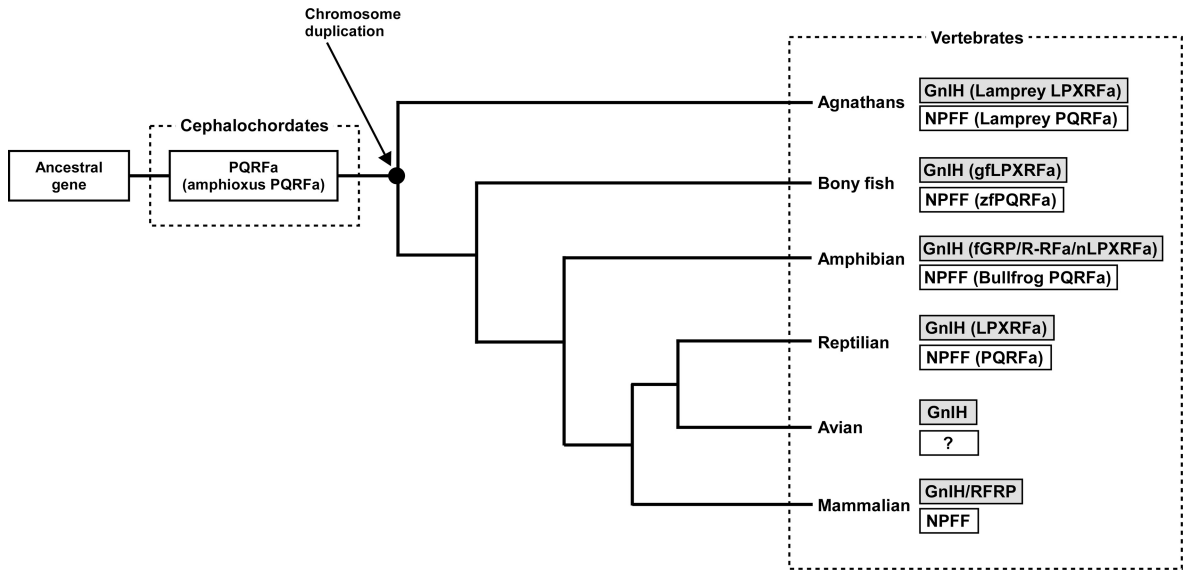

FIGURE 4 | Proposed evolutionary history of GnIH and NPFF genes. GnIH and NPFF genes may have originated from a common ancestral gene. The amphioxus which has not experienced chromosome duplication has sustained an ancestral form of GnIH and NPFF genes. GnIH and NPFF genes may have evolved through chromosome duplication that has occurred at the beginning of vertebrate evolution. ?, unknown.

may have been diversified in some lineage during the course of vertebrate evolution.

Currently, the following RF-amide peptide groups, namely, GnIH, NPFF, 26RF-amide peptide (26RFa)/pyroglutamylated RF-amide peptide (QRFP), prolactin-releasing peptide (PrRP), Kiss1, and Kiss2 are found in the brain of vertebrates (Tsutsui, 2009; Tsutsui et al., 2010b). Within these groups, GnIH and NPFF, and Kiss1 and Kiss2 are thought to be paralogous (Felip et al., 2009; Lee et al., 2009; Osugi et al., 2012, 2013, 2014; Pasquier et al., 2012). It is of interest to investigate the evolutionary relationship between RF-amide peptide groups, and search the common origin of these RF-amide peptides. Recent advances in genome database with powerful instruments, such as bioinformatic tools and next-generation sequencing, may enable us to analyze the genome data of various animals and answer to this question.

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Diversity of the RFamide peptide family in mollusks

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Since the initial characterization of the cardioexcitatory peptide FMRFamide in the bivalve mollusk *Macrocallista nimbosa*, a great number of FMRFamide-like peptides (FLPs) have been identified in mollusks. FLPs were initially isolated and molecularly characterized in model mollusks using biochemical methods. The development of recombinant technologies and, more recently, of genomics has boosted knowledge on their diversity in various mollusk classes. Today, mollusk FLPs represent approximately 75 distinct RFamide peptides that appear to result from the expression of only five genes: the FMRFamide-related peptide gene, the LFRFamide gene, the luqin gene, the neuropeptide F gene, and the cholecystokinin/sulfakinin gene. FLPs display a complex spatiotemporal pattern of expression in the central and peripheral nervous system. Working as neurotransmitters, neuromodulators, or neurohormones, FLPs are involved in the control of a great variety of biological and physiological processes including cardiovascular regulation, osmoregulation, reproduction, digestion, and feeding behavior. From an evolutionary viewpoint, the major challenge will then logically concern the elucidation of the FLP repertoire of orphan mollusk classes and the way they are functionally related. In this respect, deciphering FLP signaling pathways by characterizing the specific receptors these peptides bind remains another exciting objective.

Keywords: FaRPs, LFRFamide, luqin, NPF, CCK/SK, mollusks

INTRODUCTION

Mollusks exhibit great morphological diversity. They have adapted to marine, freshwater, and terrestrial habitats. They have distinct reproductive strategies; some species are gonochoric, simultaneous hermaphrodite, or alternative hermaphrodite. The phylum Mollusca is divided into eight classes (1): Monoplacophora (*Neopilina*), Polyplacophora (chitons), Bivalvia (clams, oysters, mussels), Gastropoda (snails, slugs), Aplacophora (worm-like mollusks), Cephalopoda (squid, cuttlefish, octopus), and Scaphopoda (tusk shells). Neuropeptides play a crucial neurotransmitter, neuromodulator, or neurohormone role in the elaboration of adapted physiological and behavioral responses to environmental constraints. As a result, phylogenetic distances, together with body plan and physiological behavior diversity probably reflect differences in the composition of mollusks' neuropeptide record and their pattern of expression. The relatively simple central nervous system (CNS) of the gastropod mollusks *Aplysia californica* and *Lymnaea stagnalis,* with large identified neurons, has made them the most widely studied species for deciphering the role of neuropeptides in the control of physiological processes and behaviors (2–4). In mollusks, among the multiplicity of neuropeptides, the cardioexcitatory neuropeptide FMRFamide was first isolated in the clam *Macrocallista nimbosa* (5). So far, FMRFamide along with its structurally related neuropeptides or FMRFamide-like peptides (FLPs) that display varying sizes but harbor the common C-terminal RFamide sequence probably represent the best investigated family of neuropeptides in mollusks. Initial studies essentially concerned immunocytochemical screening of mollusk tissues with antibodies raised against the tetrapeptide FMRFamide. Then, with the refinement of peptide purification and sequencing methodologies, many new related peptides were discovered. By rendering the structure of the precursors encoding this family of peptides accessible, the development of recombinant technology has progressively extended knowledge about the diversity of FLPs. Knowledge includes FMRFamide-related peptides (FaRPs) and other RFamide peptides, especially in mollusks where purification was difficult for anatomical reasons (**Figure 1**).

In addition to mollusks (6), the FLP family has been extensively described in all major animal phyla, from cnidarians to mammals (7, 8), and are involved in the regulation of a great variety of physiological processes (9–12). With the advent of the genomics era, bioinformatics analyses of transcriptome and genome databases now clearly suggest that the high diversity of RFamide peptides in mollusks results from the expression of only five distinct genes: the FMRF gene, the LFRFamide gene, the luqin gene, the neuropeptide F (NPF) gene, and the cholecystokinin/sulfakinin (CCK/SK)-related gene (**Figure 1**).

THE FMRF GENE CODES FOR A DIVERSITY OF FMRFamide-RELATED PEPTIDES

Since the discovery of the FMRFamide, a cardioactive neuropeptide in the clam *M. nimbosa* (5), an increasing number of studies has demonstrated the presence of this peptide and related peptides

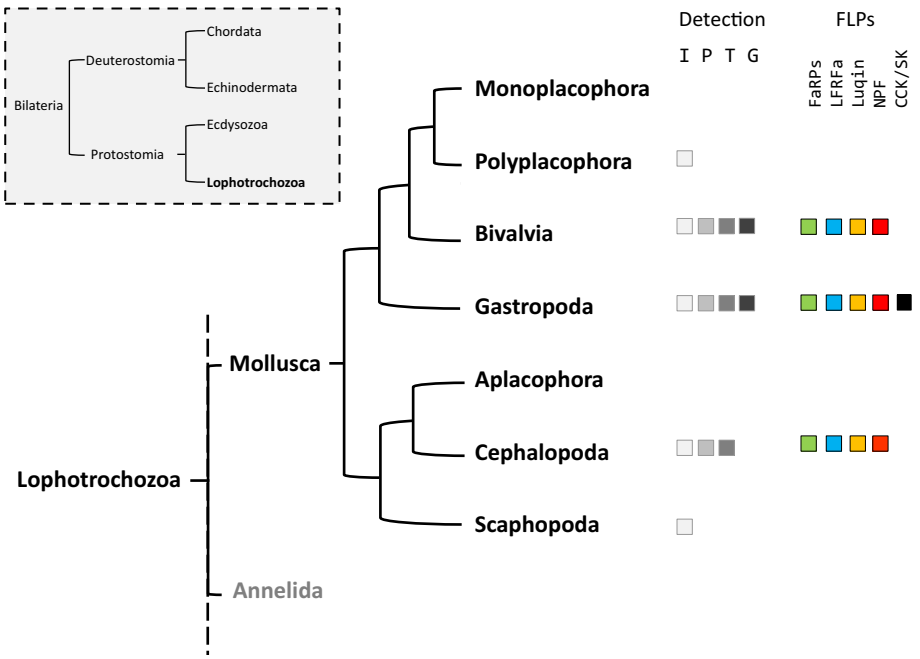

FIGURE 1 | Phylogenic tree of mollusks based on Stöger et al. (1). The occurrence of the different FMRFamide-like peptides (FLPs) in each class is detailed. F_a, FMRFa-related peptides or FaRPs; L_r, LFRFamide peptides; L_u, Luqin; N_p, NPF; C_{ck}, cholecystokinin-/sulfakinin-like peptides. The methodologies used for FLPs detection are indicated: I, immunohistochemistry; P, peptide identification; T, transcript characterization; G, genome.

(FaRPs) in other mollusks (**Table 1**) and other protostomes [for reviews, see Ref. (6, 8)].

Immunohistochemistry assays using an anti-FMRFamide serum have evidenced the expression of a diversity of RFamide-like peptides in the five classes of mollusks through a variety of nervous structures and peripheral tissues. After the initial studies demonstrating the widespread distribution of FMRF-amide immunoreactivity in perikarya and nerve fibers in the central and peripheral nervous system of the pond snail *L. stagnalis* (29, 30), presence of FMRFamide immunoreactivity was investigated in a significant collection of mollusks. In the bivalve *Placopecten magellanicus,* immunoreactivity was not only concentrated in the cerebral, pedal, and parietovisceral ganglia but was also equally localized in the peripheral organs, including the gut and gills of juveniles and adults (31). In *Dreissena polymorpha,* immunoreactivity was observed in all nervous ganglia and in the neuromuscular system associated to the siphon and mantle (32). In the gastropod *Helix aspersa*, immunoreactivity revealed a highly developed peripheral nervous system where nerves containing FMRFamide were associated to the muscular fibers of the reproductive or digestive tracts (33, 34). In the cephalopod *Sepia officinalis*, immunoreactive neurons were detected in the CNS at the olfactory and basal-dorsal lobe of the supra-esophageal brain mass as well as in the vicinity of the optic gland (35). In this species, FMRFamide-containing motoneurons innervating the fin chromatophore muscles are localized to the posterior chromatophore and fin lobes in the posterior subesophageal mass of the brain (36). The occurrence of FLPs during mollusk development was also observed in the sensory system of chiton larvae (polyplacophores) (37) or during the development of the cerebral system, the visceral loop, and the buccal nerve cords in scaphodod larvae (38). However, the use of FMRFamide antisera recognizing the antigenic major determinant RFamide likely covers the detection of different sets of peptides.

Different FLPs including FaRPs have been characterized by HPLC separation and amino acid sequencing by Edman degradation. Besides the tetrapeptide identified in several gastropod or cephalopod species, other peptides defined as FaRPs have been described; FaRPs include all the peptides with the C-terminal consensus sequence $X_{1\times2}$RFamide where X_1 is an aromatic amino acid Phenylalanine (F), Tryptophane (W), or Tyrosine (Y) and X_2 a hybrophobic amino acid Phenylalanine (F), Methionine (M), Isoleucine (I), or Leucine (L) (39). Thus, the pentapeptides AFLRFamide and TFLRFamide were identified from immunoreactive fractions of *Octopus vulgaris* vena cava (14). The heptapeptides SDPFLRFamide and GDPFLRFamide were identified from immunoreactive fractions in *L. stagnalis* (40) but also in *H. aspersa* where other heptapeptides (pQDPFLRFamide, NDPYLRFamide, and SEPYLRFamide) were isolated (13, 16). The first decapeptide (AdLAGDHFFRFamide) related to FaRPs was purified from *Mytilus edulis* based on its excitatory effect on the anterior byssus retractor muscle (ABRM) (20). This decapeptide has a D-leucine in position 2 that does not appear essential for biological activity of the peptide on ABRM (20). Surprisingly, in the venom of *Conus spurius*, two singular peptides inducing hyperactivity syndrome in mouse have been identified. The first one (GPMGWVPVFYRFamide) belongs to the FaRPs family (26), and the second one exhibits two gamma-carboxyglutamates and an IIRIa C-terminal sequence (GPMγDPLγIIRIa, with γ = gamma-carboxyglutamate) (41).

Table 1 | Diversity of FMRFamide-related peptides or FaRPs in mollusks.

Peptide sequence	Species	Reference
FMRFa	*Macrocallista nimbosa*	(5)
pQDPFLRFa	*Helix aspersa*	(13)
AFLRFa	*Octopus vulgaris*	(14)
TFLRFa	*Octopus vulgaris*	(14)
FLRFa	*Lymnaea stagnalis*	(15)
SFMRFa	*Lymnaea stagnalis*	(15)
NDPFLRFa	*Helix aspersa*	(16)
NDPYLRFa	*Helix aspersa*	(16)
SEPYLRFa	*Helix aspersa*	(16)
GDPFLRFa	*Lymnaea stagnalis*	(17)
SDPFFRFa	*Lymnaea stagnalis*	(18)
SDPFLRFa	*Lymnaea stagnalis*	(17)
SDPYLRFa	*Lymnaea stagnalis*	(17)
pQFYRFa	*Helix aspersa*	(19)
ENNNGYIRFa	*Helix aspersa*	(19)
*SYGWAEGDTTDNEYLRFa	*Helix aspersa*	(19)
AdLAGDHFFRFa	*Mytilus edulis*	(20)
DPFLRFa	*Helix pomatia*	(21)
pQGDTADNEYLRFa	*Helix pomatia*	(21)
SKPYMRFa	*Lymnaea stagnalis*	(22)
ALTNDHELRFa	*Fusinus ferrugineus*	(23)
PYMRFa	*Lymnaea stagnalis*	(18)
HDYMRFa	*Lymnaea stagnalis*	(18)
FIRFa	*Sepia officinalis*	(24)
ALSGDAFLRFa	*Sepia officinalis*	(24)
*NFLRFa	*Mytilus edulis*	(25)
GPMGWVPVFYRFa	*Conus spurius*	(26)
*ALAGDGFLRFa	*Lottia gigantea*	(27)
NFGEPFLRFa	*Haliotis asinina*	(28)
TLAGDSFLRFa	*Haliotis asinina*	(28)
*FDSYEDKAYLRFa	*Haliotis asinina*	(28)

*Peptide sequences: *, only predicted by cDNA or EST; a, amide.*

With the characterization of cDNAs, the number of FaRPs identified in mollusks increased and gave a more rational basis for this diversity. The first mRNA encoding FaRP precursors was characterized in *A. californica* (42, 43) and revealed the occurrence of 28 copies of the FMRFamide sequence associated with a single copy of FLRFamide. Thereafter, several transcripts coding for multiple copies of the FMRFamide sequence were identified in other gastropods like *L. stagnalis* (17) and *H. aspersa* (19), in the bivalve *M. edulis* (25) and in cephalopods such as *S. officinalis* (24). In cuttlefish and squid, FMRFamide transcripts encode four identical FaRPs: three tetrapeptides (FMRFamide, FLRFamide, FIRFamide) and one decapeptide (ALSGDAFLRFamide) (44). In *O. vulgaris*, partial cDNA predicts the presence of the same ALSGDAFLRFamide decapeptide associated to an uncommon FMKFamide tetrapeptide (45). A common model of FMRFamide precursor can be defined based on the analysis of all of these tetrapeptide encoding transcripts (**Figure 2**; **Table 2**). It contains a unique tetrabasic site corresponding to a furin-processing site (RXK/RR) that typically separates the precursor into two domains: the N-terminal region encoding the FL/IRFamide peptides and a decapeptide, while the C-terminal domain harbors the FMRFamides. As a result, peptides from the two separate domains may presumably be sorted differentially into distinct secretory vesicles, as suggested for *L. stagnalis* FMRF gene products (46) and demonstrated for egg-laying prohormone gene products in *A. californica* (47) and *L. stagnalis* (48).

There exists a second transcript coding for FaRPs in some gastropods. In the pulmonate mollusks *L. stagnalis* (17) and *H. aspersa* (19), this second mRNA mostly codes for heptapeptides (**Table 2**) and represents an alternatively spliced transcript that only shares the 5′exon with the tetrapeptide type transcript. In *L. stagnalis* (18), *Ls*-FMRF1 mRNA includes exons E1 and E2 and *Ls*-FMRF2 mRNA exons E1, E3, E4, and E5 (**Figure 2**). Singularly, two mRNAs were also characterized in the gastropod *Haliotis asinana.* These mRNAs share the first exon coding for the signal peptide and appear to be splice variants of a single gene, but in contrast to *Lymnaea* alternative transcripts they both encode copies of FMRFamide and FLRFamide along with a small number of new peptides. These two FaRP transcripts are differentially expressed in the gastropod nervous system. In adult *H. aspersa* snails, tetraFaRP mRNA (*Ha*-FMRF1) is located primarily in the cerebral ganglia, whereas heptaFaRP mRNA (*Ha*-FMRF2) is expressed almost exclusively in the parietal ganglia (50). In *L. stagnalis*, the two alternative mRNA transcripts are expressed in the CNS in a mutually exclusive manner at the single cell level; as a result, the distinct sets of FaRPs that they encode are differentially distributed in defined neuronal networks (51). FaRPs are expressed early during embryo development and larval development in mollusks with indirect development. In *Lymnaea* embryos, the differential localization of FaRPs could be established with specific antibodies against EFLRIa expressed by tetraFaRP mRNA (*Ls*-FMRF1) or against the acidic spacer peptide expressed by heptaFaRPs mRNA (*Ls*-FMRF2). Thus, the first neurons in *Lymnaea* embryos co-express the two transcript products, but neurons differentiate rapidly during ganglion development by expressing peptides of only one type of transcript. Otherwise, abundant EFLRIa-immunoreactive cells (corresponding to *Ls*-FMRF1) have been observed in the lip, mantle, and foot of larvae, but no peripheral cells immunoreactive to antibodies raised against the acidic peptide (corresponding to an *Ls*-FMRF2 cleaved product) have been found (52). *In H. asinina*, the two transcripts are always co-expressed in the larval ganglia but as in *Lymnaea* larvae, *Has*-FMRF1 is expressed alone in the periphery of putative sensory cells of the foot (28). In the cephalopod *S. officinalis*, expression of FaRP-encoding mRNAs was also observed during embryogenesis in the nervous system from the early steps of organogenesis (stage 16). Wider FaRP expression was observed concomitantly with brain differentiation (around stage 22) (53).

In mollusks, as in other invertebrates, FaRPS are involved in a variety of physiological processes including regulation of circulation, neuronal activity, feeding, digestion, reproduction, and osmoregulation. After the initial discovery of the excitatory effect of FMRFamide on the heart of the clam *M. nimbosa* (5), the cardioexcitatory or the cardioinhibitory effects of FaRPs on the cardiac muscle were later confirmed in other bivalves (54). In

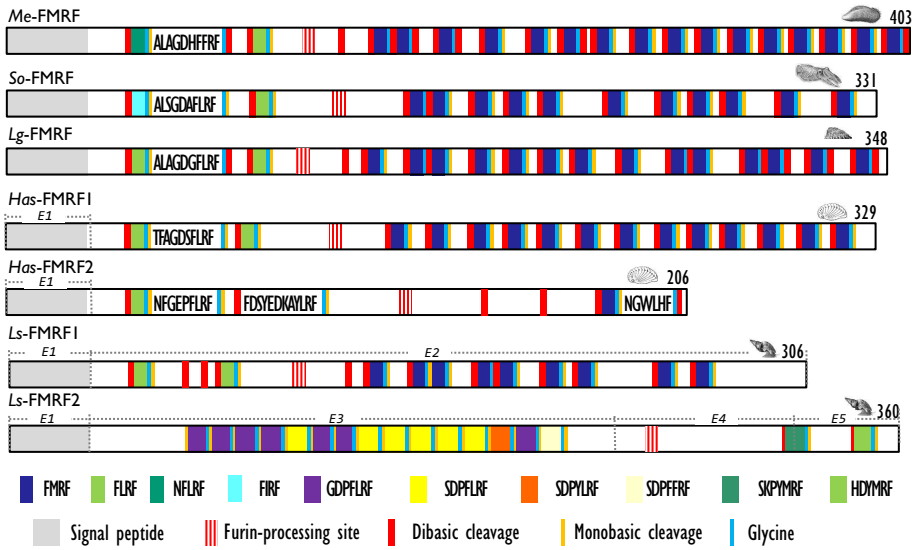

FIGURE 2 | Comparison of the linear schematic organization of FMRFa precursors in mollusks: *Me*-FMRF identified in *Mytilus edulis* (25), *So*-FMRF in *Sepia officinalis* (24), *Lg*-FMRF in *Lottia gigantea* (27), *Has*-FMRF1 and *Has*-FMRF2 in *Haliotis asinina* (28), and *Ls*-FMRF1 and *Ls*-FMRF2 in *Lymnaea stagnalis* (18). Signal peptides, proteolytic processing sites, and C-terminal glycines for amidation are indicated by distinct labels. For *Ls*-FMRF1 and *Ls*-FMRF2 precursors, the respective encoding exons are indicated as E1, E2, E3, E4, and E5.

Table 2 | FaRP precursors from different species of mollusks with indication of copy numbers for each peptide category.

Mollusk class	Transcript	Tetrapeptides			Pentapeptides	Hexapeptides	Heptapeptides	Non-apeptides	Decapeptides	Dodecapeptides	Heptadecapeptides	Reference
		FMRFa	FLRFa	FIRFa								
Gastropods	*Ac*-FMRF	28	1									(43)
	Ls-FMRF1	9	2									(18)
	Ls-FMRF2					1	16					(18)
	Ha-FMRF1	10	2		2							(19)
	Ha-FMRF2						13	1			1	(19)
	Has-FMRF1	13	2						1			(28)
	Has-FMRF2	1	1					1		1		(28)
	Lg-FMRF	14	2						1			(27)
Cephalopods	*So*-FMRF	11	1	1					1			(24)
	Lo-FMRF	11	1	1					1			(49)
	Lp-FMRF	11	1	1					1			(49)
	Ov-FMRF	7	1						1			(45)
Bivalves	*Me*-FMRF	16	2		1				1			(25)

Ac, Aplysia californica; Ls, Lymnaea stagnalis; Ha, Helix aspersa; Has, Haliotis asinana; Lg, Lottia gigantea; So, Sepia officinalis; Lo, Loligo opalescens; Lp, Loligo pealei; Ov, Octopus vulgaris; Me: Mytilus edulis.

gastropods, FaRPs induced contractions of the ventricles by triggering calcium release from internal pools (55). Besides their direct activity on the heart, FaRPs also play a role in the circulatory system by acting on vessels, as shown in *A. californica* where the FMRFamide inhibited aorta contractions in synergy with serotonin (56). FaRPs are also involved in the control of the feeding behavior in mollusks, as observed in other invertebrates and for the other FLPs in vertebrates [for a review, see Ref. (57)]. The FMRFamide inhibited motoneurons B15 and B16 but excited interneurons B4 involved in the control of the feeding behavior in *A. californica*

(58). FMRFamide affects the feeding system in other gastropods like *Lymnaea* and *Helisoma* where bath application of FMRFamide slowed down fictive feeding (59, 60). In *Lymnaea*, FMRFamide rapidly attenuated the rhythmic firing of buccal and cerebral ganglion neurons responsible for initiating and maintaining the repetitive motor outputs required for feeding (61). In addition, the tetrapeptide acts directly on muscles as described in *Aplysia.* FMRFamide modulate the activity of radula opener muscles in association with serotonin and myomodulins (62), and FaRPs play a role in digestion by modulating spontaneous gut activity or inducing contractions of the gizzard in *Aplysia* (63). In the gastropod *Helisoma*, the FMRFamide suppresses the activation of the salivary glands by directly acting on gland cells and on the effector neuron (neuron 4) (64). The effect on secretion activity was confirmed in scallop (*Pecten maximus),* where the FMRFamide exhibited a secretagogue effect on α-amylase secretion from the stomach-digestive gland complex (65). The FaRPs widely expressed in the CNS modulate neuronal activity. FMRFamide has a powerful inhibitory influence on bag cell neurons in *A. californica* by altering the properties of ion currents involved in the generation of action potentials and in the control of the resting potential (66). As bag cell neurons control egg-laying behavior, this effect on neurons can modulate a main physiological function like reproduction. Similarly, in the mollusk *L. stagnalis*, FMRFamide activates K+ currents that induce the inhibition of the caudodorsal neurons involved in the release of CDCH, the egg-laying hormone (ELH) (67). In cephalopods, FaRPs also appear to be involved in the control of reproduction regulation processes. In *Octopus,* the FMRFamide inhibits the activity of the optic gland involved in sexual maturity (45), whereas in cuttlefish, FaRPs control egg laying by directly regulating oocyte transport through the oviduct; the tetrapeptides FMRFamide and FLRFamide stimulate oviduct contractions, whereas FIRFamide and ALSGDAFLRFamide lower the tonus, the frequency, and the amplitude of the contractions and thus modulate them (68). FaRPs are also involved in specific functions in cephalopods. FMRFamide induces the contraction of chromatophore muscles involved in the control of body coloration patterning (69). The establishment of neural networks at the origin of this control was monitored during cuttlefish embryogenesis. FaRP staining throughout CNS development evidenced the implementation of neuronal networks involved in the control of coloration patterns. This suggests that the involvement of FaRPs in the chromatophore control pathway takes place early during embryonic development (53). In *Helisoma trivolvis*, the level of FaRPs in kidney, detected by radioimmunoassay, appeared to be lower in snails kept under hypoosmotic stress than in snails kept under isosmotic conditions, suggesting an involvement in gastropod osmoregulation (70).

FMRFamide-related peptides act synergistically with other mollusk-specific neuropeptides like APGWamide-related peptides. In *H. aspersa,* some cells contain both the APGWamide and FMRFamide; these cells may have dual projections in both the penial nerve and the nervus cutaneous pedalis primus dexter (71). In *Lymnaea*, different FaRPs and APGWamide have distinct actions on the penis retractor muscle; this demonstrates a complex peptidergic regulation of the male copulation behavior (72, 73). Together, these two tetrapeptides also modulate the feeding behavior in the gastropod mollusks *H. trivolvis* and *L. stagnalis* in association with other factors like biogenic amines (serotonin, dopamine, octopamine) (74). In the bivalve *M. edulis,* they act in synergy to modulate the contractile activity of the ABRM (75).

In mollusks, FaRPs appear to act via FMRFamide-gated sodium channels (FaNaC) and via G protein-coupled receptors. The first FaNaC was molecularly characterized in *H. aspersa* (76). Only gastropod FaNaCs have been characterized so far (77–79). FaNaC is a neuronal Na^+-selective channel that is directly gated by micromolar concentrations of FMRFamide and related tetrapeptides inducing a fast and partially desensitizing response, and it is thought to participate in peptidergic neurotransmission. The sensitivity of *L. stagnalis* FaNaC to acids suggests common ancestry with mammalian acid-sensing ion channels (ASICs) (80), strengthened by the study of Askwith and collaborators concerning the direct modulation of rat ASICs by FMRFamide (81). Among the variety of candidate G-protein-coupled receptors (GPCRs) encoded by mollusk genomes, no FaRP-specific GPCR has been functionally characterized yet. Receptor-binding assays in *H. aspersa* suggest the presence of two receptors, one for tetrapeptides and another for heptapeptides localized in the heart, the brain, the reproductive, and digestive systems (82, 83). In a structure–activity relationship study of FMRFmide analogs in *Lymnaea,* the occurrence of a single receptor was proposed and demonstrated that activation of this receptor requires the C-terminal RFamide sequence, whereas the N-terminal amino acids are involved in binding. This unique receptor mediates the transient hyperpolarizing response and the long-lasting depression of excitability of the neurosecretory caudo-dorsal cells (CDCs) (84). In the squid, *Loligo pealei*, a radio receptor assay stressed the critical role of the RFamide moiety for binding (85).

THE LFRFamide/SHORT NEUROPEPTIDE F-RELATED GENE

LFRFamide peptides (**Table 3**) have been identified later. The first heptapeptides GSLFRFamide and SSLFRFamide were discovered from their inhibitory activity on F2 neurons of the prosobranch gastropod *Fusinus ferrugineus* (23). In parallel, in the opisthobranch *A. californica*, GSLFRFamide peptides were characterized, along with two other LFRFamide peptides with the STLFRFamide and GGALFRFamide amino acid sequences, from their activities on the accessory radula closer (ARC) neuromuscular system (86).

For a long time, LFRFamide peptides were found only in gastropods, yet recent studies in cephalopods and oysters have evidenced this peptide family in other mollusk classes. In the cephalopod *S. officinalis*, two LFRFamide peptides (GNLFRFamide and TIFRFamide) were clearly characterized from LC-MS/MS analysis, with an experimental strategy based on the presence of the RFamide moiety (90). In oyster (*Crassostrea gigas),* the LFRF precursor was retrieved from the GigasDatabase [CU994925] (92). The first LFRFamide precursor was identified in the pulmonate snail *L. stagnalis* from its overexpression following parasitization by *Trichobilharzia ocellata* (93). The precursor contains one of several types of LFRFamide peptides in *L. stagnalis* (88) and *Lottia gigantea* (27) (**Figure 3**). Sometimes, several copies of the same peptide are present as in the case of the LFRF precursor (*Ac*-LFRF) isolated from *A. californica.* In the *Ac*-LFRF precursor, two copies of the GGALFRFamide are associated with

Table 3 | LFRFamide-related peptides.

Peptide sequence	Species	Reference
GSLFRFa	*F. ferrugineus*	(23)
	A. californica	(86)
	C. gigas	(87)
SSLFRFa	*Fusinus ferrugineus*	(23)
	C. gigas	(87)
STLFRFa	*A. californica*	(86)
GGALFRFa	*A. californica*	(86)
GGSLFRFa	*Lymnaea stagnalis*	(88)
GTLLRFGa	*Lymnaea stagnalis*	(88)
NTLFRFGa	*Lymnaea stagnalis*	(88)
QGSLFRFGa	*Lymnaea stagnalis*	(88)
TLFRFa	*L. stagnalis, A. californica*	(88, 89)
GAGTLFRFa	*Aplysia californica*	(89)
GNLFRFa	*Sepia officinalis*	(90)
TIFRFa	*Sepia officinalis*	(90)
*NSLFRFa	*Sepia officinalis*	(91)
*PHTPFRFa	*Sepia officinalis*	(91)
GALFRFa	*Crassostrea gigas*	(87)
SVDNEPTHPFRFa	*Crassostrea gigas*	(87)

*Peptide sequences: *, only predicted by cDNA or EST; a, amide.*

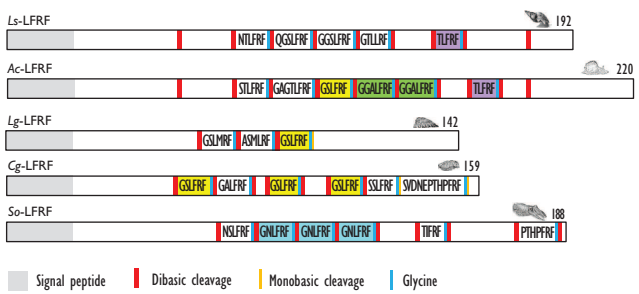

FIGURE 3 | Comparison of the linear schematic organization of LFRFa precursors in mollusks: *Ls*-LFRF identified in *Lymnaea stagnalis* (88), *Ac*-LFRF in *Aplysia californica* (86), *Lg*-LFRF in *Lottia gigantea* (27), *Cg*-LFRF in *Crassostrea gigas* (87), and So-LFRF in *Sepia officinalis* (91). Signal peptides, proteolytic processing sites, and C-terminal glycines for amidation are indicated by distinct labels.

one copy of the GSLFRFamide, TLFRFamide, STLFRFamide, and GAGTLFRFamide sequences (89) (**Figure 3**).

The characterization of the *S. officinalis So*-LFRFamide precursor revealed the presence of four different LFRF peptides with three copies of the GNLFRFamide associated to one copy of the TIRFamide, NSLRFamide, and PHTPFRFamide sequences (91) (**Figure 3**). The recent identification of LFRFamide peptides in the oyster *C. gigas* (87) confirms the widespread distribution of this peptide family in the mollusk phylum, with two of the bivalve peptides (GSLFRFamide and SSLFRFamide) previously identified in gastropods, particularly in *F. ferrugineus*. Similar to the *So*-LFRF precursor, the *Cg* precursor contains three copies of the GSLFRFamide peptide associated to one copy of the SSLFRFamide, GAFLRFamide, and SVDNEPTHPFRFamide sequences (**Figure 3**). That last sequence is located in the C-terminal moiety of the *Cg*-LFRFamide precursor and shares a common sequence with the last peptide (THPFRFamide) of the *So*-LFRFamide precursor.

Similar to FaRPs, LFRFamide peptides are involved in the regulation of main physiological functions. Studies on the localization of LFRFamide transcripts in *L. stagnalis* during parasitization show an expression in neurons in the buccal ganglia and in the cerebral ganglia and suggest an involvement in feeding and reproduction (88). Schistosome parasites adjust the physiology and behavior of their intermediate molluscan hosts to their own benefit by altering the expression of regulatory neuropeptides. The very close presence of LFRFamide-expressing neurons to *Lymnaea* CDCs and light green cells (LGCs), two cell clusters that regulate female reproduction, and growth and metabolism, respectively, together with the inhibitory activity of FLRFamide peptides on these cells suggest that the parasite induces LFRFamide gene expression to suppress host metabolism and reproduction (88). In cuttlefish, the myotropic activity of the GNLFRFamide peptide differs from that of FaRPs. Only the contractions of the rectum were modulated by this heptapeptide, but those of the oviduct were not, suggesting a role only in digestion-associated processes but not in reproduction (90). *In situ* hybridization (ISH) carried out in all parts of the cuttlefish CNS confirmed the involvement of LFRFamide peptides in feeding but also in learning and memory and in the control of body patterning (94, 95).

A recent study aimed at the functional characterization of a *C. gigas* short NPF like receptor (Cg-sNPFR-like) identified oyster LFRFamide family peptides as specific ligands (87). The *Cg*-sNPFR-like receptor was more abundantly expressed in the ganglia of females compared to males, and upregulated in starved oysters suggesting a role in reproduction and feeding. In the gonad area, highest receptor gene expression was observed at the start of gametogenesis, when glycogen storage activity is maximal. Oyster LFRFamide peptides are thought to play a role in the coordination of nutrition, energy storage, and metabolism through the *Cg*-sNPFR-like receptor by promoting storage at the expense of reproduction. The involvement of LFRFa in feeding and learning is very similar to sNPF in insects (96–98), further suggesting ancestral relationship The authors propose that mollusk FLRFamide peptides could represent functional orthologs of insect sNPFs (87).

THE LUQIN GENE

Using a differential screening strategy devoted to characterizing the neuropeptide-encoding transcripts specifically expressed in *A. californica* RF-amide immunoreactive L5 (left abdominal hemiganglion) neuron, a first precursor was characterized. L5 peptide precursor sequence predicts an RFamide decapeptide generated by signal peptide cleavage and by proteolytic cleavages at the Lys-Arg sequence. The C-terminal glycine of the resulting peptide is subsequently enzymatically converted into an amide group (99). A biochemical investigation showed that the processing of this precursor generates Luqin, a mature amidated decapeptide, together with a

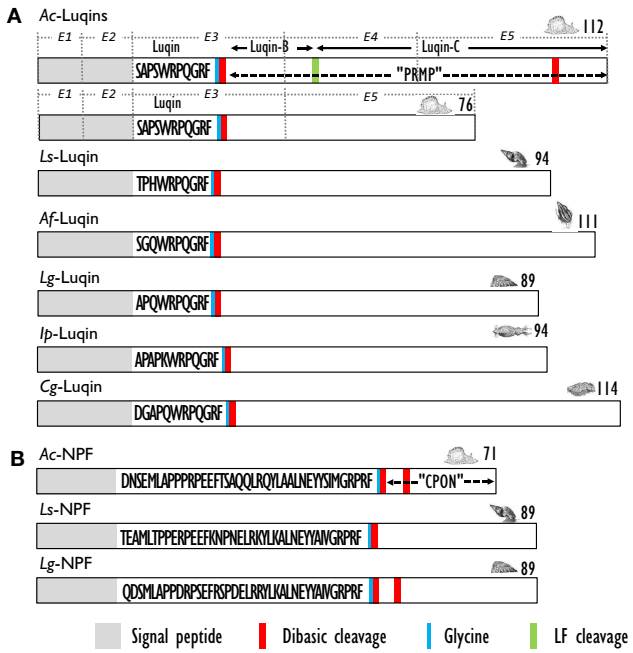

FIGURE 4 | (A) Comparison of the linear schematic organization of Luqin precursors in mollusks: *Ac*-Luqin identified in *Aplysia californica* (101), *Ls*-Luqin (LyCEP: *Lymnaea* CardioExcitatory Peptide) in *Lymnaea stagnalis* (104), *Af*-Luqin (ACEP: *Achatina* CardioExcitatory Peptide) in *Achatina fulica* (108), *Lg*-Luqin in *Lottia gigantea*, *Ip*-Luqin in *Idiosepius paradoxus*, and *Cg*-Luqin in *Crassostrea gigas* (27). **(B)** Comparison of the linear schematic organization of NPF precursors in gastropods: *Ac*-NPF in *A. californica* (109), *Ls*-NPF in *L. stagnalis* (93), and *Lg*-NPF in *Lottia gigantea* (27). Signal peptides, proteolytic processing sites, and C-terminal glycines for amidation are indicated by distinct labels. CPON, carboxy-terminal peptide of NPF; PRMP, proline-rich mature peptide. For *Ac*-Luqin precursors, the respective encoding exons are indicated as E1, E2, E3, E4, and E5.

C-terminal proline-rich mature peptide (PRMP) of 76 amino acids (100). That latter peptide is subsequently proteolytically processed to generate two products, Luqin-B and Luqin-C (101) (**Figure 4A**). Alternative splicing by exon skipping of the gene encoding this precursor results in the production of a shorter PRMP with a distinct C-terminal sequence due to a reading frame shift (102). The *A. californica* decapeptide was named Luqin because it is differentially expressed in LUQ (left upper quadrant) neurons. Luqin displays a high degree of identity with the formerly identified cardioexcitatory undecapeptide ACEP1 isolated from atria of the African giant snail *Achatina fulica* (103) and with LyCEP, a decapeptide from *L. stagnalis* CNS isolated from its ability to activate an orphan GPCR (104). In addition to sequences from gastropod mollusks such as *L. gigantea*, expressed sequence tags (ESTs) encoding Luqin precursors were also identified from bivalve and cephalopod mollusks that potentially yield structurally highly conserved mature luqin peptides with only the first three amino acids exhibiting conservative substitutions (27) (**Figure 4A**). Interestingly, annelids also express Luqin precursors with an analogous organization and a predicted mature peptide of very similar structure (105) (**Table 4**). Recent studies on the evolution of neuropeptide-signaling components in animals suggest the ancestral presence of Luqin in bilaterians. Luqin has been proposed to have evolutionary links with ecdyzoan RYamide and ambulacrian RWamide precursors, as they all harbor a proline-rich C-terminal peptide displaying a conserved domain including two cysteine residues (106, 107).

Table 4 | Luqin-like peptides.

Peptide sequence	Species	Reference
SGQSWRPQGRFa	*Achatina fulica*	(103)
SAPSWRPQGRFa	*Aplysia californica*	(100)
TPHWRPQGRFa	*Lymnaea stagnalis*	(104)
*APQWRPQGRFa	*Lottia gigantea*	(27)
*APAPKWRPQGRFa	*Idiosepius paradoxus*	(27)
*DGAPQWRPQGRFa	*Crassostrea gigas*	(27)

*Peptide sequences: *, only predicted by cDNA or EST; a, amide.*

Luqin was initially known to enhance the amplitude of the tetanic contraction of the heart ventricle, induce the tetanic contraction of the penis retractor muscle in response to electrical stimulation *in A. fulica*, or enhance the phasic contraction of the ABRM produced by repetitive electrical stimulation in *Mytilus* (103). Besides its activity as myoactive or cardioactive peptide, Luqin functional involvement was also inferred from the pattern of expression of peptide precursors or receptor transcripts by ISH or by immunocytochemical detection (ICC) of mature Luqin peptides using specific antibodies.

Luqin-encoding transcripts or mature Luqin peptides appear to be distributed in each of the central ganglia in around 20 neurons in *A. fulica* (108) and approximately 100 neurons including LUQ cells in *A. californica* (110). In *L. stagnalis*, prominent immunoreactivity was mainly found in neurons in the pedal ganglia (104). In this species, the caudodorsal cells that produce ELHs are the predominant site of Luqin- (LyCEP-) specific receptor gene expression and appear to be innervated by LyCEP-containing fibers involved in their inhibition (104). Thus, Luqin seems to inhibit the egg-laying system in that snail. In *L. stagnalis* peripheral tissues, Luqin immunoreactivity was detected in fibers ending blindly at the pericardial lumen (104). In *A. californica*, Luqin distribution was consistent with each LUQ cell sending its major axon through the pericardial nerve to innervate the kidney and the renal pore, a sphincter that controls urine efflux (111). Immunoreactive fibers were detected in specific regions of the circulatory system, such as the auricle, the ventricle, the cristae, and the anterior aorta, in the reproductive system (especially in the genital ganglion), the large and small hermaphrodite ducts, and the ovotestis (110). Intestine and kidney also display immunoreactivity in widely ramifying varicose fibers (110). In kidney, both Luqin transcripts and gene products were detected in neurites located in a large nerve associated with muscles inside the renal pore (112). Besides the conventional transcript, an alternatively spliced Luqin transcript appears to be specifically transcribed in peripheral neurons located in or in the vicinity of the kidney. Its gene products may locally play a physiological role by complementing or antagonizing the action of Luqin/PRMP peptides (102). Altogether, these data suggest a role of Luqin in the control of reproduction. Given the involvement of the target organs in fluid mobilization, absorption, and

secretion, Luqin is also probably involved in the regulation of water and/or salt balance in mollusks.

THE NEUROPEPTIDE F (NPF) GENE

The first biochemical evidence for mollusk neuropeptide Y (NPY) homologs was provided by the amino acid sequence of a peptide isolated by means of a radioimmunoassay using an antibody raised against the C-terminal hexapeptide amide (LTRPRYamide) of mammalian pancreatic polypeptide (PP), in extracts of circumesophageal ganglia of the garden snail, *H. aspersa* (113). The 39 amino acid *Helix* peptide displayed significant homology with the vertebrate NPY/PP superfamily and with the first characterized invertebrate NPY homolog identified from the tapeworm *Monieza expansa* (114). Similar to its flatworm relative, the *Helix* C-terminal tyrosine-amide peptide (Ya) is substituted for a phenylalanine-amide peptide (Fa), so it was called NPF. Soon after the initial characterization of *Helix* NPF, *A. californica* NPF, a 40 amino acid long peptide, was isolated from the abdominal ganglia by using its distinctive, prolonged inhibitory effect on neurons L3 and L6. Next, the cDNA encoding its precursor protein was cloned and sequenced (115). *A. californica* NPF prohormone revealed an organization similar to vertebrate NPY precursor proteins; it had a single copy of NPF immediately following the signal peptide, and separated from the carboxy-terminal peptide of NPF (CPON) by a GKR sequence serving as combined amidation and prohormone convertase proteolytic signals (**Figure 4B**). At the same time, a nonapeptide with high homology with the C-terminal end of the other molluscan NPFs was purified from brain extracts of squid (*Loligo vulgaris*) (116). This peptide may represent a processed form of an authentic NPF peptide whose N-terminally truncated forms were also later characterized in other animal phyla [for a review, see Ref. (117)]. In *L. stagnalis*, a partial cDNA encoding an NPF prohormone was cloned by differential screening of a CNS-specific cDNA library of snails parasitized with *T. ocellata* (93) (**Figure 4B**). Then, the mature NPF peptide was structurally characterized after its purification to homogeneity using a reverse endocrinology approach. This method is based on the ability of fractionated brain extracts to induce changes in cAMP levels following the activation of an *L. stagnalis* orphan GPCR (GRL105) expressed in CHO cells (118). This receptor shares structural and functional similarity with vertebrate NPY-receptors and currently represents the only functionally characterized NPF receptor in mollusks. That a typical NPF could be isolated from the venom of the cone snail *Conus betulinus* (119) was somewhat intriguing though neuroendocrine-like peptides used for prey catching or defense have already been described (120). Completion of mollusk genome and transcriptome sequencing projects (91, 92) has now paved the way toward the identification of novel NPFs and their putative receptors. NPF members from owl limpet *L. gigantea* (27), pygmy squid *Idiosepius paradoxus* (121), and Pacific oyster *C. gigas* (87) have been identified (**Table 5**). In *C. gigas*, bioinformatics investigation of genome and transcriptome databases yielded two additional NPY sequences with a C-terminal tyrosine-amide instead of phenylalanine-amide (personal communication). All mollusk NPFs display a high degree of identity, especially among the last 20 C-terminal residues. They also have a conserved N-terminal pattern of proline residues probably involved in the formation of a polyproline helix proved essential for the three-dimensional structure of vertebrate NPYs (122).

Expression of NPF in these different species was investigated by ISH, mass spectrometry, and ICC using antibodies specific to each mollusk NPF or antibodies raised against vertebrate NPY/PP peptides, though in this later case, a cross-reaction with other FLPs cannot be discarded.

In *L. stagnalis*, ISH and whole-mount ICC studies revealed neurons expressing the NPF gene and peptide in all ganglia of the CNS, except the two buccal ganglia that innervate the buccal mass. In the visceral ganglion, one NPY-positive neuron projects its axon into the nervus intestinalis, which also innervates the reproductive tract including the accessory sex glands. In the CNS, NPF-positive axons run as a circular band through all ganglia passing the commissures connecting the ganglia. A close association was also found between NPF-positive axons and axons from ovulation hormone-producing neurons and molluscan insulin-like peptide-producing neurons involved in the regulation of growth (123). A subset of anterior lobe neurons expressing the gene encoding the APGWamide peptide was also shown to co-express NPF mRNA and peptide (124). These cells project via the penis nerve to the penial complex and play an important role in the control of male copulating behavior. NPF immunoreactivity was also detected in axons innervating the penis retractor muscle (124).

Aplysia californica NPF-encoding mRNAs are also widely expressed in the CNS, very abundantly in the abdominal ganglion, to a lesser extent in the pleural-pedal ganglia, and at much lower levels in the cerebral ganglion. In the abdominal ganglion, *A. californica* NPF coexists with ELH and other peptides processed

Table 5 | NPF-related peptides.

Peptide sequence	Species	Reference
YAIVARPRFa	*Loligo vulgaris*	(116)
STQMLSPPERPREFRHPNELRQYLKELNEYYAIMGRTRFa	*Helix aspersa*	(113)
DNSEMLAPPPRPEEFTSAQQLRQYLAALNEYYSIMGRPRFa	*Aplysia californica*	(115)
TEAMLTPPERPEEFKNPNELRKYLKALNEYYAIVGRPRFa	*Lymnaea stagnalis*	(118)
*MFAPPNRPAEFKSPEELRQYMKALNEYYAIVGRPRFa	*Idiosepius paradoxus*	(121)
*QDSMLAPPDRPSEFRSPDELRRYLKALNEYYAIVGRPRFa	*Lottia gigantea*	(27)
*NDSLLPPNRPSRFSSPGQLRQYLKALNDYYAIVGRPRFa	*Crassostrea gigas*	(87)

*Peptide sequences: *, only predicted by cDNA or EST; a, amide.*

from its precursor in bag cell neurons involved in the control of egg-laying behavior (115). In a study investigating the role of NPF in the control of feeding, whole-mount ISH and immunocytochemistry localized NPF mRNAs and peptides in several cerebral somata and in only one buccal neuron in the *A. californica* feeding system (109). NPF was present in fibers in all major connectives and especially in buccal ganglion afferent fibers originating in the gut-innervating esophageal nerve, a nerve involved in satiation and in the generation of egestive programs (109).

The widespread distribution of NPF immunoreactivity in the CNS and in a variety of target organs of *H. aspersa* was also demonstrated by ICC using antibodies specific to Helix NPF or vertebrate NPY (125). In *O. vulgaris*, ICC studies evidenced NPF immunoreactivity in the optic lobe and peduncle complex. In the optic gland, an endocrine organ that controls gonad maturation (126), NPF was detected in varicose fibers, suggesting a role in optic gland regulation (127).

The widespread and high expression of NPF in the CNS and the variety of target cells and organs innervated by NPF-containing fibers in mollusks emphasize the involvement of NPF signaling in the control of a number of physiological processes. In mollusks, the biological function of NPF has mainly been investigated in the two model species *L. stagnalis* and *A. californica*.

In *L. stagnalis*, the gene encoding NPF is upregulated in *T. ocellata*-parasitized animals (93). A role of NPF in the control of the energy flow was thus assumed. Indeed, parasites selectively interfere with neuroendocrine mechanisms that regulate the main determinants of the energy budget in the host's reproduction and growth processes (128). NPF injection or implantation of slow-release peptide pellets in non-parasitized snails resulted in suppressed reproductive activity and reduced growth in a dose- and time-dependent manner (123). This activity appeared reminiscent of the well-known role of NPY in the regulation of the energy flow in vertebrates (129) but singularly differed by not affecting food intake (130). By contrast, NPF injection produced satiation-like effects in *A. californica*, i.e., a reduced meal-size and slower ingestion. NPF released from the esophageal nerve affects the feeding behavior; it modifies the feeding central pattern generator, a neuron network in the buccal ganglion, by eliciting egestive responses (109). Altogether, the distribution and the activity of NPF in mollusks point to a role in the coordination of the energy balance via the control of energy-consuming processes like growth and reproduction or possibly via the control of food intake. An NPF-related receptor-encoding mRNA was recently found significantly upregulated in oyster (*C. gigas*) lines with reduced reproductive investment and increased potential for energy production (131). Although care should be paid until the actual ligand of this receptor is identified, this result also suggests the control of NPF signaling in bivalve mollusk reproduction and energy management.

Table 6 | CCK/SK-like peptides.

Peptide sequence	Species	Reference
*FDYNFGGGRWa *QGAWDYDYGLGGGRFa	*Lottia gigantea*	(106)
NYGEYGFGGGRFa *QGAWSYDYGLGGGRFa	*Haliotis diversicolor*	(106)
*SYGDYGIGGGRFa *QGAWSYDYGLGGGRFa	*Aplysia californica*	(106)

*Peptide sequences: *, only predicted by cDNA or EST; a, amide.*

THE CHOLECYSTOKININ/SULFAKININ-RELATED GENE

A recent extended comparative survey of peptide signaling systems in bilaterians demonstrated the early evolutionary origin of the CCK/SK signaling system (106). In this study, *in silico* data-mining

Table 7 | Functional involvement of RFamide-like peptides in the three main mollusk classes: B, Bivalves; C, Cephalopods; G, Gastropods.

	FaRPs		LFRF	Luqin	NPF
	Tetrapeptides	Hexapeptides			
Circulation/heart activity	B (5, 54) G (40, 55, 139)	G (16, 40, 55)		G (103, 104)	
Feeding	G (58–62, 74)		G (86, 88) C (94) B (87)		G (109)
Digestion	G (63, 64) B (65)	G (64)	C (90)		
Reproduction	G (34, 72, 84, 140) C (45, 68)	G (34, 72)	G (88) B (87)	G (103, 104, 110)	G (115, 123, 124)
Osmoregulation	G (70)	G (70)		G (102)	
Neuronal activity	G (66, 67)			G (110–112)	
Chromatophore activity	C (69)		C (95)		
Energy balance			B (87)		G (128)

of *Haliotis diversicolor, A. californica, and L. gigantea* sequence resources revealed the existence of peptides with the following consensus sequence: X(1-6)(D/E)Y(G/N)(L/F/I)GGGR(F/W)-amide. These mollusk CCK/SK peptides exhibit the C-terminal RF(W)amide sequence common to insect SKs and the DY motif shared by both insect SKs and vertebrate CCKs (**Table 6**). Whether mollusk peptides are sulfated remains to be investigated. As vertebrate CCKs and insect SKs reveal similar biological functions with respect to digestive enzyme secretion, satiety (food intake), and smooth muscle contraction (132), we could expect their mollusk counterparts to have retained these basic biological activities. In this respect, it is interesting to note that FMRFamide and FLPs, including insect SKs, elicit potent stimulation of the release of the digestive enzyme α-amylase from cell suspensions of the stomach-digestive gland complex of scallop (*P. maximus*) (65, 133, 134).

CONCLUSION

FMRFamide-like peptides exert pleiotropic activities and mediate a variety of physiological and behavioral processes in mollusks (**Table 7**). Interestingly, mollusks with diverse and well-defined habitats, life styles, and behaviors do not seem to exhibit significantly marked complexity levels in their FLP repertoires. This probably means that behavioral variety and adaptation to environmental cues result not only from the structural diversity of neuropeptide components but also from the intricacy of the spatiotemporal and stimulus-dependent patterns of expression of their encoding genes. Most of the functional assignments of this family of neuropeptides were obtained from the key model species *A. californica* and *L. stagnalis* because they have a CNS with large and reproducibly identified neurons, readily accessible and amenable to *in vivo* or *in vitro* handling. Since the activity of these neurons can also be correlated with physiological states or behaviors, establishing functional circuitries has become feasible in these species (61, 109, 135). As a result, a specific neuropeptide distribution within the CNS strongly infers its physiological involvement (**Figure 5**). Although sequence information is still lacking about so far completely understudied mollusk classes, such as monoplacophora, placophora, aplacophora, and scaphopoda, substantial genomic data are now becoming available in economically important classes of mollusks such as bivalves (91, 92) and cephalopods (136, 137). Such data offer the opportunity to investigate new mollusk models. These model species often have a compact and tiny CNS, so the methodologies developed for *A. californica* and *L. stagnalis* do not apply. Therefore, access to the neuropeptide-encoding gene repertoire of these non-conventional model species will necessarily require the improvement of currently operating post-genomic functional tools such as RNA interference (138) to succeed in deciphering their biological function.

In the field of neuroendocrinology, sequence data will become crucial for unraveling the complexity of signaling systems in an evolutionary context; genome-wide analysis gives access to the complexity of the repertoire of GPCRs, which constitute the main molecular targets of neuropeptides. In parallel, exhaustive interaction between bioinformatics and mass spectrometry analyses makes the molecular identification of most mature neuropeptides produced in a given species accessible (143). In the context of FLPs, gaining insight into the structure of the mature peptides appears crucial. Indeed, some peptides like CCK/SKs might be subjected to post-translational sulfatation. Others, like NPFs, are presumed to produce distinct truncated forms, with potentially distinct biological properties that derive from the non-conventional processing of a precursor protein (116, 144). The grouping of RFamide peptides within a same family might be somewhat misleading and restrictive, especially when considering the evolutionary links between the deuterostome and protostome NPY/F signaling systems, or between the Luqin and insect RYamide signaling systems (106, 107). Phenylalanine and tyrosine display very close physicochemical properties and the shift from one residue to the other only results from a single nucleotide mutation. We suggest that mollusk neuropeptides with the C-terminal RYamide might be included. These include the newly LFRYamide peptide family identified in *Lottia* (27) or neuropeptide KY (NKY) with the C-terminal RYamide initially identified in *Aplysia* (145) and also via the identification of ESTs encoding NKY precursors in a variety of mollusks (27). In other respects, sharing a common C-terminal RFamide moiety does not entail that the peptides are functionally

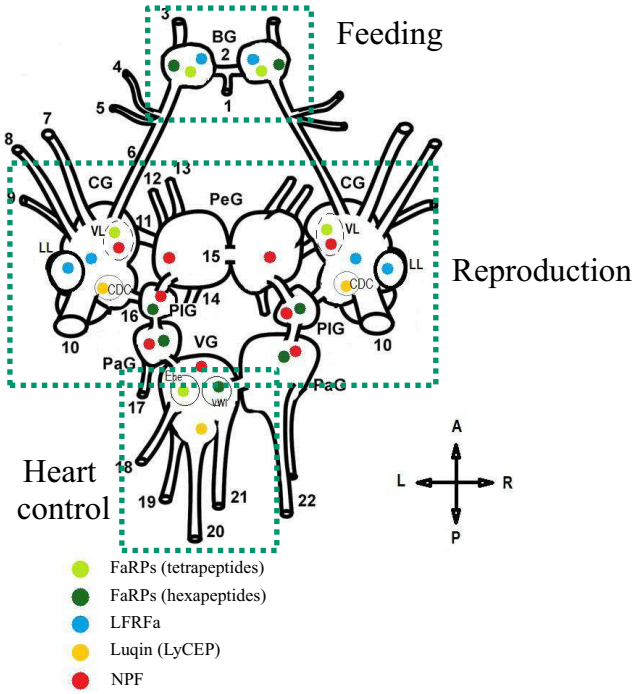

FIGURE 5 | Schematic representation of the distribution and the role of distinct RFamide-like peptides (FLPs) in the central nervous system (dorsal view) of *Lymnaea stagnalis*. BG, buccal ganglion; CG, cerebral ganglion; PeG, pedal ganglion; PlG, pleural ganglion; PaG, parietal ganglion; VG, visceral ganglion; VL, ventral lobe; LL, lateral lobe; CDC, caudo-dorsal cell. Nerves: 1, postbuccal; 2, buccal commissure; 3, dorsobuccal; 4, laterobuccal; 5, ventrobuccal; 6, cerebro-buccal connective; 7, superior lip; 8, median lip; 9, tentacular; 10, cerebral commissure; 11, cerebro-pedal connective; 12, superior medial; 13, median pedal; 14, inferior pedal; 15, pedal commissure; 16, cerebro-pleural connective; 17, left parietal; 18, cutaneous; 19, anal; 20, intestinal; 21, genital; 22, right parietal. Ehe neuron and the visceral white interneuron (VWI) [Adapted from Ref. (72, 104, 130, 141, 142)].

or evolutionarily related. Thus, the major challenge will be to get further insight into the evolution and the functional assignment of these various FLPs, especially by focusing on poorly studied mollusk classes. One way of reaching this goal will rely on the characterization of ligand/receptor pairs thanks to the development of methodologies proven efficient in mollusks (87, 104, 118), and in other animal phyla (146–149). Such an approach was efficient to demonstrate the ancestral functional relationship between mollusk LFRFamide and insect sNPF signaling pathways (87). This indicates that sequence homology or disparity alone is not sufficient to ascribe evolutionary relatedness. Within a wide comparative context, the functional characterization of FLP/receptor couples in a variety of bilaterian or eumetozoan species will obviously help define the orthologies between the genes encoding the FLP signaling components. Then, functional and synteny analyses of these orthologous genes might generate unique data to propose convincing scenarios on the evolution of this complex family of neuropeptides in Eumetazoa.

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Family of FLP peptides in *Caenorhabditis elegans* and related nematodes

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Neuropeptides regulate all aspects of behavior in multicellular organisms. Because of their ability to act at long distances, neuropeptides can exert their effects beyond the conventional synaptic connections, thereby adding an intricate layer of complexity to the activity of neural networks. In the nematode *Caenorhabditis elegans*, a large number of neuropeptide genes that are expressed throughout the nervous system have been identified. The actions of these peptides supplement the synaptic connections of the 302 neurons, allowing for fine tuning of neural networks and increasing the ways in which behaviors can be regulated. In this review, we focus on a large family of genes encoding FMRFamide-related peptides (FaRPs). These genes, the *flp* genes, have been used as a starting point to identifying *flp* genes throughout Nematoda. Nematodes have the largest family of FaRPs described thus far. The challenges in the future are the elucidation of their functions and the identification of the receptors and signaling pathways through which they function.

Keywords: neuropeptides, neural circuits, behavior, nematodes, worms

INTRODUCTION

Behavior in all animals is the output of multiple neural networks, whose individual circuits are activated, inhibited, or modulated at any one time by the combinatorial action of many neurotransmitters. In recent years, there has been a push to determine the neural connectome of organisms, including the human connectome (Human Connectome Project). The first completed neural connectome was that of the nematode *Caenorhabditis elegans* (1). In a heroic effort and with much foresight into the usefulness of the project, John White and collaborators embarked on deciphering the entire neural circuitry and mapping all chemical synapses and gap junctions between the 302 neurons of the *C. elegans* adult hermaphrodite (1); more recently, Scott Emmons and David Hall have initiated work on the *C. elegans* male neural connectome (2). The mapping of all the synaptic connections between neurons has been highly informative and helpful to researchers. However, the neural connectome, while representing a large fraction of neural network activity, underestimates the complexity of the nervous system. Layered on top of the connectome is the action of hormones and neuropeptides, whose effects can be exerted over large distances and whose actions, therefore, are not wholly represented by the neural connectome. Hence, hormones and neuropeptides add considerable complexity to neural networks to affect behavior.

In *C. elegans* and other invertebrates, neuropeptides, which are short sequences of amino acids, can act as primary transmitters as well as neuromodulators. Most neurons in *C. elegans* express at least one neuropeptide gene and in the majority of neurons, neuropeptides are co-localized with classical small molecule transmitters, such as acetylcholine, GABA, serotonin (5-HT), and dopamine (3, 4). Based on BLAST and bioinformatic screening, two major families of neuropeptides emerged in *C. elegans*: insulin-like peptides (ILPs) (5–9) and FMRFamide-related peptides (FaRPs) (10–12). In addition, *C. elegans* also has a large complement of non-insulin, non-FaRP neuropeptides (NLPs), many of which have invertebrate and vertebrate orthologs (3, 13). In this review, we will focus on the *C. elegans* FaRPs, which, by definition, all share a C-terminal Arg-Phe-NH_2 and which are more commonly referred to as FLPs in *C. elegans*. We will describe their processing and some of their functions, and compare the diversity of FLPs in other nematodes.

IDENTIFICATION OF *flp* GENES

By cDNA library screening, bioinformatic searches, and genome/transcriptome data mining for proteins with Arg-Phe-Gly sequences flanked by N- and C-terminal tri-, di-, or mono-basic residues, we and others identified 31 genes encoding FLPs in *C. elegans* (10, 12). Each *flp* gene encodes a unique set of FLPs for a total of 71 possible distinct FLPs (**Table 1**) (10, 12). Fourteen *flp* genes encode a single FLP, although seven of these genes encode multiple copies of the same FLP [*flp-6* (6 copies of KSAYMRF-NH_2), *8* (3–4 copies of KNEFIRF-NH_2), *9* (2 copies of KPSFVRF-NH_2), *10* (1 copy of QPKARSGYIRF-NH_2), *12* (1 copy of RNKFEFIRF-NH_2), *14* (4 copies of KHEYLRF-NH_2; **Figure 1A**), *20* (2 copies of AMMRF-NH_2), *21* (1 copy of GLGRPLRF-NH_2), *22* (3 copies of SPSAKWMRF-NH_2), *24* (1 copy of VPSAGDMMVRF-NH_2), *27* (1 copy of GLGGRMRF-NH_2), *28* (1 copy of APNRVLMRF-NH_2), *32* (1 copy of AMRNSLVRF-NH_2), *33* (1 copy of APLEGFEDMSGFLRTIDGIQKPRF-NH_2), and *34* (1 copy of ALNRDSVASLNNAERLRF-NH_2)]. At least nine *flp* genes encode non-FLP peptides [*flp-1*, *3* (**Figure 1A**), *7*, *11*, *20*, *23*, *26*, *27*, and *34*] and six *flp* genes (*flp-1*, *2*, *8*, *11*, *22*, and *23*) are alternatively spliced (WormBase). The organization of *flp* genes ranges from the

Table 1 | Predicted FLP neuropeptides in *C. elegans*.

***flp-1* IV**	***flp-7* X**	***flp-15* III**	***flp-24* III**
*SADPNFLRFG	x3 *SPMQRSSMVRFG	*GGPQGPLRFG	*VPSAGDMMVRFG
*SQPNFLRFG	x2 *TPMQRSSMVRFG	*RGPSGPLRFG	
*ASGDPNFLRFG	SPMERSAMVRFG		***flp-25* III**
*SDPNFLRFG	SPMDRSKMVRFG	***flp-16* II**	DYDFVRFG
*AAADPNFLRFG	SSIDRASMVRLG	x2 *AQTFVRFG	*ASYDYIRFG
*(K)PNFLRFG		*GQTFVRFG	
AGSDPNFLRFG	***flp-8* X**		***flp-26* X**
(K)PNFMRFG	x3 *KNEFIRFG	***flp-17* IV**	*(E)FNADDLTLRFG
		x2 KSAFVRFG	*GGAGEPLAFSPDMLSLRFG
***flp-2* X**	***flp-9* IV**	KSQYIRFG	NYYESKPY
*SPREPIRFG	x2 *KPSFVRFG		
LRGEPIRFG		***flp-18* X**	***flp-27* II**
	***flp-10* IV**	*DFDGAMPGVLRFG	*(EASAFGDIIGELKGK)-
***flp-3* X**	QPKARSGYIRFG	*EMPGVLRFG	GLGGRMRFG
SPLGTMRFG		x3 *(K)SVPGVLRFG	*pQPIDEERPIFME
*TPLGTMRFG	***flp-11* X**	*EIPGVLRFG	
*EAEEPLGTMRFG	*AMRNALVRFG	*SEVPGVLRFG	***flp-28* X**
NPLGTMRFG	*ASGGMRNALVRFG	*DVPGVLRFG	*APNRVLMRFG
*ASEDALFGTMRFG	*NGAPQPFVRFG		
EDGNAPFGTMKFG	NGAPQPFG	***flp-19* X**	***flp-32* X**
*SAEPFGTMRFG		*WANQVRFG	*AMRNSLVRFG
*SADDSAPFGTMRFG	***flp-12* X**	*ASWASSVRFG	
*NPENDTPFGTMRFG	*RNKFEFIRFG		***flp-33* I**
		***flp-20* X**	*APLEGFEDMSGFLR-
***flp-4* II**	***flp-13* IV**	x2 AMMRFG	TIDGIQKPRFG
PTFIRFG	*AMDSPLIRFG	AVFRMG	
*ASPSFIRFG	x2 *AADGAPLIRFG		***flp-34* I**
	x2 *APEASPFIRFG	***flp-21* V**	ALNRDSVASLNNAERLRFG
***flp-5* X**	*ASPSAPLIRFG	GLGPRPLRFG	ADISTFASAINNAGRLRYG
*GAKFIRFG	*SPSAVPLIRFG		
*AGAKFIRFG	*SAAAPLIRFG	***flp-22* I**	†Additional FLPs:
*APKPKFIRFG	*ASSAPLIRFG	x3 *SPSAKWMRFG	APNRVLMRLVRF-NH_2
			HFYNFSSESRKPNFLRF-NH_2
***flp-6* V**	***flp-14* III**	***flp-23* III**	KPXPXFIRF-NH_2
x6 *KSAYMRFG	x4 *KHEYLRFG	*VVGQQDFLRFG	
		NDFLRFG	
		EGHPEFMSQIAPP?	

Chromosomal location indicated after gene name. Blue indicates common amino acids among peptides encoded by the same gene; the C-terminal glycine donates an amide during amidation. Green indicates a non-FLP peptide. The number of copies of an encoded peptide is indicated.

**Biochemically isolated. From Rosoff et al. (14), Marks et al. (15–19), Davis and Stretton (20), Li et al. (11), Nelson et al. (10), Husson et al. (21, 22), Husson and Schoofs (23), Husson et al. (24).*

†N. Marks, A. Maule, and A. Stretton, pers. comm.

simplest at only two small exons to the largest at six exons. Similarly, the FLP protein precursors are small, ranging from 66 to 184 amino acids, sizes that include the signal peptide; the bioactive peptides vary from 5 to 24 amino acids in length (**Table 1**).

PROCESSING OF PRE-PRO-NEUROPEPTIDE PRECURSORS

As with other neuropeptides (25), the FLPs are derived from pre-propeptides (**Figure 1**), which are large precursor molecules that must be proteolytically cleaved and post-translationally modified to yield the active peptides (**Figure 1B**). The FLP precursor molecules all have a signal peptide, which is removed to yield the propeptide. The propeptides are then cleaved by proprotein convertases (PCs), serine endoproteases that preferentially recognize dibasic residues and cleave C-terminal to the basic residues (26). In *C. elegans*, PCs also cleave after tribasic and monobasic residues and very rarely, after a non-basic residue. Of the FLP precursor molecules, including different isoforms encoded by one gene, there are 184 instances of FLP sequences flanked by basic amino acids (**Table 2**). Seventy-one percent of the FLPs are flanked by the typical dibasic residues, of which the majority (83%) are

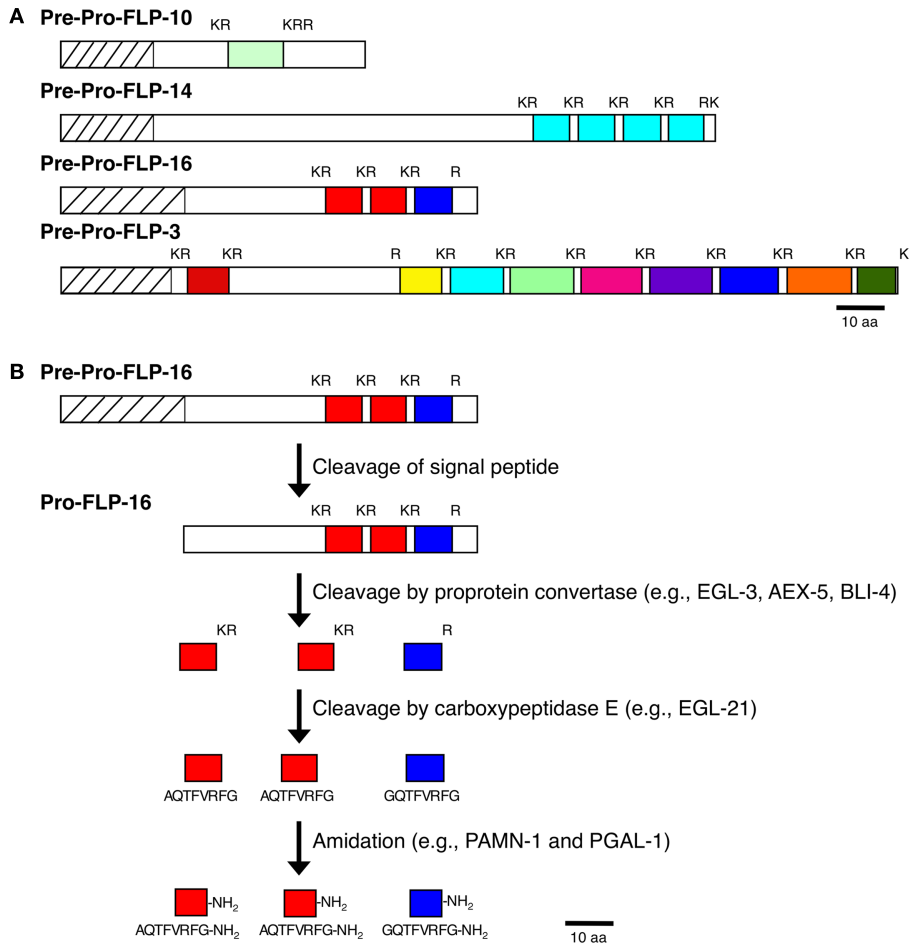


FIGURE 1 | Processing of neuropeptide precursor molecules. **(A)** Representative FLP precursor molecules that contain one FLP peptide (FLP-10), multiple copies of the same FLP peptide (FLP-14), multiple distinct FLP peptides (FLP-3), and multiple distinct FLP peptides and multiple copies of the same FLP (FLP-16) are shown. **(B)** Proteolytic pathway of the FLP-16 precursor molecule to yield the active FLP-16 peptides.

Lys-Arg, and 29% are flanked by monobasic residues of which Arg is the most common residue (70%). Five FLP peptides are directly followed by a stop codon and one FLP peptide has an unusual non-basic flanking residue (Ala).

There are four genes encoding PCs in *C. elegans*: *kpc-1*, *egl-3/kpc-2*, *aex-5/kpc-3*, and *bli-4/kpc-4*; with the exception of *aex-5/kpc-3*, all are alternatively spliced to yield isoforms that differ at the C-terminus [(27, 28); WormBase], suggesting that different isoforms may have different substrate targets or preferences. Mutant alleles of all the PC genes have been isolated (27–31). By mass spectrometry (MS) analysis of neuropeptide profiles, the major PC that cleaves FLP propeptides is EGL-3/KPC-2 (22), whose mammalian ortholog is proprotein convertase 2 (PC2) (32). The number of isolated FLP peptides is significantly decreased in *egl-3/kpc-2* mutants compared to in *kpc-1*, *aex-5/kpc-3*, and *bli-4/kpc-4* mutants (22). However, the biochemical data also indicate that besides EGL-3/KPC-2 other PCs cleave propeptides (22). Furthermore, using an antibody that only recognizes mature FLPs, anti-FMRFamide immunoreactivity was detected in *egl-3* mutants, suggesting that other PC2s function in neuropeptide processing in *C. elegans* (33). *egl-3* is widely expressed in the nervous system and acts within post-Golgi secretory vesicles (32). Knockout of *egl-3* PC2 causes several defects, including in egg-laying (30) and body mechanosensation (32).

The minor PCs also have the capability to cleave neuropeptide precursors. *kpc-1* encodes a PC similar to mammalian furin (28) and is widely expressed in the nervous system and epithelial cells (34). *kpc-1* PC mutants show slight uncoordination and slowed growth (28) and have defects in the dendritic remodeling of the IL2 neurons during dauer formation and nictation (34). While the dendritic remodeling defects may be due to lack of cleavage of semaphorins or TGFβ substrates (34), the nictation defects may be due to lack of cleavage of neuropeptide precursors to yield active peptides that regulate nictation (35). *aex-5/kpc-3* mutants show a defect in the defecation motor pattern (31). Furthermore, knockdown of *aex-5/kpc-3* by RNA-mediated interference (RNAi) feeding gives a low rate of embryonic lethality (36), suggesting other essential substrates for AEX-5/KPC-3. The genomic organization of *aex-5/kpc-3* is slightly unusual because it is part of an operon with *unc-54*, which encodes a muscle myosin heavy chain

Table 2 | Residues flanking FLP*.

Basic residues	No. sites
KRR	1
KR	108
RK	10
KK	9
RR	3
R	37
K	16
Non-basic residue	
A†	1
Stop codon	5

*Includes all alternative transcripts.

†A non-FLP peptide encoded by flp-27 is also flanked by an A.

Peptides encoded by all flp transcripts.

(28); the regulation of the operon would predict that *aex-5/kpc-3* is expressed only in muscle, similar to *unc-54* myosin. However, intestinal specific expression of *aex-5/kpc-3* can rescue the defection motor program in *aex-5/kpc-3* mutants, suggesting that the intestine also expresses *aex-5/kpc-3* (37). *bli-4/kpc-4* encodes a furin-like protease that is expressed in neurons, hypodermal cells, vulval muscles, and the intestine; loss of *bli-4/kpc-4* results in late embryonic lethality (27). Although DPY-5 is a procollagen that must be cleaved by BLI-4/KPC-4 for proper adult cuticle formation (38), BLI-4/KPC-4 must have other substrates, currently unidentified, during embryogenesis. The relevance of the different PC isoforms is unknown, but may relate to substrate specificity.

The PC2s themselves are also synthesized as precursor molecules that must be proteolytically cleaved to become activated and transported (39). SBT-1 is the *C. elegans* neuroendocrine 7B2 ortholog (40) that presumably cleaves pro-EGL-3/PC2 for its activation. In high pressure liquic chromatography (HPLC)-MS isolations, 29 FLPs were isolated from extracts of *sbt-1*/7B2 mutants compared to 53 FLPs isolated from wild-type extracts, suggesting that loss of *sbt-1* interferes with neuropeptide maturation (23). *sbt-1*/7B2 mutants also showed resistance to the effects of aldicarb (40), an acetylcholinesterase inhibitor that causes paralysis in *C. elegans* (41).

After cleavage by the PCs, the C-terminal basic residues are removed by the action of carboxypeptidase E (CPE), which cleaves N-terminal to the basic residue(s). In *C. elegans*, one CPE ortholog is *egl-21*, which is expressed widely in the nervous system (33). Loss of *egl-21* CPE results in defects in egg-laying (30), locomotion, and defecation and decreased FMRFamide-like immunoreactivity and aldicarb sensitivity (33). Because residual FMRFamide-like immunoreactivity is present in *egl-21* CPE mutants (33), other carboxypeptidases must also function to process peptide intermediates. There are two other carboxypeptidases with similarity to CPE and carboxypeptidase D (33): CPD-1/F59A3.1 and CPD-2/T27A8.1[1]. Knockdown of *cpd-2*/T27A8.1 had no phenotype, whereas knockdown of *cpd-1* by RNAi showed variable uncoordination and larval lethality (42). Elucidation of *cpd-1* and *cpd-2* function in neuropeptide processing awaits future mutant analysis.

To prolong their effects, many neuropeptides are post-translationally modified to prevent their immediate degradation. In the case of the FLPs, the peptides are amidated and this amidation is required for the bioactivity of the peptide (43). The amide at the C-terminus of each mature FLP is donated by the C-terminal glycine. In mammals, amidation occurs as the result of two sequential enzymatic steps from a bifunctional protein peptidylglycine-α-amidating monooxygenase (PAM): the peptide is subjected to the action of a peptidylglycine-α-hydroxylating monooxygenase (PHM), followed by the action of a peptidyl-α-hydroxyglycine α-amidating lyase (PAL) (44, 45). By contrast, *C. elegans* has two independent enzymes: PAMN-1/T19B4.1 and PGAL-1/F21F3.1 have similarity to PHM and PAL, respectively, and could function in neuropeptide amidation [(46); WormBase].

After release, inactivation of neuropeptide signaling is regulated by their proteolytic cleavage by the class of neprilysin (NEP) zinc metallopeptidases (47). In *C. elegans*, NEP activity cleaves the amino group of hydrophobic amino acids; for instance, FLP-1 (SDPNFLRF-NH_2) is cleaved at multiple sites (e.g., NF, FL, and RF), each including the Phe residue (48). At least 20 NEP-encoding genes are present in *C. elegans*; 10 of these are known to be expressed (48–50). *nep-1* is expressed in pharyngeal cells and a single head neuron (51), suggesting that NEPs are active at different synapses. A *nep-1* knockout showed an uncoordinated phenotype; in addition, in isolated pharyngeal preparations, *nep-1* mutants showed decreased basal activity (51). Which, if any, of these NEP genes are relevant to peptide processing is still unknown.

PACKAGING AND RELEASE OF FLPs

The processing of neuropeptide precursor molecules into bioactive peptides starts in the endoplasmic reticulum (ER), when the signal peptide is first removed. Subsequent cleavages of the propeptide and post-translational modifications of the cleaved peptides occur in the ER and Golgi network or as the vesicle, which in the case of neuropeptides is large, dense core vesicles (DCVs), is transported to the nerve terminal. In *C. elegans* transport of DCVs to the nerve terminals is dependent on UNC-104/KIF1A/kinesin-3 (33, 52, 53) and UNC-116/kinesin-1 (53).

Small molecule transmitters and neuropeptides are packaged into distinct vesicles, small, clear synaptic vesicles (SVs) versus large, DCVs, respectively. Calcium triggers the release of their contents with a latency of 4.5 ms after the peak of an action potential for SVs or 16.7 ms for DCVs, which show better release after repeated or prolonged stimulation (54). In addition, the contents of the vesicles are significantly different; one SV releases about 4,700 transmitter molecules with a time constant of decay of transmitter discharge of 260 μs, while one DCV releases about 18,000 molecules with a time constant of 1.6 ms (54). DCVs are marked by the presence of IDA-1/IA-2/phogrin, which is a receptor protein tyrosine phosphatase found specifically on DCVs and which regulates levels of phosphatidylinositol phospholipids (PIPs) (55, 56). Regulated release of either type of vesicle is a

[1]http://www.wormbase.org

multi-step process: vesicle docking, priming, fusion, and release. DCVs are primarily found in *C. elegans* axons and not in cell somas; activity of the UNC-43 CaMKII regulates DCV exocytosis from the axon rather than the cell soma (57). The core SNARE complex, which includes vesicular SNB-1/synaptobrevin-1 (v-SNARE) and plasma membrane proteins RIC-4/SNAP-25 and UNC-64/syntaxin (t-SNAREs), is essential for this process and interacts with a number of other proteins, some specific to DCVs, to facilitate vesicular release (58). Presumably, v-SNARE SNT-1/synaptotagmin (59) has a similar function with DCVs and acts as a calcium sensor. Both types of vesicles also require the cytoplasmic protein UNC-18 for vesicular release; UNC-18/Munc-18 has multiple functions: (1) it helps in the efficient trafficking from the ER and anterograde transport of UNC-64/syntaxin (60); (2) it binds UNC-64/syntaxin and mediates the conformational switch of UNC-64/syntaxin from a closed to open state to allow SNARE complex assembly, thereby priming the vesicle (61–63); and (3) it allows vesicle and plasma membrane fusion (62). Unlike SVs that are docked and released from active zones, DCVs are released at sites distinct from active zones (64). These DCV docking sites may be determined by the cytoplasmic protein RIC-7, which is a nematode-specific protein with no known homologies (65). UNC-31/CAPS, a calcium-dependent activator protein that contains a DCV-binding domain and pleckstrin-homology domain, serves as a bridge between DCVs and the plasma membrane (66, 67) to promote DCV docking and release (64, 68, 69). UNC-13, which promotes SNARE complex assembly (69, 70), and PKC-1 both contain C1 domains that bind phorbol esters to regulate vesicular release, although PKC-1 is only involved in DCV release (69). UNC-13/Munc13 stabilizes the open conformation of syntaxin to promote vesicle priming and this activity is antagonized by TOM-1/tomosyn, which associates with UNC-64/syntaxin and RIC-4/SNAP-25 to form an inhibitory SNARE complex (71) and prevent DCV release (68). Unlike small molecule transmitters, neuropeptides are not recycled and excess neuropeptides are cleared from the synaptic regions by degradation (see above); new neuropropeptides must be synthesized *de novo* in the cell body, processed and packaged into DCVs, and transported to the nerve terminals.

ISOLATION OF FLPs AND EXPRESSION PATTERNS

Among the 31 *flp* genes, there are 71 predicted FLPs. The initial isolation of FLPs was through laborious extracts from grams worth of animals (14–19, 21). Fortunately, the advent of new technologies with high pressure liquid chromatography in tandem with MALDI-TOF mass spectrometry have greatly decreased the amount of starting materials necessary for peptidomic studies and increased the resolution of peptide identities. Hence, microgram amounts of animals are now sufficient to isolate even low abundance peptides. The group of Liliane Schoofs has taken on the challenge to decipher the FLP peptidome in *C. elegans* and other nematodes (22, 24). Fifty-four of the 71 predicted FLPs representing 25 of the 31 *flp* genes have now been isolated (**Table 1**) (22, 24). However, there are also three FLPs, APNRVLMRLVRF-NH_2, HFYNFSSESRKPNFLRF-NH2, and KPXPXFIRF-NH_2, that have been biochemically isolated, but the corresponding gene has not been identified (N. Marks, A. Maule, and A.O. Stretton, pers. comm.). These results suggest that most, if not all, predicted FLPs are produced and additional *flp* genes have yet to be identified. Given that many of the peptides encoded by one gene are highly similar, we predict that these peptides have slightly different binding affinities to receptors to allow fine tweaking of a circuit. For instance, *flp-18* encodes six distinct FLPs that share a common C-terminal PGVLRF-NH_2; NMR analysis of two of the peptides DGDGAMPGVLRF-NH_2 and EMPGVLRF-NH_2 revealed that the N-terminal aspartates of DGDGAMPGVLRF-NH_2 form long-range electrostatic interactions with the C-terminal arginines, thereby forming a loop that decreases peptide binding to one of its receptors, NPR-1 (72). Similarly, other N-terminal extensions can form secondary structures that decrease the affinity of the peptide to its receptors, thereby modulating the biological effects of the peptides.

cDNAs from all *flp* genes have been isolated, indicating that all *flp* genes are expressed [(4, 10, 73, 74); WormBase]. Because FLPs are relatively small, ranging from 5 to 24 amino acids, and all share a C-terminal Arg-Phe-NH_2 motif, making antibodies against specific FLPs have been difficult. A general anti-FaRP antiserum that recognizes FaRPs encoded by multiple *flp* genes showed that roughly 10% of the neurons were immunoreactive (75). This figure, however, appears to grossly underestimate the widespread expression of the *flp* genes. Using transcriptional reporters, over 50% of the nervous system was found to express one or more *flp* genes (4, 76–78). Transcriptional reporters, however, have many inherent caveats, such as incorrect/incomplete promoter regions used or intronic sequences with regulatory elements missing; in addition, researchers injecting similar constructs have sometimes reported different expression patterns (4, 77). Using a monoclonal antibody specific for FLP-8 (79, 80), we showed that the immunoreactivity pattern matched that of the reporter expression pattern, suggesting that transcriptional reporters can reflect protein expression patterns. Although each *flp* gene is expressed in a distinct set of neurons, there is considerable overlap in the expression pattern of the different *flp* genes and a single neuron can show a wide diversity of *flp* expression. For instance, the chemosensory neuron ASE and oxygen-sensor URX express five and four *flp* genes, respectively (4). In addition, most *flp* genes are expressed in neurons that also express a small molecule transmitter. Some *flp* genes are also expressed in non-neuronal cells, such as the intestine or gonad (4, 77). The complex *flp* expression pattern allows exceptionally intricate modulation of neural networks to generate behavior.

FUNCTION OF FLPs IN *C. ELEGANS*

FLP neuropeptides have been shown to function in various behaviors in *C. elegans* (**Table 3**). A common functional modality among FLPs is that these neuropeptides appear to inhibit circuit activity in most, but not all, behaviors tested. Pharmacological application of FLPs, however, shows that FLPs also has excitatory effects (**Table 3**).

LOCOMOTION

Wild-type animals move in a sinusoidal waveform on a solid surface and initiate swimming in liquid. *flp-1* mutants displayed

Table 3 | Function of FLP Neuropeptides in *C. elegans*.

***flp* gene**	**Phenotypes in mutants or by RNAi**	**Phenotypes due to overexpression**	**Pharmacology of FLPs on pharynx**
flp-1	Loopy waveform (1); suppressed paralysis due to increased levels of dopamine (2); enhanced convulsive locomotion and aldicarb hypersensitivity in synergy with *flp-18* (3); decreased entry into the active phase of egg-laying (4)	Flattened waveform (1)	Decreased pharyngeal activity at 1 μM (5, 6)
flp-2			Increased pharyngeal activity at 1 μM (6)
flp-3			Decreased pharyngeal activity at 1 μM (5, 6)
flp-4			Increased pharyngeal activity at 1 μM (6)
flp-5			Increased pharyngeal activity at 1 μM (5, 6)
flp-6			Increased pharyngeal activity at 1 μM (5, 6)
flp-8	Increased repetitive turning during male mating (7)		Increased pharyngeal activity at 100 nM (5, 6)
flp-9			Decreased pharyngeal activity at 1 μM (5, 6)
flp-10	Increased repetitive turning during male mating (7)	Inhibits egg-laying (8)	
flp-11			Decreased pharyngeal activity at 100 nM (5, 6)
flp-12	Increased repetitive turning during male mating (7)		
flp-13			Decreased pharyngeal activity at 100 nM (4, 5)
flp-14			Increased pharyngeal activity at 1 μM (5, 6)
flp-15			Decreased pharyngeal activity at 1 μM (5, 6)
flp-16			Decreased pharyngeal activity at 1 μM (5, 6)
flp-17		Inhibits egg-laying (8)	Increased pharyngeal activity at 100 nM (6)
flp-18	Decreased odor response, increased reversals after starvation, enhanced dauer formation in *daf-7* TGFβ mutants, reduced oxygen consumption, increased intestinal fat storage (9); enhanced locomotory activity of *npr-1(g320)* mutants during lethargus (10)		
flp-19			Decreased pharyngeal activity at 1 μM (4, 5)
flp-20	Loss of massed training-induced memory for tap habituation and decreased number of synaptic vesicles (11); increased repetitive turning during male mating (7)		
flp-21	Blocked hypoxia-induced 5-HT stress signals from the pharynx to head neurons (12); enhanced locomotory activity of *npr-1(g320)* mutants during lethargus (10); increased thermal thresholds for heat avoidance (13); displayed aggregation behavior (14)		Decreased pharyngeal activity at 1 μM (5, 6)
flp-22			Increased pharyngeal activity at 1 μM (6)

5-HT = serotonin; 1, Nelson et al. (76); 2, Wani et al. (81); 3, Stawicki et al. (82); 4, Waggoner et al. (83); 5, Rogers et al. (84); 6, Papaioannou et al. (85); 7, Liu et al. (86); 8, Ringstad and Horvitz (77); 9, Cohen et al. (87); 10, Choi et al. (88); 11, Li et al. (89); 12, Pocock and Hobert (90); 13, Glauser et al. (91); 14, Rogers et al. (78).

an exaggerated waveform on a solid surface; conversely, overexpression of *flp-1* caused a flattening of the waveform and extreme sluggishness, suggesting that *flp-1* modulates the locomotory circuits (76). *dat-1* encodes a dopamine transporter and mutations in *dat-1* caused paralysis due to increased synaptic dopamine concentration (92). Chase and coworkers showed that a *flp-1* mutation fully suppressed this locomotion defect of *dat-1* mutants (81). Although the exact mechanism has not been elucidated, these results further indicate a role of *flp-1* in locomotory circuits (76).

ACR-2 is a nicotinic acetylcholine receptor subunit expressed in cholinergic motoneurons (93). *acr-2* gain-of-function (*gf*) mutants exhibited excitation–inhibition imbalance due to an increase in cholinergic excitation and a decrease in GABAergic inhibition in the locomotory circuit, resulting in spontaneous convulsive behavior and increased aldicarb sensitivity (93). Loss of *egl-3* PC2 decreased GABAergic inhibition and enhanced the convulsion phenotype of *acr-2*(*gf*), suggesting that neuropeptides were involved (82). After testing multiple neuropeptide mutants, *flp-1*; *flp-18* double mutants but not single mutants, were identified as enhancing convulsive locomotion and aldicarb hypersensitivity of *acr-2*(*gf*) by further reducing GABAergic inhibition (82). In addition, *acr-2*(*gf*) mutants showed increased expression of *flp-18* in the ventral cord cholinergic B-type motoneurons and overexpression of *flp-18* decreased convulsion phenotype of *acr-2*(*gf*) (82). NPR-1 and NPR-5 G protein-coupled receptors (GPCRs) appeared to mediate the action of FLP-18 in *acr-2* (*gf*) mutants (82). These results indicate that *flp-1* and *flp-18* play

important roles in suppressing overexcitation of the locomotory circuit.

EGG-LAYING

C. elegans egg-laying behavior is regulated by the actions of both small molecule neurotransmitters, such as acetylcholine and 5-HT, and neuropeptides (94). The HSN and VC4/5 serotoninergic neurons control the temporal switch between active/inactive states of egg-laying; during the active phase, release of acetylcholine triggers the egg-laying event (95). *flp-1* functions in parallel with serotonin to promote entry into the active phase of egg-laying (83). A different peptidergic circuit regulating egg-laying behavior was revealed by analysis of *egl-6* mutants, which were isolated on the basis of their egg-laying defects (30). EGL-6 is a GPCR; a gain-of-function mutation or overexpression of *egl-6* exclusively in the HSN motoneurons inhibited egg-laying, while *egl-6* deletion mutants did not exhibit egg-laying defects (77). However, *egl-6* deletion mutants fully suppressed egg-laying defects caused by overexpression of *flp-10* or *flp-17* (77). Surprisingly, the effects of the FLP-10 peptide were conferred by expression in non-neuronal cells, including vulval cells and spermatheca, as well as the CO_2-sensing BAG neurons, whereas *flp-17* appeared to function primarily in the BAG neurons (77). The FLP-10/FLP-17 peptidergic circuit acted redundantly with a cholinergic circuit distinct from HSN/VCs to inhibit egg-laying (77). Using the *Xenopus* oocyte heterologous system, FLP-10 and FLP-17 were verified as cognate ligands for EGL-6 GPCR (77).

METABOLISM

de Bono and coworkers found that *flp-18* deletion mutants showed a multitude of defects, including decreased odor response, increased reversals and turns after 1 h of starvation, enhanced dauer formation in *daf-7* TGFβ mutant background, reduced oxygen consumption, and increased intestinal fat storage (87). These *flp-18* phenotypes were rescued by expression of *flp-18* in the AIY interneurons (or additionally RIG interneurons for the fat phenotype), which are major synaptic targets of multiple chemosensory neurons, indicating that the AIY neurons may integrate and process food information to determine release of FLP-18 (87). FLP-18 activated two GPCRs, NPR-4 and NPR-5, in *Xenopus* oocytes; furthermore, activation of NPR-4 in the intestine and NPR-5 in a set of chemosensory neurons mediated fat storage, activation of NPR-4 in the AVA and RIV interneurons mediated reversals and turns, and activation of NPR-5 in the ASJ chemosensory neurons mediated dauer formation (87). Fat storage was also increased in two other NPY-like receptor mutants, *npr-1* or *npr-7*, suggesting additional roles of non-FLP-18 neuropeptides in fat metabolism (87).

STRESS RESPONSES

Pocock and Hobert noticed that hypoxic stress increased levels of serotonin (5-HT) in a subset of gustatory neurons (ASG and ADF) of *C. elegans* via direct regulation by the hypoxia-inducible transcription factor HIF-1 and enhanced response to NaCl (90). Serotonin released from the ASG and ADF neurons activated the M4 pharyngeal motoneuron via its SER-7 5-HT receptor; activation of M4 resulted in the release of the FLP-21 peptide to relay these hypoxia-induced 5-HT signals from the pharynx to NPR-1-expressing neurons in the head (AQR, PQR, and URX), which may act upstream of the gustatory circuit (90). Thus, FLP-21 and its receptor NPR-1 play roles in transmitting stress signals.

AGGREGATION

The wild-type laboratory strain N2 var. Bristol displays solitary feeding behavior (96). However, under stressful conditions, such as high population density, low food, or low O_2 concentration, animals will aggregate (96–98). In addition, a mutation in the Bristol *npr-1* GPCR gene that corresponded to an NPR-1 isoform found in many naturally isolated wild strains caused aggregation even under non-stressful conditions, indicating that low activity of NPR-1 GPCR may represent stress-related behavioral states (96, 99, 100); expression of *npr-1* in the AQR, PQR, and oxygen-sensor URX neurons regulates aggregation behavior (101). Animals lacking FLP-21, a cognate ligand of NPR-1 GPCR, also displayed aggregation behavior, providing genetic evidence for interactions between FLP-21 and NPR-1 (78, 102). Recently, Bargmann, Sengupta, and coworkers found a "hub-and-spoke" circuit motif that generates behavioral differences depending upon NPR-1 activity (103, 104). In this circuit, the RMG command interneuron/motoneuron, which also expresses *npr-1* (101), serves as the hub of seven spoke neurons, including two pheromone-sensing neurons ASK and ADL and an oxygen-sensing URX, which are connected to the RMG hub via gap junctions; high RMG activity is required for aggregation behavior (103, 104). The ASK neuron is involved in male attraction and hermaphrodite repulsion at low and high concentrations of pheromone, respectively (105). High NPR-1 activity in solitary N2 hermaphrodites resulted in decreased RMG activity, leading to an avoidance response due to the enhanced ADL (repulsion) and reduced ASK (attraction) pheromone response (103, 104). Conversely, low or absent NPR-1 activity resulted in increased RMG activity, leading to an increased ASK–RMG mediated attraction and decreased ADL mediated repulsion, resulting in hermaphrodites being neutral to pheromone (103, 104). Thus, NPR-1 signaling in RMG regulates its activity level to integrate environmental signals, such as pheromones and oxygen.

SLEEP-LIKE BEHAVIOR

During each of the four larval molts, *C. elegans* exhibits a sleep-like behavioral state during a period called lethargus, during which wild-type animals show reduced sensory and motor activity and feeding behaviors (106). By contrast, *npr-1* GPCR mutants exhibited increased locomotive activity compared to that of quiescent wild-type animals; this activity was further enhanced in *flp-18* or *flp-21* single or double mutants (88). FLP-18/21 activation of NPR-1 signaling in the RMG hub-and-spoke interneuron was found to decrease sensory activity of the ASK sensory neurons, resulting in decreased PDF-1 secretion from the ASK neurons (88). Decreased PDF-1 secretion led to decreased activation of the PDF-1 receptor in mechanosensory neurons and muscles, resulting in decreased arousal (88). Thus, *flp-18* and *flp-21* have roles in decreasing neuronal and circuit activities underlying lethargus.

LEARNING AND MEMORY

C. elegans have been shown to habituate to mechanical stimuli generated by tapping the worm plate; repeated tap stimulation caused decremented responses to taps (107). Massed training-induced memory for tap habituation lasted at least 12 h (89). This 12-h memory retention of tap habituation training was abolished in *flp-20* mutants and the increased number of SVs in mechanosensory neurons after massed training was also reduced in *flp-20* mutants, indicating that FLP-20 is required for the 12-h memory retention (89). Since *flp-20* was expressed in and acted in the mechanosensory neurons, FLP-20 may regulate the number of DCVs in mechanosensory neurons via a feedback circuit (89).

HEAT AVOIDANCE

Goodman and coworkers developed new assays to identify genes involved in heat avoidance (91). Mutations in either *flp-21* or *npr-1* were found to increase thermal thresholds of heat avoidance. These effects may be due to FLP-21 activation of the RMG interneurons to decrease thermal thresholds (91).

MALE MATING

During mating, males undergo a stereotypic series of movements that includes hermaphrodite contact, backing, turning, locating the vulva, spicule insertion, and sperm transfer (108, 109). The male tail contains many specialized ray neurons that function in concert with core neurons for efficient male mating. In particular, glutamatergic mechanosensory neurons are required independently of ray neurons for turning behavior (86). In addition, loss of *flp-8*, *10*, *12*, and *20*, all of which are expressed in all or subsets of the mechanosensory neurons (4), increased repetitive turning behavior, suggesting that these FLPs are necessary for the accurate timing of turning (86).

PHARYNGEAL PHARMACOLOGY

Holden-Dye's group has made excellent use of a dissected pharyngeal preparation that contains the pharynx and nerve ring; the pharyngeal musculature has myogenic properties that can be stimulated with 500 nM serotonin (84). By recording from the pharyngeal muscle with either an intracellular electrode or a suction pipette, the group found that numerous FLP peptides increased (FLP-17 and 8 at 100 nM; FLP-2, 4, 5, 6, 14, and 22 at 1 μM) or decreased (FLP-11 and 13 at 100 nM; FLP-1, 3, 9, 15, 16, 19, and 21 at 1 μM) pharyngeal activity (84, 85). Several of the *flp* genes with bioactive peptides are expressed within pharyngeal neurons, but many of the genes encoding the bioactive peptides are expressed in neurons outside the pharyngeal system (4). Whether the peptides are acting directly on pharyngeal muscle or indirectly via other neurons remains to be determined. In addition, because some of the peptides were bioactive only at high concentrations, the physiological roles of the peptides await further confirmation.

IDENTIFYING G PROTEIN-COUPLED RECEPTORS THROUGH WHICH FLPs SIGNAL

With one exception (110), neuropeptides signal through GPCRs. In *C. elegans*, there are an estimated 1,100 GPCRs (111) of which most have been classified as chemoreceptors and 50–125 are likely to correspond to neuropeptide GPCRs (111, 112). Knocking down activity of a subset of the identified neuropeptide receptors suggest that signaling through these receptors affect reproduction and locomotion (113).

A current initiative by several labs has been to de-orphanize the GPCRs by identifying their respective ligand(s) and determining the pathways in which they function. This task is daunting in any system, because GPCRs are promiscuous and can bind more than one ligand; similarly, neuropeptides are also promiscuous and one peptide may bind multiple GPCRs with different affinities. Whether these differing binding affinities reflect a biological function, whereby activation by a specific peptide may only occur after low or high frequency stimulation, or are an artifact of the experimental system remains to be clarified.

Several heterologous systems have been used to de-orphanize FLP receptors: expression of receptors in *Xenopus laevis* oocytes or in cell lines and use of different signaling readouts, including whole cell voltage-clamp, GTPγS binding, cAMP levels, and calcium indicators, to determine receptor activation. The EC_{50} (concentration which produces 50% maximal activation) varies substantially among the different readouts (nanomolar to micromolar), suggesting different sensitivity among the readout systems. These studies have revealed that as with vertebrate and other invertebrate neuropeptide receptors, *C. elegans* FLP receptors are generally activated by multiple FLPs, which may be encoded by the same or, more likely, distinct *flp* genes (**Table 4**). FRPR-18/T19F4.1, is the only receptor identified thus far that is activated by peptides encoded by only one *flp* gene, *flp-2*, with a physiological EC_{50} (114). Most receptors are activated by peptides encoded by multiple *flp* genes. For instance, NPR-3/C10C6.2 was activated at an EC_{50} in the nM range by both peptides encoded by *flp-15* (115) and the single *flp-21* peptide, which all share a C-terminal PLRFamide (116). FRPR-3/C26F1.6 was activated by one FLP-11 and one FLP-7 peptide, both of which share a C-terminal VRFamide, while other closely related FLP-7 and FLP-11 peptides did not activate the receptor (117). EGL-6/C46F4.1 was activated by two C-terminally related peptides (YIRFamide), FLP-10, and FLP-17 (77). Several receptors can be activated by a large number of FLP peptides that do not appear to share structural similarities except for the C-terminal RFamide: NPR-22/Y59H11AL.1 binds 15 peptides encoded by six *flp* genes (*flp-1*, *7*, *9*, *13*, *11*, and 22) with an EC_{50} ranging from 25 nM to 5 μM (116, 118), NPR-4/C16D6.2 binds 13 peptides encoded by seven *flp* genes (*flp-1*, *3*, *4*, *11*, *14*, *15*, and *18*) with an EC_{50} ranging from 5 nM to >10 μM (116), and NPR-11/C25G6.5 binds eight FLP peptides encoded by five genes (*flp-1*, *5*, *14*, *18*, and *21)* with an EC_{50} ranging from 1 nM to 8 μM (116). Despite being able to activate receptors at high concentrations (e.g., $EC_{50} > 10$ μM), such peptides may not be physiological ligands. For instance, FLP-1 activates multiple receptors, but all at an EC_{50} that is unlikely to be physiological; hence, the FLP-1 receptor has yet to be identified. One receptor, CKR-2, has been found to bind both FLP and non-FLP peptides. CKR-2 binds NLP-12 and NLP-13 at high affinity (nanomolar range), but also binds a FLP-1 peptide at low affinity (>10 μM) (119). The complexity with which the peptides binds receptors and the receptors bind peptides suggests that behaviors can be subtly refined by modulating peptide levels.

Table 4 | Receptors that bind FLP peptides.

Receptor	Cosmid	Activates/binds receptor		
		EC_{50} in nM range	**EC_{50} in mM range**	**(EC_{50} > 10 mM)**
NPR-1	C39E6.6	**FLP-18** (EMPGVLRFa, DFDGAMPGVLRFa, SVPGVLRFa, EIPGVLRFa) **FLP-21** (GLGPRPLRFa)	**FLP-18** (SEVPGVLRFa, DVPGVLRFa)	
NPR-3	C10C6.2	**FLP-15** (GGPQGPLRFa, RGPSGPLRFa) **FLP-21** (GLGPRPLRFa)		
NPR-4	C16D6.2	**FLP-4** (ASPFIRFa) **FLP-18** (DVPGVLRFa, (K)SEVPGVLRFa, SVPGVLRFa, DFDGAMPGVLRFa, EIPGVLRFa)	**FLP-1** (KPNFLRFa) **FLP-3** (SPLGTMRFa, SAEPFGTMRFa) **FLP-11** (NGAPQPFVRFa) **FLP-15** (GGPQGPLRFa)	
NPR-5	Y58G8A.4	**FLP-18** (DVPGVLRFa, (K)SEVPGVLRFa, SVPGVLRFa, DFDGAMPGVLRFa, EIPGVLRFa) **FLP-21** (GLGPRPLRFa)	**FLP-1** (KPNFLRFa, SQPNFLRFa) **FLP-3** (SPLGRTMRFa, SAEPFGTMRFa, SADDSAPFGTMRFa, EDGNAPFGTMRFa)	
NPR-6	F41E7.3			**FLP-18** (DVPGVLRFa, (K)SVPGVLRFa) **FLP-21** (GLGPRPLRFa)
NPR-10	C53C7.1	**FLP-3** (SPLGTMRFa, SAFPFGTMRFa, SADDSAPFGTMRFa, ASEDALFGTMRFa, EDGNAPFGTMRFa, EAEEPLGTMRFa) **FLP-18** (SVPGVLRFa)	**FLP-18** (KSVPGVLRFa, DVPGVLRFa, SEVPGVLRFa, DFDGAMPGVLRFa, EIPGVLRFa)	
NPR-11	C25G6.5	**FLP-18** (SVPGVLRFa) **FLP-21** (GLGPRPLRFa)	**FLP-1** (KPNFLRFa) **FLP-5** (AGAKFIRFa) **FLP-14** (KHEYLRFa) **FLP-18** (KSVPGVLRFa)	
NPR-22	Y59H11AL.1		**FLP-7** (SPMERSAMVRFa)	**FLP-1** (KPNFMRYa) **FLP-7** (TPMQRSSMVRFa, SPMQRSSMVRFa, SPMDRSKMVRFa) **FLP-9** (KPSFVRFa) **FLP-11** (AMRNALVRFa, NGAPQPFVRFa) **FLP-13** (AADGAPLIRFa, ASPSAPLIRFa, SPSAVPLIRFa, ASSAPLIRFa, SAAAPLIRFa) **FLP-22** (SPSAKWMRFa)
FRPR-3	C26F1.6		**FLP-7** (TPMQRSSMVRFa) **FLP-11** (AMRNALVRFa)	**FLP-7** (SPMQRSSMVRFa, SPMERSAMVRFa)
FRPR-18	T19F4.1	**FLP-2** (SPREPIRFa, LRGEPIRFa)		**FLP-10** (QPKARSGYIRFa) **FLP-11** (AMRNAVLRFa) **FLP-14** (KHEYLRFa)

(Continued)

Table 4 | Continued

Receptor	Cosmid	Activates/binds receptor		
		EC_{50} in nM range	**EC_{50} in mM range**	**(EC_{50} > 10 mM)**
EGL-6	C46F4.1	**FLP-10** (QPKARSGYIRFa) **FLP-17** (KSQYIRFa, KSAFVRFa)		
CKR-2	Y39A3B.5	**NLP-12** (DYRPLQFa, DGYRPLQFa)		**FLP-1** (SADPNFLRFa) **NLP-13** (pQPSYDRDIMSFa) **NLP-14** (ALDGLDGSGFGFD)

a, amidated, p, pyroglutamic acid.

From Ringstad and Horvitz (77); Rogers et al. (78); Cohen et al. (87); Kubiak et al. (102), Mertens et al. (114); Kubiak et al. (115); Lowery et al. (116); Mertens et al. (117); Mertens et al. (118); Janssen et al. (119); Kubiak et al. (120); Larsen et al. (121).

DIVERSITY OF FLPs IN NEMATODES

The accessibility of *C. elegans*, ease of manipulation and maintenance in the laboratory, and wealth of previous research have made *C. elegans* a useful model in the study of FLPs in other nematodes, and, in particular, parasitic nematodes. Parasitic nematodes infect over 1 billion people worldwide (University of Cambridge) and contributes to greater than $126 billion of crop damage globally (122). The recent sequencing of multiple nematode genomes and transcriptomes has greatly enhanced our understanding of the FLP complement in different nematodes. In addition to *C. elegans*, there are now 16 nematode genomes and/or transcriptomes that have been completed, although some are not as well annotated as others; this collection includes both parasitic and non-parasitic nematodes[2] . Other nematode sequences have also been deposited in the NCBI database, allowing us to use bioinformatic techniques to scan for *flp* genes among these genomes. By comparison to the *C. elegans flp* genes, there are 30 parasitic and 6 free-living nematodes that have *flp* orthologs [(123); unpubl. obs.].

Some striking observations can be noted among this collection of sequences. First, most nematodes, including free-living and parasitic (animal and plant), have a large complement of *flp* genes (**Table 5**). The most frequently appearing genes (>65%) among the scanned genomes are *flp-1*, *11*, *14*, and *18*; other common genes (>50%) include *flp-6*, *12*, *13*, *16*, *19*, *21*, *22*, and *24*. There were also some genes that appear infrequently. For instance, *flp-10* has only been identified in free-living nematodes (both hermaphrodite and male/female species), whereas, *flp-31* only appears in plant parasitic nematodes (123). Among the nematodes whose genomes have been completely determined, free-living and animal parasitic nematodes express *flp-8*, *15*, *23*, *24*, and *33*; none of the plant parasitic nematodes have been found to express these *flp* genes thus far. By contrast, we have not identified any *flp* gene that is expressed in free-living and plant parasitic nematodes, but not in animal parasitic nematodes. Some *flp* genes appear in free-living and parasitic nematodes, but are poorly represented, appearing in <25% of the genomes scanned (e.g., *flp-9*, *26*, and *28*). As the genomes/transcriptomes become better annotated, these numbers may change. However, the diversity and number of FLPs suggest that FLPs are widely used as neuromodulators in many, if not all, nematodes.

[2]http://www.nematodes.org

FUNCTION OF FLPs IN NON-*C. ELEGANS* NEMATODES

Because of the difficulty of propagating some of the parasitic nematodes in the lab, the function of peptides has been difficult to query. Furthermore, many genetic tools, such as isolating mutants or generating transgenic strains, are not available. However, the larger sizes of some nematodes allow the ability to do dissections and perform electrophysiological recordings and *in situ* hybridization techniques (128–130). Hence, the *flp* expression patterns described in many pathogenic nematodes based on *in situ* data may more accurately reflect the expression patterns of the genes than in *C. elegans*, where expression patterns are generally determined by use of transcriptional reporters (12). In addition, while the decreased expression of the SID-1 receptor in neurons hampers analysis of neuropeptides in *C. elegans* (131, 132), RNAi by soaking has been effective in neurons of some pathogenic nematodes (133). However, as in *C. elegans*, there are many caveats with the use of RNAi, particularly as there are no mutants among parasitic nematodes that can be used to confirm RNAi phenotypes (134). For simplicity in the discussion below, any *C. elegans* FLP peptide that is identical and conserved through different nematode species will be referred to by the *C. elegans* name, while peptides that are specific to a certain species will be indicated as such.

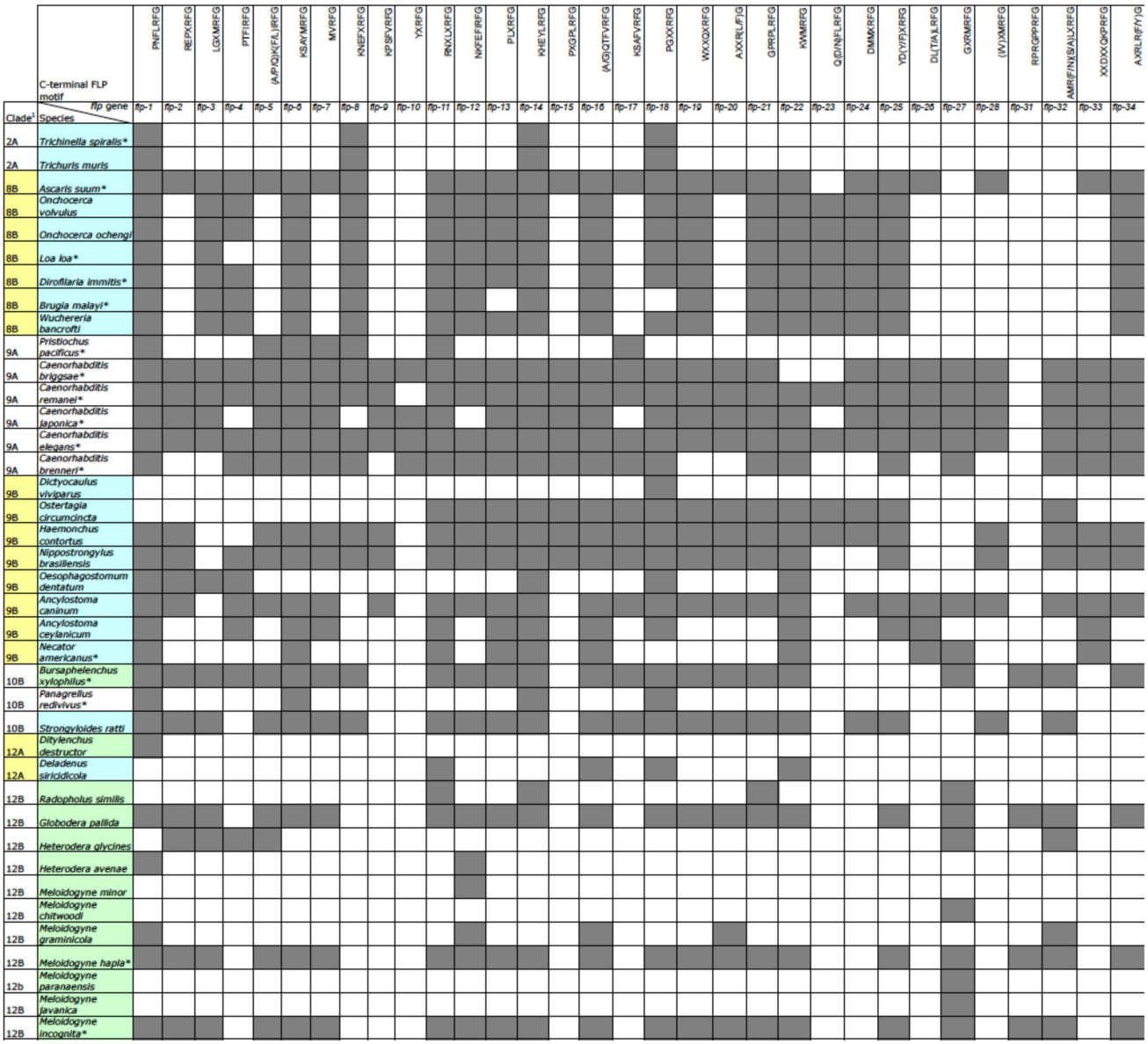
LOCOMOTION

The neuromuscular system is highly sensitive to disruptions in FLP peptide levels. In a sand column motility assay, knockdown of *Gp-flp-1* and *18* and *flp-6*, *12*, and *14* inhibited motility of second-stage juvenile (J2) *Globodera pallida* animals by 70–100% after 24 h of dsRNA soaking; the effects were transitory and by 6 days post-treatment, 27.1% of the animals recovered 39.4% of their normal activity (133). By contrast, when *Gp-flp-32* was knocked down, *G. pallida* and *Meloidogyne incognita* juveniles became more mobile, which translated to an enhanced ability to infect potato plant and other plant roots (135).

CHEMOSENSATION

When infecting the roots of tomato plants, RNAi knockdown of *Mi-flp-18* in J2 *M. incognita* dramatically decreased chemotaxis to tomato plant roots, thereby decreasing rates of infection (136). The exudate of *Chrysanthemum coronarium*, which is often planted with tomato, contains lauric acid, which decreases *Mi-flp-18* expression, hence providing a mechanism to alleviate crop damage (136).

Table 5 | FLP diversity in nematodes.



[1]Clade designations according to van Megen et al., 2009. Modified from McCoy et al., in press. Gray box indicates presence of gene; green indicates a plant parasitic nematode; blue indicates an animal parasitic nematode.
*Genome/transcriptome has been completed. From Geary et al., 1992; Maule et al., 1994; Schinkmann & Li, 1994; McVeigh et al., 2005; Husson et al., 2006, 2009; Thakur et al., 2012; McCoy et al., in press; NCBI database and WormBase.

PHARMACOLOGY OF FLPs

Muscle preparations of some of the larger nematodes have allowed the pharmacological application of different FLPs at nanomolar to micromolar concentrations. For instance, *Ascaris suum* somatic muscle strips can be isolated, muscle tension recorded, and the effects of different FLPs examined (126). *As*-FLP-18 (0.1 μM) caused contraction and occasionally rhythmic activity; this action could be reversed by FLP-1 (SADPNFLRF-NH_2; 0.1 μM) (137). *As*-FLP-1, FLP-1 (SADPNFLRF-NH_2, SDPNFLRF-NH_2; 1 nM), FLP-13 (APEASPFIRF-NH_2;10 μM) caused relaxation of muscle strips whether innervated or denervated (16, 138). Furthermore, *As*-FLP-1 (1 nM) inhibited contractions of spontaneously active preparations (138) and hyperpolarized muscle membrane (139). Some of the FLPs had more complex actions and produced biphasic responses. FLP-8 (0.1–1 μM) and FLP-14 initially caused relaxation, followed by rhythmic contractions; FLP-14 (nanomolar) was 10–100 times more potent on the muscle strips than FLP-8 (138, 140). FLP-6 (0.1–10 μM) caused a rapid (within 30 s)

increase in muscle tension in ventral muscle strips, but relaxation of dorsal muscle strips (126, 138). Intracellular recordings from *A. suum* muscle support the effects of some FLPs on muscle strips. Namely, FLP-1 (SDPNFLRF-NH_2 and SADPNFLRF-NH_2; EC_{50} ~300 nM) caused a slow hyperpolarization and decreased amplitude of the excitatory junction potentials (141).

The reproductive system of *A. suum* also provides a robust biological system to examine the activity of multiple FLPs. In particular, the ovijector contains circular muscle that regulates egg release and sperm influx; the muscle is innervated by a nerve plexus, which is FMRFamide-like immunoreactive (142). When the ovijector was hooked to a photo-optic transducer, the ovipositor revealed rhythmic spontaneous contractions (143). Most FLPs induced an inhibitory response, whereby there was a decrease in contraction frequency and amplitude; these peptides included FLP-3 (SPLGTMRF-NH_2; 0.1–10 μM), 4 (ASPSFIRF-NH_2; 0.01 μM), 7 (SPMERSAMVRF-NH_2; 0.1 μM), 10 (QPKARSGYIRF-NH_2; 1 μM), 11 (NGAPQPFVRF-NH_2; 1 μM), 12 (1 μM), 13 (ASSAPLIRF-NH_2; 1 μM), 15 (GPSGPLRF-NH_2; 0.1 μM), FLP-16 (GQTFVRF-NH_2; 0.1 μM), 17 (KSQYIRF-NH_2; 0.1 μM), and 20 (AMMRF-NH_2; 10 μM) (143). A few FLPs caused an excitatory response [FLP-2 (SPREPIRF-NH_2; 10–100 nM), 18 (SVPGVLRF-NH_2; 10 nM–10 μM), 19 (WANQVRF-NH_2; 10 nM–10 μM; ASWASSVRF-NH_2; 10 μM), and 21 (1–10 μM)] or showed a shortening of the ovipositor before an increase in contraction frequency [FLP-11 (AMRNALVRF-NH_2; 100 nM)] (143). The activities of some peptides were only a transient contraction [FLP-2 (LRGEPIRF-NH_2; 0.1–10 μM)] or a transient contraction followed by an extended period of inactivity [FLP-5 (AGAKFIRF-NH_2, APKPKFIRF-NH_2), 8, and 22 (SPSAKWMRF-NH_2)] (143). Whether these FLP responses are physiological are unclear, particularly as some activities were only induced at high, micromolar concentrations.

To determine the effect of peptides in intact animals, peptides were directly injected into the *Ascaris* pseudocelom (injected at 100 μM; final concentration within pseudocelom estimated at 10 μM). FLP-8 and 14 inhibited locomotion, caused shallower waveforms with no propagation, and shortened body length, while injection of FLP-4 (ASPSFIRF-NH_2), 6 (KSAYMRF-NH_2), 7 (SPMQRSMVRF-NH_2), 13 (APEASPIRF-NH_2), and 16 (AQTFVRF-NH_2) and *As*FLP-13 abolished body waves and increased body length; *As*-FLP-1 produced a more severe phenotype by causing a complete paralysis (144, 145). By contrast, injection of *As*-FLP-18 increased the number of body waves, but the body waves did not propagate and body length was decreased (145). FLP-6 and 9 caused ventral coils, while FLP-12 caused uncoordination (145).

PHYSIOLOGY OF FLP PEPTIDES ON *A. SUUM* MOTONEURONS

Several FLPs are expressed in the motoneurons of many nematodes. *A. suum* presents a relatively accessible system in which DE2 excitatory and DI inhibitory motoneurons can be exposed *in situ* and recorded while saline containing test solutions (generally 10 μM except where noted) are superfused; recorded responses can be direct actions of peptides on motoneurons or indirect effects via presynaptic neurons (128, 146). FLP-8 (1–100 nM) abolished slow oscillatory membrane potentials and reduced input resistances (147). *As*-FLP-4 and FLP-8 induced strong depolarizations in DE2 and weak depolarizations in DI motoneurons and generally decreased input resistances; FLP-16 elicited strong depolarizations in DE2 and DI (144). FLP-14 induced a stronger depolarization, but showed a transient increase in input resistance followed by a longer decreased input resistance in DE2 neurons; although FLP-14 caused a modest decrease in the input resistance of DI motoneurons, there was no membrane potential effect (144). The depolarizations elicited by the FLPs were in many cases the result of increased frequency of EPSPs. FLP-21 caused a complex response in which there was an initial hyperpolarization, followed by a sustained depolarization in DE2 neurons, but only a hyperpolarization in DI neurons; no change or a decrease was seen in the input resistance of DE2 and DI neurons, respectively. Application of different *As*-FLP-18 peptides or *As*-FLP-1 elicited weak depolarizations in DE2. FLP-6 elicited a weak hyperpolarization in DE2, but a strong depolarization, followed by miniature IPSPs in DI (144). *As*-FLP-13 elicited weak hyperpolarizations in DE2 and DI motoneurons. No effect was seen with application of *As*-FLP-28 in DE2 or DI neurons. As seen in other *Ascaris* preparations, FLPs exert a diversity of effects, but the physiological relevance has yet to be determined.

SUMMARY

The diversity and plethora of neuropeptides that can act over long distances allows invertebrates to greatly increase the complexity of their neural networks despite the relatively small number of neurons. A single neuron may mediate response to several sensory modalities and activate different or conserved downstream pathways to ensure correct motor outputs. Despite the relatively simple nervous system of *C. elegans*, the clever use of neuropeptides, gap junctions, hub neurons, and conventional synaptic connections allows the animal to have a wide behavioral repertoire that can be finely tuned in response to different environmental stimuli. Understanding how these simple neural networks determine behavior remains the challenge for the future.

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The FMRFamide-like peptide family in nematodes

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In the three decades since the FMRFamide peptide was isolated from the mollusk *Macrocallista nimbosa*, structurally similar peptides sharing a C-terminal RFamide motif have been identified across the animal kingdom. FMRFamide-like peptides (FLPs) represent the largest known family of neuropeptides in invertebrates. In the phylum Nematoda, at least 32 *flp*-genes are classified, making the FLP system of nematodes unusually complex. The diversity of the nematode FLP complement is most extensively mapped in *Caenorhabditis elegans*, where over 70 FLPs have been predicted. FLPs have shown to be expressed in the majority of the 302 *C. elegans* neurons including interneurons, sensory neurons, and motor neurons. The vast expression of FLPs is reflected in the broad functional repertoire of nematode FLP signaling, including neuroendocrine and neuromodulatory effects on locomotory activity, reproduction, feeding, and behavior. In contrast to the many identified nematode FLPs, only few peptides have been assigned a receptor and there is the need to clarify the pathway components and working mechanisms of the FLP signaling network. Here, we review the diversity, distribution, and functions of FLPs in nematodes.

Keywords: FMRFamide-like peptides, nematodes, *C. elegans*, neuropeptide, G protein-coupled receptor, feeding behavior, reproduction

INTRODUCTION

FMRFamide-like peptides (FLPs) are the largest and most diverse family of neuropeptides known (1, 2). Since the identification of the founder sequence FMRFamide from the clam *Macrocallista nimbosa* (3), structurally similar peptides have shown to be present in animals of all major phyla (4, 5). Sequence variants of the authentic tetrapeptide have been mainly identified in lophotrochozoans (6). In most phyla and especially in nematodes, however, a diverse repertoire of extended peptides sharing the C-terminal RFamide motif is found (5, 6). Though, they are thought to have a common eumetazoan origin, the relatedness of subfamilies of FLPs remains unclear because of the large sequence diversity (7, 8). Some peptides show high sequence similarity to FMRFamide suggesting homology to the tetrapeptide, and are therefore often referred to as FMRFamide-related peptides (FaRPs). FaRPs are broadly defined as peptides containing the C-terminal sequence X_1 X_2 RFamide, with X_1 generally representing an aromatic amino acid, whereas X_2 denotes a hydrophobic residue (6, 7). As many described RFamides differ from the tetrapeptide core for which evolutionary relationships are difficult to determine, the more general term FLP will be used here to address all peptides with a C-terminal RFamide sequence.

FMRFamide-like peptides are intimately involved in a broad pattern of biological processes as diverse as feeding, cardiovascular function, and water homeostasis (4–6, 9). Despite the large sequence diversity, typified by more than 70 family members in the nematode *Caenorhabditis elegans*, several functions of FLPs in the control of energy balance, feeding behavior, reproduction, and neuromodulation emerge consistently throughout evolution (10, 11). Biochemical and genetic studies, exploiting mainly *C. elegans*, have provided insight into the FLP-coordinated regulation of these processes in nematodes. The central role of FLPs in nematode biology including reproduction and locomotory activity has also boosted research on FLP signaling as a target for parasite control in pathogenic nematodes (12–14). However, a lack of data on functional nematode FLP-receptor couples slows down the progress in understanding and exploiting the FLP signaling system. Here, we focus on the evolutionary aspect of FLPs, discussing both the sequence conservation and diversity in the phylum Nematoda, and review our knowledge of conserved FLP signaling functions in nematodes.

FLP REPERTOIRE OF NEMATODES

Initial attempts to identify FLPs relied on molecular cloning of *flp*-genes (15, 16), and biochemical characterization of immunoreactive peptide fractions by Edman degradation or gas-phase sequencing [reviewed by Maule et al. (17) and Day and Maule (18)]. The first nematode FLP, named AF1 (KNEFIRFa), was biochemically isolated in this way from the parasite *Ascaris suum* (19). The completion of the *C. elegans* genome sequence revealed a large diversity in the nematode FLP system, boosting the prediction of neuropeptides through *in silico* data-mining (20–23). To date, at least 31 *flp* precursor genes are predicted in *C. elegans* that give rise to around 70 distinct FLPs [**Table 1**; Ref. (24)]. Expression has been confirmed for the majority of these peptides (**Table 1**), mainly by peptidomic strategies enabling a comprehensive analysis of the whole peptide content of organisms (25–27). In addition, these approaches allow determining the presence of posttranslational modifications and the exact processing into bioactive peptides, which may be difficult to accurately predict when multiple or non-conventional cleavage sites are present (25). Peptidomic techniques have also been successfully adopted

Table 1 | Neuropeptide genes encoding FLPs in nematodes.

flp gene[a]	Species[b]	(C-terminal) peptide consensus sequence[c]	*C. elegans* FLPs[d]	*C. elegans flp* expression[e]	*C. elegans* receptor interaction (EC_{50} range)[f]	Reference
flp-1	*A. caninum*, *A. ceylanicum*, *A. suum*, *B. malayi*, *B. xylophilus*, *C. elegans*, *C. vulgaris*, *D. immitis*, *G. pallida*, *G. rostochiensis*, *H. concortus*, *H. schachtii*, *L. loa*, *M. arenaria*, *M. chitwoodi*, *M. hapla*, *M. incognita*, *M. javanica*, *M. paranaensis*, *N. brasiliensis*, *N. americanus*, *O. onchengi*, *O. volvulus*, *P. redivivus*, *P. trichosuri*, *S. ratti*, *S. stercoralis*, *T. muris*, *T. spiralis*, *W. bancrofti*	-[P/N/Q/A/] [N/T/D/S/K][F/Y]LRFa	**SADPNFLRFa**, **SQPNFLRFa**, **ASGDPNFLRFa**, **SDPNFLRFa**, **AAADPNFLRFa**, (K)PNFLRFa, AGSDPNGLRFa, *(K)PNFMRYa	AIA, AIY, AVA, AVE, AVK, RIG, RMG, M5	NPR-22 (100 nM), NPR-4 (~0.4–9 μM), NPR-11 (~1–8 μM)	(13, 15, 21, 23–26, 33–44)
flp-2	*A. caninum*, *A. suum*, *B. xylophilus*, *C. elegans*, *G. pallida*, *H. concortus*, *M. chitwoodi*, *M. hapla*, *M. incognita*, *M. javanica*, *N. americanus*, *N. brasiliensis*, *O. ostertagi*, *S. ratti*	[L/F/V/S/Q][P/R/M][G/R]EP[I/L]RFa	LRGEPIRFa, **SPREPIRFa**	AIA, RID, PVW, I5, MC (ASI, M4, head muscles, an extra pair of cells in the head	FRPR-18 (~50 nM)	(13, 14, 21, 23, 24, 43, 45)
flp-3	*A. suum*, *B. malayi*, *B. xylophilus*, *C. elegans*, *D. immitis*, *G. pallida*, *H. glycines*, *L. loa*, *M. arenaria*, *M. chitwoodi*, *M. hapla*, *M. incognita*, *O. volvulus*, *O. onchengi*, *S. ratti*, *W. bancrofti*	-[S/A/E/T/N][P/L][L/F/P]GTMRFa	SPLGTMRFa, **TPLGTMRFa**, **SAEPFGTMRFa**, **NPENDTPFGTMRFa**, **ASEDALFGTMRFa**, EDGNAPFGTMRFa, **EAEEPLGTMRFa**, **SADDSAPFGTMRFa**, NPLGTMRFa	IL1, PQR, SP, CP9	NPR-10 (~60–300 nM), NPR-4 (≥10 μM)	(13, 21, 23–25, 27, 34, 40, 43, 44, 46, 47)
flp-4	*A. caninum*, *A. ceylanicum*, *A. suum*, *B. malayi*, *B. xylophilus*, *C. elegans*, *D. immitis*, *H. glycines*, *N. brasiliensis*, *O. ochengi*, *O. volvulus*, *W. bancrofti*	-[A/T/G][Q/N/S/K][P/S][T/S]FIRFa	PTFIRFa, ASPSFIRFa	ADL, ASEL, AVM, AWC, FLP, PHA, PHB, PVD, I5, I6, NSM	NPR-4 (~5–80 nM)	(13, 21, 23, 24, 32, 40, 43, 46)

(Continued)

Table 1 | Continued

flp gene[a]	Species[b]	(C-terminal) peptide consensus sequence[c]	*C. elegans* FLPs[d]	*C. elegans flp* expression[e]	*C. elegans* receptor interaction (EC_{50} range)[f]	Reference
flp-5	*A. caninum*, *A. suum*, *B. xylophilus*, *C. elegans*, *G. pallida*, *G. rostochiensis*, *H. concortus*, *H. glycines*, *M. arenaria*, *M. hapla*, *M. javanica*, *M. incognita*, *N. brasiliensis*, *N. americanus*, *P. penetrans*, *S. ratti*	-[G/A/N/K][A/Q/P]KFIRFa	APKFIRFa, AGAKFIRFa, **GAKFIRFa**	ASE, PVT, RMG, I4, M4, pharyngeal muscle, amphidial neuron (PB, I2), rays 1,5,7, HOB, P8	NPR-11 (~1–8 μM)	(13, 21, 23–25, 34, 40, 43, 44, 46)
flp-6	*A. caninum*, *A. ceylanicum*, *A. suum*, *B. malayi*, *B. xylophilus*, *C. elegans*, *D. immitis*, *G. pallida*, *G. rostochiensis*, *H. concortus*, *H. glycines*, *L. loa*, *M. chitwoodi*, *M. hapla*, *M. incognita*, *M. paranaensis*, *N. brasiliensis*, *N. americanus*, *O. ochengi*, *O. ostertagi*, *O. volvulus*, *P. redivivus*, *S. ratti*, *S. stercoralis*, *T. circumcincta*, *W. bancrofti*	KS[A/S]YMRFa	**KSAYMRFa** (6x), *pQQDSEVEREMM	ASE, AFD, ADF, ASG, PVT, I1 (one or two pairs of head cells), rays 2, 5, 6, 7		(13, 21, 23–25, 31, 34, 43, 44, 46, 48, 49)
flp-7	*A. caninum*, *A. suum*, *B. xylophilus*, *C. elegans*, *G. pallida*, *G. rostochiensis*, *H. concortus*, *H. glycines*, *M. hapla*, *M. incognita*, *M. javanica*, *N. brasiliensis*, *O. ostertagi*, *S. ratti*, *S. stercoralis*	[A/T/S]P[F/L/M/I][D/Q/A/E]R[S/A/T], [S/A/T/K][M/L/I][A/V/I]RFa	**TPMQRSSMVRFa** (2x), **SPMQRSSMVRFa** (3x), SPMERSAMVRFa, SPMDRSKMVRFa	ALA, AVG, PHB, PDA, PVW, RIC, SAA (RMDV/SMDV, PHA)	NPR-22 (0.025–5 μM), FRPR-3 (>1 μM)	(13, 21, 24, 26, 34, 35, 43, 44, 46, 50)
flp-8	*A. ceylanicum*, *A. suum*, *B. malayi*, *B. xylophilus*, *C. elegans*, *D. immitis*, *H. concortus*, *L. loa*, *N. americanus*, *N. brasiliensis*, *O. ochengi*, *O. volvulus*, *S. ratti*, *T. muris*, *T. spiralis*, *W. bancrofti*, *X. index*	KNEF[I/V]RFa	**KNEFIRFa** (3x)	AUA, PVM, URX (RMG, ADA, an extra pair of cells in the head), CP9		(13, 19, 21, 23, 24, 29, 34, 43, 46, 51)
flp-9	*A. caninum*, *A. ceylanicum*, *C. elegans*, *H. concortus*, *N. americanus*, *N. brasiliensis*, *O. ostertagi*	KPSFVRFa	**KPSFVRFa**		NPR-22 (5 μM)	(13, 21, 24–26, 35, 52)

(Continued)

Table 1 | Continued

flp gene[a]	Species[b]	(C-terminal) peptide consensus sequence[c]	*C. elegans* FLPs[d]	*C. elegans flp* expression[e]	*C. elegans* receptor interaction (EC_{50} range)[f]	Reference
flp-10	*A. ceylanicum*, *C. elegans*, *X. index*	-[A/T/M][R/A][S/G][G/S/K]Y[I/L]RFa	QPKARSGYIRFa	AIM, ASI, AUA, BAG, BDU, DVB, PQR, PVR, URX, vulD	EGL-6 (11 nM)	(13, 21, 23, 53)
flp-11	*A. suum*, *A. caninum*, *A. ceylanicum*, *B. malayi*, *B. xylophilus*, *C. elegans*, *D. immitis*, *G. pallida*, *G. rostochiensis*, *H. concortus*, *H. glycines*, *L. loa*, *M. hapla*, *M. incognita*, *M. paranaensis*, *N. americanus*, *N. brasiliensis*, *O. ochengi*, *O. ostertagi*, *O. volvulus*, *P. penetrans*, *R. similis*, *S. ratti*, *S. stercoralis*, *T. circumcincta*, *W. bancrofti*	-M/I/G/A/S][R/A][N/P][A/S/Q/E][P/L], VRFa	**AMRNALVRFa**, **ASGGMRNALVRFa**, **NGAPQPFVRFa**, ***SPLDEEDFAPESPLQa**	AUA, BAG, VD, DA, DD, DVB, LUA, PHC, PVC, SAB, URX, uvl, head muscle (socket cells), ray 4	NPR-22 (0.75–2.5 μM), FRPR-3 (~1 μM), NPR-4 (≥10 μM)	(13, 21, 23–26, 28, 29, 35, 40, 43, 46, 50, 54)
flp-12	*A. caninum*, *A. suum*, *B. malayi*, *B. xylophilus*, *C. elegans*, *D. immitis*, *G. pallida*, *G. rostochiensis*, *H. concortus*, *H. glycines*, *L. loa*, *M. arenaria*, *M. chitwoodi*, *M. hapla*, *M. incognita*, *M. javanica*, *M. minor*, *M. paranaensis*, *N. americanus*, *N. brasiliensis*, *O. ochengi*, *O. volvulus*, *S. ratti*, *W. bancrofti*	(K)[R/K/N]NKFEFIRFa	RNKFEFIRFa	AVA,AVJ, AVH, BAG, PDA, PVR, SAA, SDQ, SMB (BDU), rays 1, 4, 5, 7, CP9		(13, 21, 23, 24, 29, 37–39, 43, 44, 51, 55)
flp-13	*A. caninum*, *A. ceylanicum*, *A. suum*, *B. xylophilus*, *C. elegans*, *D. immitis*, *G. pallida*, *G. rostochiensis*, *H. concortus*, *H. glycines*, *L. loa*, *M. chitwoodi*, *M. hapla*, *M. incognita*, *M. javanica*, *N. americanus*, *N. brasiliensis*, *O. ochengi*, *O. ostertagi*, *O. volvulus*, *P. penetrans*, *P.pacificus*, *S. ratti*, *S. stercoralis*, *W. bancrofti*	-P[F/L/I][I/L/M/V]RFa	**AMDSPFIRFa**, **AADGAPFIRFa**, **APEASPFIRFa** (2x), AADGAPLIRFa, **ASPSAPFIRFa**, **SPSAVPIRFa**, **SAAAPLIRFa**, ASSAPFIRFa	ASE, ASG, ASK, BAG, DD, I5, M3, M5 (an extra pair of cells in the head), VSP	NPR-22 (2.5–5 μM)	(21, 23–26, 31, 35, 43, 44, 46, 51, 56, 57)

(Continued)

Table 1 | Continued

flp gene[a]	Species[b]	(C-terminal) peptide consensus sequence[c]	*C. elegans* FLPs[d]	*C. elegans flp* expression[e]	*C. elegans* receptor interaction (EC_{50} range)[f]	Reference
flp-14	*A. caninum*, *A. ceylanicum*, *A. suum*, *B. malayi*, *B. xylophilus*, *C. elegans*, *D. immitis*, *G. pallida*, *G. rostochiensis*, *H. concortus*, *L. loa*, *M. arenaria*, *M. chitwoodi*, *M. hapla*, *M. incognita*, *M. javanica*, *M. paranaensis*, *N. americanus*, *N. brasiliensis*, *O. ochengi*, *O. volvulus*, *P. redivivus*, *P. trichosuri*, *P. penetrans*, *P.penetrans*, *R. similis*, *S. ratti*, *S. stercoralis*, *T. circumcincta*, *T. muris*, *T. spiralis*, *W. bancrofti*	KH[E/D][Y/F][L/V/I]RFa	**KHEYLRFa** (4x)		NPR-4 ($\geq$ 10 μM), NPR-11 (~1 – 8 μM)	(13, 22, 24, 26, 29, 31, 34, 37–40, 43, 44, 51, 55, 58, 59)
flp-15	*A. ceylanicum*, *A. suum*, *C. elegans*, *H. concortus*, *N. americanus*, *N. braziliensis*, *O. ostertagi*, *T. circumcincta*	[R/D/G/A][G/V]P[T/S/Q]GPLRFa	**GGPQGPLRFa**, **RGPSGPLRFa**	PHA, I2, socket/sheath cells (pharyngeal muscle, several cells in the head)	NPR-3 (~100–600 nM), NPR-4 ($\geq$10 μM)	(13, 22–24, 35, 40, 46, 60)
flp-16	*A. caninum*, *A. ceylanicum*, *A. suum*, *B. malayi*, *B. xylophilus*, *C. elegans*, *D. immitis*, *G. pallida*, *G. rostochiensis*, *H. concortus*, *H. glycines*, *L. loa*, *M. hapla*, *M. incognita*, *N. americanus*, *N. brasiliensis*, *O. ochengi*, *O. volvulus*, *O. ostertagi*, *P. trichosuri*, *P. penetrans*, *P. vulnus*, *R. similis*, *S. ratti*, *W. bancrofti*	[A/G]QTFVRFa	**AQTFVRFa** (2x), **GQTFVRFa**			(13, 24, 43, 44, 46)
flp-17	*A. caninum*, *A. suum*, *B. xylophilus*, *C. elegans*, *H. contortus*, *N. americanus*, *N. brasiliensis*, *O. ostertagi*, *S. ratti*, *S. stercoralis*, *X. index*	KS [A/S/Q][F/Y/L][V/I]RFa	KSAFVRFa (2x), KSQYIRFa	BAG, M5 (an extra pair of cells in the head), rays 1, 5, 7	EGL-6 (1–28 nM)	(13, 22–24, 32, 43, 46, 53)

(Continued)

Table 1 | Continued

flp gene[a]	Species[b]	(C-terminal) peptide consensus sequence[c]	*C. elegans* FLPs[d]	*C. elegans flp* expression[e]	*C. elegans* receptor interaction (EC_{50} range)[f]	Reference
flp-18	*A. caninum*, *A. ceylanicum*, *A. suum*, *B. xylophilus*, *C. elegans*, *D. immitis*, *G. pallida*, *G. rostochiensis*, *H. concortus*, *L. loa*, *M. chitwoodi*, *M. hapla*, *M. incognita*, *M. javanica*, *N. americanus*, *N. brasiliensis*, *O. ochengi*, *O. ostertagi*, *O. volvulus*, *P. pacificus*, *S. ratti*, *S. stercoralis*, *T. muris*, *T. spiralis*, *W. bancrofti*	-[P/Q/A][G/Q/D/A], [V/M/F/L][V/M/F/L]RFa	(DFD)GAMPGVLRFa, EMPGVLRFa, (SYFDEKK)SVPGVLRFa (3x), EIPGVLRFa, SEVPGVLRFa, DVPGVLRFa	AVA, AIY, RIG, RIM, M2 (M3, two extra pairs of cells in the head), rays 2, 6	NPR-4 (~5–80 nM), NPR-10 (~60 nM–4.6 μM), NPR-1 [(−32.2)–(−6.8)]**, NPR-5a (~20–70 μM), NPR-5b (~30–800 nM), NPR-11 (~80 nM–8 μM)	(13, 16, 24–26, 29, 37, 39, 40, 43, 44, 46, 61, 62)
flp-19	*A. caninum*, *A. suum*, *B. malayi*, *B. xylophilus*, *C. elegans*, *D.immitis*, *G. pallida*, *H. concortus*, *H. glycines*, *L. loa*, *M. hapla*, *M. incognita*, *N. americanus*, *N. brasiliensis*, *O. ochengi*, *O. volvulus*, *P. penetrans*, *S. ratti*, *T. circumcincta*, *W. bancrofti*	-W[A/S][N/S/T][Q/K/S][V/L]RFa	**WANQVRFa**, **ASWASSVRFa**	AIN, AWA, BAG, HSN, URX (an extra pair of cells in the tail), rays 5, 7, 9, CEM		(13, 22–26, 32, 34, 43, 44, 46)
flp-20	*A. suum*, *A. caninum*, *B. xylophilus*, *C. elegans*, *G. pallida*, *H. concortus*, *M. hapla*, *M. incognita*, *N. brasiliensis*, *P. trichosuri*, *S. ratti*	[A/V]MMRFa	AMMRFa (2x)	ALM, ASEL, AVM, LUA, PLM, PVC, PVM, PVR, RIB, AIB (PVT)		(13, 22–24, 43, 44)
flp-21	*A. caninum*, *A. suum*, *B. malayi*, *B. xylophilus*, *C. elegans*, *D. immitis*, *G. pallida*, *H. concortus*, *L. loa*, *M. hapla*, *M. incognita*, *N. americanus*, *N. brasiliensis*, *O. ochengi*, *O. ostertagi*, *O. volvulus*, *P. penetrans*, *P. pacificus*, *R. similis*, *S. ratti*, *S. stercoralis*, *T. circumcincta*, *W. bancrofti*	-[G/A/S/L][L/A]GPRPLRFa	GLGPRPLRFa	ADL, ASI, ASEASH, ASJ, ASK, FLP, URA, MC, M4, M2, SP, DVF, P6, P7, P9	NPR-1 (~2.5–100 nM), NPR-11 (~1–10 nM), NPR-5a (~0.6–5 μM), NPR-5b (~200–1500 nM)	(13, 24, 40, 43, 44, 47, 61–63)

(Continued)

Table 1 | Continued

flp gene[a]	Species[b]	(C-terminal) peptide consensus sequence[c]	*C. elegans* FLPs[d]	*C. elegans flp* expression[e]	*C. elegans* receptor interaction (EC_{50} range)[f]	Reference
flp-22	*A. caninum*, *A. ceylanicum*, *A. suum*, *B. malayi*, *B. xylophilus*, *C. elegans*, *D. immitis*, *G. pallida*, *G. rostochiensis*, *H. concortus*, *H. glycines*, *L. loa*, *M. hapla*, *M. incognita*, *N. brasiliensis*, *O. ochengi*, *O. ostertagi*, *O. volvulus*, *P. trichosuri*, *P. penetrans*, *P. pacificus*, *R. similis*, *S. ratti*, *S. stercoralis*, *T. circumcincta*, *W. bancrofti*	-[P/E/A/T/S][P/Q/G/E/N/S][S/G/V/A], KWMRFa	**SPSAKWMRFa** (3x)	AIM, ASG, AVA, AVG, AVL, CEP, PVD, PVW, RIC,AIZ, RIV, SMD, URA, uvl, 6 out of 9 CP	NPR-22 (1 μM)	(13, 24–26, 35, 43, 44)
flp-23	*B. malayi*, *C. elegans*, *D. immitis*, *L. loa*, *O. ochengi*, *O. volvulus*, *T. circumcincta*, *W. bancrofti*	-[V/I/T][V/D/K][G/D/F][Q/G/F]QDFLRFa	VVGQQDFLRFa, TKFQDFLRFa			(13, 23, 24, 46)
flp-24	*A. caninum*, *A. ceylanicum*, *A. suum*, *B. malayi*, *C. elegans*, *D. immitis*, *H. concortus*, *L. loa*, *N. americanus*, *O. ostertagi*, *O. ochengi*, *O. volvulus*, *S. ratti*, *W. bancrofti*	VP[S/N][A/P][G/A]DMM[V/I]RFa	**VPSAGDMMVRFa**			(13, 23, 24, 31, 46)
flp-25	*A. caninum*, *A. suum*, *B. malayi*, *C. elegans*, *D. immitis*, *G. pallida*, *G. rostochiensis*, *H. concortus*, *L. loa*, *M. chitwoodi*, *M. hapla*, *M. incognita*, *M. javanica*, *N. americanus*, *N. brasiliensis*, *O. ochengi*, *O. volvulus*, *S. ratti*, *S. stercoralis*, *W. bancrofti*	-[D/A/S/N/T]YD[Y/F][V/I]RFa	DYDFVRFa, **ASYDYIRFa**	ASE		(13, 24, 26, 44, 46, 64)
flp-26	*A. caninum*, *A. ceylanicum*, *A. suum*, *C.elegans*, *N. americanus*	-[G/S][G/E][G/E/P][L/M/I][A/E]F[H/S/N], [P/A][N/D][D/M]L[A/S/T]LRFa	**(E)FNADDLTLRFa**, **GGAGEPLAFSPDML-SLRFa**, *__FRLPFQFFGANEDFNSGLT__, *__NYYESKPY__			(13, 24, 26, 46)

(Continued)

Table 1 | Continued

flp gene[a]	Species[b]	(C-terminal) peptide consensus sequence[c]	*C. elegans* FLPs[d]	*C. elegans flp* expression[e]	*C. elegans* receptor interaction (EC_{50} range)[f]	Reference
flp-27	*A. caninum*, *C. elegans*, *H. glycines*, *M. chitwoodi*, *M. hapla*, *M. incognita*, *M. javanica*, *M. paranaensis*, *N. americanus*, *R. similis*	[G/T/S/A][K/L/M]G[G/S]RMRFa	GLGGRMRFa, *pQPIDEERPIFME			(13, 24, 26, 44, 46)
flp-28	*A. suum*, *A. caninum*, *C. elegans*, *H. concortus*, *N. brasiliensis*, *O. ostertagi*, *P. penetrans*, *S. ratti*	-[V/I][L/F]MRFa	VLMRFa, **APNRVLMRFa**			(13, 24, 26)
flp-31	*B. xylophilus*, *G. pallida*, *M. chitwoodi*, *M. hapla*, *M. incognita*, *P. penetrans*	LYRPRGPPRFa				(13, 24, 44)
flp-32	*A. caninum*, *B. xylophilus*, *C. elegans*, *G. pallida*, *H. concortus*, *M. hapla*, *M. incognita*, *N. brasiliensis*, *S. ratti*		AMRNSLVRFa			(13, 24, 43, 44, 46)
flp-33	*A. suum*, *A. caninum*, *B. xylophilus*, *C. elegans*, *H. concortus*, *N. brasiliensis*		**APLEGFEDMSGFLRTIDGIQ**, **KPRFa**			(24, 43, 46, 65)
flp-34	*A. suum*, *A. caninum*, *B. malayi*, *B. xylophilus*, *C. elegans*, *D. immitis*, *G. pallida*, *H. concortus*, *M. hapla*, *M. incognita*, *L. loa*, *N. brasiliensis*, *O. onchengi*, *O. volvulus*, *W. bancrofti*		ALNRDSLVASLNNAERLRFa, *ADISTFASAINNAGRL-RYa			(24, 46)

[a] *The flp-coding genes flp-29 and flp-30 were recently suggested to represent orthologs of C. elegans flp-28 and flp-2, respectively, and have been accordingly included in this table (24).*

[b] *Species: Ascaris suum, Ancylostoma caninum, Ancylostoma ceylanicum, Brugia malayi, Bursaphelenchus xylophilus, Caenorhabditis elegans, Caenorhabditis vulgaris, Dirofilaria immitis, Globodera pallida, Globodera rostochiensis, Haemonchus concortus, Heterodera glycines, Heterodera schachtii, Loa loa, Meloidogyne arenaria, Meloidogyne incognita, Meloidogyne javanica, Meloidogyne hapla, Meloidogyne paranaensis, Necator americanus, Nippostrongylus braziliensis, Onchocerca ochengi, Onchocerca volvulus, Ostertagia ostertagi, Panagrellus redivivus, Parastrongyloides trichosuri, Pratylenchus penetrans, Pristionchus pacificus, Radolphus similis, Strongyloïdes ratti, Strongyloïdes stercoralis, Teladorsagia circumcincta, Trichinella spiralis, Trichuris muris, Wuchereria bancrofti, and Xiphinema index.*

[c] *Sequences that start with a hyphen have variable N-terminal extensions.*

[d] *Peptides indicated in bold have been isolated from C. elegans. Peptides indicated with an asterisks are non-FLPs encoded by the indicated flp gene. The copy number of peptides encoded by the gene is indicated between brackets.*

[e] *Expression patterns were adapted from Ref. (46, 64) and Wormbase (http:// www.wormbase.org).*

[f] *The approximate EC_{50} range for receptor activation is indicated between brackets and includes receptor activation by all peptides encoded by this precursor.*

***Values represent alteration of current in response to neuropeptide application in Xenopus assay.*

for characterizing and localizing FLPs in other nematodes, mostly in *A. suum* (28–32).

Although the FLP repertoire of nematodes has been best studied in *C. elegans*, evidence emerges on the distribution of FLPs in parasitic and free-living species across the nematode phylum (**Table 1**). Complete genome sequences have been determined for few phylum members so far, but transcriptome data is available for over 60 nematode species (66, 67). In 2005, McVeigh and co-workers performed a systematic BLAST analysis of EST databases to investigate FLP sequence diversity within the phylum Nematoda identifying more than 500 FLPs across 46 species (13). The FLP complement of other nematodes seems to be generally similar to that of *C. elegans* (13, 30, 44). This finding is confirmed by a very recent study of McCoy and colleagues, who re-investigated the available genome and transcriptome resources for 17 pathogenic nematodes (24). Many nematode FLPs display a high degree of inter-species structural conservation that is independent of their parasitic or free-living lifestyle [**Table 1**; Ref. (13)], supporting a fundamental role of FLPs in nematode biology. Corroborating this, only one nematode *flp* gene is thought to be parasite-specific; the *flp-31* gene is absent from the *C. elegans* genome, but occurs in several plant parasitic nematodes suggesting a function specific in phytoparasitism (13, 24, 44). Although *flp-31* was previously predicted from *A. suum* (32), this gene is considered to be a sequelog of *C. elegans flp-15* (24). Initially, *flp*-29 and *flp*-30 were also found to be parasite-specific (13), but a recent investigation of their C-terminal motif and genomic location suggests that these genes should be re-designated to respectively *flp*-28 and *flp*-2 (24).

Whereas most FLPs are likely widespread throughout the nematode phylum, variable conservation has been reported for some family members. Highly conserved nematode *flp*-genes include *flp-1*, *flp-6*, *flp-11*, *flp-12*, *flp-14*, *flp-16*, *flp-18*, *flp-19*, *flp-21*, and *flp-22*; other genes such as *flp-2* and *flp-10* have shown to be more restricted and structurally diverse (13, 24). Interestingly, parasitic nematodes appear to possess variable proportions of the *C. elegans flp*-gene complement and variation is highest among distinct clades (24). Genome-wide analysis of the parasite *Meloidogyne incognita* showed that its FLP complement is reduced to about 60% compared to that of *C. elegans* (44). Two other nematodes, *Trichuris muris* and *Trichinella spiralis*, were shown to display a dramatically reduced complement of only 13%, whereas *A. suum* possesses 84% of *C. elegans flp*-genes (24) The finding that fewer *flp*-genes are expressed in parasitic nematodes as compared to free-living species has been postulated to be an indication of the more contained repertoire of stimuli these nematodes encounter during their endoparasitic stage (68). Furthermore, more FLPs seem to be present in the animal parasitic datasets compared to plant parasitic nematodes (68). Our view on the diversity of FLPs in nematodes however strongly depends on the available sequence data. In depth analyses of the increasing number of completed genome sequences and transcriptome resources should further expand our understanding of the nematode FLP repertoire in the near future.

Recent studies estimate the presence of 32 distinct *flp*-genes in nematodes (24). Among them are 15 genes that code for N-terminally extended peptides carrying the classical FaRP motif, whereas most others peptides share the restricted RFamide core (**Table 1**). Although the relatedness of FLPs across metazoans is often unclear, sequences of the neuropeptide F (NPF) family have been identified in several invertebrate groups and predicted in the nematode phylum as well (69). NPF-like peptides are encoded by the *flp-27* precursor that is highly conserved in nematodes, and contains the C-terminal RXRFamide motif characteristic of the invertebrate NPF family (70). The plethora of FLPs in nematodes is high, given the structural simplicity of their nervous system harboring around 300 neurons (2, 5, 6). This diversity of neural messengers is magnified by classical neurotransmitters and a broad range of other neuropeptides of insulin (*ins*) and neuropeptide-like protein (*nlp*) families, of which about 200 peptides are predicted in *C. elegans* (46, 71).

LOCALIZATION OF FLPs

Immunocytochemical localization of FLPs has been performed in various nematode species, mainly using antibodies raised against synthetic FMRFamide or the RFamide motif (18, 37, 55). These studies suggest that FLPs are widely expressed in the nervous system of all nematodes, supporting a general role for FLP signaling in nematode biology. The broad distribution of immunoreactive neurons, including in motor neurons, fueled the research effort to decipher nematode FLP signaling and its role in neuromotor function, which had already proven to be a successful target for parasite control (12). Although the vast patterns of FLP immunoreactivity are generally similar between nematode species, HPLC-ELISA studies have identified qualitative differences between free-living and plant parasitic nematodes, suggesting that the distinct peptides present in plant nematodes are structurally different (72, 73).

Despite immunocytochemistry being immensely useful to study gross patterns of FLP localization, most of the C-terminally directed antibodies used were incapable of reliably discriminating between structurally related FLPs. Gene-specific *flp* expression has been mainly investigated in *C. elegans*, using reporter transgenes in which LacZ or a fluorescent protein gene is placed under the control of the endogenous promoter region. Li and co-workers applied this molecular approach to map the specific expression patterns of *flp-1* to *flp-23* genes (22, 23). Just over 50% of the total number of neurons were found to express *flp*'s, a wide distribution in stark contrast to earlier immunochemical studies in which only 10% of all *C. elegans* neurons showed FLP reactivity (74). Expression could be detected in all neuronal cell types, including interneurons, sensory neurons, and motor neurons. Six *flp*-genes were also expressed in non-neuronal cells, including in head muscle (*flp-2* and *flp-11*), pharyngeal muscle (*flp-5* and *flp-15*), socket and/or sheath cells (*flp-11* and *flp-15*), vulval cells (*flp-10*), and uterine cells (*flp-11* and *flp-2*). Although the expression of each *flp* gene can be precisely delineated, there is a considerable overlap with many cells expressing more than one *flp* gene (23). Most *flp*-genes are also expressed in multiple neurons suggesting that some FLPs have overlapping functions, unlike others fulfilling unique roles.

In situ hybridization (ISH), which uses nucleotide probes complementary to specific gene transcripts, offers an attractive alternative to delineate *flp* gene expression in other nematodes

that are less amenable to transgenesis than *C. elegans* (38, 42, 54, 75). Furthermore, as immunochemistry allows the determination of neurite morphology facilitating neuronal identification, several antibodies highly specific to certain *A. suum* FLPs were recently generated (42, 76). Another approach for FLP localization consists of direct mass spectrometric analysis on dissected neuronal tissue without the need of an extensive extraction process (29, 76). Importantly, this technique can identify previously unknown peptides as no prior sequence information is required. Yew et al. (29) subjected individually dissected nerve ganglia from *A. suum* to mass spectrometric analysis, producing a peptidomic map of the individual anterior ganglions. FLP distribution appeared to be much less restricted to specific cells as compared to the gene expression studies in *C. elegans* or *G. pallida* using reporter constructs and ISH, thus supporting the notion that FLP expression differs among nematode species. The authors however also stress the sampling biases inherent in the use of mass spectrometry. In large nematodes such as the foot-long *A. suum*, cell-specific FLP content can also be rapidly determined by precisely dissecting individual cells (31, 42).

Taken together, abovementioned studies paint a picture in which FLPs widely occur in all known nematode neuronal subtypes and even in non-neuronal tissues. Whereas the gross patterns of FLP distribution remain consistent across the phylum, more recent studies indicate that the cellular expression of homologous FLPs can substantially differ between nematode species (2, 42). This is remarkable, given that both the general FLP complement and the basic nervous architecture are conserved, with *C. elegans* even considered as a miniature version of *A. suum* at the level of neuronal morphology (2, 24, 51, 77, 78). Caution is however warranted as dissimilarities could be attributed to experimental differences, with each technique suffering from inherent caveats. Besides the limited specificity of the commonly used antibodies, transgenic reporter constructs may not contain all regulatory sequences necessary to recapitulate endogenous gene expression. Moreover, variability of cellular expression patterns of different gene products has repeatedly been observed in *A. suum*, partially due to genetic differences since the worms are not isogenic (31, 42, 75, 76).

FLP-RECEPTORS IN NEMATODES

Most FLPs are known to act through binding of G protein-coupled receptors (GPCRs) (79–81). Although the early work on nematode FLPs primarily focused on peptide identifications, *in vitro* and functional studies have started to address the biology of their receptors and mode of action. In *C. elegans*, sequence similarity or homology to the FLP-receptor family has been postulated for several of the more than 100 peptide GPCR genes predicted in the genome (81, 82). The neuropeptide receptor NPR-1 was previously suggested as a member of the invertebrate NPF receptor (NPFR) family and related neuropeptide Y receptors (NPYRs) in mammals (83). Sequence similarity and phylogenetic clustering suggests additional NPFR/NPYR-like family members are likely to be present in *C. elegans*, as well as representatives related to vertebrate neuropeptide FF receptors, and *Drosophila* myosuppressin and FMRFamide receptors (8, 82, 84). Peptides that functionally activate these GPCRs, with exception of NPR-1, unfortunately remain unknown.

In general, few nematode FLPs have been matched to their receptor(s) and the identification of FLP-receptor couples has only been undertaken in *C. elegans* (2, 81). Activation by FLPs has been reported for 13 *C. elegans* receptors encoded by 10 genes (**Table 1**), all of which are members of the rhodopsin family of GPCRs [reviewed in Ref. (81)]. Deorphanization, i.e., the identification of receptor ligand(s), is typically done by expressing GPCRs in a heterologous cellular system such as *Xenopus* oocytes, mammalian cells or yeast. Receptor activation can then be detected by monitoring downstream steps in the GPCR signaling pathway including levels of secondary messenger molecules or GTP exchange upon G protein activation (85). When heterologous GPCRs are challenged with a peptide library, multiple FLPs are generally found to activate a single receptor. Peptide motifs essential for receptor activation are often shared by FLPs derived from the same precursor protein. For example, Kubiak and co-workers showed that all peptides from the FLP-15 precursor carrying the highly similar GPXGPLRFamide motif, recognize the neuropeptide receptor NPR-3 (60). Likewise, two structurally similar FLPs processed from the FLP-2 precursor were found to activate *C. elegans* receptors encoded by the *frpr-18* locus (45). By monitoring intracellular calcium levels, Mertens et al. showed that both FLP-2 peptides activate two isoforms of the receptor FRPR-18 though with different potencies. Whereas SPREPIRFamide (FLP-2A) was active with nanomolar half-maximal effective concentrations (EC_{50} values), FRPR-18 receptors were only activated at micromolar concentrations by LRGEPIRFamide (FLP-2B). In contrast, Larsen and co-workers found FLP-2A and FLP-2B to be equipotent on the FRPR-18b isoform using a similar calcium mobilization assay in a different type of cells (14). Receptor pharmacology can thus vary dependent on the heterologous system, which may be due to differences in the available G protein signaling machinery or folding properties that affect the functional expression of a GPCR. Although *in vitro* expression systems may not fully reflect endogenous settings, most ligand-receptor couples identified in *C. elegans* are supported by functional studies on FLPs and their receptors (61, 62, 81). Functional evidence on peptide GPCRs and putative FLP ligands is also emerging in other nematodes (86, 87), which may serve as a lead in the search for FLP-receptors in these species.

In *C. elegans* and likely other nematodes, the FLP signaling network is highly expanded by GPCRs able of binding multiple FLPs that, can even originate from different precursor proteins (**Table 1**). The neuropeptide receptor NPR-1 was the first FLP-receptor to be deorphanized in *C. elegans*, and shown to recognize both FLP-18 and FLP-21 peptides (61, 63). Interestingly, this GPCR exists in two variants differing by a single amino acid at position 215, NPR-1.215F or NPR-1.215V that is likely implicated in G protein coupling. Substitution of this residue is sufficient for affecting ligand binding and potency resulting in the differential regulation of feeding behavior (61, 63, 83). Both receptor variants are activated by the FLP-21 peptide that is, however, 10-fold more potent in binding NPR-1.215V than NPR-1.215F (61, 63). In addition, Rogers and co-workers found that the NPR-1.215V

variant expressed in *Xenopus* oocytes can be activated by peptides from the FLP-18 precursor, albeit with lower potencies than FLP-21 (61). A second study reported by Kubiak et al. did not identify FLP-18 peptides as the ligands of NPR-1.215V (63), but the receptor variant was expressed in mammalian cells and differences in expression system may account for the discrepancy in identified ligands in both studies. Both FLP-21 and FLP-18 peptides have been found to activate other *C. elegans* receptors as well, including NPR-11 and NPR-5 (40, 62, 88). In addition, FLP-18 peptides were also identified as ligands of the receptors NPR-4 and NPR-10 (40, 62). An unusual structure-activity relationship has been suggested for the *C. elegans* receptor FRPR-3 (47, 50). Ligands identified for this GPCR include a FLP-7 (TPMQRSSMVRFamide) and FLP-11 (AMRNALVRFamide) peptide, whereas structurally similar peptides encoded on the same precursor proteins were ineffective at activating the receptor (50). However, it should be noted that EC_{50} values for both peptides reside in the micromolar range (50), and other functional ligands might activate FRPR-3 with higher potency. FLP-7 and FLP-11 peptides were also shown to activate another receptor, NPR-22, together with an array of FLPs including FLP-1, FLP-9, FLP-13, and FLP-22 peptides (35). The potencies of receptor activation varied from the nanomolar to the micromolar range (**Table 1**). Finally, the EGL-6 receptor found to be involved in *C. elegans* egg-laying has been coupled to its FLP-10 and FLP-17 ligands in two ways, by making use of an *in vitro* assay but also by screening neuropeptide-encoding transgenes for the ability to inhibit egg-laying (53).

Although knowledge has been gathered on the receptor biology of several *C. elegans* FLPs, our view of nematode FLP-receptors is far from complete. Deorphanization of GPCRs has been successful in matching some FLP-receptor couples; however, often a sub-set of the predicted peptide repertoire is tested such that the array of ligands acting on a receptor remains incomplete. FLPs are thought to exert most of their effects through the activation of GPCRs, but some family members are capable of eliciting fast responses by gating ion channels (89–91). This mode of action likely also applies for several nematode FLPs (90–94). The coupling of multiple peptides to a single GPCR and vice versa greatly enhances the complexity of FLP signaling in *C. elegans*. However, the characterization of all functional FLP-receptor couples will be crucial to further expand our understanding of nematode FLP signaling, and will uncover whether promiscuity of FLP-receptors can be generalized in nematodes.

FLP-MEDIATED MODULATION OF NEMATODE PHYSIOLOGY AND BEHAVIOR

Despite the apparent simplicity of the nematode nervous system, harboring around 300 cells, a surprisingly rich behavioral repertoire has been described (86, 87, 95, 96). The structural and spatiotemporal gene expression diversity of the nematode FLP system is reflected in the range of FLP-induced physiological responses. The role of FLPs has been extensively described in previous reviews (2, 5, 6); here, we focus on FLP signaling functions emerging consistently throughout evolution to illustrate some of the general principles of FLP signaling gleaned from the study of nematode peptides. Although *C. elegans* has been heavily exploited to investigate the basic biology of FLP signaling, we highlight some pharmacological and behavioral studies performed on related nematodes.

NEMATODE FLPs IN THE CONTROL OF FEEDING BEHAVIOR

Although FLPs display a tremendous diversity in structure and biological activity, their involvement in the regulation of energy balance and feeding behavior has been described in both invertebrate and vertebrate lineages (11). Feeding state is a paramount environmental factor that guides *C. elegans* behavior, with a central role for FLP signaling in for instance the regulation of locomotory activity, foraging and food intake (5, 6, 46).

C. elegans NPR-1 signaling regulates food-dependent aggregation behavior

The best characterized example of FLP-modulated behavior in *C. elegans* is food-related aggregation. Certain wild-type isolates, including the standard laboratory strain N2, mainly show a "solitary feeding" phenotype in which worms disperse to feed alone. Others have a propensity to aggregate into clumps in areas of high food density, a behavior that is termed "social feeding" (83). This behavioral polymorphism can be attributed to a single amino acid difference in the *npr-1* gene, which encodes a member of the NPYR/NPFR family (83). Worms expressing the partial loss-of-function isoform with a phenylalanine, NPR-1.215F, are social feeders, whereas strains bearing the *npr-1* allele encoding the version with a valine, NPR-1.215V, are solitary. Since chemically generated null mutations of *npr-1* convert the solitary wild-type N2 lab strain into an aggregating one, NPR-1 activity is suggested to repress aggregating behavior (83).

Both loss-of-function and gain-of-function studies confirm that FLP-21 acts as the endogenous NPR-1 ligand required for its activation and consequent suppression of food-dependent aggregation (61). Whereas transgenic overexpression of *flp-21* rescues the social feeding phenotype of NPR-1.215F worms, genomic deletion of *flp-21* further enhances worm clumping. However, loss of *flp-21* only slightly increases aggregation in animals bearing the Val-215 allele, suggesting that another ligand most likely encoded by the *flp-18* gene may functionally substitute for the loss of FLP-21 ligands (61, 95). FLP-21 furthermore does not appear to act in NPR-1 dependent acute ethanol tolerance, once again suggesting that FLP-18 may be a physiological active ligand (97).

The food-dependent aggregation of social *npr-1* mutant worms relies on chemosensory responses in a number of different sensory neurons exposed to the environment and the pseudocoelomic body fluid. Due to their specific localization in *C. elegans*, these cells are able to detect various adverse or stressful conditions (98, 99). Despite expression of *npr-1* in at least 20 neurons (99), the inter/motorneuron RMG seems to be the cellular hub of the NPR-1 mediated feeding behavior (100). Anatomical gap junctions connect RMG to five sensory neurons known to promote aggregation, including the nociceptive ASH and ADL neurons and the URX oxygen sensor (101). In a hub-and-spoke model in which RMG functions as the central hub, RMG is suggested to integrate signals from various sensory neurons to stimulate aggregation using its own chemical synapses. Furthermore, due to the bidirectionality of the gap junctions, RMG in turn modulates the responses of

its associated sensory neurons having their own synaptic outputs (100). NPR-1 however inhibits the gap junction driven activation of RMG, either by downregulating the gap junctions or by altering RMG excitability (99, 100).

NPR-1 functions as a modulator of many neurons and behavioral responses, not only in response to food but also to other key environmental parameters of which ambient O_2 and CO_2 levels appear to have a major influence. NPR-1 modulates aerotaxis as well as the integration of sensory cues of food availability, internal metabolic state and O_2 levels (100, 102–106). NPR-1 also alters the sensitivity to environmental repellents (e.g., pheromones, CO_2), innate immune responses, and tolerance to ethanol (107–113). The fact that *flp-18* and *flp-21* mutants often display more subtle phenotypes in these studies indicates that NPR-1 might have additional ligands. Furthermore, the expression patterns of *flp-18* and *flp-21* have limited overlap and it is not known how the expression of both genes or release of their peptide products is regulated. Despite the wide neuronal expression pattern of *npr-1*, RMG can be pinpointed as the cellular locus of NPR-1 function for a number of behaviors. Besides "social feeding," *npr-1* expression in RMG acts synergistically with the primary heat sensing machinery to regulate aversive behaviors at high temperature, with *npr-1* or *flp-21* loss-of-function animals showing an increased threshold for heat avoidance (112). Similarly, RMG-specific rescue of *npr-1* restores pheromone avoidance defects in the *npr-1* mutant background (114). Furthermore, NPR-1 and its FLP-18 and FLP-21 ligands are required for locomotion quiescence during lethargus, a quiescent behavioral state occurring before each of the four molts, with increased activity in the RMG circuit promoting locomotion arousal (115). The RMG-hub-and-spoke circuit therefore appears to be a multifunctional sensory circuit integrating various stimuli that heavily depends on FLP neuropeptidergic signaling in order to coordinate behavioral output.

Other FLPs modulating food-related behaviors

Besides aggregation, FLPs are implicated in other feeding behaviors such as the regulation of energy balance and metabolism according to perceived food availability (11). In *C. elegans*, loss-of-function of the *flp-18* precursor gene causes defects in chemosensation, foraging, and formation of the arrested dauer developmental stage that is induced by stress conditions (62). In addition, these mutants display increased levels in intestinal fat and reduced aerobic metabolism, strongly suggesting that FLP-18 neuropeptides are involved in fat storage and metabolism (62). FLP-18 peptides activate the neuropeptide receptors NPR-4 and NPR-5, and loss-of-function of these GPCRs recapitulates some of the phenotypic effects observed in *flp-18* mutants (62, 88). Cohen and co-workers found that *npr-4* is expressed in a number of sites including the intestine, whereas NPR-5 is present in several sensory neurons and head, neck, and body wall muscles. FLP-18 signaling through activation of NPR-4 in intestinal muscle was shown to regulate the accumulation of intestinal fat. NPR-5 however modulates the activity of a number of amphid sensory neurons that directly sense environmental cues, of which the chemosensory ASJ neurons are critical in dauer formation (**Figure 1A**) (116).

Environmental food availability also strongly influences *C. elegans* food-seeking behavior. When feeding on a bacterial lawn, *C. elegans* spends most of its time slowly moving within a restricted area. Upon removal from their food source, animals however initiate an intensive local search behavior characterized by repetitive bursts of reversing and turning in a restricted immediate area. After prolonged food withdrawal ($\geq$15 min off-food), FLP-18 neuropeptides released from the primary interneuron AIY and to a lesser extent from RIG interneurons, activate a switch in behavioral state from this local search to dispersal in which turning events are suppressed (62, 117). FLP-18 peptides were found to act on the neuropeptide receptor NPR-4 in AVA interneurons and RIV motor neurons that regulate reversal frequency and turning bias, respectively (**Figure 1B**) (117, 122). NPR-4 signaling by FLP-18 peptides may therefore reduce local search behavior by modulating the activity of these neurons (62). Upstream in the circuit, AIY interneurons, which release FLP-18, receive synaptic input from various sensory neurons. As such, they presumably play an integrative role enabling the regulation of locomotory behaviors in response to environmental perception. Among the presynaptic partners is the AWC olfactory neuron pair that is a prominent player in local search behavior (117). Both neurons are stimulated following the removal of an attractive odorant that serves as a cue for food presence (119). Upon odorant removal, the AWC neurons provide glutamatergic input to downstream interneurons that will accordingly reorient locomotory behavior by stimulating local search behavior. Glutamate release was found to hyperpolarize AIY neurons that express FLP-18 and suppress turning, via the glutamate-gated Cl^- channel GLC-3. On the other hand, AIB interneurons that promote turning are depolarized by glutamate via the AMPA/kainate-like glutamate receptor GLR-1, resulting in directed chemotaxis behavior along odor gradients (119, 120, 123). Taken together, these observations fit within a model in which sensory detection of food availability can coordinately regulate adequate responses such as foraging behavior and energy metabolism via FLP signaling (**Figure 1**).

Interestingly, there is evidence for a neuropeptide-mediated sensorimotor feedback loop that dampens the odor-evoked activity of the AWC neurons, hereby limiting local search behavior (120). When odor is sensed, the AWC neurons release buccalin-related NLP-1 peptides, which in turn act upon the NPR-11 receptor on AIA to modulate INS-1 peptide secretion. Closing the feedback loop, INS-1 acts on the AWC sensory neurons to modulate their responsiveness to sensory stimuli (**Figure 1C**). Although strong evidence from mutant and other studies demonstrate a functional NLP-1/NPR-11 relationship, other peptides have been shown to activate the receptor with EC_{50} values in the nanomolar range, including FLP-21 (1–10 nM) and FLP-18 [(SYFDEKK)SVPGVLRFa, 80–800 nM] (40). As mentioned above, the FLP-21 peptide modulates behavior in the context of food and other environmental parameters through activation of the receptor NPR-1, and is expressed in ADL, ASE, and ASH sensory neurons among others (61, 63, 111). ASE neurons in particular are implicated in food-dependent behavior, as they are mainly responsible for chemotaxis to water-soluble

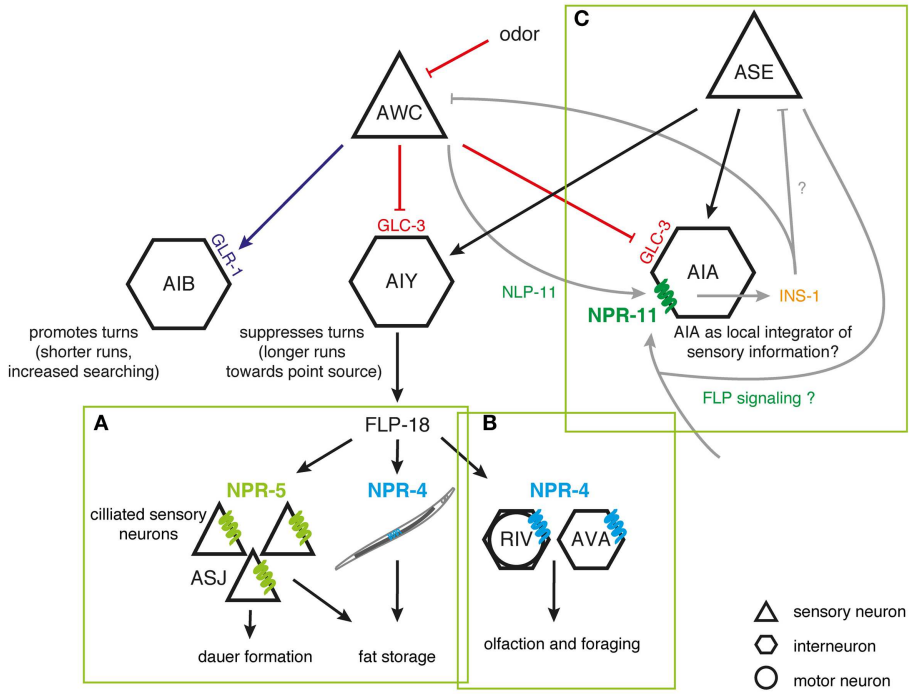

FIGURE 1 | FLP signaling regulates *C. elegans* foraging and metabolism. **(A)** FLP-18 peptides are released from AIY in response to sensory cues relaying food availability. By acting on the receptors NPR-4 in the intestine and NPR-5 in ciliated neurons, FLP-18 peptides control fat storage; while activation of NPR-5 in ASJ neurons regulates dauer formation. **(B)** To regulate odor responses and foraging strategy, FLP-18 peptides signal through NPR-4 in AVA and RIV neurons that control reversal frequency and turning bias, respectively. **(C)** Peptidergic feedback modulates sensory responses in *C. elegans*. In response to odor, the AWC olfactory neuron releases NLP-1 neuropeptides, which act on the NPR-11 receptor on AIA to modulate INS-1 peptide secretion. INS-1 subsequently closes the feedback loop by modulating AWC's responsiveness to sensory stimuli. AIA could act as a local integrator of sensory information, with FLP sensory peptides driving similar neuropeptidergic feedback loops to modulate the responsiveness to sensory stimuli [adapted from Ref. (62, 117–121)].

attractants (116, 124). Which neurons functionally act downstream of ASE in water-soluble chemotaxis has not been fully understood. AIA interneurons are prominent targets of ASE, hereby hinting on a functional FLP-21/NPR-11 interaction consistent with the observed *in vitro* data. Although this interaction has not been uncovered in previous studies, it remains interesting to investigate whether NPR-11 signaling in AIA by FLP sensory peptides can activate a neuropeptidergic feedback loop to modulate the gain or temporal properties of the sensory activation process, analogous to that for AWC olfactory neurons (118, 120). AIA could in that respect act as a local integrator of sensory information (**Figure 1**).

In addition to the regulation of foraging behavior and metabolism, feeding in *C. elegans* is closely linked to pharyngeal pumping activity (125). Pumping activity is regulated by an intrinsic pharyngeal nervous system (126), but neurohormones released from neurons extrinsic to this cellular system can also influence pumping behavior (127). Several FLPs act on pharyngeal muscle to either excite or inhibit pumping (34, 128, 129). Despite the disadvantage of its size, numerous electrophysiological studies have been performed to reveal the effect of FLPs on pharyngeal preparations in *C. elegans*. Surprisingly, many of the tested FLPs modulate action potential frequency, suggesting an impressive neurochemical complexity of the feeding circuit (34, 128, 129). Different FLPs have been found to exert opposite effects on action potential frequencies of pharyngeal muscles. Stimulatory peptides include FLPs derived from the *flp-5*, *6*, *8*, and *14* precursor genes, whereas others elicit inhibitory effects on serotonin-induced depolarization of pharyngeal muscles like *flp-1*, *3*, *9*, *13*, and *16* encoded peptides. By using wild-type worms and mutants with deficits in synaptic signaling, it was shown that FLP-13 (APEASPFIRFa) acts directly on the pharyngeal muscle, while FLP-8 acts via the pharyngeal neuronal circuit (34). These results are consistent with the fact that the majority of excitatory and inhibitory peptides were encoded on genes shown to be expressed in the *C. elegans* pharyngeal nervous system (23). It therefore appears that multiple FLPs are involved in feeding behavior by modulating pharyngeal activity, as supported by findings in *A. suum* (54, 130–132). Using a modified pressure transducer, Brownlee and colleagues measured changes in intrapharyngeal pressure to monitor the contraction of the *Ascaris* radial pharynx muscle. PF3 (AF8, KSAYMRFa) causes a biphasic response in the pharynx of *A. suum*, with hyper-contraction following an initial relaxation. AF1 (KNEFIRFa), however, leaves the muscle in a more relaxed state (130, 132).

Effects of FLPs on locomotion

Besides locomotory activity, the control of the neuromuscular junctions that drive locomotion is certainly also to be considered in the context of nematode feeding, as it enables the worm to migrate toward food sources. Diverse inhibitory and excitatory activities have been reported in *A. suum* on body wall muscles upon the application of FLPs (54, 91, 133–136). Data obtained from this type of studies do, however, not always facilitate a better understanding of *in vivo* physiological functions. Although an array of FLP peptides clearly shows muscle-based effects, denervation of somatic muscle strips can alter or even abolish the activity of many FLPs indicating that FLP-receptors do not only reside on muscles (1, 92). Electrophysiological studies indeed demonstrate that FLPs, such as AF1, can act through modulation of neuronal conductance of motor neurons in addition to their muscle-based effects (137). Contrary to AF1, the effects of AF8 (KSAYMRFa) on somatic muscle of *A. suum* are uniquely differential and context-dependent. While application to dorsal muscles causes slow relaxation, AF8 has profound excitatory effects on ventral muscles (49). Remarkably, this is the only known nematode peptide to show such differential neuromusculatory activity. Stretton et al. have characterized the effects of *C. elegans* and *Ascaris* FLPs on the synaptic activity of *Ascaris* motor neurons (138). They identified five major neuronal response types, theoretically corresponding to at least five FLP-receptor subtypes. These differences might possibly be attributed to different receptors, second messengers, or the combination of both.

In order to understand the *in vivo* functions of neuropeptides, comprehensive analyses on locomotory behaviors of intact nematodes have been carried out. In *A. suum*, direct injection of synthetic FLPs into the body cavity elicits diverse behavioral responses including effects on body waveforms, body length, and paralysis (19, 96, 138, 139). Similarly, the normal locomotory behavior is severely disrupted when *flp*-coding genes are silenced by RNAi, as was shown for *flp-14* and *flp-32* in *G. pallida* (87, 140). FLPs also have profound impacts on the migrational abilities of parasitic nematodes toward their host, as illustrated by RNAi silencing of *flp-14* and *flp-18* in *M. incognita* (86, 141). Host delivered RNAi of *flps* as non-chemical based control strategy for parasitic nematodes is therefore gaining importance (142).

When on a solid surface, *C. elegans* lays on its side and moves in a sinusoidal fashion by undulating contractions and relaxations of dorsal and ventral longitudinal body wall muscles. These muscles use acetylcholine (ACh) and GABA as their primary excitatory and inhibitory neurotransmitters, respectively, and disruption of either of these transmitter biosynthetic pathways leads to severely uncoordinated locomotion (143–145). FLP-1 peptides are also required for the smooth sinusoidal movement of the animals, as inactivation of *flp-1* in *C. elegans* causes hyperactive movement (146). FLP-1 has been found to modulate ACh signaling (147), hereby providing a possible direct link to the regulation of locomotion. FLP-1 as well as FLP-18 peptides were also recently implied in the homeostatic balance of excitation-inhibition coupling in the locomotor circuit that drives body wall muscle contractions (148). This neuropeptide modulation primarily acts on the GABAergic neural transmission at the neuromuscular junctions, where FLP-18 peptides act directly on muscles via the NPR-5 receptor to either inhibit contraction or to promote relaxation. However, the FLPs also appear to have an effect on other cell types to coordinate locomotory output. In addition, Wani and co-workers performed a large-scale RNAi screen to identify genes that mediate endogenous dopamine signaling in *C. elegans*, an important system controlling worm locomotion (149). The identification of FLP-1 peptides in this study suggests that FLP signaling may be required for dopamine synthesis and release from dopaminergic neurons or for modulating dopamine signaling in dopamine-receptive neurons.

FLP-COORDINATED REGULATION OF FEEDING AND NOCICEPTION

One salient feature of neuropeptide modulation common to both vertebrates as invertebrates is their role in gating and controlling the gain of peripheral sensory inputs (150, 151). In vertebrates, FLP signaling has been repeatedly linked to the modulation of opioid signaling and nociception, whereas the opioid system participates in the regulation of feeding (11, 152, 153). This recurrent interplay makes it conceivable to state that FLP and opioid systems could interact to integrate feeding with stress. Such coordinated regulation would enable animals to decide whether to engage in feeding-related behaviors when presented with an attractive food source in the presence of aversive or noxious stimuli (11). Furthermore, the primary FMRFamide sequence is embedded within an endogenous mammalian opioid peptide derived from the Met-enkephalin precursor, suggesting that enkephalins and FLPs may have coevolved from a common ancestral peptide and share functional links (154). These findings imply that synergistic pathways between stress and feeding behavior might have been evolutionary conserved.

The coordinated regulation of food-dependent behavior (aggregation) and stress perception (nociception) has been thoroughly documented in *C. elegans*. The manifestation of aggregating behavior involves multiple pathways linking the RMG hub neuron by gap junctions to nociceptive (ASH and ADL), oxygen-sensing (URX), and chemosensory neuron spokes (98–100). Simultaneous ablation of ASH and ADL attenuates aggregation, implying that this behavior may be a response to repulsive or stressful environmental stimuli (98). Aggregation could supply a defense to the animal, with group feeding stimulating dauer formation or prompting the secretion of enzymes that inactivate bacterial toxins (98). The induction of solitary behavior by the FLP receptor NPR-1 hints that its actions may antagonize responses of ASH and ADL to stressful cues. As both neuron types synthesize FLP-21 (61), they are believed to be able to induce solitary behavior under certain conditions. Given that NPR-1 is expressed in ASH nociceptors, it may also directly modulate their sensory responses correlated to feeding state and food availability (99).

Surprisingly, NPR-1 is able to uncouple two overlapping circuits downstream of the ASH nociceptor (151). ASH utilizes glutamatergic synapses to signal to interneurons that control backward locomotion associated with the avoidance response to noxious stimuli (155, 156). In contrast, aggregation is driven by electrical gap junction signaling between ASH and the RMG hub neuron. Neuromodulation of RMG by NPR-1 uncouples the aggregation circuitry thus making it functionally silent, while sparing the function of the ASH-mediated avoidance circuit. This organization

allows ASH to differentially generate behaviors depending on the neuromodulatory state, with aggregation occurring only when NPR-1 activity is low, and avoidance occurring regardless of modulation. One attractive hypothesis is that the dynamics of this circuit is differentially regulated by distinct sensory cues. A high intensity aversive cue might trigger the release of FLP-21 peptides and subsequently suppress the aggregation behavior, hereby facilitating efficient escape from highly noxious stimuli without the interference of motor programs for aggregation. The polymodal ASH nociceptor is exquisitely suited to detect various aversive stimuli, but other (FLP releasing) sensory neurons may also impinge on the RMG circuit. The RMG-hub-and-spoke circuit perfectly illustrates how information flow through worm circuits depends on neuromodulatory states defined by neuropeptides (**Figure 2**). The principle of circuit flexibility relying on connectivity modulation also extends to vertebrates as exemplified by stress-induced analgesia, an acute suppression of pain generated mediated by opioids (151, 157, 158).

NEMATODE REPRODUCTION: FLP MODULATION OF EGG-LAYING AND SEXUAL BEHAVIOR

FMRFamide-like peptide signaling modulates nematode reproductive behaviors such as egg-laying and copulation. Neuropeptides encoded by the *C. elegans flp-1* gene are suggested to modulate egg-laying rates, since *flp-1* deletion mutants show a defect in the timing of these events (159). This FLP-1-dependent regulation is furthermore dependent on food abundance (159). In a genome-wide RNAi study, Keating et al. (160) reported that knockdown of the FLP receptor FRPR-3 increases brood size and the rate of egg-laying (160). Besides genetic studies, FLPs have been directly tested for activity on muscles associated with the female reproductive system (134, 161). When applied to the ovijector of *A. suum*, for example, AF1 causes a biphasic effect transiently relaxing and then contracting the tissue, whereas both AF2 (KHEYLRFa) and PF3 (AF8, KSAYMRFa) have inhibitory effects (133).

Egg-laying in *C. elegans* is also modulated by *flp-10* and *flp-17* encoded peptides (53). These FLPs are able to activate the EGL-6 receptor that is present in the HSN motor neurons innervating the vulval musculature, hence regulating egg-laying behavior (53, 101, 162). In comparison to wild-type *C. elegans*, *egl-6* overexpression and gain-of-function mutants display slower egg-laying rates, suggesting an inhibitory receptor function (53). Both peptides encoded by *flp-17* are expressed in a pair of BAG sensory neurons, whereas *flp-10* is expressed in several neuronal and non-neuronal tissue. Laser-ablation and overexpression experiments suggest that the vulva and spermatheca are the principal source of the endogenous FLP-10 peptide acting on EGL-6 (23, 53). This leads to a simple model in which relevant sensory cues control FLP-10/FLP-17 secretion, hereby directly modulating the activity of the egg-laying motor neurons to suppress egg-laying in unsuitable environments (**Figure 3**). Inhibition of the HSN motor neuron by EGL-6 seems to be synergistically to cholinergic inhibition of egg-laying upon unfavorable conditions (53).

Although *C. elegans* populations almost entirely consist of self-fertilizing hermaphrodites, males arise infrequently under certain environmental conditions. Males strikingly differ from their hermaphrodite counterparts in their complex mating behavior in which males turn backwards along the hermaphrodite body until their tail contacts the vulva, after which copulation is engaged.

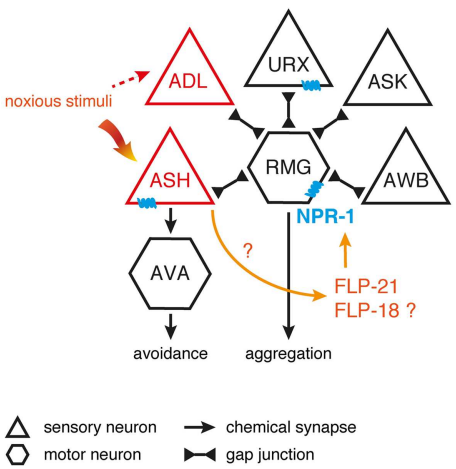

FIGURE 2 | Inhibition of the RMG interneuron by NPR-1. Signaling from ASH and ADL neurons induces aggregation through gap junctions with RMG. RMG is the hub neuron of a gap junction network connecting various sensory neurons known to trigger aggregation. ASH and ADL also mediate acute avoidance behavior through synaptic signaling. Both types of connections are differentially regulated by the NPR-1 receptor, with FLP signaling inhibiting the gap junction driven activation of RMG and not being essential to ASH-mediated avoidance [adapted from Ref. (100, 151)].

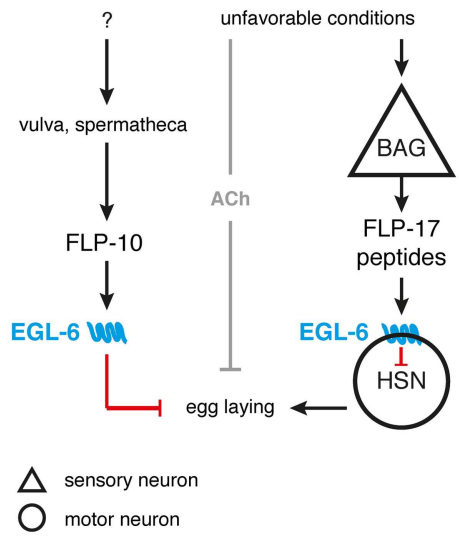

FIGURE 3 | FLP signaling suppresses egg-laying in unsuitable environments. BAG neurons release FLP-17 neuropeptides in response to unfavorable conditions. These peptides are able to activate the EGL-6 receptor on HSN motor neurons, hereby inhibiting egg-laying. Release of FLP-10 by the vulva and spermatheca along with subsequent EGL-6 signaling further inhibits egg-laying, with the exact triggering stimuli still uncharacterized. Under unfavorable conditions, cholinergic signals (ACh) may be independently invoked by other sensory circuits to synergistically inhibit egg-laying [adapted from Ref. (53, 81)].

Peptidergic signaling by FLP-8, FLP-10, FLP-12, and FLP-20 is required for the sensory transduction in male turning behavior (163). Loss-of-function mutations in corresponding genes each induce repeated turning, with males continually circling the hermaphrodite instead of initiating copulation after a single turn. Although these *flp*-genes are somewhat dispersedly expressed in various sensory neurons and interneurons, *flp-20* expression in the mechanosensitive cells completely rescues the mutant's turning phenotype (163). FLP-20 is therefore hypothesized to convey somatosensory information to terminate the turning program and initiate copulation. How gender-specific modifications of the shared touch circuitry of male and hermaphrodite nervous systems contribute to copulatory behaviors still remains unknown.

FLP SIGNALING IN LEARNING BEHAVIOR

A growing body of evidence, including from studies on mollusks and arthropods, implicates FLPs in the regulation of learning behavior (164–166). *C. elegans* displays a remarkable level of behavioral plasticity similar to that observed in higher organisms (95, 167, 168), including non-associative (adaptation, habituation) and associative learning behaviors (169). For example, *C. elegans* can learn to approach or avoid tastes, odors, oxygen, or temperatures that predict the presence or absence of food. Both short-term and long-term forms of memory have been demonstrated in *C. elegans* (95).

In *C. elegans*, FLP-20 is involved in tap habituation, a type of non-associative learning behavior (170). *C. elegans* reverses its locomotion in response to a non-localized mechanical stimulus generated by tapping the culture plate containing the animal, a behavior known as the tap withdrawal response. Repeated taps result in habituation as measured in a decrement of both the amplitude and the frequency of this reversal (171). Mutants for the *flp-20* gene show deficits in the relatively short-term 12-h memory following a massed training session. On the other hand, *flp-20* is not required for long-term memory of tap habituation that lasts up to 48-h after temporally spaced training in which the same amount of training is presented with interval resting periods (172). This and other studies illustrate how two types of memory within the same learning paradigm are induced by distinct molecular mechanisms that are differentially initiated depending on the temporal pattern of the training regimen. The *flp-20* gene is specifically required within the mechanosensory neurons that presumably release FLP-20 peptides to activate downstream neurons required for short-term memory consolidation. This type of memory correlates with a *flp-20*-dependent increase of synaptic vesicles in the terminals of the mechanosensory neurons (170). This and other studies suggest that the molecular changes underlying short-term memory arise and are maintained at the level of the sensory neurons. Pre-synaptic changes in particular seem indispensable, and likely entail differential release of signaling molecules to dampen the reversal response in the context of tap habituation.

CONCLUSION

The nematode FLP system comprises an intertwined signaling network with a broad array of neuropeptides operating within an anatomically small nervous system. FLP diversity translates into a central role of this neuropeptide family in various aspects of nematode biology. Functional studies in nematodes support the evolutionary continuity of FLPs as key regulators of energy balance, feeding behavior, reproduction, and sensory modulation. In general, the FLP complement has shown to be widely conserved throughout the phylum though some peptides show a more restricted distribution, with the latter potentially as a consequence of adaptation to a specific lifestyle such as parasitism (2, 24, 42). The particular cellular distribution of FLPs appears not to be fully conserved across nematodes, in contrast to the slow rate at which the nematode nervous system evolves at the cellular level. Rapidly evolving peptide expression could therefore reveal to be an essential factor in the generation of species-specific behavior, furthermore facilitating the radiation of nematodes into a variety of habitats including as parasites of both animals and plants (12).

In *C. elegans*, the *flp*-genes have overlapping expression patterns, with at least half of all neurons expressing one or more FLPs (173). This implies that some neurons use a repertoire of FLP peptides in addition to other messengers, which may be deployed in a context-dependent way rendering these cells multifunctional. Such multiplexing could contribute to increase the complexity of information processing in a numerally simple nervous system, hereby supporting the rich behavioral palette of nematodes. Given their broad diversity and expression, neuropeptides are exquisitely suited to actively recruit particular cellular circuits depending on the environmental and internal context. This type of neuromodulation appears to be an irreducible part of circuit flexibility in the nematode nervous system (174).

The considerable amounts of data on nematode FLP function derived from neuronal and neuromuscular bioassays demonstrate an impressive complexity in the FLP signaling system. On the other hand, the knowledge of FLP-receptor interplay remains sparse, and most of our current understanding is derived from *C. elegans* in which several FLP-receptors have been coupled to their peptide ligands by *in vitro* assays and *in vivo* functional studies. A common theme in these studies is that a single receptor can be activated by multiple FLPs encoded by one or more genes. However, this apparent receptor promiscuity will need to be proven physiologically relevant, as a whole layer in the control of FLP signaling may reside in the spatiotemporal expression patterns of both receptor and ligand molecules. With the increasing number of completed genome projects and transcriptome resources, putative FLP-receptors can readily be identified using bioinformatics, and *C. elegans* data as a scaffold, broadening our view on FLP signaling in other nematodes. In addition, further receptor deorphanization and subsequent localization of these proteins will, together with the extensive data regarding FLP distribution, shed light on specific FLP functioning within the modulation and coordination of nematode behavior and physiology.

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Involvement of mammalian RF-amide peptides and their receptors in the modulation of nociception in rodents

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Mammalian RF-amide peptides, which all share a conserved carboxyl-terminal Arg–Phe–NH_2 sequence, constitute a family of five groups of neuropeptides that are encoded by five different genes. They act through five G-protein-coupled receptors and each group of peptide binds to and activates mostly one receptor: RF-amide related peptide group binds to NPFFR1, neuropeptide FF group to NPFFR2, pyroglutamylated RF-amide peptide group to QRFPR, prolactin-releasing peptide group to prolactin-releasing peptide receptor, and kisspeptin group to Kiss1R. These peptides and their receptors have been involved in the modulation of several functions including reproduction, feeding, and cardiovascular regulation. Data from the literature now provide emerging evidence that all RF-amide peptides and their receptors are also involved in the modulation of nociception. This review will present the current knowledge on the involvement in rodents of the different mammalian RF-amide peptides and their receptors in the modulation of nociception in basal and chronic pain conditions as well as their modulatory effects on the analgesic effects of opiates.

Keywords: pain, nociception, GPCRs, RF-amide, opiates, opioid-induced hyperalgesia

INTRODUCTION

A tetrapeptide Phe–Met–Arg–Phe–NH_2 (FMRF-NH_2) has been isolated in 1977 by Price and Greenberg and was described as having cardioexcitatory properties in the neverid clam *Macrocallista nimbosa* (1). Thereafter, using specific antibodies for the COOH-terminus of this peptide, FMRF-NH_2 immunoreactive peptides were identified in several species including mouse (2, 3) and the first two mammalian FMRF-NH_2-like peptides, neuropeptides FF and AF, were further isolated from bovine brain (4). They represent the first members of the family of mammalian RF-amide peptides, which all share a conserved carboxy-terminal Arg–Phe–NH_2 sequence. Additional members of this family and their receptors were identified and cloned in the late 90s and early 2000s (5). At present, five groups of mammalian RF-amide peptides have been described including neuropeptide FF (NPFF), RF-amide related peptide (RFRP), pyroglutamylated RF-amide peptide (QRFP), prolactin-releasing peptide (PrRP), and kisspeptin groups. The sequences of human and rodents RF-amide peptides, their different names as well as the name of their receptors are listed in **Table 1**.

Several RF-amide peptide receptors have been discovered: NPFFR1 (alias GPR147), NPFFR2 (alias GPR74), QRFPR (alias GPR103), prolactin-releasing peptide receptor (PrRPR) (alias GPR10), and Kiss1R (alias GPR54). They all belong to the G-protein-coupled receptor family, and share approximately 50% homology. Each receptor binds mostly one group of RF-amide peptide: NPFFR1 binds RFRP peptides, NPFFR2–NPFF group, QRFPR–QRFP group, PrRPR–PrRP group, and Kiss1R–kisspeptin group. However, *in vitro* it has been shown that NPFFR1/2 display a good affinity for all mammalian RF-amide peptides, whereas, QRFPR, PrRPR, and Kiss1R show a high level of discrimination for their endogenous peptides (6).

In this review, we will focus on the involvement of the five mammalian RF-amide peptide groups and their respective receptors in the modulation of nociception in rodents.

Abbreviations: ASIC, acid-sensing ion channels; CNS, central nervous system; DOR, δ-opioid receptor; DRG, dorsal root ganglia; i.c.v., intracerebroventricular; i.th., intrathecal; KOR, κ-opioid receptor; MOR, μ-opioid receptor; NPAF, neuropeptide AF; NPFF, neuropeptide FF; PrRP, prolactin-releasing peptide; QRFP, pyroglutamylated RF-amide peptide; RFRP, RF-amide related peptide.

NPFF, RFRP, AND NPFF RECEPTORS 1/2

Neuropeptide FF group of peptides includes NPFF and neuropeptide AF (NPAF), while RFRP group comprises RFRP-1 and -3 (also called NPSF and NPVF). NPFF and NPAF are derived from the same precursor, whereas RFRP-1 and RFRP-3 are generated from another precursor (7–10). These different peptides can all activate NPFFR1 and NPFFR2, but RFRP group displays better activity for NPFFR1, and NPFF group preferentially activates NPFFR2 (5). NPFFR1 and NPFFR2 are both coupled to G_i (9, 11–13). They display around 50% amino acid identity, and are encoded by two different genes concomitantly identified in 2000 by different research teams (9, 11, 12). NPFFR1 and NPFFR2 are also called OT7T022/GPR147 and HLWAR77/GPR74, respectively (see **Table 1**). They are expressed in all vertebrate species examined and are highly conserved, underlining their important role (14).

NPFF, NPAF, AND NPFFR2

Localization in the pain pathways

NPFF and NPAF. Neuropeptide FF and NPAF were first isolated from bovine medulla oblongata and described as having anti-opioid activity on morphine-induced analgesia in the rat (4, 15). The distribution of NPFF/NPAF peptides and mRNA

Table 1 | Summary of nomenclature and sequences for mammalian RF-amide peptides and their receptors.

Group	Peptide	Species	Uniprot accession number	Sequence	Receptor name
NPFF	NPFF	Human	O15130	SQ**AFLFQPQRF-NH2**	NPFFR2
		Rat	Q9WVA9	NP**AFLFQPQRF-NH2**	GPR74
		Mouse	Q9WVA8	SP**AFLFQPQRF-NH2**	HLWAR77
	NPAF (NPSF for the last eight residues)	Human	O15130	AGEGLNSQ**FWSLAAPQRF-NH2**	
		Rat	Q9WVA9	E**FWSLAAPQRF-NH2**	
		Mouse	Q9WVA8	Q**FWSLAAPQRF-NH2**	
RFRP	RFRP-1 (NPSF, GnIH)	Human	Q9HCQ7	MPHSF**ANLPLRF-NH2**	NPFFR1
		Rat	Q9ESQ9	VPHSA**ANLPLRF-NH2**	GPR147
		Mouse	Q9ESQ8		OT7T022
	RFRP-3 (NPVF)	Human	Q9HCQ7	VPN**LPQRF-NH2**	
		Rat	Q9ESQ9	ANMEAGTMSHFPS**LPQRF-NH2**	
		Mouse	Q9ESQ8	VNMEAGTRSHFPS**LPQRF-NH2**	
QRFP	QRFP43 (43RFa, P518)	Human	P83859	<EDEGSEATGFLPAAGEKTSGPLGNLAEELNG**YSRKKG**	QRFPR
				GFSFRF-NH2	GPR103
		Rat	P83860	<EDSGSEATGFLPTDSEKASGPLGTLAEELSS**YSRRKGG**	AQ27
				FSFRF-NH2	SP9155
		Mouse	Q8CE23	<EDGSSEAAGFLPADSEKASGPLGTLAEELSS**YSRRKGG**	
				FSFRF-NH2	
	QRFP26 (26RFa)	Human	P83859	TSGPLGNLAEELNG**YSRKKGGFSFRF-NH2**	
		Rat	P83860	ASGPLGTLAEELSS**YSRRKGGFSFRF-NH2**	
		Mouse	Q8CE23		
PrRP	PrRP31	Human	P81277	SRTHRHSMEIRTPDINPAWYAS**RGIRPVGRF-NH2**	PrRPR
		Rat	P81278	SRAHQHSMETRTPDINPAWYTG**RGIRPVGRF-NH2**	GPR10
		Mouse	B7U2G4		hGR3
	PrRP20	Human	P81277	TPDINPAWYAS**RGIRPVGRF-NH2**	UHR-1
		Rat	P81278	TPDINPAWYTG**RGIRPVGRF-NH2**	
		Mouse	B7U2G4		
Kisspeptin	Kisspeptin-54 (metastin)	Human	Q15726	GTSLSPPPESSGSPQQPGLSAPHSRQIPAPQGAVLVQR	Kiss1R
				EKDLPN**YNWNSFGLRF-NH2**	GPR54
	Kisspeptin-52	Rat	Q7TSB7	TSPCPPVENPTGHQRPPCATRSRLIPAPRGSVLVQREKDMSA	OT7T175
				YNWNSFGLRY-NH2	AXOR12
		Mouse	Q6Y4S4	SSPCPPVEGPAGRQRPLCASRSRLIPAPRGAVLVQREKDLS	
				T**YNWNSFGLRY-NH2**	
	Kisspeptin-10	Human	Q15726	**YNWNSFGLRF-NH2**	
		Rat	Q7TSB7	**YNWNSFGLRY-NH2**	
		Mouse	Q6Y4S4		

Sequences in bold correspond to the conserved carboxyl-terminal sequence between human, rat and mouse peptides.

has been extensively reviewed by Yang and collaborators (14). In this review, the authors pointed out that one must remain vigilant regarding the NPFF distribution by immunohistochemistry or radioimmunoassay, because the antibodies first used were against the COOH-terminus of NPFF, which is shared by other RF-amide peptides, thus results may be non-specific to NPFF. Nevertheless, NPFF presence in rodents has largely been reported in discrete central nervous system (CNS) areas by radioimmunoassay, immunohistochemistry, or *in situ* hybridization studies. The highest levels recorded were in the dorsal horn of the spinal cord and in the posterior lobe of the pituitary gland [see Ref. (14, 16)]. Fibers containing NPFF in the spinal cord seems to have intrinsic spinal origin (17). Most studies have reported the absence of NPFF mRNA or immunoreactivity in dorsal roots ganglia (DRG) except Allard et al. (18) who showed the presence of NPFF immunoreactivity in DRG, but at low level and only after blocking axonal transport [see Ref. (16)].

Neuropeptide AF (also called NPSF in rodents; see **Table 1**) has been detected in mouse and rat spinal cord as well as in human cerebrospinal fluid [see Ref. (14, 19–21)].

NPFFR2. The detailed distribution in rodents of both protein and mRNA for NPFFR2 is reviewed in Ref. (14–16, 22, 23). NPFFR2 mRNA has been detected by *in situ* hybridization and qRT-PCR in several brain regions of rodents including thalamic nuclei, hypothalamus, and superficial layers of spinal cord (10–12, 24). Using immunohistochemistry and western blot, the presence of NPFFR2 has also been detected in ventral tegmental area, hippocampus, hypothalamus olfactory tubercle, and spinal cord (25). NPFFR2 distribution in rodents has been widely described by using binding experiments (11, 26–32). The highest NPFFR2 binding sites are found in the olfactory bulb (of mice but not rat), in several thalamic nuclei, and in superficial layers of the spinal cord. However, it must be noted that, in these studies, the radiolabeled ligands used for the detection of NPFFR2 are not highly selective for this receptor and could therefore limit the relevance of the conclusions.

Like for NPFF, results concerning the presence of NPFFR2 and its mRNA in DRG or in primary afferent terminals in the spinal cord are controversial, with some studies supporting their presence and other their absence in this region [see Ref. (14–16)]. NPFFR2 mRNA has been detected in rat dorsal root ganglia and trigeminal ganglia (11). Different studies have shown a decrease of NPFFR2 binding in rats after dorsal rhizotomy, neonatal capsaicin treatment, sciatic nerve section, or spinal cord ligation (29, 33) while Lombard and collaborators did not see any decrease of binding sites after neonatal capsaicin treatment or dorsal rhizotomy suggesting that, in the rat spinal cord, NPFF receptors are mostly post-synaptically expressed (34).

Overall, the plurality of techniques leading to the same distribution pattern supports the presence of NPFFR2 in different brain structures and spinal cord and is consistent with its potential role in the modulation of nociception and sensory input.

Modulation of nociception

Effects of NPFF/NPAF on basal nociception and opiate analgesia. The modulation of nociception by NPFF/NPAF has been largely studied and reviewed in Ref. (5, 14–16, 22, 23, 35). **Table 2** summarizes the effect of the different RF-amide peptides on nociception. NPFF has been described as having two different effects on pain perception depending on the site of administration. When administrated by intrathecal (i.th.) injection, NPFF showed anti-nociceptive effect, which may be considered opioid-like effect as it provoked analgesia and potentiated opioid effects. When administrated at the supra spinal level via intracerebroventricular (i.c.v.) injection, NPFF had a pro-nociceptive effect characterized by a reversal of morphine analgesia, indicating that it displays anti-opioid properties [for details, see Ref. (15)]. Furthermore, inhibition of morphine analgesia induced by i.c.v. injection of NPFF in mice was blocked when NPFF was co-administered with RF9 (either i.c.v. or subcutaneously), a selective antagonist of NPFFR1/2 (6, 36–38). RF9, also potentiated opiate analgesic effects and blocked opioid-induced hyperalgesia and analgesic tolerance both in mice and rats (38, 39). Altogether, these results support that NPFF action on nociception and opiate analgesia is mediated by NPFF receptors and that this peptide and its receptor are part of an anti-opioid system that is involved in the homeostatic control of opiates action. Hypothesis explaining NPFF anti-opioid properties are further described below. We can notice that in most reports, NPFF alone had no effect on basal nociceptive threshold but efficiently reversed morphine analgesia, suggesting that NPFF anti-opioid properties depend on opioid receptors stimulation and points to their interconnected mechanisms of action. However, it is noteworthy that in few studies, NPFF administered alone by i.c.v. lowered the nociceptive threshold measured by the tail flick test in rat, which is consistent with anti-opioid properties of this peptide (4, 40). Interestingly, in PrRPR lacking mice, NPFF administration did not reverse morphine analgesia any more, indicating that at least some NPFF actions require a functional PrRPR [see below; (41)]. Finally, NPFF-related peptides delayed the rate of acid-sensing ion channels (ASIC) desensitization causing an enhancement of acid gated currents (42), which are known to have pain modulatory properties (43). In addition, it has been demonstrated that expression of ASIC3 increased under inflammatory conditions (44). Therefore, NPFF may also modulate pain through ASICs. However, these results were obtained with high concentrations of RF-NH_2 peptides, thus questioning the physiological relevance of these observations.

Neuropeptide AF has been shown to display NPFF-like bioactivity. NPAF injection, in the lateral ventricle of mice, increased or decreased morphine-induced analgesia in the tail flick test depending on NPAF and morphine amounts used (50) while another study showed that i.c.v. administration of NPAF in mice potently reversed morphine-induced analgesia in the tail flick test (19). I.th. administration of a low dose of NPAF in the rat potentiated morphine anti-nociception in tail flick and paw-pressure tests and efficiently reversed morphine analgesic tolerance (49). Altogether, these results demonstrate NPAF implication in opioid-modulating system. Like NPFF, NPAF can increase the amplitude of the sustained current of ASIC (57) indicating its eventual role in neuron excitability and then in nociception. However, in ASIC3 knockout mice, nociceptive behavior induced by NPAF subcutaneous injection is similar than in wild type animals, suggesting that ASIC3 is not involved in this effect (58).

Anti-opioid properties. Tolerance is defined by the loss of efficacy of a given compound after prolonged treatment, which leads to the necessity to increase the dose to reach the same effect. Two different hypotheses have been proposed to explain tolerance to morphine. One is based on different molecular mechanisms including functional selective desensitization of receptor signaling, receptor endocytosis, and degradation (59–63). The second one proposes the existence of a homeostatic equilibrium between the anti-nociceptive opioid system and pro-nociceptive anti-opioid systems (64, 65). In this model, activation of the opioid system by an exogenous opiate produces analgesia, but also stimulates the release of endogenous anti-opioid molecules, which produces a pro-nociceptive effect. This pro-nociceptive effect ramps up during chronic administration of the opiate and thus opposes to its analgesic effect. It is then necessary to increase opiate doses to overcome the activation of anti-opioid systems and produce an analgesic effect, thus explaining the tolerance described following chronic opioid treatment. Moreover, upon cessation of the opiate treatment, the opioid system is no longer activated while the anti-opioid system remains activated by

Table 2 | Summary of the effects of mammalian RF-amide peptides on the modulation of nociception.

Group	Peptide	Injection	Basal nociception	Inflammatory pain	Neuropathic pain
NPFF	NPFF (or analog)	i.th.	Analgesia and potentiation of opioid effects [see Ref. (15)]	Attenuates allodynia and thermal hyperalgesia (45, 46)	Attenuates allodynia (45–47)
		i.c.v.	No effect or decreases basal nociceptive threshold and reverses morphine analgesia [see Ref. (15)]		Attenuates tactile allodynia (46, 47)
		Intraperitoneal		Attenuates flinching behavior in formalin test (48)	Decreases mechanical hypersensitivity (48)
	NPAF	i.th.	Potentiates morphine analgesia and reverses morphine analgesic tolerance (49)		
		i.c.v.	Increases/decreases morphine analgesia (19, 50)		
RFRP	RFRP-1 (NPSF, GnIH)	i.th.			Thermal anti-nociception and tactile anti-allodynia (51)
		i.c.v.	Decreases morphine analgesia (10)	Decreases morphine analgesia (10)	
	RFRP-3 (NPVF)	i.c.v.	Decreases thermal nociceptive threshold (6); potentiates/ decreases/no effect on morphine analgesia (6, 37, 50)		
QRFP	QRFP26 (26RFa)	i.th.	No effect on mechanical or thermal nociception (52, 53)	Decreases mechanical allodynia; inhibits agitation behavior in formalin test (52, 54)	Antiallodynic effect (53)
		i.c.v.	No effect on thermal nociception (54); decreases thermal nociceptive threshold (6)	Inhibits agitation behavior in formalin test (52, 54)	
PrRP	PrRP20	i.th.	No effect (55)		
		Intracerebral	Analgesia [nucleus tractus solitary; (55)]		Attenuates tactile allodynia (periaqueductal gray, nucleus tractus solitarius) or no effect [caudal ventrolateral medulla; (55)]
		i.c.v.	Hyperalgesia (6, 41, 55)		
Kisspeptin	kisspeptin-54	Intraplantar	Nocifensive response; decreases thermal pain threshold (56)		
	kisspeptin-10	i.c.v.	Hyperalgesia and anti-morphine activity (6)		

endogenous anti-opioid molecules, which explains the decrease in the basal nociceptive threshold or hyperalgesia observed following disruption of chronic morphine treatment. This phenomenon is called opioid-induced hyperalgesia.

Neuropeptide FF has been shown to participate in the adaptive processes that counteract the opioid effects or change in the nociceptive threshold leading to opioid-induced hyperalgesia and tolerance. Indeed, increase in NPFF-like immunoreactivity has been observed in the brain and spinal cord during chronic or acute morphine treatment (66–68), supporting the key role of NPFF in these processes [see Ref. (35, 64)]. It has also been shown that reducing NPFF expression by central administration of antibodies or antisense oligonucleotides attenuated the tolerance to morphine analgesia (69, 70). Moreover, when NPFFR1/2 were pharmacologically blocked in rats or mice with RF9, there was a potentiation of opiate analgesic effects, a prevention of the development of opiate tolerance and an abolition of hyperalgesia induced by acute or chronic administration of different opiates including, fentanyl, heroin, and morphine (38, 39). Furthermore, triple knockout mice for μ-, δ-, κ-opioids receptors (corresponding to MOR, DOR, and KOR, respectively) are hyperalgesic, which supports the idea that MOR/DOR/KOR deletion certainly affects the balance between opioid and anti-opioid systems, and then, may lead to an increase in NPFF system efficacy.

Accordingly, MOR/DOR/KOR triple knockout mice showed an increase in NPFFR2 binding sites in several brain region and spinal cord (71). Finally, it has been shown that chronic i.c.v. infusion of NPFF downregulated MOR binding sites in the rat brain (72), whereas injection of antiserum against NPFF (anti-NPFF IgG) upregulated MOR binding sites (73). This suggests that density of MOR is regulated by NPFF, raising another possible way to induce anti-opioid effects.

Regarding morphine withdrawal and rewarding effects, NPFF also shows anti-opioid properties. NPFF i.c.v. injection produced an abstinence syndrome in morphine-dependent rats (66), and IgG i.c.v. injection from an antiserum against NPFF attenuated naloxone-induced withdrawal syndrome (70, 74), suggesting that NPFF modulates the different effects of opioids. I.c.v. injection of NPFF analog (dNPA) in mice counteracted the c-Fos expression induced by morphine in the shell of nucleus accumbens (75) known to be required for the acquisition of morphine-conditioned place preference (76). NPFFR1/R2 blockade with RF9 increased morphine-induced conditioned place preference and decreased naltrexone-precipitated withdrawal syndrome (38). Accordingly, injection of the NPFF analog (1DMe)-NPYF inhibited the rewarding effect of morphine (77), and acute administration of NPFF inhibited morphine-induced hyperlocomotion (78).

Overall, these results suggest that NPFF/NPFFRs system opposes to opioid effects, highlighting its role as an anti-opioid system.

Cellular effect. Hypothesis to explain the activation of NPFF/NPFFRs anti-opioid system, by the stimulation of the opioid system, may be that the neuronal circuitry implicated in both systems is interconnected, or it may exist a crosstalk between receptors in the same neurons involving a correlation/crosscheck in the cellular effects (79). In agreement with this second hypothesis, it has been shown that in rat spinal ganglion neurons the NPFF analog (1DMe)-NPYF reversed the inhibition by DAMGO of depolarization-evoked rise in intracellular Ca^{2+} (80), suggesting that NPFF could block the inhibition of morphine analgesia by reversing the effect of mu agonists on Ca^{2+}channels. Another possibility to explain anti-opioid activity of NPFFR2 receptors is based on physical interaction between NPFFR2 and MOR. In fact, it has been shown that NPFF agonist promoted a heteromeric association between NPFFFR2 and MOR that changed the lateral diffusion of MOR, which seems to move MOR away from its signaling partners, thus reducing response to opioids (81). Moreover, NPFF have been shown to modify the G-protein environment of MOR, which may also participate in the mechanism by which this peptide reduces the inhibitory activity of opioids (82). More generally, stimulation of NPFF receptor seems to provoke changes in MOR-associated signaling that could occur by receptor heteromerization and/or alteration in signaling transduction pathways [see Ref. (35)].

Concerning the pro-opioid properties, Mollereau et al. have shown that, similarly to opioid agonists, NPFF increased voltage-dependent potassium outward currents in F-11 DRG cell line (hybridoma derived from rat DRG and mouse neuroblastoma), which may explain the similar anti-nociceptive actions of NPFF and opioid agonists at the spinal level (83). Regarding the opioid potentiating effect of intrathecally injected NPFF, it has been proposed that activation of NPFF receptors could result in a functional blockade of δ opioid autoreceptors in the spinal cord, which are involved in an inhibitory feedback on Met-enkephalin release (84). However, the molecular mechanisms involved in this effect are still unclear and this hypothesis is not supported by studies in Chinese hamster ovary cell line showing that NPFF analog (1DMe)-NPYF had no intrinsic activity but enhanced DOR-mediated inhibition of forskolin-stimulated cAMP accumulation and phosphorylation of ERK2 (85).

Involvement of NPFF system in persistent pain. Neuropeptide FF and NPFFR2 seem to be implicated in inflammatory pain. Indeed, several studies, using different models of inflammatory pain, have shown that inflammation-induced modulation of NPFF or NPFFR2 at the mRNA and protein levels [see Table 3 in Ref. (14)]. For instance, persistent pain induced by carrageenan inflammation upregulated NPFF and NPFFR2 gene expressions in the rat spinal cord, suggesting an involvement of spinal NPFF system in inflammatory pain (8, 24, 86). In the rat, it has also been shown that during acute colonic inflammation there is an up-regulation of supraspinal NPFFR2 (86). Moreover, in the spinal cord of rats with tibio-tarsal joint inflammation (induced by *Mycobacterium butyricum* in Freund's adjuvant injected into the tibio-tarsal joint), there is an increase of [^{125}I]-1DMe-NPYF binding (87) while carrageenan induced an increase of NPFF immunoreactive cell bodies in spinal cord (88) and an increase of NPFFR2 immunoreactivity in primary afferent terminals (24). Otherwise, it has been shown that NPFF administration (i.th. or i.c.v.) attenuated allodynia induced by injection of Freund's Complete Adjuvant in the rat paw (46) while (1DMe)-NPYF i.th. administration decreased mechanical allodynia and thermal hyperalgesia induced by carrageenan inflammation (45). More recently, another study showed that an intraperitoneal injection of the NPFFR2 agonist AC-263093 attenuated flinching behavior in formalin test, and thermal hyperalgesia induced by carrageenan injection in hotplate test (48). Very recently, an *in vitro* study using LPS-stimulated macrophages has shown that NPFF suppressed the production of nitric oxide, an important signal transmitter during inflammatory processes, suggesting a possible anti-inflammatory action of this peptide (89). This effect was blocked by a pretreatment with RF9, indicating that it is mediated by NPFF receptors. Finally, in mice with chronic inflammatory pain induced by complete Freund's adjuvant, the decrease of morphine rewarding properties were associated with an increase of NPFFR2 agonist [^{125}I]-EYWSLAAPQRF-NH_2 binding in several brain regions involved in morphine reward including the shell of the nucleus accumbens, the major islands of Calleja and the ventral endopiriform nucleus. Altogether, these data indicate that NPFF and NPFFR2 are probably upregulated in different models of inflammatory pain in rodents and that NPFF displays antiallodynic and anti-hyperalgesic effects in these models.

In rats with neuropathic pain, NPFF mRNA was not upregulated neither in the spinal cord nor in the brainstem, while NPFFR2 mRNA was slightly upregulated only in the brainstem (8, 86). NPFF administrations either i.c.v., i.th. or directly into the periaqueductal gray attenuated tactile allodynia induced by

chronic neuropathy in rats (46, 47). Moreover, i.th. administration of the NPFF analog (1DMe)-NPYF decreased cold and mechanical allodynia observed in rats with spinal nerve ligation (45) and intraperitoneal injection of NPFFR2 agonist AC-263093 attenuated mechanical hypersensitivity in the same model of neuropathy (48). In summary, although NPFF and NPFFR2 mRNA do not seem to be strongly regulated in neuropathic pain models, NPFF and NPFF-related compounds display antiallodynic properties in neuropathic animals.

In a model of cancerous pain induced by melanoma cells injected in the hind paw of the mouse, the binding of NPFFR2 agonist [^{125}I]-EYWSLAAPQRF-NH_2 increased in several brain areas involved in the rewarding properties of opiates, similarly to what was observed in a model of chronic inflammation (see above). This increase of binding was associated with a decrease of morphine's motivational properties tested with the place preference paradigm. These data suggest that NPFF contributes to the suppression of morphine rewarding effects in this model of cancerous pain (90).

Overall, NPFF and NPFF-related compounds have been shown to display antiallodynic/hyperalgesic properties in different models of chronic pain, which contrast with the pro-nociceptive anti-opioid properties of NPFF that have been observed in animals that were chronically treated with opiates.

RFRP-1, RFRP-3, AND NPFFR1

The gene encoding RFRP-1 and RFRP-3 have been identified and cloned in the beginning of 2000s by two research teams through databases searches (9, 10). These two different peptides, also known as NPSF and NPVF, derived from the same precursor protein and constitute the RFRP group [(9, 10); see **Table 1**]. RFRP-1 seems to be the mammalian homolog of LPLRF-amide, a peptide isolated in 1983 in avian brain (3) and of gonadotropin-inhibitory hormone (GnIH) characterized from quail brain (91). RFRP-1 and RFRP-3 activate preferentially NPFFR1, which triggers the $G_{i/o}$ signal transduction pathway (9, 11).

Localization in the pain pathways

RFRP-1 and RFRP-3. Neurons containing RFRP-1 and RFRP-3 proteins and RFRP mRNA have been found by using immunohistochemical analyses and *in situ* hybridization in rat and mouse CNS (9, 10, 92–96). Their highest level has been found in the hypothalamus (periventricular and ventromedial nucleus), and fibers containing RFRP-1 and RFRP-3 are widely distributed in the brain (94). These results were confirmed very recently by the analysis of transgenic rats expressing an enhanced green fluorescent protein tagged to the RFRP promoter (97). Neurons containing RFRP immunoreactivity and mRNA are distinct from those containing NPFF (94). In mice, RFRP-immunoreactive fibers were found in the superficial layer of spinal trigeminal nucleus and dorsal horn of the spinal cord (93), while in rats, low levels of RFRP-1 immunoreactivity and no mRNA were detected in these regions (51, 94). From these observations, Pertovaara et al. (51) suggested that RFRP neurons localized in rat hypothalamus may contribute to well-known descending pain modulatory pathways (98), which project to nucleus of the solitary tract and spinal cord.

NPFFR1. NPFFR1 transcript was identified both in RT-qPCR and *in situ* hybridization experiments in several structures of the rat CNS. Highest signals were detected in the lateral septum and in various hypothalamic and thalamic nuclei (9–11). Accordingly, a high level of NPFFR1 binding sites was found in these regions both in the rat and mouse (30–32). NPFFR1 mRNA and binding sites were weakly or not detectable in the superficial layers of the spinal cord, depending on the strains of rodents used (10, 11, 31). Overall, there is a good correlation between the distribution of NPFFR1 receptor and its endogenous ligands RFRP-1/-3.

Modulation of nociception

Modulatory effects of both RFRP-1 and RFRP-3 on nociception have been reported (see **Table 2**). I.c.v. injection of RFRP-1, when given alone, had no effect on hot plate latencies in the rat but when co-administrated with morphine, it decreased or blocked morphine analgesia in hot plate and formalin tests (10). These data suggest that, like NPFF, RFRP-1 action depends on opioid receptors activation, supporting its role in an anti-opioid system related to the opioid system. In neuropathic rats, RFRP-1 produced pain suppressive effects depending on the modality of the noxious test and the localization of the injection (51). Indeed, i.th. injection of RFRP-1 induced thermal anti-nociception (tail flick test) and tactile anti-allodynia (Von Frey filament), while injection in the solitary tract nucleus produced only mechanical anti-hyperalgesia (paw-pressure test). Furthermore, tactile anti-allodynia produced by RFRP-1 was attenuated by naloxone, indicating that opioid receptors are involved in the spinal action of RFRP-1.

In mice, i.c.v. injection of VPNLPQRF-NH_2 (human RFRP-3) in the lateral ventricle has been reported to either potentiate morphine analgesia at high doses [10–32 nmol; (50)] or decrease morphine analgesia at similar doses [20 nmol; (37)]. Conversely, i.c.v. injection in mice of RFRP-3 from mouse origin (10 nmol) had no effect on morphine analgesia but significantly decreased thermal nociceptive threshold when injected alone and this effect was reversed by RF9 (6). In SH-SY5Y cells transfected with NPFFR1 and endogenously expressing mu and delta opioid receptors, RFRP-3 acted like opioids by inhibiting adenylyl cyclase activity and voltage-gated Ca^{2+} currents, whereas preincubation of those cells with RFRP-3 induced an anti-opioid activity characterized by a decrease of response to opioids on calcium signaling (99). Finally, NPFFR1 was shown to be downregulated in several brain regions of triple MOR/DOR/KOR knockout mice, suggesting an interaction between opioid and RFRP/NPFFR1 systems (71).

Altogether, these results indicate that RFRP-1/3 play a role in the modulation of nociception and opioid effects, presumably via their receptor NPFFR1. However, if we consider that no NPFFR1 has been found in the spinal cord of rodents and that RFRP-1/-3 peptides can bind to NPFFR2 subtype, this receptor may be involved in the spinal nociceptive modulating effect of these peptides (6). Moreover, RFRPs as well as NPFF and NPAF have also been shown to bind and activate some Mrgs (mas-related genes), which are G-protein-coupled receptor expressed in DRG by a specific subset of sensory neurons (100, 101), suggesting that these receptors could also represent relevant targets for nociceptive effects of these peptides.

QRFP AND QRFP RECEPTOR

Pyroglutamylated RF-amide peptide and its receptor QRFPR are the most recently discovered mammalian RF-amide peptide and receptor, respectively (102–104). QRFPR is also known as GPR103, AQ27, SP9155, and QRFP as P518, 26RFa/43RFa (see **Table 1**). 43RFa is a NH_2-terminally elongated form of 26RFa. Both peptides arise from the same precursor and were isolated from human hypothalamus and spinal cord, while only 43RFa was isolated from rat brain (102, 105). The primary sequences of human and rodent 26RFa display more than 80% identity (102). The nomenclature QRFP26 and QRFP43 will be used throughout the text for 26 and 43RFa, respectively. QRFPR have been shown to activate the G_q/G_i signal transduction pathways in heterologous cells (103, 104), while QRFP26 has been shown to stimulate cAMP production by rat pituitary cells (102). In rodents, two receptor subtypes have been identified QRFPR1 and QRFPR2 (105, 106). Human QRFPR shares 83/84 and 79/82% amino acid identity with mouse and rat QRFPR1 and QRFPR2, respectively, while mouse QRFPR1 and R2 share with each other 75% amino acid identity (105). QRFP26 and 43 have also been shown to display high affinity for and agonist activity at both NPFF receptor subtypes (6, 107).

LOCALIZATION IN THE PAIN PATHWAYS

Pyroglutamylated RF-amide peptide mRNA distribution has been studied in detail in mouse and rat CNS by *in situ* hybridization (102, 103, 105, 106). QRFP transcript was detected almost exclusively in the hypothalamus including ventromedial hypothalamic nucleus, the lateral hypothalamic area, the arcuate nucleus, the retrochiasmatic area, and periventricular nucleus [see Ref. (108)]. In mouse, low levels of QRFP transcript were identified by RT-qPCR in spinal cord (105). In naive rat, QRFP-like immunoreactivity was observed in the L5 DRG (52, 53) where it was mainly located in small to medium sized neurons suggesting a relationship with C-fibers and therefore nociceptive transmission (53). Similar observations were made after a partial spinal nerve ligation (53).

Conversely to QRFP transcript, QRFPR1 and R2 mRNAs are widely distributed in the CNS of rodents including spinal cord [see Ref. (108)]. High expression levels were observed in several different hypothalamic and thalamic nuclei as well as brainstem nuclei including dorsal raphe nucleus and the locus coeruleus that are critical regions involved in pain control. In mice, the mRNA distribution of QRFPR1 and R2 displayed no overlap with each other (105). However, the transcript for both receptor subtypes has been detected in the spinal cord (105, 109). Like QRFPR transcripts, QRFP binding sites are widely distributed in the brain and the spinal cord with a high density in the superficial layers of the dorsal horn [see Ref. (108)]. QRFP binding sites have been described in nuclei involved in processing of pain, such as the parafascicular thalamic nucleus, the locus coeruleus, the dorsal raphe nucleus, the parabrachial nucleus. However, in the rat, QRFPR mRNA-containing cells are particularly expressed in the midbrain, the pons, and the medulla oblongata, while QRFP binding sites are widely distributed throughout the brain and the spinal cord. These differences may be explained by the fact that QRFP also bind to NPFFR2 (110). QRFPR-like immunoreactivity was observed in the superficial lamina of the spinal dorsal horn but not in DRG (52). Taken together, these data suggest the involvement of QRFP-QRFPR system in the nociceptive transmission and/or integration.

MODULATION OF NOCICEPTION

As summarized in **Table 2**, in naïve rats, i.th. injection of QRFP26 had no effect neither on mechanical nor on thermal nociception [tested with the hotplate test and Von Frey filaments, respectively; (52, 53)], and i.c.v. injections had no effect on thermal nociception (54). However, a recent study showed that i.c.v. administration of QRFP26 in mice provoked a decrease of thermal nociceptive threshold (6). This discrepancy could be explained by several differences between these studies including the species (rat versus mouse) and nociceptive test used (hot plate versus tail immersion) as well as amount of QRFP injected (three or four times higher in the rat study than in the mouse study).

The effect of QRFP26 has also been examined in different models of persistent pain in rats including inflammatory pain (carrageenan and formalin tests) and neuropathic pain (partial sciatic nerve ligation). Concerning inflammatory pain, it has been shown that i.th. injection of QRFP26 attenuated the level of mechanical allodynia induced by paw carrageenan injection, while i.th. or i.c.v. injections of this peptide inhibited the phase 1 and phase 2 of the agitation behavior induced by paw formalin injection (52, 54). Similarly, i.th. and i.c.v. injections of QRFP produced an antiallodynic effect in a model of partial sciatic nerve ligation (53).

These data together with the observations made on naïve animals in the same studies suggest that QRFP modulates nociceptive transmission only during inflammation or neuropathy and has no effect on physiological nociceptive transmission. In addition, Yamamoto et al. pointed out that i.th. but not i.c.v. injection of QRFP suppressed the expression of Fos-like immunoreactivity induced by paw formalin injection in the superficial layers of the spinal dorsal horn, suggesting thus, an implication of QRFP in spinal sensitization induced by paw formalin, carrageenan injection, or partial spinal nerve ligation (52).

Recently, a novel QRFP peptide named TC26RFa has been identified from Chinese tree shrews (111). I.p. injection of TC26RFa in mice induced analgesia in thermal nociceptive tests (tail flick and hot plate tests), in a model of visceral pain, and in the formalin test, and stimulated secretion of anti-inflammatory factors from RAW 264.7 cells (111). In the future, it will be important to determine whether these effects occur through the direct activation of mouse QRFPR and which receptor subtype is involved in these effects.

PrRP AND PrRP RECEPTOR

Prolactin-releasing peptide, named as such for its prolactin-releasing activity, has been first isolated by Hinuma and collaborators as stimulator of arachidonic acid release from cells expressing an orphan G-protein-coupled receptor cloned from human pituitary and called hGR3 (112). This receptor is nearly identical to a previously cloned orphan receptor called GPR10 (113) and its rat ortholog was cloned from hypothalamus and called UHR-1 (114). Its official name is presently prolactin-releasing peptide receptor (PrRPR). Both short (PrRP20) and NH_2-terminally elongated (PrRP31) forms of PrRP originate from the same precursor protein of 98 amino acids [see **Table 1** and Ref. (115)]. Several results suggest that PrRPR is coupled to either G_q or $G_{i/o}$. Indeed, in

CHO cells expressing PrRPR, PrRP induced intracellular Ca^{2+} influx and a decrease of forskolin-stimulated intracellular cAMP production (112). It has also been shown that PrRP, in rat pituitary tumor cells, stimulated the activation of ERK that was blocked by inactivating $G_{i/o}$ by pertussis toxin (116) while in HEK293 cells, PrRPR stimulation induced intracellular Ca^{2+} influx but had no effect on cAMP production (117). Interestingly, Lin et al. have shown that PrRPR can associate with PSD-95/Disks-large/ZO-1 (PDZ) domain proteins (118). PDZ domain proteins are involved in mechanisms of scaffolding and targeting of proteins to specific subcellular domains [see Ref. (119)]. It has also been shown that PDZ domain proteins can interact with AMPA receptors, suggesting that PrRPR may modulate neurotransmission at glutamatergic synapses (118). Both PrRP20 and 31 bind with very high affinity to PrRPR, but they have also been described to display high affinity for and agonist activity at both NPFFR1 and NPFFR2 (6, 107, 120).

LOCALIZATION IN THE PAIN PATHWAYS

Prolactin-releasing peptide and PrRPR are widely distributed in the nervous system. Their detailed distribution in rodent CNS has been recently reviewed [see Ref. (23, 115, 121)]. In the rat (and human) PrRP mRNA is expressed in several regions that have been involved in the processing of nociceptive inputs including the medulla oblongata and the nucleus of solitary tract as well as dorsomedial hypothalamic nucleus. PrRP-immunoreactive fibers are present in many brain regions including several areas important for pain control including ventrolateral medulla, basolateral amygdaloid nucleus, and bed nucleus of the stria terminalis. No PrRP has been detected in the spinal cord using immunohistochemistry or *in situ* hybridization (55).

Prolactin-releasing peptide receptor mRNA is broadly expressed in the brain and the highest expression is in the anterior lobe of the pituitary (122). PrRPR mRNA has also been shown to be highly expressed in lateral and medial parabrachial nuclei, which are key region involved in the central processing of nociceptive inputs from spinal lamina I–II and spinal trigeminal cells (123). Autoradiographic binding experiments with [^{125}I]-PrRP31 only identified a strong signal in the reticular nucleus of the thalamus as well as light binding in the periventricular nucleus (124).

Overall, there is a good correlation between the localization of PrRP fibers and its receptor. However, there are some discrepancies that suggest that PrRPR might not be the only receptor for PrRP. As *in vitro* binding experiments have shown that PrRP also display high affinity for NPFFR1 and NPFFR2 (6, 107, 120), it is tempting to speculate that these receptors could also represent endogenous targets of PrRP in some brain areas. In agreement with this hypothesis, we have shown that in rats the increase in blood pressure and heart rate induced by i.c.v. administration of PrRP is blocked by the NPFFR1/R2 selective antagonist RF9, while they are still present in Otsuka Long-Evans Tokushima Fatty (OLETF) rat strain, in which the GRP10 receptor gene was mutated (125).

MODULATION OF NOCICEPTION

Prolactin-releasing peptide modulates nociception in various ways (see **Table 2**). Indeed, when administered in rats by i.th. injection, PrRP20 produced no significant anti-nociception, while intracerebral injection of PrRP20 in the nucleus tractus solitarius provoked a strong analgesia in tail flick and paw-pressure tests (55). Conversely, PrRP induced significant hyperalgesia in tail immersion test when administered i.c.v. in mice (6, 41) and provoked a weak hyperalgesia in paw-pressure test when injected in the rat caudal ventrolateral medulla (55), a structure known to be involved in descending control of pain (126–128). These data indicate that PrRP modulates nociception in rats and mice under basal condition and nociceptive or anti-nociceptive actions of this peptide depend on the site of injection.

It has also been shown that PrRP plays a role in the modulation of nociception under neuropathic conditions. Indeed, in rats with spinal nerve ligation, PrRP attenuated tactile allodynia when it was injected in the periaqueductal gray or in the nucleus tractus solitarius, but had no effect when administered in the caudal ventrolateral medulla (55).

Based on the hypothesis that PrRPR participates to an anti-opioid system, Laurent et al. studied the consequence of PrRPR deletion on opiate response (41). They showed that, compared to wild type, PrRPR-knockout mice have higher nociceptive thresholds and stronger stress-induced analgesia, and these differences are suppressed by naloxone. Moreover, the hyperalgesic effect of i.c.v. administration of PrRP as well as its capacity to block morphine analgesia was absent in PrRPR-knockout mice. These animals also displayed a potentiation of morphine induced analgesia, a reduction of morphine tolerance, an enhancement of acquisition of morphine-induced conditioned place preference and a decrease of the severity of naloxone precipitated morphine withdrawal syndrome. These results suggest that PrRP–PrRPR system participate in an anti-opioid system (41). More recently, we have shown that the hyperalgesic and anti-morphine effects of PrRP in wild type mice were blocked by the selective NPFFR1/R2 antagonist RF9, indicating that NPFF receptors are also critically involved in these effects. Altogether, these data show that PrRPR and NPFF receptors are both mandatory for the action of PrRP on nociception. Similarly, these receptors seem both essential for the action of NPFF since its anti-morphine effect was absent in PrRPR-knockout animals (41) and was blocked in wild-type mice by RF9 (6).

Overall, these data indicate the existence of a functional interaction between NPFFR1/2 and PrRPR that is critical for the effect of NPFF and PrRP on nociception. Whether this interaction occurs through direct heterodimerization of these receptors or whether it is due to expression of both receptors in different cells from the same circuit remains to be investigated.

KISSPEPTIN AND Kiss1 RECEPTOR

Kisspeptin is a 54-amino acid peptide first discovered as a metastasis suppressor, hence was also named metastin (129). In the human, Kiss1 gene codes for a 145-amino acid precursor protein, which is cleaved to the 54-amino acid protein (kisspeptin-54) or into shorter products (kisspeptin-10, -13, -14) that all share the same COOH-terminal Arg–Phe–NH_2 sequence (130). In rodents, the largest proteolytic product of the kisspeptin precursor is composed of 52 amino acids (kisspeptin-52), and the RF-amide sequence is substituted by an Arg–Tyr–NH_2 motif (see **Table 1**). Kisspeptin activates Kiss1R (also named GPR54, AXOR12, or

OT7T175). This receptor has first been cloned from the rat brain (131) and was deorphanized in 2001 by three independent research groups (130, 132, 133). GPR54 is known to act via G_q/G_{11} signal transduction pathways [see Ref. (134)]. Kisspeptin has also been described as capable to activate different set of interconnected signals in a cell type-dependent manner [see Ref. (135–138)]. It has also been shown that kisspeptin-10, -13, and -54 display high affinity binding and activate both NPFF receptor subtypes (6, 139, 140). Although Kisspeptin was originally identified as a metastasis suppressor, this peptide and its receptor Kiss1R are now largely recognized to have a central effect on neuroendocrine regulation of reproduction. This aspect has recently been reviewed in details by Pinilla and collaborators (141).

LOCALIZATION IN THE PAIN PATHWAYS

In the mouse and rat brain, kisspeptin transcript and protein have been detected in several hypothalamic nuclei that are involved in the control of gonadotropin secretion [see Ref. (142)]. Outside of hypothalamus, kisseptin-immunoreactive fibers were identified in relatively few regions of mouse brain, some of which being critically involved in the control of nociception including the paraventricular thalamic nucleus, periaqueductal gray, and locus coeruleus (143). In this study, the antisera specificity was controlled with brains of Kiss1 knockout mice thus insuring that the immunoreactivity observed is due to kisspeptin and not other RF-amide peptides. In the rat, kisspeptin mRNA and immunoreactivity have been found in the dorsal horn of the spinal cord and in L4/L5 DRG (144, 145). In DRG, kisspeptin is expressed in large-sized neurons and also in unmyelinated small- and medium-sized neurons, which are considered like nociceptors (145).

Kiss1 receptor mRNA has been detected in several regions of rodents' brain [see Ref. (142)]. In the mouse, by using X gal histochemistry in a transgenic Kiss1RLacZ knock-in mouse, Herbison et al. identified Kiss1R positive cells mainly in hypothalamus and hippocampus, and in several discrete brain regions such as periaqueductal gray and cuneate nucleus of brainstem, which are involved in pain control (146). Kiss1 immunostaining have been found in rat DRG neurons and in lamina I and II of the dorsal horn of the spinal cord. In DRG, Kiss1R distribution is similar to that of kisspeptin (145). Kiss1R has also been found, by immunohistochemical analysis, in peripheral nerve endings of PGP9.5-positive sensory fibers in the mouse skin (56).

All these data suggest that kisspeptin/Kiss1R system may play a role in the modulation of nociception.

MODULATION OF NOCICEPTION

There are few reports that describe the effect of kisspeptin on nociception in rodents (see **Table 2**). In naïve mice, intraplantar injection of kisspeptin induced a small nocifensive response and a decrease of thermal pain threshold in the hot plate test (56) while i.c.v. administration of kisspeptin-10 induced hyperalgesia and anti-morphine activity (6). However, these latter effects were blocked by the NPFF1R/2R antagonist RF9 indicating that these receptors are important for the effects of kisspeptin-10 on nociception. If we consider that kisspeptins display high affinity for Kiss1R and NPFFR1/R2, the receptor(s) that constitutes the direct target of kisspeptins in these effects still remains to clarify.

In addition to its involvement in basal nociception, i.th. and intraplantar injections of kisspeptin have been shown to provoke hyperalgesia in the first and second phases of the formalin test and to enhance TRPV1 phosphorylation at Ser800 at the site of injection and ERK1/2 phosphorylation in the dorsal horn (56). Moreover, in formalin test, the blockade of the endogenous action of kisspeptin using an antagonist of Kiss1R, p234 (intraplantar and i.th.), caused analgesia. From these results, Spampinato et al. proposed that kisspeptin, by activating Kiss1R, may lead to peripheral nociceptive sensitization through the modulation of TRPV1 phosphorylation as well as to central sensitization via the activation of the ERK/MAPK pathway in the dorsal horn of the spinal cord (56). Finally, in a model of chronic inflammatory pain (complete Freund adjuvant), kisspeptin and Kiss1R transcripts and proteins were upregulated in DRG and dorsal horn neurons (145).

Altogether, these data attest that kisspeptin regulates pain and/or nociceptive sensitization in mice, and may be predominantly implicated during inflammatory pain.

CONCLUSION

Mammalian RF-amide peptides and their receptors have been implicated in the modulation of several functions including feeding, reproduction, and cardiovascular regulation. Data from the literature, including tissue distribution and *in vivo* activity of RF-amide peptides, now provide emerging evidence that, not only NPFF/NPFFR2 system but all RF-amide peptides and their receptors are involved in the modulation of nociception, either in basal or chronic pain conditions. Moreover, several of these peptides and their receptors seem to participate to an anti-opioid pro-nociceptive system that controls anti-nociceptive and possibly other functions of opiates. Future studies, by using genetic and selective pharmacological tools, will be necessary to clearly delineate the role of each of these systems in the modulation of nociceptive inputs. Ultimately, these studies should lead to the identification of novel targets for pain treatment and for improving the efficacy of opiates and limit the development of tolerance following chronic treatment.

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Structural and functional divergence of gonadotropin-inhibitory hormone from jawless fish to mammals

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Gonadotropin-inhibitory hormone (GnIH) was discovered as a novel hypothalamic peptide that inhibits gonadotropin release in the quail. The presence of GnIH-homologous peptides and its receptors (GnIHRs) have been demonstrated in various vertebrate species including teleosts, suggesting that the GnIH-GnIHR family is evolutionarily conserved. In avian and mammalian brain, GnIH neurons are localized in the hypothalamic nuclei and their neural projections are widely distributed. GnIH acts on the pituitary and gonadotropin-releasing hormone neurons to inhibit reproductive functions by decreasing gonadotropin release and synthesis. In addition, GnIH-GnIHR signaling is regulated by various factors, such as environmental cues and stress. However, the function of fish GnIH orthologs remains inconclusive because the physiological properties of fish GnIH peptides are debatable. This review summarizes the current research progress in GnIH-GnIHR signaling and their physiological functions in vertebrates with special emphasis on non-mammalian vertebrate species.

Keywords: LPXRFa, GnRH, reproduction, teleosts, gonadotropin

INTRODUCTION: DISCOVERY OF GnIH

When the reproductive axis is triggered, gonadotropin-releasing hormone (GnRH), a neuropeptide involved in regulating vertebrate reproduction, is released from the hypothalamus. The released GnRH then enters into the anterior pituitary gland and triggers the release of gonadotropins: luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (1, 2). These gonadotropins act on the gonads to stimulate the synthesis and release of gonadal steroids (3). Kisspeptin, the peptide product of *KISS1*/*Kiss1* gene and its cognate receptor (GPR54 = kisspeptin receptor) has been well recognized as a potent regulator of GnRH release in vertebrates (4, 5). In mammals, kisspeptin immunoreactive fibers are seen in close apposition with GnRH neurons (6, 7) and with GnRH axons in the median eminence (ME) in the primates (8). Furthermore, GPR54 expression has been demonstrated in GnRH neurons from a non-mammalian species, the cichlid fish, tilapia (9), suggesting that kisspeptin plays stimulatory role via its action on GnRH neurons. In 2000, Tsutsui and his colleagues discovered a novel hypothalamic neuropeptide, termed gonadotropin-inhibitory hormone (GnIH) in the Japanese quail, *Coturnix japonica* that directly acts on the pituitary gland, thus impeding gonadotropin release (10). This was the first illustration of a hypothalamic neuropeptide demonstrating inhibitory effects on reproduction in any vertebrate (10).

STRUCTURE OF GnIH AND GnIH RECEPTOR ORTHOLOGS IN VERTEBRATES

GnIH AND GnIH ORTHOLOGS

GnIH belongs to the RFamide family of peptides as it contains RFamide motifs (Arg-Phe-NH_2) at its C-terminus. The amino acid sequence of GnIH and its orthologs in various vertebrates and their phylogenetic relationship are demonstrated in **Table 1** and **Figure 1**.

Jawless and jawed fish

In jawless fish species, GnIH orthologs have been identified and characterized in the lamprey (11) and the hagfish (12).

In jawed fish, teleosts GnIH orthologs have been identified and characterized in several species including the goldfish (13), sockeye salmon (48), grass puffer (15), tilapia (16), stickleback, tetraodon, medaka, Takifugu, and the zebrafish (14).

In this review article, all LPXRFa family of peptides (GnIH, RFRP3, and LPXRFa) are designated as GnIH orthologs based on their "GnIH peptide-like" structure. In most fish species, GnIH gene sequence encodes three putative peptide sequences (LPXRFa-1, -2, and -3), while only two putative sequences (LPXRFa-1 and -2) are present in some teleosts such as the stickleback, tetraodon, and takifugu (14). This suggests that the structures of GnIH family of peptides are evolutionarily conserved in vertebrates.

Amphibians

In the bullfrog, frog GH-releasing peptide (fGRP) has been identified as the amphibian GnIH orthologous peptide (17). In addition, using the molecular approach, another three fGRP-related peptides (fGRP-RP-1, -RP-2, and -RP-3) have been identified (19). In the European green frog, *Rana* RFamide (R-RFa) with LPXRFa motif has been identified (20). In the newt, four LPXRFa peptides (nLPXRFa-1, -2, -3, and -4) are predicted to be encoded in the newt LPXRFa cDNA. HPLC analysis further confirmed the existence of all four mature LPXRFa peptides in the newt brain (21).

Table 1 | Comparison of amino acid sequences of GnIH and its homologous peptides from jawless fish to mammals.

Animal	Species	Name	Amino acid sequence	Distribution (mRNA or peptides)[a]	Mode of action	Reference
JAWLESS FISH						
Sea lamprey	*Petromyzon marinus*	LPXRFa-1a	SGVGQGRSSKTLF**QPQRFa**	B, T, O		(11)
		LPXRFa-1b	AALRSGVGQGRSSKTLF**QPQRFa**			(11)
		LPXRFa-2	SEPFWHRT**RPQRFa**			(11)
Hagfish	*Myxine glutinosa*	LPXRFa	A**LPQRFa**			(12)
JAWED FISH						
Goldfish	*Carassius auratus*	gfLPXRFa-1	PTHLHAN**LPLRFa**	B		(13)
		gfLPXRFa-2	AKSNIN**LPQRFa**			(13)
		gfLPXRFa-3	SGTGLSAT**LPQRFa**			(13)
Zebrafish	*Danio rerio*	zfLPXRFa-1	PAHLHAN**LPLRFa**	B, E, T, O,		(14)
		zfLPXRFa-2	APKSTIN**LPQRFa**	M, K, SP, G		(14)
		zfLPXRFa-3	SGTGPSAT**LPQRFa**			(14)
Grass Puffer	*Takifugu niphobles*	LPXRFa-1	SLDMERINIQVSPTSGKVSLP TIVRLYPPTLQPHHQHVN**MPMRFa**	B, P, E, K, SP		(15)
		LPXRFa-2	DGVQGGDHVPNLNPN**MPQRFa**			(15)
Nile tilapia	*Oreochromis niloticus*	LPXRFa-1	Ac-TLLSSNDGTYSVRKQPHQETKNEIHRSLDL ESFNIRVAPTTSKFSLPTIIRFYPPTVKPLHLHAN**MPLRFa**	B, P, T, O		(14, 16)
		LPXRFa-2	p-QSDERTPNSSPN**LPQRFa**			(14, 16)
		LPXRFa-3	Ac-APNQL**LSQRF**E			(14, 16)
AMPHIBIAN						
Bullfrog	*Rana catesbeiana*	fGRP/R-Rfa	SLKPAAN**LPLRFa**	B		(17, 18)
		fGRP-RP-1	SIPN**LPQRFa**			(19)
		fGRP-RP-2	YLSGKTKVQSMAN**LPQRFa**			(19)
		fGRP-RP-3	AQYTNHFVHSLDT**LPLRFa**			(19)
European green frog	*Rana esculenta*	R-RFa	SLKPAAN**LPLRFa**	B		(20)
Japanese red-bellied newt	*Cynops pyrrhogaster*	nLPXRFa-1	SVPN**LPQRFa**	B		(21)
		nLPXRFa-2	MPHASAN**LPLRFa**			(21)
		nLPXRFa-3	SIQPLAN**LPQRFa**			(21)
		nLPXRFa-4	APSAGQFIQTLAN**LPQRFa**			(21)
BIRD						
Japanese Quail	*Coturnix japonica*	GnIH	SIKPSAY**LPLRFa**	B, T, O	GnRH1	(10, 22, 23)
		GnIH-RP-1	SLNFEEMKDWGSKNFMKVNTPTVNKVPNSVAN**LPLRFa**			(24)
		GnIH-RP-2	SSIQSLLN**LPQRFa**			(24)
Chicken	*Gallus gallus*	GnIH	SIRPSAY**LPLRFa**	B		(25)
		GnIH-RP-1	SLNFEEMKDWGSKNFLKVNTPTVNKVPNSVAN**LPLRFa**			(25)
		GnIH-RP-2	SSIQSLLN**LPQRFa**			(25)
Gambel's white-crowned sparrow	*Zonotrichia leucophrys gambelii*	GnIH	SIKPFSN**LPLRFa**	B	GnRH2	(26, 27)
		GnIH-RP-1	SLNFEEMEDWGSKDIIKMNPFTASKMPNSVAN**LPLRFa**			(26)
		GnIH-RP-2	SPLVKGSSQSLLN**LPQRFa**			(26)
European starling	*Sturnus vulgaris*	GnIH	SIKPFAN**LPLRFa**	B, T, O	GnRH1, GnRH2	(28)
		GnIH-RP-1	SLNFDEMEDWGSKDIIKMNPFTVSKMPNSVAN**LPLRFa**			(28)
		GnIH-RP-2	GSSQSLLN**LPQRFa**			(28)

(Continued)

Table 1 | Continued

Animal	Species	Name	Amino acid sequence	Distribution (mRNA or peptides)[a]	Mode of action	Reference
Zebra finch	*Taeniopygia guttata*	GnIH	SIKPFSN**LPLRFa**	B	GnRH1	(29)
		GnIH-RP-1	SLNFEEMEDWRSKDIIKMNPFAASKMPNSVAN**LPLRFa**			(29)
		GnIH-RP-2	SPLVKGSSQSLLN**LPQRFa**			(29)
MAMMAL						
Human being	*Homo sapiens*	RFRP-1	MPHSFAN**LPLRFa**	B		(30)
		RFRP-3	VPN**LPQRFa**	B	GnRH1	(30)
Rhesus macaque	*Macaca mulatta*	RFRP-1	MPHSVTN**LPLRFa**	B		(31)
		RFRP-3	SGRNMEVSLVRQVLN**LPQRFa**	B	GnRH1, GnRH2, dopamine, β-endorphin	(31–33)
Mouse	*Mus musculus*	RFRP-1	SVSFQELKDWGAKKVIKMSPAPANKVPHSAAN**LPLRFa**	B		(34)
		RFRP-3	ANMEAGTRSHFPS**LPQRFa**	B	GnRH1, kisspeptin	(34, 35)
Rat	*Rattus norvegicus*	RFRP-1	SVTFQELKDWGAKKDIKMSPAPANKVPHSAAN**LPLRFa**	B, E		(36)
		RFRP-3	ANMEAGTMSHFPS**LPQRFa**	B	GnRH1, kisspeptin	(34, 37–39)
Syrian golden hamster	*Mesocricetus auratus*	RFRP-1	SPAPANKVPHSAAN**LPLRFa**	B		(34)
		RFRP-3	TLSRVPS**LPQRFa**	B	GnRH1	(34, 40)
Cow	*Bos taurus*	RFRP-1	SLTFEEVKDWAPKIKMNKPVVNKMPPSAAN**LPLRFa**	B		(41)
		RFRP-3	AMAHLPLRLGKNREDSLSRWVPN**LPQRFa**	B, P		(42)
Sheep	*Ovis aries*	RFRP-1	SLTFEEVKDWGPKIKMNTPAVNKMPPSAAN**LPLRFa**	B		(43, 44)
		RFRP-3	VMAHLPLRLGKNREDSLSRRVPN**LPQRFa**	B, P	GnRH1, NPY, POMC, orexin, MCH	(43–46)
Pig	*Sus scrofa*	LPXRF-1	SLNFEELKDWGPKNVIKMSTPVVNKMPPLAAN**LPLRFa**	B, M, O, E, K, A, U, Pg		(47)
		LPXRF-3	AIASLPLRFGRNTEDSMSRPVPM**LPQRFa**			

[a] *B, brain; P, pituitary; E, eye; T, testis; O, ovary; M, muscle; K, kidney; SP, spleen; Gl, gill; A, adrenal gland; U, uterus; Pg, parotid gland.*
The identical C-terminal LPXRFamide (X = Leu or Gln) motif sequences are in bold font.

Birds

GnIH peptides have been identified in various avian species such as chicken, zebra finches, starlings, and sparrows (10, 24, 28, 29).

Mammals

Orthologs of GnIH have also been determined in the mammalian species (43, 49, 50). In mammals, three different RFamide-related peptides (RFRP), including RFRP-1, -2, and -3, were initially identified from the bovine and human brain cDNA, whereas only two RFRPs (RFRP-1 and/or RFRP-3) were discovered in rodents (51, 52). The mammalian GnIH orthologs, RFRP-1 and -3, possess the LPXRFamide (X = Leu or Gln) peptide, which is absent in the RFRP-2 ortholog (53). Therefore, it has been concluded that RFRP-1 and RFRP-3 serve as the functional mammalian GnIH orthologs.

GnIH RECEPTOR

The receptor for GnIH family of peptides belongs to the seven transmembrane G protein-coupled receptor (GPCR or GPR) family. Two potential GnIH receptors (GPR147 and GPR74) have been identified in vertebrates and GPR147 has been accepted as a potent receptor for GnIH. The summary of GnIH-homologous peptides and its receptor (GnIHR = GPR147) and its orthologs in various vertebrates and their phylogenetic relationship are demonstrated in **Table 2** and **Figure 2**.

Jawless and jawed fish

In jawless fish, there is no report on identification of GnIH receptor to date. In jawed fishes, GnIH receptors have been identified in several species; where GPR147 has been identified in the grass puffer (15), goldfish (66), zebrafish (14), and tilapia (16), and GPR74 has been identified in several teleosts species (14, 16). In most

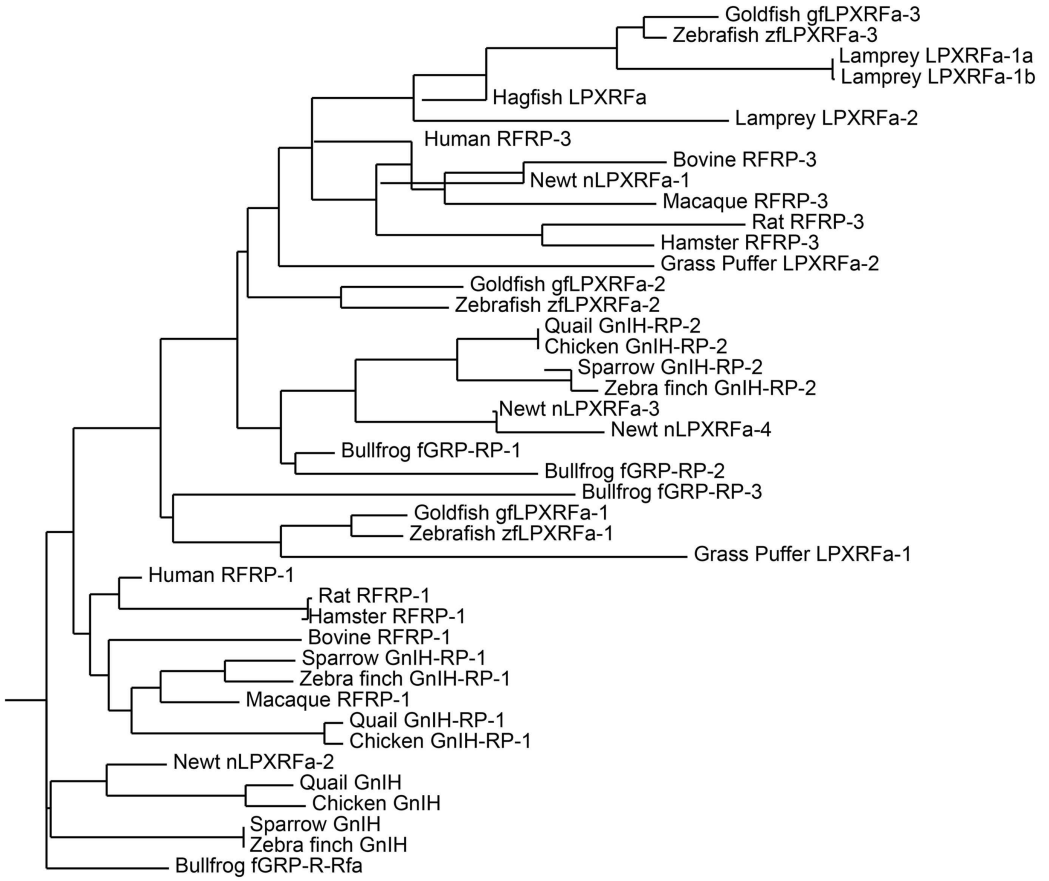

FIGURE 1 | Phylogenetic tree of GnIH and its homologous peptides sequences in vertebrates. The phylogenetic tree was constructed by MEGA 3.1 using the neighbor-joining method. The amino acid sequences analyzed for the phylogenetic tree construction are listed in **Table 1**.

teleosts, only one GnIH receptor gene has been identified, while in the zebrafish, three different GnIH receptor gene types (*gnihr1*, *gnihr2*, and *gnihr3*) have been isolated (14). However, the binding affinities of teleost GPR147 and GPR74 to GnIH peptides have not been characterized. Our recent study has shown that tilapia GPR147 (tiLPXRFa-R) has strong affinity to tilapia LPXRFa-2 peptides through both cAMP/PKA and Ca^{+2}/PKC pathways (16).

Birds

In the avian species, two receptors (GPR74 and GPR147) have been identified and further characterization has revealed GPR147 as the potent receptor for the avian GnIH based on their binding affinity to GnIH and RFRP-3 peptides (25, 59).

Mammals

In mammals, two receptors (GPR74 and GPR147) have been identified (36, 44, 67, 68). GPR147 couples to $G_{\alpha i}$ protein, which is involved in inhibiting the production of cAMP (36). Therefore, GPR147 is generally accepted as the candidate receptor for GnIH and RFRP-3 in birds and mammals because of its stronger inhibitory effect on $G_{\alpha i}$ mRNA expression in COS-7 cells, as compared to that of GPR74 (25, 52, 69). However, other studies have shown that GPR147 receptor also tends to bind to $G\alpha_{i3}$ and $G\alpha_s$ proteins, while GPR74 binds to $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$, and $G\alpha_s$ proteins (70).

DISTRIBUTION OF GnIH AND GnIHR

DISTRIBUTION OF GnIH NEURONS IN THE BRAIN

Compared to mammals and birds, in other non-mammalian vertebrate species, studies describing the distribution of GnIH expression are very few due to limited GnIH gene sequences and the lack of specific antibodies to non-mammalian GnIH orthologous peptides. The distribution pattern of GnIH neurons in the brain of various vertebrate species are illustrated in **Figure 3** (71).

Jawless and jawed fish

In the brain of sea lamprey, the expression of lamprey LPXRFa mRNA as well as lamprey LPXRFa-immunoreactive cells has been detected in the bed nucleus of the tract of the postoptic commissure (nTPOC) in the hypothalamus (11). Lamprey LPXRFa-immunoreactive fibers are widely seen in the brain and a few fibers are seen in the neurohypophysis (11).

In jawed fish species, such as the goldfish, *in situ* hybridization study has shown the expression of GnIH mRNA in the nucleus posterioris periventricularis (NPPv) in the hypothalamus (13). Using antibodies to avian GnIH and fGRP, the distribution of

Table 2 | List of GnIH receptor (GPR147) and its homologous sequences found or predicted from jawless fish to mammals.

Animal	Species	Name	GenBank accession number	Distribution[a]	Expression in GnRH or other neurons	Reference
JAWED FISH						
Coelacanth	*Latimeria chalumnae*	Neuropeptide FF receptor 1	XP_005991458			Predicted
Spotted gar	*Lepisosteus oculatus*	Neuropeptide FF receptor 1 like	XP_006630407			Predicted
Goldfish	*Carassius auratus*	G-protein couple receptor IHR1/GnIHR1	AFY63167	B, P, T, O		(54, 55)
		G-protein couple receptor IHR2/GnIHR2	AFY63168	B, P, T, O		(54, 55)
		G-protein couple receptor IHR3/GnIHR3	AFY63169 AER11372	B, P		(54)
Zebrafish	*Danio rerio*	GnIHR1 (neuropeptide FF receptor 1 like 1)	ADB43133 NP_001165167	B, P, T, M, K, SP, H, GI, E		(14)
		GnIHR2 (neuropeptide FF receptor 1 like 2)	ADB43134 NP_001165168	B, T, K, SP, H, L, GI, E		(14)
		GnIHR3 (neuropeptide FF receptor 1)	ADB43135 NP_001082858	B, T, O, M, K, SP, IN, H, GI, E		(14)
Takifugu	*Takifugu rubripes*	RFamide-related peptide receptor	BAF34887	B, P, E, K		(25)
Mexican tetra	*Astyanax mexicanus*	Neuropeptide FF receptor 1 like	XP_007255089			Predicted
Rainbow trout	*Oncorhynchus mykiss*	Unnamed protein product	CDQ96641			(56)
Bicolor damselfish	*Stegastes partitus*	Neuropeptide FF receptor 1 like	XP_008295983			Predicted
AMPHIBIAN						
Xenopus	*Xenopus laevis*	Neuropeptide FF receptor 1	NP_001084551			(57)
REPTILE						
Green anole	*Anolis carolinensis*	Neuropeptide FF receptor 1	XP_008104865			Predicted
King cobra	*Ophiophagus hannah*	Neuropeptide FF receptor 1	ETE63534			(58)
Chinese alligator	*Alligator sinensis*	Neuropeptide FF receptor 1	XP_006027961			Predicted
American alligator	*Alligator mississippiensis*	Neuropeptide FF receptor 1	XP_006265135			Predicted
Western painted turtle	*Chrysemys picta bellii*	Neuropeptide FF receptor 1 like	XP_005286579			Predicted
Green sea turtle	*Chelonia mydas*	Neuropeptide FF receptor 1 like	XP_007053537			Predicted
BIRD						
Japanese quail	*Coturnix japonica*	GnIH receptor	BAD86818	B, T, O		(23, 59)
European starling	*Sturnus vulgaris*	GnIH receptor	EF212891	B, P, T, O	GnRH1, GnRH2	(23, 28)
Budgerigar	*Melopsittacus undulatus*	Neuropeptide FF receptor 1	XP_005154065			Predicted
Chicken	*Gallus gallus*	Neuropeptide FF receptor 1	NP_989693 BAE17050	B, P, T, O		(25, 60, 61)

(Continued)

Table 2 | Continued

Animal	Species	Name	GenBank accession number	Distribution[a]	Expression in GnRH or other neurons	Reference
MAMMAL						
Human being	*Homo sapiens*	Neuropeptide FF receptor 1	NP_071429	B, P		(30, 36)
Mouse	*Mus musculus*	Neuropeptide FF receptor 1	NP_001170982		GnRH, kisspeptin	(35, 62, 63)
Rat	*Rattus norvegicus*	Neuropeptide FF receptor 1	NP_071627	B, E	GnRH, kisspeptin, dopamine	(36, 39)
Syrian golden hamster	*Mesocricetus auratus*	GPR147	ACY39880	B, P, T		(64, 65)
Sheep	*Ovis aries*	Neuropeptide FF receptor 1	ABW08098	B		(44)
Pig	*Sus scrofa*	Neuropeptide FF receptor 1	HQ681286	B, P, O, K, E, U, A, IN, S		(47)

[a] *B, brain; P, pituitary; E, eye; T, testis; O, ovary; M, muscle; K, kidney; SP, spleen; Gl, gills; H, heart; L, liver; IN, intestine; A, adrenal gland; U, uterus.*

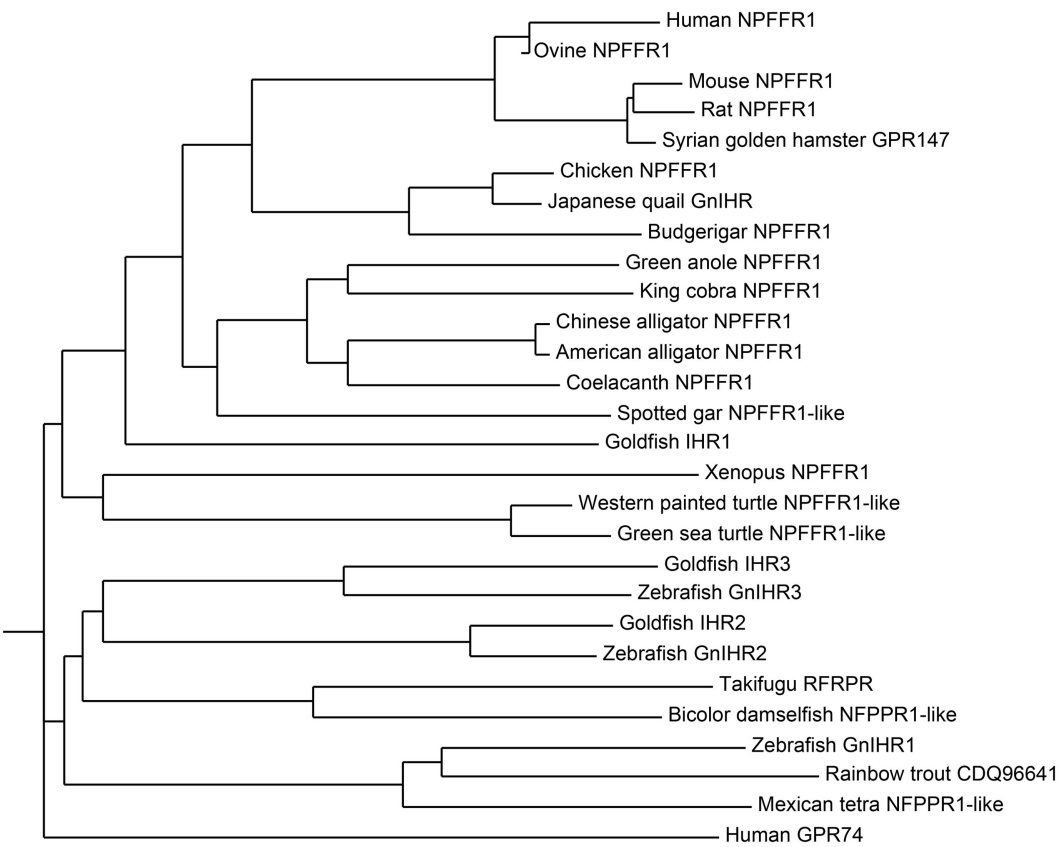

FIGURE 2 | Phylogenetic tree of GnIH receptor (GPR147) and its homologous sequences in vertebrates. The phylogenetic tree was constructed by MEGA 3.1 using the neighbor-joining method. GenBank accession numbers for the sequences are listed in **Table 2**.

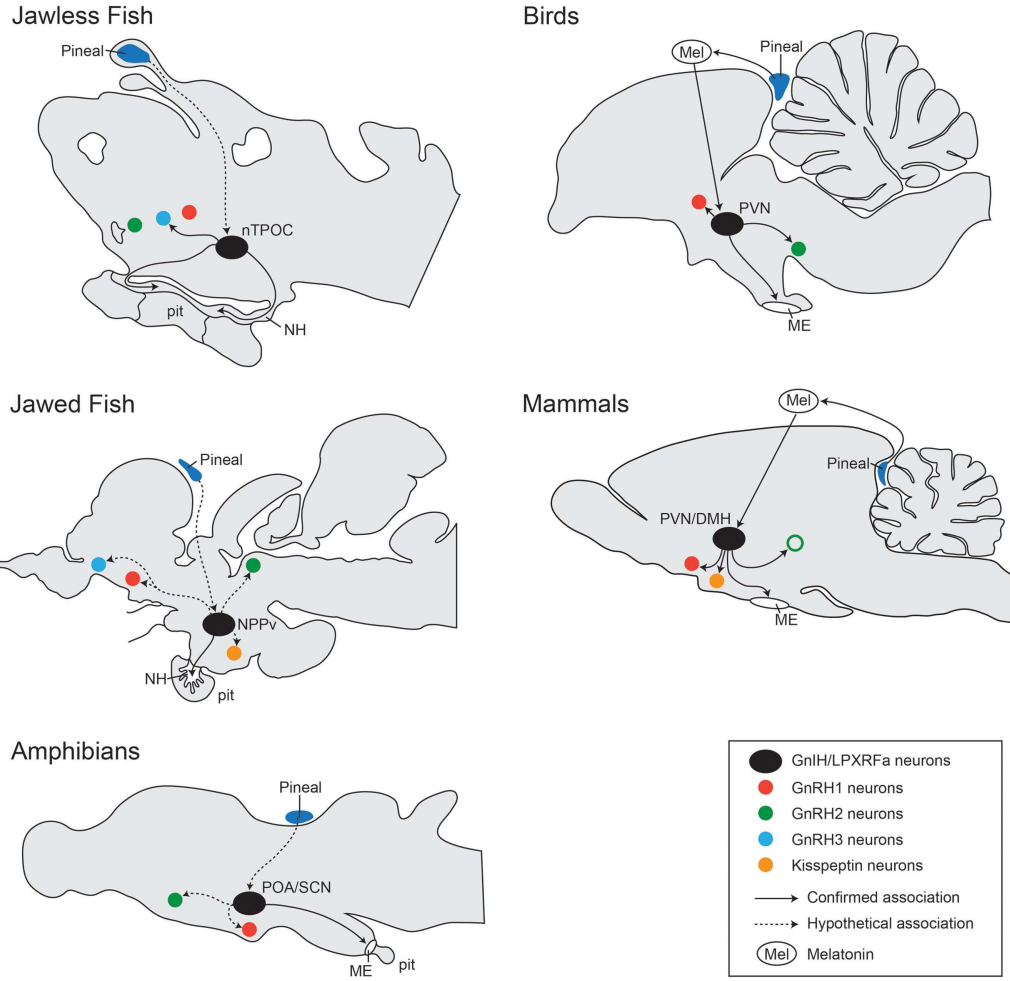

FIGURE 3 | Comparison of localization of GnIH cells and their associations with other neural systems in the brain from jawless fish, jawed fish, amphibians, birds, and mammals. In jawless fish (sea lamprey), LPXRFa-cells are localized in the bed nucleus of the tract of the postoptic commissure (nTPOC) in the hypothalamus (11). In jawed fish (goldfish, salmon, and carp), LPXRFa-immunoreactive cells are seen in the nucleus posterioris periventricularis (NPPv) (13, 48, 72). In amphibians (bullfrog and newt), LPXRFa-neurons are seen in the anterior preoptic area (POA) and/or the suprachiasmatic nucleus (SCN) (17, 18, 20, 21). In birds, the GnIH neurons are present in the PVN. In mammals and birds, GnIH neurons project to the median eminence (ME). In mammals, GnIH neurons are localized in the dorsomedial nucleus of the hypothalamus (DMH) and in the paraventricular nucleus (PVN). In jawless fish (sea lamprey), LPXRFa-immunoreactive fibers are seen in the neurohypophysis (NE), suggesting action of GnIH on the pituitary (pit) cells. In jawed fish, LPXRFa-immunoreactive fibers are present in the pituitary. In amphibians, birds, and mammals, LPXRFa/GnIH fibers are terminated in the ME. In birds and mammals, GnIH cells (*black circle*) associated with several other neurons such as GnRH neuron types: GnRH1 (*red circle*), GnRH2 (*green circle*), and GnRH3 (*blue circle*) neurons. The open circle with green indicates the presence of GnRH2 neurons only in certain mammalian species such as primates but not in rodents (73). In some mammals, GnIH fibers are also closely associated with kisspeptin neurons (*yellow circle*). GnIH neural functions are regulated by melatonin (Mel) derived from the pineal gland (dark blue) or eyes. In jawed and jawless fish, the effect of melatonin on GnIH neurons is still unknown, but there might be direct projection from the pineal gland to GnIH neurons in the hypothalamus (74–76). Confirmed association is indicated by the line, and unconfirmed hypothetical association is indicated by the dotted line.

GnIH orthologs-like immunoreactivity has been examined in the brain of several teleosts including the goldfish (13), sockeye salmon (48), and the Indian major carp (72). In the sockeye salmon and the Indian major carp, the distribution pattern of fGRP/GnIH-immunoreactive cells is similar to GnIH mRNA expression in the NPPv of the goldfish (13, 48, 72), suggesting that the presence of GnIH neurons in the NPPv is a common pattern in teleosts.

The presence of fGPR/GnIH-immunoreactive fibers have been reported in several brain regions including in the olfactory bulb, telencephalon, optic tectum, mesencephalon, diencephalon, and the spinal cord (13, 48, 72). In the goldfish and sockeye salmon, the presence of fGPR-immunoreactive fibers has also been noted in the pituitary (13, 48). In the pituitary of the Indian major carp, GnIH-immunoreactive cells and fibers have been detected in the proximal pars distalis region only during the early developmental stage, but not in adults (72). However, in the Indian major carp, GnIH-immunoreactive cells are also seen in several mesencephalic regions, such as the nucleus of medial longitudinal

fascicle and the occulomotor nucleus (72), which needs further verification by *in situ* hybridization with specific GnIH gene sequence in the Indian major carp. Similarly, in the goldfish, fGRP-immunoreactive cells have been reported in the terminal nerve of the olfactory bulb, where no GnIH mRNA is expressed (13), which indicates the fGRP antibody has cross reactivity to other unknown RFamide peptides. Therefore, to identify the targets of GnIH neurons in the brain and in the pituitary more precisely, a specific antibody to fish GnIH orthologs peptide needs to be generated.

Amphibians

In the brain of the European green frog, R-RFa-containing neurons are localized in the hypothalamus, which includes the anterior preoptic area (POA), the suprachiasmatic nucleus (SCN), and the dorsal and ventral hypothalamic nuclei (20). R-RFa-containing fibers are widely distributed throughout the brain from the olfactory bulb to the brainstem, and are particularly abundant in the external layer of the ME (20). In the bullfrog, fGRP neurons are mainly seen in the telencephalon and the diencephalon including the medial septum, nucleus of the diagonal band of Broca, anterior POA and the SCN (17, 18). fGRP-immunoreactive fibers are widely distributed throughout the brain including mesencephalic and rhombencephalic regions, and are terminate in the ME (17). In the newt brain, nLPXRFa mRNA and the peptide (with anti-fGRP serum) are expressed only in the SCN in the hypothalamus (21). Similar to frogs, fGRP-immunoreactive fibers are seen in the mesencephalic and rhombencephalic regions and terminate in the ME (21).

Reptiles

In the Japanese grass lizard, GnIH-immunoreactive neurons are seen in the nucleus accumbens, paraventricular nucleus (PVN), and upper medulla, and GnIH fibers are distributed in the third ventricle, the paraventricular organ, and the ME (77).

Birds

In the avian species, majority of the hypothalamic GnIH neuronal cell bodies are present in the PVN, with the main projections extending to the ME (10, 26, 78, 79). However, in the ME of Rufous-winged sparrows, there are no GnIH fibers (80), although expression of GnIH receptors has been shown in the pituitary (69). Additionally, the diencephalic and mesencephalic regions of the avian brain have extensive distribution of GnIH fibers.

Mammals

In rodents, GnIH neurons are concentrated within the dorsomedial nucleus of the hypothalamus (DMH), where abundant fibers project to the hypothalamic and limbic structures (34). In the ovine species, GnIH neurons are widespread in the brain, where they are present throughout the DMH, PVN, and the mediobasal hypothalamus (43). Recently, using transgenic rats carrying an enhanced green fluorescent protein (EGFP) tagged to the GnIH promoter, another population of smaller EGFP-positive neurons were seen in the ventromedial hypothalamus (VMH), which was not detected previously by GnIH immunohistochemistry (81). The mammalian GnIH fiber terminals project to the external layer of the ME (30, 31, 43, 64), suggesting the action of GnIH on the pituitary via the blood vasculature, which is supported by the measurement of GnRH peptide concentration in hypophyseal portal blood in ewes (82). However, GnIH-immunoreactive fibers are absent in the ME of hamsters (34, 40) and Wistar rats (83).

DISTRIBUTION OF GnIH RECEPTORS IN THE BRAIN AND PITUITARY

In most vertebrates, GnIH receptors (GPR147) are mainly expressed in the pituitary and in several brain regions including the hypothalamus and the spinal cord (14, 25, 30, 59, 84), most of which have been examined mainly by RT-PCR or Southern-blot analysis. However, to date, detail neuroanatomical information of GnIH receptor localization in the vertebrate brain is very limited (28).

Jawless and jawed fish

There is no report demonstrating the distribution of GnIH receptor in jawless species. However, in jawed fish species, the zebrafish, the expression of three GnIH receptors have been detected in the brain by RT-PCR (14). In the zebrafish, two GnIH receptors genes (*gnihr1* and *gnihr3*) are expressed in the pituitary (14). In the grass puffer and the tilapia, both GnIH and GnIH receptor genes are expressed in the brain and pituitary (15, 16). Furthermore, our recent study in the tilapia has shown the co-expression of GnIH receptor gene (*lpxrf-r*) in LH and FSH cells by double *in situ* hybridization (16).

Birds and mammals

In the quail, RT-PCR has shown GnIH receptor mRNA expression in the cerebrum, diencephalon, mesencephalon, and the spinal cord (59). In human beings, the expression of GnIH receptor gene has been shown in the hypothalamus and in the pituitary by RT-PCR (30). In the human pituitary, gene expression of GnIH receptors in LH cells has been shown by *in situ* hybridization (30).

DISTRIBUTION OF GnIH AND GnIH RECEPTORS IN THE GONADS

In several vertebrate species, the expression of GnIH and GnIH receptors has been reported in some peripheral tissues including the gonadal tissues (69) (**Tables 1** and **2**), indicating the role of GnIH in ovarian or testicular maturations (65, 85). Expression of GnIH and/or GnRH receptor has been shown in the gonadal tissues by RT-PCR, *in situ* hybridization, and immunohistochemistry (32, 86).

Jawless and jawed fish

In the sea lamprey, LPXRFa mRNA is expressed in the testis and ovary (11).

In the zebrafish, GnIH and three GnIH receptor genes (*gnihr1*, *gnihr2*, and *gnihr3*) are expressed in the testis, and GnIH and GnIH receptor gene (*gnihr3*) are expressed in the ovary (14). Similarly, in the goldfish, two out of three GnIH receptor types (*gnrh1* and *gnrh2*) are expressed in the testis and ovary (55). In the tilapia, LPXRFa and LPXRFa-R (GPR147) mRNAs are expressed in the gonads (16). However, in the grass puffer, there is no expression of LPXRFa and LPXRFa-R mRNAs in the gonads (15). *In situ* hybridization study in the goldfish has shown expression of *gnrh1* and *gnrh2* genes in the oocytes only before the cortical alveolus stage, but not at the vitellogenic stage (55). In the testis of

goldfish, expression of two GnIH receptor gene types have been reported in the interstitial tissue (55). *In vitro* treatment of goldfish gonadal cell culture with GnIH peptides (gfLPXRFa-2 and gfLPXRFa-3) has no effect on the mRNA expression of genes involved in steroidogenesis in ovarian cells, while in testicular cell culture, GnIH peptides significantly upregulate the expression of genes involved in testosterone biosynthesis, but suppress the CYP9 gene, which is responsible for aromatization of testosterone (55).

Amphibians and reptiles

There is no report demonstrating the presence of either GnIH or GnIH receptors in gonadal tissues of amphibian species.

In reptiles, the garden lizard, *Calotes versicolor*, has GnIH-immunoreactivity in the granulosa cells of previtellogenic follicles and stroma cells, which is relatively higher during inactive phase, but lower during the active preovulatory phase suggesting inverse correlation with circulating estradiol level (87).

Birds

In birds, GnIH and GnIH receptor gene expression has been shown in the testis and ovary by RT-PCR (23, 60, 88). Furthermore, *in situ* hybridization and immunohistochemical approaches have revealed the presence of GnIH mRNA and peptides in the ovarian thecal and granulosa cells, testicular interstitial and germ cells, and pseudostratified columnar epithelial cells in the epididymis (23, 88). GnIH receptor is also localized in the ovarian thecal and granulosa cell layers, and testicular interstitial, germ cells, and spermatocytes (23, 60, 88). In the European starlings, melatonin upregulates the expression of GnIH mRNA in the gonads. Furthermore, GnIH and melatonin significantly decrease testosterone secretion from LH/FSH-stimulated testes (89), suggesting that GnIH is involved in the seasonal regulation of testicular maturation.

Mammals

In the mammalian species, the expression of GnIH and GnIH receptors and the role of GnIH in gonadal maturation have been well demonstrated (32, 85). In the Syrian hamster, the presence of GnIH and GnIH receptor has been shown in spermatocytes and in spermatids, but not in the Leydig cells of the testis (65). In the rhesus macaque, GnIH and GnIH receptors are expressed in the Leydig cells, spermatogonia, and spermatocytes, and in the ovarian preantral follicles and granulosa cells (88). In the ovary of mice, GnIH is expressed in the granulosa cells, antral follicles, and the luteal cells (90). Similarly, in the pig, GnIH and GnIH receptor immunoreactivity has been shown in the luteal cells and in the granulosa and theca cells of the antral follicles during proestrus and estrus (47). In human beings, the expression of GnIH and GnIH receptor has been shown in the granulosa cell layer of large preovulatory follicles and the corpus luteum as well as in the primary cultures of human granulosa-lutein cells (91). A very recent study in mice has reported that GnIH (RFRP-3) treatment reduces germ cell proliferation and survival but increases apoptosis with a reduction of testosterone synthesis in the testis in a dose-dependent manner (92). Similarly, mice treated *in vivo* with GnIH for 8 days show dose-dependent changes in ovarian follicular morphology, reduction in the number of healthy antral follicles, an increase in the number of atretic follicles with low dose of GnIH (100 ng/day), and appearance of abnormal follicles at high doses (2 μg/day) (93). *In vitro* treatment of mice ovary with GnIH suppresses the production of ovarian progesterone synthesis and reduces steroidogenic enzymes such as 3β-hydroxysteroid dehydrogenase (93).

ASSOCIATION OF GnIH SYSTEM WITH OTHER NEURAL SYSTEMS

Based on the morphological distribution of GnIH and GnIH receptors in the brain and pituitary, their potential role as well as their mechanism of action have been well demonstrated in the avian and the mammalian species. In birds and mammals, GnIH fibers are seen in close proximity to the GnRH neurons in the POA (22, 28, 30, 45, 78–81) (**Figure 3**). Furthermore, the expression of GnIH receptor has been shown in GnRH1 neurons (28, 40, 94–96). In monkeys and birds, GnIH neurons send projections to midbrain GnRH2 neurons that express GPR147 (28, 30, 78). However, in ray-fin fishes, neural associations between GnIH with other hypothalamic neurons are very limited due to the lack of specific antibody.

Jawless and jawed fish

In the sea lamprey, lamprey GnIH (LPXRFa-2) immunoreactive fibers have been observed in close apposition to GnRH-III neurons (11).

A recent study in the dwarf gourami demonstrated that medaka GnIH (RFRP2 = LPXRFa-2) inhibits the pacemaker activity of GnRH3 neurons in the terminal nerve (97), suggesting the functional association of GnIH fibers with non-hypothalamic GnRH3 neurons. This suggests the action of GnIH on GnRH neurons could be evolutionarily conserved in vertebrates, which remains to be further confirmed in other fish species with fish-specific GnIH antibodies.

Birds

Interactions of GnIH with GnRH1 (c-GnRH-I) neurons are seen in several avian species including the Japanese quail, European starling, song sparrow, house sparrow, and the zebra finch (22, 28, 29, 34, 78). In Gambel's white-crowned sparrow and European starling, GnIH fibers are also closely associated with GnRH2 (c-GnRH-II) neurons (27, 28). Furthermore, expression of GnIH receptor mRNA has been identified in GnRH1 and GnRH2 neurons in the brain of the European starling (28).

Mammals

In the rhesus macaque, GnIH fibers are observed in close proximity to GnRH1 and GnRH2 neurons (31). A morphological study in the sheep using a retrograde tracer has shown fiber projection of GnIH neurons to several other hypothalamic neuropeptides-containing neurons, such as to neuropeptide Y, pro-opiomelanocortin (POMC), orexin, melanin-concentrating hormone, corticotrophin-releasing hormone, and oxytocin neurons (46). Similarly, GnIH fibers are seen in close association with POMC neurons in mice (98). In rats, GnIH fibers are closely associated with kisspeptin neurons in the rostral periventricular region of the third ventricle region (39), and in the arcuate nucleus of mice (35), which is supported by the expression of GPR147 mRNA in

kisspeptin neurons (35, 95). On the other hand, very few GnIH cells (3–7%) receive kisspeptin fibers in mice (35). Interestingly, in mice, GnIH neurons also co-express neurokinin B (*Tac2*) and its receptor (*Tacr3*) mRNAs (35).

In addition to neuropeptides, GnIH neurons are also associated with neurotransmitters. In the rhesus macaque, GnIH fibers are closely associated with dopamine and β-endorphin neurons (31). In mice, morphological and electrophysiological studies have revealed functional interactions between GnIH with glutamatergic neurons but not with cholinergic or GABAergic neurons (99). In rats, GPR147 is expressed in dopamine neurons (36). In addition, a recent report in rats has shown no co-expression of GnIH neurons with GABA (39). In rats, GnIH neural population in the DMH express 11 types of serotonin receptors (63). Similar observation has been reported in the Japanese grass lizard (77). These results indicate multiple functions of the GnIH system, in addition to its inhibitory action on reproduction.

FUNCTION OF GnIH-GnIHR SIGNALING IN VERTEBRATE REPRODUCTION

ROLE OF GnIH IN GONADOTROPIN SYNTHESIS AND RELEASE

As the name of the peptide indicates, GnIH peptides act as inhibitory factor in the control of reproduction mainly in birds and mammals (10, 34). Similar findings have been reported in various vertebrate species (52) (**Table 3**). On the contrary, in ray-fin fishes, the role of GnIH peptides in the control of gonadotropin release has been debatable.

Jawless and jawed fish

In female lampreys, treatment of lamprey GnIH (LPXRFa-2) stimulates the expression of lamprey GnRH-III protein in the hypothalamus and GTHβ mRNA expression in the pituitary (11).

The first physiological study demonstrating the role of teleost GnIH peptides (goldfish LPXRFa-1, -2, and -3 peptides) was reported in the sockeye salmon, in which goldfish LPXRFa peptides increase the release of LH, FSH, and growth hormone (GH) from cultured pituitary cells (48). Similarly, an *in vivo* study in the goldfish has shown that GnIH significantly increases pituitary levels of mRNAs for LHβ and FSHβ in a reproductive stage-dependent manner (100). Goldfish GnIH (gfLPXRFa-1) peptide treatment to the grass puffer significantly stimulates FSHβ and LHβ gene expression (15). Our recent study in the female tilapia has shown that tilapia LPXRFa-2 peptides positively increase LH and FSH release *in vitro* and *in vivo* (16).

In contrast, intraperitoneal administration of zebrafish GnIH (LPXRFa-3) to goldfish decreases the plasma LH levels (14). Similarly, inhibitory effects of GnIH on circulating serum LH levels have been demonstrated during the early to later stages of recrudescence in the goldfish (66, 100). These differences in gonadotropin responses to GnIH seen in different and in the same fish species (summarized in **Table 3**) can be explained by a recent physiological study conducted in the goldfish (54). Intraperitoneal injections of goldfish GnIH-II peptide and GnIH-III peptide significantly decreases FSHβ mRNA levels, whereas *in vitro* application of GnIH has no effect on gonadotropin synthesis. However, an inhibition of GnRH-stimulated LHβ and FSHβ synthesis has been observed when GnIH-III was applied to primary pituitary cell cultures (54). Collectively, these reports in ray-fin fish species suggest that the inhibitory action of GnIH on gonadotropin synthesis/release is closely associated with the reproductive stages in fish, which can be modulated by GnRH-dependent mechanism of action as in birds and mammals (26, 95).

Birds and mammals

In birds and mammals, GnIH reduces gonadotropin release from the anterior pituitary (10, 34), which has been extensively reviewed previously. RFRP-3 inhibits the synthesis and/or release of gonadotropins across various mammalian species, and recently, it has also been found that RFRP-1 is capable of inhibiting the release of gonadotropins in hamsters (40). Indeed, in sheep, GnIH (GnIH-3) peptide levels in the portal blood are around 2–3 pg/ml during the breeding season but increase to 4–8 pg/ml during the non-breeding season (82). In rats, the central administration of GnIH (RFRP3–8) peptides has shown to suppress the circulating LH levels at the dose of 1 nmol/injection *in vivo*, and GnIH suppresses gonadotropin secretion from pituitary culture at the concentration of 10^{-8} M *in vitro* (108). However, in rufous-winged sparrows (*Aimophila carpalis*), there is no effect of peripheral injections of GnIH on basal plasma LH levels and on GnRH-elicited LH secretion (104). This could be due to the shorter half-life of GnIH peptides *in vivo* compared with *in vitro*. In ewes, the half-life of peripherally injected GnIH in portal blood is 6.03 ± 0.30 min *in vivo* (82). While under *in vitro* condition, the half-life of GnIH (RFRP3–8) peptides is 14.3 min in rat serum (108).

ROLE OF GnIH IN SOCIO-SEXUAL BEHAVIORS

Gonadotropin-inhibitory hormone is also involved in the regulation of reproductive and social behaviors (**Table 3**) (109).

Jawless and jawed fish

The role of GnIH orthologs in socio-sexual behaviors has not been demonstrated in jawless and jawed fish species. Nevertheless, a recent study has suggested GnIH as a regulator of neuroestrogen synthesis (110) and the potential involvement of neuroestrogen in socio-sexual behaviors has been demonstrated in several jawed fish species. In a sex-changing fish (*Lythrypnus dalli*), socially induced decrease in brain aromatase levels correspond with increased aggression (111). Male Endler guppy (*Poecilia reticulata*) treated with the aromatase inhibitor show reduce of courtship activities (112). In the African cichlid fish (*Astatotilapia burtoni*), males treated with aromatase inhibitor show decrease aggressive, but not reproductive behaviors (113).

Birds

Female white-crowned sparrows injected with GnIH show inhibition of copulation-solicitation with the reduction of circulating LH levels (27). In the European starlings, there is close association between social and breeding status and GnIH levels in the brain (114). Indeed, bird pairs (male and female) with nest (winner) have significantly different numbers of GnIH peptide-producing cells than those without nest (losers), suggesting that GnIH may

Table 3 | Functions of GnIH and its homologous peptides from jawless fish to mammals.

Animal	Species	GnIH types	Functions	Reference
JAWLESS FISH				
Sea lamprey	*Petromyzon marinus*	LPXRFa-2	Stimulation of GnRH-III synthesis and GTHβ mRNA expression	(11)
JAWED FISH				
Goldfish	*Carassius auratus*	gfLPXRFa-1	Stimulation of GTH and GH release	(48)
		gfLPXRFa-2	Stimulation of GTH and GH release	(48)
			Inhibition of LH and FSH synthesis	(54)
		gfLPXRFa-3	Stimulation of GTH and GH release	(48)
			Inhibition of LH synthesis	(54)
			Inhibition of GnRH-elicited FSH synthesis	(54)
			Stimulation of GTH synthesis and release in prespawning fish	(66, 100)
			Inhibition of GTH synthesis in early to later stages of gonadal recrudescence	(100)
			Inhibition of GnRH-elicited GTH synthesis in early and mid gonadal recrudescence	(66)
Zebrafish	*Danio rerio*	gfLPXRFa-3	Inhibition of GTH release	(14)
Grass puffer	*Takifugu niphobles*	gfLPXRFa-1	Stimulation of GTH synthesis	(15)
Nile tilapia	*Oreochromis niloticus*	LPXRFa-2	Stimulation of LH and FSH release (*in vivo* and *in vitro*)	(16)
AMPHIBIAN				
Bullfrog	*Rana catesbeiana*	fGRP	Stimulation of GH release	(17)
		fGRP-RP-2	Stimulation of GH/PRL release	(19)
BIRD				
Japanese quail	*Coturnix japonica*	GnIH	Inhibition of GTH synthesis and release	(10, 101)
Chicken	*Gallus gallus*	GnIH	Inhibition of GTH synthesis and release	(102)
			Inhibition of LH release in immature but not mature chickens	(60)
			Stimulation of feeding behavior	(103)
		GnIH-RP-1	Stimulation of feeding behavior	(103)
		GnIH-RP-2	Stimulation of feeding behavior	(103)
Gambel's white-crowned sparrow	*Zonotrichia leucophrys* gambelii	GnIH	Inhibition of GnRH-elicited GTH release Inhibition of reproductive behavior	(26, 27)
Song sparrows	*Melospiza melodia*	GnIH	Inhibiting GnRH-induced LH release	(26)
Rufous-winged sparrow	*Aimophila carpalis*	GnIH	No effect on LH release and GnRH-elicited LH secretion	(104)
MAMMALS				
Human being	*Homo sapiens*	RFRP-1	Stimulation of PRL release	(36)
Mouse	*Mus musculus*	RFRP-3	Suppressive action on the excitability of GnRH neurons	(105)
Rat	*Rattus norvegicus*	RFRP-1	Stimulation of ACTH and oxytocin release	(106)
		RFRP-3	Stimulation of ACTH and oxytocin release	(106)
			Stimulation of GH secretion	(38)
			Inhibition of GTH release	(38, 49)
			Inhibition of GnRH-elicited GTH release	(49)
			Inhibition of reproductive behavior	(38)
			Stimulation of feeding behavior	(38, 49)
			No effect on basal LH secretion, but inhibition of GnRH-elicited LH release	(83)

(Continued)

Table 3 | Continued

Animal	Species	GnIH types	Functions	Reference
Syrian golden hamster	*Mesocricetus auratus*	RFRP-1	Inhibition of GTH release	(34)
		RFRP-3	Inhibition of GTH release	(34, 40, 64)
Bovine	*Bos taurus*	RFRP-3	Inhibition of GnRH-elicited GTH release	(50)
Ovine	*Ovis aries*	RFRP-3	Inhibition of GnRH-elicited GTH synthesis and release Reduction of the amplitude of LH pulses	(43, 107)

play a key role in the switch from mating and aggressive behaviors to those of parental care (114). Similarly, in the male quail, the role of GnIH in aggressive and sexual behaviors has been demonstrated (115), which has been suggested to be regulated by increasing neuroestrogen synthesis (110).

Mammals

In rats, GnIH injections suppress male sex behaviors (38). On the contrary, in a study in non-human primates, ewes, and rats, there is no effect of GnIH on sexual behavior (116), which could be due to different injection conditions (109). In the female Syrian hamsters, GnIH treatment inhibits sexual motivation and precopulatory behavior, but has no effect on copulatory behavior (117). GnIH is critical for the regulation of socio-sexual arousal, motivation, and performance in vertebrates (110). Therefore, changes in socio-sexual behaviors that are influenced by neuroestrogen levels can be modulated by GnIH in fish as in birds.

REGULATORS OF GnIH SYSTEM

In addition to the role of GnIH, its regulatory mechanism has also been well examined (52, 85, 118). For example, GnIH neurons express steroid receptors (ERα and AR), which are responsible for steroid response in GnIH neurons (34, 119). There are numerous factors that suppress reproduction and these have been demonstrated as regulators of the GnIH system. GnIH system is known to be regulated by environmental cues particularly seasonal- and diurnal-rhythmicity (120–122). Furthermore, seasonal or photoperiod-dependent alterations of GnIH neurons indicate the modulatory role of melatonin in GnIH expression and synthesis (123).

Seasonal regulation

Jawless and jawed fish. Seasonal effect on GnIH orthologs has not been demonstrated in jawless fish species. In the goldfish, the effect of GnIH injections on the reduction of circulating LH levels is closely associated with seasonal dependent gonadal maturation stages (100). Interestingly, in the grass puffer, GnIH and GnIH receptor gene expression patterns are synchronized with diurnal and circadian rhythmicity, which indicates the involvement of GnIH system in the regulation of lunar-synchronized spawning (15). Furthermore, the potential neuronal mechanism of seasonal-dependent change in GnIH system has been demonstrated (15). However, there is no direct evidence that demonstrates melatonin action on GnIH in fish, although the role of melatonin in the regulation of fish reproduction has been well recognized (124, 125). Nevertheless, in some teleosts species, there is direct projection from the pineal organ to the NPPv in the hypothalamus (74, 75), where GnIH neurons exist in teleost species. These results indicate that the GnIH system plays an important role to transmit photoperiodic cues via melatonin signaling in vertebrate reproduction.

Amphibians. In newts, peripheral treatment (intraperitoneal injection) of melatonin (at 1 h post-injection) or treatment in water containing melatonin (for 2 weeks) induces LPXRFa gene expression in the brain (21). Similarly, in bullfrogs, fGRP neurons in the SCN express Mel_{1b}, a melatonin receptor subtype (18). Furthermore, the expression of fGRP precursor mRNA is photoperiodically controlled, which increases under short-day photoperiods, when the nocturnal duration of melatonin secretion increases (18), suggesting stimulatory action of melatonin on fGRP secretion.

Birds. In the song sparrows, GnIH peptide levels are highest at the end of the breeding season (78). Similarly, in the Rufous-winged sparrow (*A. carpalis*), male birds during the breeding season have fewer, less densely labeled GnIH cell bodies than birds before the breeding season (80). While in the Australian zebra finches (*Taeniopygia guttata*), GnIH cell number and size, as well as GnIH mRNA levels are similar in the breeding and the non-breeding conditions (126). In the Japanese quail, GnIH mRNA levels decrease significantly in the pinealectomized birds (127). Furthermore, melatonin administration causes a dose-dependent increase in the expression of GnIH precursor mRNA as well as the production and release of mature peptide, which is modulated via Mel_{1c} receptor subtype (127, 128). Interestingly, in the song birds, the pineal gland conveys photoperiodic information to the vocal control system to regulate song behavior (129). Furthermore, a recent study in female great tits (*Parus major*) has shown that melatonin treatment delays clutch initiation (130). Interestingly, one of the song-control nucleus in the telencephalic area, called area X is sensitive to melatonin (131) and GnIH neurons may have association with the area X (79). Therefore, it would be interesting to look into the possible association between GnIH system and song behavior.

Mammals. Similar to other vertebrates, mammalian GnIH is also influenced by seasonal change. In the sheep, lower expression of RFRP levels in the brain is concurrent with the breeding season (45). In Syrian and Siberian hamsters, RFRP mRNA and the number of RFRP-immunoreactive cell bodies decrease under short-day photoperiod (132). Furthermore, in the Syrian hamsters

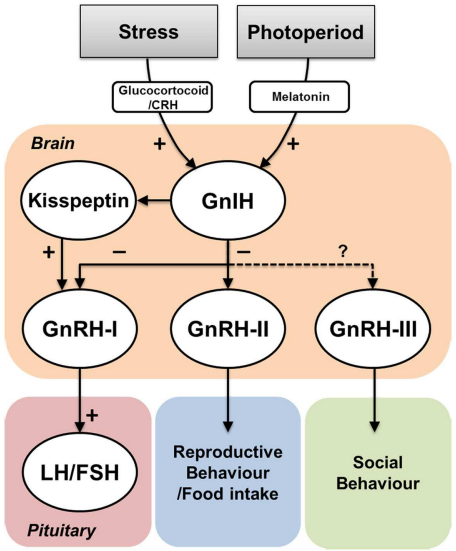

FIGURE 4 | Effect of environmental actions on GnIH system and its potential functions. Environmental cues such as social stress or seasonal/diurnal change influence on GnIH neurons via hormonal mediators such as corticosterone or melatonin. GnIH neurons negatively act on GnRH-I and GnRH-II neurons, which influence on gonadotropin (LH and FSH) secretion in the pituitary and reproductive and/or food intake behaviors, respectively. In jawless and jawed fish, GnIH neurons send projection to GnRH-III neurons, which may regulate social behaviors. In mammals, GnIH neurons are also closely associated with kisspeptin neurons. However, the role of GnIH in kisspeptin neurons remains unknown.

treated with melatonin (60 days), RFRP mRNA levels significantly decrease in the brain (132).

Stress regulation

GnIH has also been demonstrated as a modulator linking stress and reproduction in several vertebrate species. In addition, in birds and mammals, GnIH neurons are sensitive to stress hormones such as glucocorticoid or corticotropin-releasing hormone (CRH) (96, 133, 134).

Jawless and jawed fish. There is no report demonstrating the involvement of GnIH in stress response in jawless and jawed fish. However, our promoter prediction search with the ALGGEN PROMO with TRANSFAC database v. 8.3 (135, 136) reveals the presence of a putative glucocorticoids response elements (GRE) at −983 bp upstream of the zebrafish GnIH gene promoter sequence. In addition, there are several putative GRE sites within −2,000 bp upstream of zebrafish GnIHR genes (GnIHR1: at −1,755 and −1,976 bp; GnIHR2: at −30, −260, −265, −344, −1,642, and −1,942 bp; GnRHR3: at −909 and −1,294 bp). These results indicate that the role of GnIH signaling could be evolutionarily conserved in the vertebrates.

Birds. In the house sparrows (*Passer domesticus*), there is a significant increase in GnIH positive neurons in stressed birds (137). In the European starlings, plasma corticosterone concentration is positively correlated with GnIH mRNA abundance at the middle of the breeding season (114). In the Japanese quail, corticosterone treatment increases GnIH mRNA expression in the diencephalon (134). Furthermore, glucocorticoid receptor (GR) is expressed in quail GnIH neurons (134).

Mammals. In male rats, acute and chronic immobilization stress leads to an upregulation of GnIH gene expression (133). Furthermore, corticosterone treatment increased GnIH mRNA expression in a GnIH-expressing cell line, rHypoE-23, derived from the rat hypothalamus (138), which can be blocked by GR antagonist (134, 139). In male rats, 53% of GnIH neurons co-express GR, and 11.8% of GnIH neurons co-express CRH receptor1 (133). Furthermore, one functional GRE has recently been identified in the promoter region of rat GnIH gene (134), suggesting that corticosterone directly induces GnIH transcription via GR.

SUMMARY

GnIH is an inhibitory hypothalamic RFamide neuropeptide that has been characterized in various vertebrates including in the fish species (10, 14, 34, 52, 54). GnIH fibers and GnIH receptors are widely distributed in the brain as well as in the pituitary to regulate gonadotropin release (10, 34, 59, 81). GnIH fibers are also seen in close association with cells expressing other reproductive neuropeptides such as GnRH and kisspeptin neurons. GnIH and GnIH receptor signaling is also involved in several reproductive and non-reproductive functions, such as socio-sexual behaviors, appetite regulation, and stress response. Although the structure and function of the GnIH system is highly conserved in birds, mammals, and non-mammalian vertebrate species (**Figure 4**), there are still several questions that remain to be addressed in the case of fish GnIH because fish utilize a variety of reproductive strategies (140). For example, since the fish pituitary lacks the portal system of the ME and it is directly innervated by neurosecretory fibers (141), it would be interesting to know how GnIH acts on gonadotropes, whether directly or indirectly via other hypophysiotropic neurons such as GnRH neurons or the pineal gland. To understand the functional and physiological significance of vertebrate GnIH, further studies of GnIH system in a variety of vertebrates in particular in fish species would be very important.

AUTHOR CONTRIBUTIONS

Satoshi Ogawa wrote the paper. Ishwar S. Parhar edited the paper.

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RFamide peptides in early vertebrate development

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RFamides (RFa) are neuropeptides involved in many different physiological processes in vertebrates, such as reproductive behavior, pubertal activation of the reproductive endocrine axis, control of feeding behavior, and pain modulation. As research has focused mostly on their role in adult vertebrates, the possible roles of these peptides during development are poorly understood. However, the few studies that exist show that RFa are expressed early in development in different vertebrate classes, perhaps mostly associated with the central nervous system. Interestingly, the related peptide family of FMRFa has been shown to be important for brain development in invertebrates. In a teleost, the Japanese medaka, knockdown of genes in the Kiss system indicates that Kiss ligands and receptors are vital for brain development, but few other functional studies exist. Here, we review the literature of RFa in early vertebrate development, including the possible functional roles these peptides may play.

Keywords: RFa, NPFF, PrRP, Kiss, GnIH, 26RFa/QRFP, early development, brain development

INTRODUCTION

Neuropeptides with an arginine (R) and an amidated phenylalanine (F)-motif at its C-end (called RFamides or RFa) were first described in mollusks in the 70s [FMRFamide (FMRFa)] (1). Soon after, an RFa was also found in a vertebrate (2). Since then, many RFa have been identified in different vertebrate species, with the most recent group of vertebrate RFa found as late as 2002 (3). Vertebrate RFa are currently divided into five groups: (i) neuropeptide FF (NPFF) group or PQRFa group, consisting of NPFF, neuropeptide AF (NPAF), and neuropeptide SF (NPSF); (ii) prolactin-releasing peptide (PrRP) group, consisting of PrRP20 and PrRP31, crucian carp RFamide (C-RFa), and salmon RFa; (iii) gonadotropin-inhibitory hormone (GnIH) group, including mammalian RFa-related peptides (RFRP-1 and RFRP-3), frog growth hormone-releasing peptide (fGRP), and goldfish LPXR-Famide peptide; (iv) kisspeptin group also known as metastin; and finally (v) 26RFa/QRFP, including the peptides 26RFa and 43RFa (QRFP) (4, 5). These peptides have been studied in several different physiological contexts, and are found to have a role in a wide range of processes in vertebrates, as in reproductive behavior and in control of the reproductive axis (6), in pain modulation (7), and in control of feeding (8). However, much more research is needed to fully comprehend the function of RFa in different processes.

Interestingly, FMRFa are expressed in the nervous system at very early developmental stages in several phyla of metazoans, as mollusks (cephalopods and gastropods) (9, 10), cnidarians (11), and annelids (a polychete) (12). This may indicate an evolutionary ancient role of FMRFa in the development of the nervous system. It has also been shown that regeneration of flatworm anterior body fragments are stimulated by RFa (13), further supporting a role for RFa in development of tissues in lower metazoans. Also in vertebrates, the developmental studies of RFa show exciting results. Common for all the peptides is that they seem to be expressed at a very early stage in most vertebrate species studied, ranging from fishes to mammals. It seems that RFa could have important roles in development not yet discovered. This review aims to sum up what is known about the temporal and spatial expression pattern, as well as potential functional roles of the different RFa in vertebrate development, using a comparative approach.

NPFF GROUP

The first RFa to be identified in vertebrates was NPFF (also known as F8F-amide) and NPAF (2). Also belonging to this group is the peptide NPSF, and all three peptides are transcribed from the same gene in mammals (5). Genes encoding for NPFF have been identified in many different vertebrate classes, from hagfish to mammals, and members of this group bind to the receptor NPFFR2 (also called GPR74, NPFF2) (5).

Few studies have investigated the expression, location, or function of NPFF during development. However, one study in teleosts (14), one in amphibians (15), one in birds (16), and two studies in mammals (17, 18) show that this peptide is expressed early in embryonic life in different vertebrate classes. In addition, many studies have used polyclonal antibodies against FMRFa that also seem to label the NPFF peptide (see later, and **Table 1**), providing additional information regarding NPFF expression patterns. Below, a brief description of where NPFF is expressed in adult vertebrates is included, followed by a description of the studies performed during development.

NPFF IN ADULT VERTEBRATES

In adult agnathans, NPFF RFa has been found expressed in the hypothalamus. Furthermore, it has been shown that NPFF stimulates the expression of the gonadotropin-β gene in the pituitary of hagfish, which suggests that NPFF can have a role in control

Table 1 | Overview of studies of NPFF in vertebrate development.

RFa and/or receptors	Species	Method	Antibody (or radioligand)	Embryonic stages	Location of peptide/mRNA in early developing central nervous system	Putative functions in early development	Reference
NPFF	Zebrafish (*Danio rerio*)	ISH	–	24, 30, 36 hpf, 2, 3, 4, 7 dpf, adult	Exclusively in large cells of the developing terminal nerve	–	(14)
FMRFa (NPFF + ?)	Zebrafish and sterlet (*Acipenser ruthenus*)	ir	Pol 1:1000-1:20000 rabbit anti-FMRFa (Phoenix/Incstar)	24–60 hpf and 5 dpf zebrafish, juvenile sterling	Developing terminal nerve, hyp	Involvement in brain functions	(19)
FMRFa (NPFF + ?)	Brown trout (*Salmo trutta fario*)	ir	Pol 1:500 rabbit anti-FMRFa (Chemicon/Incstar)	Embryos, alevins, fry	Developing terminal nerve, hyp (NAPv, NPPv)	Regulation of neural centers related to analgesia, feeding	(20)
FMRFa (NPFF + ?)	Lungfish (*Neoceratodus forsteri*)	ir	Pol 1:10000 anti-FMRFa, Phoenix	Just before hatching to juvenile stages	Paraventricular organ in hyp, terminal nerve at hatching	–	(21)
FMRFa (NPFF + ?)	Frog (*Rana esculenta*)	ir	Pol FMRFa antiserum (Peninsula labs)	Posthatching	tel and diencephalon (newly hatched)	Modulation of GnRH-neurons?	(22)
NPFF	African clawed frog (*Xenopus laevis*)	ir	Pol 1:1000 rabbit anti-NPFF serum (from Dr. H.Y.T. Yang, Elisabeth's Hospital, Washington, DC, USA)	E30–45, and through metamorphosis	Olfactory bulbs and ventral tel, hyp, NTS, and spinal cord in embryo	Regulation of α-MSH release? Spinal embryogenesis?	(15)
FMRFa (NPFF + ?)	Toad (*Bufo bufo*)	ir	Pol 1:30000 rabbit anti-FMRFa (Phoenix)	Embryonic and larval stages	Suprachiasmatic area in embryo (stage III_6). Olfactory bulb, tel, suprachiasmatic hyp in early larvae	Neuromodulator/ neurohormone during development	(23)
FMRFa (NPFF + ?)	Skink (*Chalcides chalcides*)	ir	Pol 1:10000, 1:30000 anti-FMRF (Phoenix)	7 –70 dpf (birth)-neonatal	Fore- and hindbrain (terminal nerve, OB, hyp lateral preoptic area, suprachiasmatic area, and NAPv), MRF (35 dpf), plus NTS and vagus nerve close to birth	Regulation of blood pressure? Control of pituitary?	(24)
FMRFa (NPFF + ?)	Chicken (*Gallus domesticus*)	ir	Pol 1:4000 FMRFa antiserum (Peninsula labs)	E11–19	TN	–	(25)

(Continued)

Table 1 | Continued

RFa and/or receptors	Species	Method	Antibody (or radioligand)	Embryonic stages	Location of peptide/mRNA in early developing central nervous system	Putative functions in early development	Reference
FMRFa (NPFF + ?)	Japanese quail (*Coturnix japonica via*)	ir	1:5000 Anti-FMRFa (26), 1:3000 anti-FMRFa (Cambridge Research Biochemicals), 1:3000 anti-bovine F8F (2)	E2.5–12	Fibers in diencephalon (hyp), brain stem, olfactory nerve, and cell bodies in septum at early stages. OB at later stages	–	(16)
FMRFa (NPFF + ?)	African clawed frog	ir	Pol 1:1000 rabbit anti-FMRFa (Diasorin, Stillwater, MN)	Through metamorphosis	Olfactory nerve, tel, suprachiasmatic hyp (prometamorphic stage 56)	–	(27)
NPFF and receptors	Mouse	Quantitive autoradiography	Radioligand: [^{125}I](1DME)Y8Famide	Post-natal	Almost all brain areas at P14	Pro-opioid (P14) and anti-opioid effect (P21) of NPFF	(28)
NPFF	Rat	ISH, qPCR	–	E14-birth	Spinal cord, medulla (caudal NTS; E14), MRF (P0), pituitary	Sensory projection development in MRF, lactrotrope differentiation?	(18)
NPFF	Rat	ir	Pol rabbit anti rat F8Fa (FLFQPQRF)	E16, E18, E20, and post-natal	Fibers in median eminence (E20), cells in medulla (P1)	Role in homeostatic mechanisms, food intake in neonatals?	(17)
FMRFa (NPFF + ?)	Tree shrew (*Tupaia belangeri*)	ir on pituitary	Pol 1:1000 rabbit anti-FMRFa (Incstar, Stillwater, MN, USA)	E20–E41	Pars intermedia of pituitary from E27	Involved in early hormone secretion and releasing factor regulation?	(29)
FMRFa (NPFF + ?)	Tree shrew	ir	Pol 1:1000 rabbit anti-FMRFa (Incstar, Stillwater, MN, USA)	E19–E43	Developing TN from E23	–	(30)

The brain areas are generally named according to the original article. α-MSH, α-melanocyte-stimulating hormone; dpf, days post-fertilization; E, embryonic day; hpf, hours post-fertilization; hyp, hypothalamus; ir, immunoreactivity; ISH, in situ hybridization; M, monoclonal; MRF, medullary reticular formation; NAPv, anterior periventricular nucleus; NPP, periventricular preoptic nucleus; NPPv, posterior periventricular nucleus; NTS, nucleus of the solitary tract in medulla; OB, olfactory bulb; P, post-natal day; Pol, polyclonal; qPCR, quantitative PCR; tel, telencephalon.

of reproduction in lower vertebrates (31). In adult teleost fishes, NPFF seems to be exclusively expressed in gonadotropin releasing hormone 3 (GnRH3) neurons of the terminal nerve (TN) that stretches parallel to the olfactory nerve from the olfactory organ to the nucleus olfactoretinalis in the telencephalon (14). The TN, also known as the cranial nerve 0 (or N), was first described in sharks over 100 years ago, and later it has become clear that most vertebrates possess this nerve, from teleosts to primates, although the function is still not fully understood (32). One characteristic of these cells is that they express one variant of GnRH (32), which, as mentioned above, is GnRH3 in fish. The TN cell bodies are located parallel to the olfactory nerve, through the olfactory

bulb to the telencephalon, but their axons project throughout the brain, affecting many different behaviors, especially reproduction-related behavior (33–35). Since it has been shown that NPFF can inhibit pacemaker activity of TN GnRH-cells, NPFF is believed to be involved in the regulation of reproductive behavior in fishes (36). In amphibians, NPFF-immunoreactive (NPFFir) cells have been identified in the preoptic area and hypothalamus (primarily the suprachiasmatic region), and extensive networks of NPFFir fibers are found throughout the brain, such as in the telencephalon, hypothalamus, medulla, and dorsal spinal cord (15, 37–39). In addition, many amphibians show NPFFir of the TN cells and fibers, similar to the findings in fishes. However, there seem to be species-specific variations regarding expression of NPFF in this site in amphibians (40). To our knowledge, the roles of NPFF in amphibians, reptiles, and birds are unknown. In rodents, NPFF cell bodies have not been identified in anterior brain regions, but are instead found in the hypothalamus, medulla, and spinal cord (41, 42). However, a dense network of NPFF fibers extends throughout most of the brain, and also to the pituitary, suggesting that NPFF can be involved in a range of different processes in mammals. In addition, there seem to be species-specific differences in the location of NPFF in mammals, since bovine cortex and hippocampus contains NPFFir (43). The known effects of NPFF are very diverse in mammals; most importantly NPFF has been found to act as a neuromodulator in the opioid system, but has also been found to increase arterial blood pressure, reduce water intake, inhibit the release of vasopressin from the neurohypophysis, and influence food intake (6, 44).

NPFF IN FISH DEVELOPMENT

In zebrafish (*Danio rerio*), NPFF expression first appears already at 30 h post-fertilization (hpf; faringula period) in a small cell cluster just ventral to the olfactory placode (*in situ* hybridization) (14). The NPFF-positive cells co-express GnRH3, a marker of ganglion cells of the TN. These cells are also marked with FMRFa polyclonal antibodies, both in 30 hpf zebrafish embryos and juvenile sturgeon (sterlet, *Acipenser ruthenus*) (19), as well as in early embryos of trout (*Salmo trutta fario*) (20) and lungfish (*Neoceratodus forsteri*) (21). However, in the latter three studies, at least one additional cluster of cell bodies was marked with FMRFa immunoreactivity (FMRFir), situated in the diencephalon (more specifically, the periventricular hypothalamus in zebrafish and trout, circumventricular regions of hypothalamus in sterlet, and the paraventricular organ in lungfish). These cells were not labeled with *in situ* hybridization in zebrafish. Thus it seems that the FMRFa polyclonal antibodies bind one or several other RFa in addition to NPFF, making it challenging to interpret immunohistochemical data for FMRFa (14, 19). At 2 dpf, NPFF-expressing cells are breaking off from the small cell cluster near the olfactory placode and start caudal migration, forming a chain along the TN trajectory in zebrafish (*in situ* hybridization) (14). This pattern is also seen with FMRFa-immunohistochemistry in zebrafish, sturgeon, and lungfish (19, 21), and is proposed to visualize a migration route for cells of the TN from the olfactory placode to the nucleus olfactoretinalis in the telencephalon (19). The origin of the TN cells was believed to be the olfactory placode, but more recent studies done in zebrafish have shown that the cells of the TN originate from the neural crest and then invade the olfactory placode and later migrate from the olfactory placode area to anterior brain regions (45, 46). The hypothalamus and the spinal cord of the zebrafish did not show any NPFF-labeled cells with *in situ* hybridization, neither in the embryo nor the adult (14). This pattern seems to be in contrast to the pattern in other vertebrates, where NPFF-expressing cell bodies can be found in other brain areas during development (see NPFF in Amphibian, Reptile, and Avian Development and NPFF in Mammalian Development).

NPFF IN AMPHIBIAN, REPTILE, AND AVIAN DEVELOPMENT

Similar to teleosts and lungfish, FMRFa-immunohistochemistry labels neurons of the TN of African clawed frog (*Xenopus laevis*), also during development. Using an anti-NPFF serum produced in rabbit, Lopez et al. (15) observed NPFFir cells in the embryonic olfactory placode, which is attached to the developing telencephalon at stage 40 in *Xenopus*. Later (stage 43), NPFFir cells could also be seen in the ventral part of olfactory bulb, and the developing telencephalon, rostral to the anterior commissure. Similar labeling of the developing TN is also found with less specific antibodies for FMRFa in *Xenopus* (27) and also in other amphibians, such as European green frog (*Rana esculenta*) (22) and toad (*Bufo bufo*) (23), in the reptile skink (*Chalcides chalcides*) (24), and in the birds Japanese quail (*Coturnix japonicavia*) (16) and chicken (25). Similar to zebrafish, the cells of the TN in these studies migrate from the olfactory placode, along the olfactory bulbs, to the telencephalon. Destruction of the olfactory placode in the toad embryo leads to elimination of FMRFa cells in the olfactory bulbs, ventral telencephalon, and anterior preoptic area, but not the cells in the hypothalamus (see next paragraph), showing that as in fish, the NPFF neurons of the TN migrate from the olfactory placode (23). Also in Japanese quail, the migration of FMRFa (F8Fa) neurons of the TN is similar to fish and amphibians (16), showing that this is an evolutionary conserved feature of the TN.

In contrast to the apparent situation in teleosts, the developing and the adult brains of amphibians and birds show presence of NPFF mRNA and protein also in brain areas other than the TN (15, 16). The main population of NPFFir cells in *Xenopus* embryos is found in the suprachiasmatic region in the hypothalamus, and these cells appear earlier than the neurons of the TN (15). This area projects to the intermediate lobe of the pituitary and is involved in the control of body color in *Xenopus* through the control of α-melanocyte-stimulating hormone (α-MSH) that stimulates the melanophores in the skin (47). The suprachiasmatic cells were immunoreactive very early in development (stage 30), before production of α-MSH starts, suggesting that NPFF may be involved in the control of melanotrope cell development (15). The hypothalamic neurons seem to innervate the tectum, torus semicirculris, and tegmentum in the mesencephalon, and the innervation increases during development in *Xenopus* (34). The suprachiasmatic region is also labeled with FMRFa antibodies early in embryonic *Xenopus*, toad, European green frog, and skink (22–24, 27, 39). Placodectomy studies in both birds and amphibians have shown that this population of cells has a different developmental origin than the FMRFir TN cells (16, 23, 39, 48).

Interestingly, neurons in the nucleus of the solitary tract in the medulla show NPFFir at an early stage in *Xenopus* (20). This

area projects to the parabranchial region and the innervation is involved in the control of feeding in mammals (49). The cell bodies in the nucleus of the solitary tract seem to decrease in their immunoreactivity during development, but the projection to the parabranchial area persists and this area is heavily innervated with NPFFir fibers when the tadpoles start feeding (15). Also in developing mammals, NPFF cells are found in the nucleus of the solitary tract (see next section). At the climax of metamorphosis, NPFFir cells are detected in the reticular formation of the brain stem (15), also similar to findings in mammals. In the spinal cord, NPFFir elements were detected early in development, first in rostral spinal segments and later in the thoracic and upper lumbar segments. Interestingly, the NPFFir intensity was higher in the *Xenopus* spinal cord than in the adult, suggesting that NPFF has a developmental role in spinal cord embryogenesis. At the end of metamorphosis, the adult pattern of NPFFir structures in *Xenopus* is established (15). The brain stem and the spinal cord is not labeled with FMRFa antibodies in frog or toad (22, 23), but the skink shows FMRFir in these locations during development (24).

In the Japanese quail, the first NPFFir (F8F) fibers appear in the diencephalon (later hypothalamus) and the brain stem at embryonic stage (E) 6 (16). Later, also fibers in the olfactory nerve and the septum are labeled. In later developmental stages (E12), fibers and cell bodies are seen in the already mentioned areas in addition to the olfactory bulb. The location of NPFFir cells is similar to GnRHir cells.

NPFF IN MAMMALIAN DEVELOPMENT

Using an antibody against rat NPFF (F8F-amide), Kivipelto et al. showed the presence of fibers and terminal-like structures as early as E20 in the rat (*Rattus norvegicus*) (17). The labeling was seen in the internal layer of the median eminence in hypothalamus, an area important for control of pituitary hormone secretion. However, no labeled cell bodies were detected anywhere in the brain at this stage. At birth, NPFFir cells were found in the caudal part of the medial nucleus of the solitary tract in medulla, parallel to findings in *Xenopus*. As mentioned, this area is involved in control of feeding through its innervation to the lateral parabranchial nucleus (an area associated with feeding control) (50). In accordance with the findings in *Xenopus*, a relatively dense area of NPFFir terminals and fibers were found in the parabranchial nucleus in the rat (17). In addition, fibers and terminals were found in numerous other parts of the brain; for instance paraventricular hypothalamic area, supraoptic nucleus, optic decussation, and the periventricular hypothalamic area. Dense networks of fibers and terminals were seen in the internal layer of median eminence and infundibular stem. Further caudally, scattered fibers were found in the central gray and the inferior colliculus. At post-natal day (P) 3, also some cell bodies were found in the caudal spinal nucleus of the trigeminal nerve (which is the place all the pain and temperature fibers from the face terminate) and the dorsal horn of the spinal cord. By the age of 4 weeks, the distribution of immunoreactivity was similar to adults, where cell bodies could be seen in the periventricular area of the medial hypothalamus in addition to more intense labeling of the previously mentioned areas (17, 51).

In an *in situ* hybridization study on developing rat, Nieminen et al. (18) found expression of NPFF at E14, much earlier than the presence of NPFF protein seen with immunohistochemistry [at E20; (17)]. E14 embryos showed NPFF expression in the medulla and spinal cord (18). Later (E17), distinct neurons expressed NPFF in the spinal cord, and at birth NPFF expression was seen in neurons in the rostral nucleus of the solitary tract in the medulla, similar to what was found with immunohistochemistry. In addition, reticular nucleus (corresponding to lateral medullary reticular nucleus in adult rats) was found to express NPFF at birth, parallel to findings in developing *Xenopus* (15). This expression was transient in the rat, indicating that NPFF may be involved in development of the sensory trajectories passing through this nucleus (18). Expression of NPFF was also seen in the pituitary of the embryonic rat, but no NPFF-expressing cells were observed here. Finally, NPFF expression was found in the developing lung and spleen.

Using an NPFF radioligand, Desprat et al. showed the presence of receptors for NPFF in regions of the developing mouse brain and spinal cord involved in the analgesic effects of opiates (28). During post-natal development, they found that NPFF affected the morphine-induced analgesia in different ways in the neonatal, but in adults NPFF had only anti-opioid effect. From birth, they could detect binding of NPFF in the olfactory bulb, and from P7 in the ventral pallidum and nucleus ventral endopiriform in the telencephalon. Also in the diencephalon in the nucleus reuniens NPFF binding appeared at P7, and in the mesencephalon a few binding sites was visible at birth. This study shows that the interplay between NPFF receptors and opioid receptors is established at early stages in mice. However, embryonic stages were not studied, so the pattern of NPFF binding in the early developing mouse is not known.

Interestingly, the TN of the mammal tree shrew (*Tupaia belangeri*) shows FMRFir (30). This mammal is closely related to primates. The FMRFir pattern was similar to the pattern in fish, amphibians, reptiles, and birds, with FMRFa cells appearing early in embryogenesis (E20) near the olfactory epithelium, and later along the migrating route for the TN. Thus, it seems that NPFF expression in the TN is a feature conserved from fishes to mammals.

In summary, NPFF is detected early in embryonic development in all vertebrates studied, see overview in **Table 1**. In fishes, it seems to be exclusively expressed in the TN, also during development. In amphibians, reptiles, birds, and mammals NPFF can in addition be found in the suprachiasmatic region in hypothalamus at very early developmental stages. In *Xenopus* and mammals, medullary reticular formation also shows NPFF labeling in embryos. No function has been demonstrated for NPFF during development, but its expression pattern in the brain may suggest it could be involved in development of neurons of the TN, and nerve circuits involved in control of feeding.

PrRP GROUP

The PrRP group includes the peptides PrRP31 and PrRP20. A new member of this family, C-RFa is found in Japanese crucian carp (*Carassius cuvieri*), zebrafish, *Xenopus*, and chicken (*Gallus gallus*), but this variant is not found in mammals (52). PrRP peptides bind the receptor GPR10 (prolactin-releasing hormone receptor; PRLHR; also named GR3), but they also bind NPFFR2. Three

different receptors for PrRP peptides exist in some vertebrates, while only one is found in mammals [see receptor synteny in Wang et al. (52)].

PrRP IN ADULT VERTEBRATES

It is believed that PrRP is involved in the control of pituitary function in fishes. Firstly, in many species of adult fishes, PrRP fibers project to and terminate on prolactin-producing cells of the pituitary. Secondly, C-RFa injections in rainbow trout and tilapia cause release of prolactin and somatostatin (53–56). Furthermore, PrRP cell bodies are found in the nucleus lateralis tuberis pars posterioris in guppy (*Poecilia reticulata*), rainbow trout (*Oncorhynchus mykiss*), and goldfish (*Carassius auratus*) (52, 56, 57), an area suggested to be important for control of pituitary function in teleosts (58). There seems to be some variation in PrRP expression between species. Some fish species have PrRP cell bodies also in other brain areas, while others do not seem to have PrRP fibers projecting to the pituitary (57).

In mammals, PrRP was thought to act on the pituitary, because of the high expression of its receptor GPR10 in the anterior pituitary (59). Preliminary studies in rats showed that the peptide could promote prolactin release from pituitary cells and from these studies the peptide got its name (60). However, later studies have shown that this pathway may not be physiologically relevant in mammals. Instead, it has been shown that PrRP is involved in control of food intake and energy balance in rats and mice, and that it can affect the stress response by elevating circulating levels adrenocorticotropic hormone (ACTH) (59). Further, it has been shown that PrRP has an effect on the cardiovascular system and on circadian rhythms in mammals. Interestingly, it has been suggested that endogenously produced PrRP peptide has an autocrine role in cell-cycle progression and growth (61), processes that are closely linked to development. PrRP is expressed in the nucleus of the solitary tract, ventrolateral medulla, and in the caudal portion of the dorsomedial hypothalamic nucleus in adult mammals (59). PrRP fibers are found in many areas of the forebrain, as in preoptic area, periventricular nucleus of the thalamus, and in periventricular nucleus and paraventricular nucleus of hypothalamus (62). In contrast to the situation in fish and amphibians, no PrRPir can be detected in the median eminence or in the hypophysiotropic cells of the hypothalamus (63, 64). In peripheral tissues, PrRP mRNA is found in the adrenal gland, pancreas, placenta, and testis (59).

PrRP IN VERTEBRATE DEVELOPMENT

Very few studies have looked at the possible role of PrRP in development. However, the few that exist show that this peptide is expressed at an early stage in *Xenopus* (65), chicken (52), and rat (18, 62).

In the teleost guppy, PrRPir cells were detected in the nucleus lateralis tuberis pars posterioris in the hypothalamus already at the day of birth (57). However, innervation of PrRP fibers to the prolactin cells of the pituitary was not seen at birth day, but appeared later. Earlier developmental stages were not investigated, so the role of this peptide in teleost development is not clear.

In *Xenopus*, the expression of PrRP mRNA is detected at an early stage (stage 54; measured with qPCR) (65). PrRP mRNA levels were highest in early premetamorphic stages (stage 57), and decreased during prometamorphosis. This coincides with prolactin starting to appear in the pituitary. In the chicken pituitaries, the PrRP receptor PrRPR1 was expressed at E8 (measured with qPCR) (52), and the expression increased at later developmental stages (E12, E16, and E20). However, earlier stages were not studied.

In rat, PrRP mRNA and PrRPir cells are found in the nucleus of the solitary tract at E18, and in the ventral and lateral reticular nucleus of the caudal medulla oblongata at E20 (62). The hypothalamus first showed PrRP expression and PrRPir at P13. Similar to sexually mature rats, P6 animals had PrRPir fibers in paraventricular hypothalamic nucleus, periventricular hypothalamic nucleus, medial preoptic area, basolateral amygdaloid nucleus, dorsomedial hypothalamus, ventromedial hypothalamus, periventricular nucleus of the thalamus, and bed nucleus of the stria terminalis. However, also areas not showing PrRPir in the adult had PrRP in the developing rat brain at P6 and P9, like optic chiasm, dorsal endopiriform nucleus, cingulum, intermediate reticular nucleus, and caudal ventrolateral reticular nucleus. This transient expression could indicate a role in development of these brain areas.

The presence of mRNA of PrRP and its receptor GPR10 has also been investigated with *in situ* hybridization in rat embryos in a different study. Nieminen et al. (18) found a similar pattern as shown with immunohistochemistry. However, they found expression of PrRP in the reticular formation at a much earlier stage [E17 compared to P6 in (62)]. The receptor GPR10 was expressed at very early stages; at E15 in the pallium, at E16 in the hippocampus, and at E19 in the reticular formation. Interestingly, this is before any PrRP mRNA can be detected in these locations (18, 62), which may suggest that the receptor has an alternative ligand. In the pituitary, the expression of both PrRP and GPR10 starts at E18, at the same time as the lactotrops first appear (18). This is the opposite of the situation in *Xenopus*, where PrRP expression decreases when prolactin starts to appear in the pituitary (65). In the periphery, PrRP expression was seen in the developing liver, and to some extent in the spleen and kidney (18).

Studies of PrRP in vertebrate development are summarized in **Table 2**.

GnIH GROUP

GnIH was first described by Tsutsui et al. (67). This RFa was found to inhibit gonadotropin release in the quail through binding to the G-protein-coupled receptor GPR147. It has since been found that in birds, GnIH can act directly on gonadotrope cells to inhibit both synthesis and release of gonadotropins, e.g., Ref. (68–70). Moreover, it may also act on GnRH-neurons to inhibit GnRH release and thereby indirectly inhibit gonadotrope cells [e.g., Ref. (71)]. Since its first discovery in birds, GnIH orthologs have been found in most vertebrate classes [see review by Tsutsui and Ubuka (72) and references therein]. Alternative names are sometimes used in different vertebrate classes – the mammalian ortholog being named RFa-related peptide (RFRP) with the RFRP gene encoding two bioactive peptides, RFRP-1 (or NPSV) and RFRP-3 (or NPVF) (73). The mammalian receptor is sometimes referred to as NPFFR1 or NPFF1. In amphibians, GnIH is sometimes referred to as GRP, GRP-RPs, or R-RFa [e.g., Ref. (74)], while in teleosts the

Table 2 | Overview of studies of PrRP in vertebrate development.

RFa and/ or receptors	Species	Method	Antibody	Embryonic stages	Location of peptide/mRNA in early developing CNS	Putative functions in early development	Reference
PrRP	Guppy (*Poecilia reticulata*)	ir	Pol rabbit anti-salmon PrRP (56)	0-P14	Hyp, pituitary pars distalis at birth	Developmental role?	(57)
PrRP	*Xenopus laevis*	qPCR	–	Premetamorphosis-climax (54–65)	Transiently increased expression in brain at prometamorphosis	–	(65)
PrRP	Chicken pituitary	RT-PCR	–	E8–20	Expressed in pituitary at all stages studied	–	(52)
PrRP	Rat	ISH, RT-PCR, ir	M 40 μl/ml P2L-1C (mature PrRP)/P2L-1T (prepro-PrRP) mouse anti human PrRP (66)	E15, E18, E20, and post-natal	NTS (E18), MRF (E20), hyp (P13)	Role in embryonic brain development?	(62)
PrRP + GPR10	Rat	ISH, qPCR	–	E14-birth	PrRP: MRF, pituitary (E19), GPR10: pallidum, hippocampus, and MRF (E15–17)	Lactrotrope differentiation?	(18)

The brain areas are generally named according to the original article. E, embryonic day; hpf, hours post-fertilization; hyp, hypothalamus; ir, immunoreactivity; ISH, in situ hybridization; M, monoclonal; MRF, medullary reticular formation; NTS, nucleus of the solitary tract in medulla; P, post-natal day; Pol, polyclonal; qPCR, quantitative PCR; RT-PCR, reverse transcription PCR.

term LPXRFa may be used [e.g., Ref. (75)]. In the following, we will use the common name GnIH regardless of vertebrate class.

GnIH IN ADULT VERTEBRATES

In adult vertebrates, GnIH positive cells are found in different regions of the brain, notably in hypothalamic regions like the avian paraventricular nucleus, from where they send their projections to GnRH1 neurons in the preoptic region or to gonadotrope cells in the pituitary. GnIH terminals and GnIH receptors have also been identified on GnRH2 neurons in birds and mammals, e.g., Ref. (71, 76). A recent paper shows that GnIH inhibits socio-sexual behavior of male quail through a direct activation of aromatase and thereby increased neuroestrogen synthesis in the preoptic area (77). Both GnIH and its receptor are also expressed in the pituitary in different vertebrate classes, indicating auto- or paracrine regulation [e.g., Ref. (78)]. In addition, various studies have revealed GnIH positive cells in the gonads (both testis and ovary), while PCR experiments have revealed gene expression in peripheral tissues like muscle, spleen, eye, and kidney [e.g., Ref. (79)]. The expression of both ligand and receptor in the avian gonads (80) again points to an auto- or paracrine role during gametogenesis.

The spatial expression pattern should indicate potential functions, although much remains to be discovered when it comes to GnIH functions in general and during development in particular. Similar to in birds, GnIH in mammals have been shown to inhibit gonadotropin synthesis and release, either directly in the pituitary or via inhibition of hypothalamic GnRH-neurons. The situation seems different in frogs and teleost fish where GnIH can either inhibit or stimulate gonadotropin (and also growth hormone and prolactin) release, depending on reproductive stage, species, and sex, e.g., Ref. (74, 79, 81–84). Also in an agnathan species (sea lamprey; *Petromyzon marinus*), GnIH stimulates expression of GnRH and gonadotropin β-subunit (85), indicating that this neuropeptide may have experienced a shift in function during vertebrate evolution.

As most interest has focused on its role as an inhibitor of GnRH and gonadotropin release during reproduction, very little is known about GnIH during vertebrate development. Apart from some studies looking at pre-pubertal stages, the information we have is mostly limited to studies on the spatio-temporal expression pattern in mammalian and avian (post-natal) development, and some very few in teleost early development.

GnIH IN VERTEBRATE DEVELOPMENT

A recent article from Biswas and colleagues (78) provided some interesting information regarding the spatial expression pattern of GnIH peptides in Indian major carp (*Labeo rohita*), although detailed origin of their antibodies are missing from the paper. For instance, they found GnIH expression in the olfactory system (epithelium and bulb) in newly hatched larvae, indicating a non-reproductive function. Expression in hypothalamic nuclei such as the periventricular preoptic nucleus and the posterior periventricular nucleus, usually related to gonadotropin regulation in the adult, were also found in newly hatched larvae of the Indian major

carp. Moreover, GnIH was found expressed in the anterior part of the carp pituitary [rostral pars distalis (RPD) and proximal pars distalis (PPD)] already from hatching, although no staining could be found at the adult stage, again pointing to an autocrine or paracrine function. Another recent study provides information of the temporal gene expression pattern of GnIH and its receptors during zebrafish early development (79). Whereas each of the three GnIH receptor paralogs could be found expressed (RT-PCR) already at the blastula stage and all the way through to the adult stage, although with differential expression patterns, the GnIH ligand was found only from a later embryonic stage; at 24 hpf (early pharyngula period). Nevertheless, these results indicate a functional GnIH ligand/receptor system active already from early embryonic stages in zebrafish. This is supported by recent results from our own lab, where we find expression of medaka (*Oryzias latipes*) *gnih* and *gnihr1* already from 1 hpf and throughout the larval period, indicating maternal transfer in medaka (own unpublished data). The other two receptor paralogs in medaka, *gnihr2* and *gnihr3* were also expressed from early stages and throughout the larval period, although not until after the mid-blastula transition. The expression profile of medaka *gnih* resembled that seen in GnIH neurons in post-natal mice (86) with an initial increase followed by a steady decrease in expression levels. There is no existing data on the spatial expression of GnIH ligand or receptor during early development in fish.

In birds, where GnIH was first characterized, the existing literature focuses on the function of the GnIH system during sexual development, especially during the pre-pubertal period. For instance, circulating gonadotropin levels have been found to be negatively correlated with hypothalamic GnIH content (87). In immature male quail, chronic injections of GnIH suppressed normal testicular development, including reduced plasma testosterone levels and suppression of germ cell proliferation and seminiferous tubule development (70).

As mammalian model systems are less suited for studies of early embryogenesis, the few papers dealing with GnIH in mammalian development starts from late gestational stages. Yano et al. (88) found both GnIHs (RFRP-1 and -3) expressed in rat fetal hypothalamus from E15–E16 (mRNA) and embryonic day 16–17 (protein), showing first evidence of the existence of a functional system at the fetal stage also in mammals. Using a combination of GnIH *in situ* hybridization and BrdU immunohistochemistry, Legagneux et al. (89) identified GnIH producing neurons in male and female rat exclusively in the tuberal hypothalamus. These neurons to a large extent developed early, around E13–E14. In male and female rats, both Quenell et al. (90) and Iwasa et al. (73) found progressive gene expression of both GnIH and its receptor from early post-natal stages (from 4 days) all the way through puberty to the adult stage. Iwasa et al. also measured GnIH peptide levels and found a profile similar to that of GnIH gene expression, suggesting that the GnIH system indeed is active and plays a role during sexual development in rats (73). In accordance with these results, Poling et al. (86) also detected early gene expression of GnIH. mRNA levels increased in both sexes during post-natal and pre-pubertal development, before a decline was seen between post-natal day 20 and adulthood. During development in male rats, sustained knockdown of GnIH led to increased plasma levels of LH and increased testicular growth (91). These data indicate that GnIH act as inhibitor of gonadal maturation and puberty, similar to the situation in birds. However, Iwasa et al. (73) found increased GnRH gene expression levels concomitant with increased GnIH ligand and receptor expression during development in rat. This suggests that GnIH alone is not sufficient as inhibitor, but that regulation of sexual development is more complex, probably including additional factors such as other RFa, in addition to energy-related factors like ghrelin or leptin, or other.

In summary, the expression of a seemingly functional GnIH system in fish, birds, and mammals already from early development suggests important developmental function(s) of this RFa in vertebrates. If these include more than the above mentioned regulatory (inhibitory/modulatory) effects on sexual development, remains to be seen. See **Table 3** for an overview over developmental studies of GnIH.

KISSPEPTIN GROUP

Kisspeptins are RFa encoded by the *Kiss* gene. The resulting protein is further processed into bioactive peptides of variable lengths (94), while their receptors belong to the rhodopsin family of G-protein-coupled receptors (95–98).

KISSPEPTIN IN ADULT VERTEBRATES

The product of the *Kiss* gene was first discovered as a metastasis suppressor and therefore termed metastin (99). However, kisspeptins and their putative receptors (Kissr or Gpr54) have during the last decade emerged as major gatekeepers of reproduction because of their central role in regulating the brain–pituitary–gonadal (BPG) axis [reviewed by Ref. (100)]. The importance of the Kiss system as a regulator of the BPG-axis came after observations that mutations in the *Gpr54-1* lead to idiopathic hypogonadotropic hypogonadism (101, 102). Besides its role as a tumor suppressor and regulator of the BPG-axis, several studies report additional roles of the Kiss system, including vasoconstriction (103, 104), neuronal migration, and increased synaptic transmission (105, 106). For example, Fiorini et al. (107) showed that stimulation with KISS increased neurite growth in GnRH-positive neurons *in vitro*. Although detailed mechanisms of action are still lacking, these seemingly pleiotropic roles may reflect the diversity of intracellular signaling pathways that can be triggered by Kiss receptor activation (100, 108). The current understanding in mammalian systems suggests that major endogenous and environmental signals act through Kiss neurons, which then directly or indirectly provide an integrated signal to the hypophysiotropic GnRH neurons.

KISSPEPTINS DURING DEVELOPMENT

Despite the accumulating data of the role of kisspeptins in adult vertebrates, less is known about kisspeptins during post-natal/pre-pubertal development, and very little is known regarding the potential expression and function of the kiss system during embryogenesis/early development. This could at least partly be due to the lack of a suitable model system. Because *Kiss* or *Gpr54* KO mice are infertile, homozygous offspring need to be established from heterozygous parents. This means that the possibility of maternal transfer of transcripts, including those of *Kiss*

Table 3 | Overview of studies of GnIH in vertebrate development.

RFa (and/ or receptors)	Species	Method	Antibody (or radioligand)	Embryonic stages	Location of peptide/mRNA in early developing CNS	Putative functions in early development	Reference
GnIH	Indian major carp (*Labeo rohita*)	ir	?	Hatchling-fry-juvenile	Cells in olfactory system, NPP, NPPv, and fibers in optic tectum, PPD in pituitary, and MRF (P0)	–	(78)
GnIH + receptors	Zebrafish (*Danio rerio*)	RT-PCR	-	Blastula-juvenile	GnIH first detected at 5-prime stage, receptors at all stages	Role in early development?	(79)
GnIH	Rat	ISH, RT-PCR, ir	M 10 μg/ml 1F3 anti-RFRP-1, P 16 μg/ml antisera anti – FRP-1 (92, 93)	E15, E18, E20, and post-natal	Caudal portion of hyp (E16), many areas at E18 and E20	Modulation of pain, response to stress during development?	(88)
GnIH and GPR147	Rat	qPCR,ELISA	Pol rabbit anti-avian GnIH (67)	Pre-pubertal (P4–20) and peripubertal	GnIH and receptor mRNA and peptide present in hyp from P4	–	(73)
GnIH	Rat	ISH + BrdU	–		Cell bodies generated at E13/E14 in tuberal hyp	–	(89)
GnIH	Mouse	ISH	–	P1, P10, P20	mRNA and protein in dorsal-medial nucleus of hyp from P1	–	(86)

The brain areas are generally named according to the original article. E, embryonic day; hyp, hypothalamus; ir, immunoreactivity; ISH, in situ hybridization; M, monoclonal; NPP, periventricular preoptic nucleus; NPPv, posterior periventricular nucleus; P, post-natal day; Pol, polyclonal; PPD, proximal pars distalis in adenohypophysis; qPCR, quantitative PCR; RT-PCR, reverse transcription PCR.

and *Gpr54*, cannot be excluded in this system. Furthermore, and common to all RFa families discussed here, studying embryonic development in mammals *in vivo* is difficult due to their viviparity.

The few existing data on kisspeptins during early development come from studies in medaka and zebrafish. We recently performed a study of kisspeptin ligand and receptor expression pattern and function during early development in medaka, exploiting the advantages of the teleost model system (109). qPCR gene expression profiles (**Figure 1**) revealed maternally provided Kiss systems involving the two *kiss* ligands (*kiss1* and *kiss2*) and one of the receptors (*gpr54-1*), indicating the possibility of functional Kiss receptor-ligand systems at very early stages. *gpr54-2*, on the other hand, was not detected until after the zygotic phase, at stage 15, with a significant increase in expression levels between stage 30 and stage 36. In zebrafish, *kiss1* and *kiss2* gene expression was reported in 24 hpf (30 somite stage) embryos (110), but earlier stages were not investigated. In another teleost, the cobia (*Rachycentron canadum*) *gpr54-1* expression was detected at 1 day post hatching (111). The early expression of *kiss* and *gpr54* also coincides with the early expression of gonadotropins in fish (112), indicating a potentially functional BPG-axis already during early embryogenesis.

In Hodne et al., we performed a series of knockdown experiments that indicated several independent kiss systems during medaka embryonic development (109). Both maternally and zygotically expressed *kiss1* and *gpr54-1* seemed critical for proper development (**Figure 2**). However, the apparent functions of the maternally and zygotically expressed transcripts were quite distinct, as explained below.

Knockdown of maternal *kiss1* and *gpr54-1* led to developmental arrest and subsequent death around the blastula stage (stage 10–11), suggesting that this early expressed system could be involved either in regulation of early asynchronous cell division or early cell migration. The downstream factors controlled by *kiss1*/*gpr54-1* signaling are not known. However, cell migration during blastulation and gastrulation is dependent on *sdf1*/*cxcr4* chemotaxis. This signaling pathway is also known to be important during bone-directed migration of GPR54-positive breast cancer cells (113) and kisspeptin can indirectly regulate *sdf1*/*cxcr4* through desensitization of *cxcr4* by preventing rise in intracellular Ca^{2+} levels after *sdf1* stimulation (114, 115). In zebrafish, knockdown of *sdf1*/*cxcr4* inhibits migration of endodermal cells during gastrulation (115). Moreover, *sdf1* signaling is crucial for survival in mice, and individuals lacking either receptor or ligand have defective hematopoiesis,

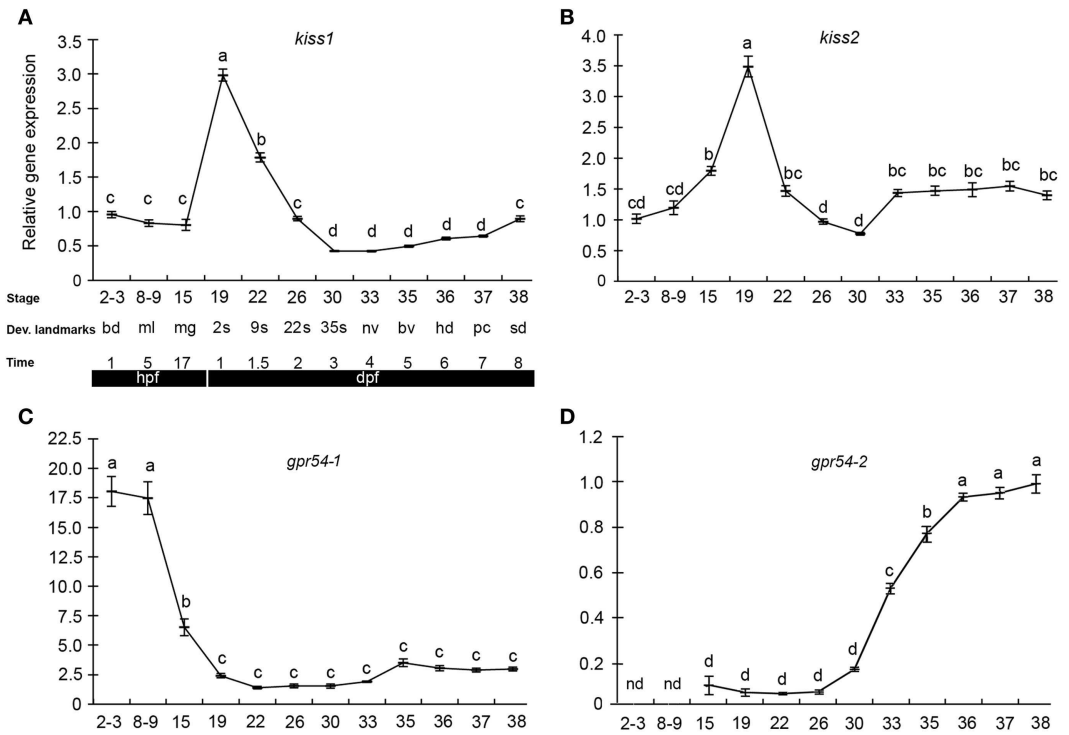

FIGURE 1 | Kiss and kiss receptors are expressed at very early stages in medaka embryos. Relative gene expression of *kiss1* **(A)**, *kiss2* **(B)**, *gpr54-1* **(C)**, and *gpr54-2* **(D)** was analyzed at different developmental stages (mean ± SEM; $n = 7$). Key developmental stages are given above the age of the hours (h) or days (d) post-fertilization (hpf). The gene expression levels are given relative to a reference gene (β-actin). Different letters indicate significant differences ($P < 0.05$). Figure from Ref. (109).

developmental lymphoid tissue, vascularization of gastrointestinal tract, migration of neuronal cells, and patterning in the central nervous system, and they die prenatally (116, 117).

Zygotic knockdown of *kiss1* and *gpr54-1*, on the other hand, allowed the embryos to survive gastrulation and a seemingly normal development continued until completion of neurulation (stage 18). At this point, early eye development is normally observed. However, after zygotic knockdown with either morpholino or low dose of peptide nucleic acid (PNA), eye development was interrupted, and further brain development was severely disrupted (**Figure 2**). Surprisingly, knockdown of *kiss2*, which was expressed at similar levels as *kiss1*, did not produce any increased mortality or malformed embryos. Unless *kiss1* takes over the role of *kiss2* following *kiss2* knockdown, *kiss2* does not seem to be critical for proper development. If *kiss2* is translated and active before zygotic activation, our results indicate that it does not work through *gpr54-1* (which is not expressed at this point), but possibly through *gpr54-2* (although knockdown of this receptor leads to developmental arrest and 100% mortality), or through other, currently unknown, RFa receptors. The different effects observed following *kiss1* and *kiss2* knockdown suggest the possibility of a very early separation of two functional systems during embryonic development. One system, comprised of Kiss1 and Gpr54-1, has a functional role important for survival during the maternal stage of development. This system continues to function throughout embryonic development, although it seems more important for regulating brain development at later embryonic stages. A second system seems to be comprised of Kiss2 binding to either Gpr54-2 or other unknown RFa receptors. The possible function of this second system remains to be clarified.

Contrary to the observed phenotypes following zygotic knockdown of *kiss1* and *gpr54-1* outlined above, a moderate knockdown of medaka *gpr54-2* arrested further development at stage 16 (late gastrula). This phenotype resembled that of maternal *kiss1* and *gpr54-1* knockdowns. However, as *gpr54-2* is first detected after transition to zygotic gene expression, new questions arise as to why a similar phenotype was not also observed after zygotic *kiss1* and *gpr54-1* knockdown. One explanation could be that the two receptors are functionally separated. If they are involved in similar functions, our results indicate that the actions of Gpr54-1 may be partly compensated for by Gpr54-2, whereas Gpr54-2 cannot be functionally replaced by Gpr54-1.

A recent work by Zhao et al. has investigated the role of kiss on GnRH neuron development in zebrafish (118). In line with Kitahashi et al. (110), *kiss1* and *kiss2* mRNA could be detected by qPCR from 24 hpf. Furthermore, it was shown that both kiss ligands stimulated GnRH3 neuron proliferation peripherally, while only *kiss1* stimulated proliferation and synaptic contact points of GnRH3 neurons in the TN and hypothalamic regions.

The existence of a functional kisspeptin system in birds is not clarified [see in Ref. (119)], and consequently, there are no data on this during bird development.

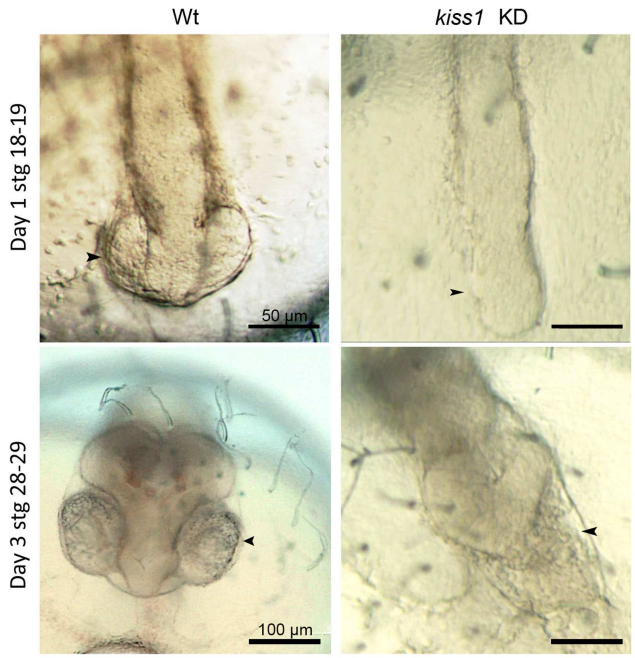

FIGURE 2 | Low dose antisense knockdown (KD) lead to impaired brain and eye development. *kiss1* knockdown embryos showed impaired eye development at 1 dpf (top right). At 3 dpf, there was no development of the forebrain after *kiss1* knockdown (bottom right). Scale bars correspond to 50 µm (top images) or 100 µm (bottom images). Arrowheads point to the area of the developing eyes. Figure from Ref. (109).

In mammals, the kisspeptin system has been intensively investigated during the last decade. Whereas, most literature covers the key role of Kiss in regulating GnRH neuron around and after puberty, pre- and early neonatal stages have been looked into more closely during recent years [see reviews in Ref. (120, 121) and references therein]. For instance, *Kiss1* gene expression has been detected in hypothalamic areas during the late fetal period in mice [stage E13.5; (122)] and both gene expression and peptide have been detected in rats [stage E14.5, (123)]. There are two hypothalamic areas expressing *Kiss*; the arcuate nucleus (ARC), and the preoptic AVPV (anteroventral periventricular nucleus) and PeN (rostral periventricular nucleus). Whereas, Kiss neurons appear in the ARC prenatally, Kiss expression in the AVPV/PeN is not seen until neonatal stages. There seems to be clear sex differences in the expression of Kiss in rodents, with females expressing higher levels than males in both the ARC and the AVPV/PeN. *Gpr54-1* gene expression has also been detected in stage E13.5 prenatal mice (122), indicating the possibility of a functional ligand–receptor system from this stage on. Also in second trimester human fetuses, KISS and GPR54 immunoreactivity was detected in the hypothalamus. Maternally provided Kiss ligands or receptors have, however, not been reported.

It seems that the early kisspeptin systems are functional in rodents in that Kiss neurons already are in close contact with GnRH-neurons prenatally, and that GnRH-neurons are able to respond to kisspeptins by enhanced GnRH secretion during prenatal life (120, 122, 124, 125). Based on these and several other studies, a more generalized picture is starting to appear with a seemingly functional kisspeptin system in place during the last part of gestation. The Kiss neurons increase in number and activity and reach a peak prenatally, before a decreased activity around birth, and then a new increase again during early neonatal life before the activity decreases to low levels until the pre-pubertal stage. The prenatal and early neonatal peaks in Kiss neuron activity seemingly coincide with similar peaks in GnRH and pituitary gonadotropin secretion [see in Ref. (120, 121, 126–132)]. However, the function of this early expression of Kiss remains elusive.

In line with the more severe phenotypes observed in medaka following receptor knockdown (109), Lapatto et al. (133) also described a more severe phenotype following *Gpr54-1* knockout- compared with *Kiss1* knockout mice. As mice possess only the one Gpr54 paralog, one of several suggestions was a possible weak activation of Gpr54-1 by other ligands. The results of Hodne et al. (109) and those of Lapatto et al. (133) suggest that Kiss and possibly other RFa may promiscuously bind to different RFa receptors [see also in Ref. (134–136)]. Interestingly, Mayer and Boehm (137) found that female mice with genetically ablated kisspeptin neurons underwent puberty and became fertile. In contrast, acute ablation in adult mice inhibited fertility. These results clearly indicate compensatory mechanisms for early loss of kisspeptins. Whether maternally transferred kiss is crucial for mouse development has not been investigated.

Although more data are available regarding the role of kisspeptins during vertebrate development compared to the role of other RFa, there are still much work to be done. One important aspect probably will be to elucidate their role in neuronal migration/development, where they seemingly play a major role, at least in fish. See **Table 4** for an overview over developmental studies of kisspeptins.

26RFa/QRFP GROUP

The 26RF/QRFP group is the newest member of the RFa family, first described in 2003 in the brain of European green frog (140). The gene for 26RFa/QRFP is found in genomes of many species, from teleost fish to human, with preserved synteny in human, mouse, chicken, and *Xenopus* (4). Mature peptides generated from this gene are 26RFa, 43RFa in rat, mouse, human, and frog, 9RFa in human and frog, and 26RFa and 7RFa in fish (4, 141). 26RFa/QRFP binds the receptor GPR103/26RFaR (142, 143). In addition, the peptide has affinity for NPFFR2 (144).

26RFa/QRFP IN ADULT VERTEBRATES

In adult goldfish, *26RFa/qrfp* mRNA is found in the hypothalamus, optic tectum-thalamus, and testis (141). Because the expression in the hypothalamus was significantly reduced after 4 days of starvation, and intraperitoneal injections of the 26RFa/QRFP peptide increased LH levels, 26RFa/QRFP has been suggested to play a role in the regulation of energy homeostasis and regulation of the BPG-axis in fish (141). Also in birds, 26RFa/QRFP is expressed in the diencephalon; in the anterior hypothalamic nucleus in chicken, and in the anterior-medial area, the ventromedial nucleus and the lateral hypothalamic area in zebra finch (*Taeniopygia guttata*), areas involved in hypothalamic control over feeding behavior (145, 146). The findings are similar in mammals,

Table 4 | Overview of studies of kiss in vertebrate development.

RFa (and/or receptors)	Species	Method	Antibody	Embryonic stages	Location of peptide/mRNA in early developing CNS	Putative functions in early development	Reference
Kiss	Zebrafish (*Danio rerio*)	qPCR, kiss treatment, electrophysiology	–	1–7 dpf	Kiss1 and 2 mRNA detectable in brain from 1 dpf, increasing during development	Kiss1 stimulates GnRH neuron development, Kiss2 involved in development of trigeminal neurons	(118)
Kiss	Zebrafish	qPCR	–	1, 3, 7, 30, 45 dpf, adult	*kiss1* and *kiss2* detected from 1 dpf	–	(110)
GPR54	Cobia (*Rachycentron canadum*)	qPCR	–	Post hatching-adult	*gpr54* present at all stages	–	(111)
Kiss and receptors	Medaka (*Oryzias latipes*)	qPCR+ knockdown	–	From fertilization to newly hatched	–	Essential for brain and eye development	(109)
Kiss	Rat	qPCR, ISH ir, BrdU birth dating	Pol sheep anti-kiss (N-ter) AC067	Embryonic rats from E11.5 to E21.5	Kiss1 neurons in arcuate nucleus born from E12.5	Involved in embryonic activation of the hypothalamic–hypophyseal–gonadal axis	(123)
Kiss	Rat	ISH	–	Post-natal (neonate to adult)	Anteroventral periventricular nucleus (P7 in males, P21 in females), arcuate nucleus (P3)	–	(131)
Kiss	Rat	ISH	–	Post-natal (P0–P19)	Anterior hyp (P11), arcuate nucleus (P0)	Role in sexual differentiation of neonatal brain	(130)
Kiss	Rat	Kiss stimulation (*in vivo* and *ex vivo*)	–	Post-natal	–	Stimulating GnRH release in neonatals (5P)	(126)
Kiss + GPR54	Rat	qPCR on hyp	–	Post-natal (P1–75) + adults	*Kiss* and *Gpr54* present at all stages	–	(138)
Kiss + GPR54	Mouse	Transgenic mice	–	E12.5, E13.5, E14.5, and E16.5	*Kiss*: arcuate nucleus in hyp (E13.5), *Gpr54*: restricted to GnRH-neurons in anterior forebrain (E13.5-post-natal)	Regulating fetal GnRH activity?	(124)

(Continued)

Table 4 | Continued

RFa (and/or receptors)	Species	Method	Antibody	Embryonic stages	Location of peptide/mRNA in early developing CNS	Putative functions in early development	Reference
Kiss + GPR54	Mouse	RT-PCR, kiss treatment++		E12.5, E13.5, E14.5, and E15.5	*Kiss1*: mediobasal hyp (E13.5), *Gpr54*: preoptic area (E13.5)	Stimulates GnRH neurite growth	(107)
Kiss + GPR54	Mouse	ISH, qPCR, ir	Pol 1:10000 rabbit anti-rodent-kiss 1 (139)	E13, E15, E17 to P35	*Kiss1*: near median eminence (E13); preoptic periventricular nucleus (P12), *Gpr54*: from nasal compartment to forebrain in migrating GnRH-neurons (E13)	Involved in sexual differentiation of the brain during embryonic development?	(132)
GPR54	Mouse	ISH, single cell qPCR, Ca^{2+} imaging	–	E12.5, E13.5, E14.5, E17.5, and adult	In GnRH-cells in nasal region and nasal forebrain junction (E13.5)	–	(122)
Kiss	Mouse	ir	Pol 1:5000 rabbit anti-kisspeptin-10 (no. 566) (139)	Post-natal-adults (P10–P61)	Anteroventral periventricular nucleus, preoptic periventricular nucleus in hyp (P25) and arcuate nucleus in hyp at all stages	Kiss neurons in anteroventral periventricular nucleus and preoptic periventricular nucleus in hyp involved in the sexually differentiated functioning of GnRH-neurons	(127)
Kiss	Mouse	ISH	–	Post-natal (P1–P16)	Anteroventral periventricular nucleus and preoptic periventricular nucleus in hyp from P10	Involved in the sexually differentiated functioning of GnRH-neurons	(129)
Kiss	Mouse	ir	Pol 1:10000 rabbit anti-kisspeptin-10 (no. 566) (139)	Post-natal (P15–P30) + adults	Preoptic periventricular nucleus in hyp from P15	–	(128)

The brain areas are in general named according to the original article. dpf, days post-fertilization; E, embryonic day; hpf, hours post-fertilization; hyp, hypothalamus; ir, immunoreactivity; ISH, in situ hybridization; P, post-natal day; Pol, polyclonal; qPCR, quantitative PCR; RT-PCR, reverse transcription PCR; tel, telencephalon.

where the presence of 26RFa/QRFP has been shown in regions that are important for regulation of food intake and energy homeostasis. In rodents, high levels of 26RFa/QRFP are found in dorsolateral and mediobasal hypothalamic areas, and in humans 26RFa/QRFP-cells are found in the paraventricular and ventromedial nuclei of the hypothalamus (140, 144, 147). It has been found that injections of 26RFa/QRFP have an orexigenic effect (increased food intake) in rodents (148). 26RFa/QRFP has also been found to affect aldosterone secretion, insulin secretion, adipogenesis, bone formation, nociceptive transmission, blood pressure and, as in fish, to stimulate pituitary hormone secretion in mammals (4).

26RFa/QRFP IN DEVELOPMENT

The developmental expression of 26RFa/QRFP and GPR103 has only been studied in human adrenal gland and rat adrenals (149). Both the receptor and ligand were present from early stages of

adrenal development, mostly expressed in the adrenal cortex, but also in the medulla in human fetus. Some clues for the potential role of 26RFa/QRFP in development are also obtained from knockout studies of the receptor for 26RFa/QRFP in mice (150). Homozygous mice was viable, but they suffered from osteopenia (reduced bone density), and the investigations suggested that the bone formation was arrested at an early stage. It remains unclear if this effect is due to lack of hypothalamic signaling of 26RFa/QRFP, because mRNA of the receptor is found in bone and in osteoblast cell lines in addition to the hypothalamic expression (150). The knockout mice seemed to behave normally and were fertile. However, studies of the brain and the behavior of the animals was not included in the paper, thus it is unknown if knockout of the 26RFa/QRFP receptor can affect specific behaviors.

CONCLUSION

The RFa form a complex family with many different members acting in various physiological processes, with one peptide seemingly having several functions in the same animal in some cases. Common to all the peptides seems to be that they could have a role in appetite regulation, pain modulation, and reproduction. With the newest member of the RFa family found only a little over 10 years ago, the field of RFa is relatively new and requires much more research. Especially, how these peptides can influence development is poorly understood. However, the few studies of RFa in developing vertebrates show interesting results that may indicate that many of the RFa could have a separate role in development. Interestingly, it seems that all RFa are expressed early in development in many different groups of vertebrates. However, most of our knowledge of RFa comes from *in situ* hybridization and immunohistochemistry experiments. Very few functional studies have been conducted, so it is difficult to assess the role of this early expression. One knockdown study on medaka performed in our laboratory shows that *kiss1*, *gpr54-1*, and *gpr54-2* play vital roles in early development in this fish species, and that these genes are probably important for proper brain development. It will be very interesting to see if loss or gain of function could reveal novel functions of the other RFa.

The cellular pathways of RFa are poorly understood, and more research is required to find out how RFa can act on developmental processes. However, some RFa (26RFa and Kiss) have been shown to affect migration in cancer cells (114, 151). RFa may also be important for the proper migration of neurons in the developing brain. However, more research is needed to clarify the role of RFa in neuronal migration. Another interesting aspect of RFa is the fact that many of them can affect apoptosis and cell-cycle progression, possibly through affecting opioid receptors (152). PrRP is also found to influence human lymphocyte proliferation (153). In invertebrates, FMRFa has been found to inhibit apoptosis in a snail, indicating that the link between RFa and apoptosis is an evolutionary conserved mechanism (154). Interestingly, NPFF gene expression in mammals is regulated by transcription factors also involved in cell-cycle regulation and apoptosis (155).

The field of RFa in vertebrates is exiting and rapidly expanding. The few developmental studies that have been done show promising and important results. Taken together, these studies indicate that RFa may have a role in development of the nervous system not yet identified. More research is needed, especially functional studies that can give insight into the role these peptides play in development.

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Could FaRP-like peptides participate in regulation of hyperosmotic stress responses in plants?

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The ability to respond to hyperosmotic stress is one of the numerous conserved cellular processes that most of the organisms have to face during their life. In metazoans, some peptides belonging to the FMRFamide-like peptide (FLP) family were shown to participate in osmoregulation via regulation of ion channels; this is, a well-known response to hyperosmotic stress in plants. Thus, we explored whether FLPs exist and regulate osmotic stress in plants. First, we demonstrated the response of *Arabidopsis thaliana* cultured cells to a metazoan FLP (FLRF). We found that *A. thaliana* express genes that display typical FLP repeated sequences, which end in RF and are surrounded by K or R, which is typical of cleavage sites and suggests bioactivity; however, the terminal G, allowing an amidation process in metazoan, seems to be replaced by W. Using synthetic peptides, we showed that amidation appears unnecessary to bioactivity in *A. thaliana*, and we provide evidence that these putative FLPs could be involved in physiological processes related to hyperosmotic stress responses in plants, urging further studies on this topic.

Keywords: *Arabidopsis thaliana*, drought, FaRP-like peptides, osmotic stress, stomata

INTRODUCTION

Most of the living organisms from bacteria to metazoans, fungi, and plants have to face hyperosmolarity (i.e., an external osmolarity that is higher than the physiological range) during their lifetime, and the establishment of an appropriate response can be a matter of life or death. Whatever the cell types, they are generally able to counteract volume perturbations following a shift in extracellular osmolarity by rapidly modulating the activities of their plasma membrane ion transport systems (1, 2). Several major hormones that respond to osmotic stress have been identified in metazoans, vertebrates to arthropods, and plants (3–9), but it is only more recently that the importance of small peptides in different regulatory mechanisms has been pointed out in metazoans and plants (10, 11). In numerous metazoans (mollusks, annelids, nematodes, and vertebrates), peptides belonging to the FMRFamide-like peptides (FLPs) family have been shown to participate in osmoregulation (12, 13). Moreover, FLPs were shown to target various ion channels, among them the membrane sodium channels, such as the amiloride-sensitive FMRFa-activated sodium channel (FaNaCh) in invertebrates (13), or the structurally related acid-sensing sodium channels (ASICs) in vertebrates (14). These ligand-gated or pH sensitive-Na^+ channels are involved in Na^+ permeability and associated water transport, which makes them critical determinants of cell volume regulation (14). In sensory neurons of *Aplysia*, chloride currents are evoked by FMRFa via the cGMP cascade (15). In the same type of neurons, FMRFa also modulates the probability of opening and closing of S-type K^+ channels, a stretch-activated channel involved in response to osmotic shock (16).

For plant, drought-induced osmotic stress and salinity represent some of the major constraints that adversely affect growth, development, and biomass production. Numerous cellular responses and proteins have been reported to be conserved between plant and animal cells (17–19). Among them are ion channels (20), and their involvement in response to hyperosmotic stress (2, 21–23). Recent works implicate small signaling peptides in developmental processes in plants (11), but to our knowledge, until now, no study has described the presence of FLPs in viridiplantae. Thus, we addressed the hypothesis that FLPs exist in plants and participate in their physiological responses to hyperosmotic stress. Moreover, since many discoveries with direct relevance to animal biology have been elaborated using plants (17), this topic could also be relevant for metazoan biology by bringing new insight in FLPs structures, functions, and evolution.

Abbreviations: FaRPs, FMRFamide related peptides; FLPs, FaRP-like peptides; PM, plasma membrane; V_m, plasma membrane potential.

MATERIALS AND METHODS

CELL CULTURE CONDITIONS

Arabidopsis thaliana L. cell suspensions were freshly prepared from calli of the cell line T87 (24), which was generated from the ecotype Columbia plant. They were maintained in Gamborg

culture medium complemented with 20 g L^{-1} sucrose, 2 mg L^{-1} 2,4 D, 0.1 mg L^{-1} kinetin at 22 ± 2°C under continuous white light (40 µE m^{-2} s^{-1}) with continuous shaking (gyratory shaker at 120 rpm), as previously described (24, 25). Cell suspensions were sub-cultured weekly using a 1:10 dilution. All experiments were performed at 22 ± 2°C using log-phase cells (4 days after sub-culture). Cell density was about 3.10^4 cells mL^{-1}.

ELECTROPHYSIOLOGY

Cells were impaled in the culture medium with borosilicate capillary glass (Clark GC 150F) micropipettes (resistance: 50 MΩ when filled with 600 mM KCl). Main ion concentrations in the medium after 4 days were 9 mM K^+, 11 mM NO_3^- (26). Individual cells were voltage-clamped using an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA, USA) as previously described (24).

HYPEROSMOSIS TEST AND CELL VIABILITY ASSAYS

Pretreatments of 15 min with the various plants putative FLPs were done prior to the induction of a hyperosmotic stress by a 400 mM sorbitol exposure (duration: 6 h). Hyperosmosis-induced cell death in the cell suspension culture was determined after staining the dead cells with Evans blue (0.005%, w/v) for 10 min. Cells were counted under a microscope and cells accumulating Evans blue were considered to be dead. At least 500 cells were counted for each independent treatment and the procedure was repeated at least three times for each condition.

MEASUREMENT OF INTRACELLULAR ROS LEVEL

For measuring reactive oxygen species (ROS) generation, we used the CellROX® Deep Red Reagent (Molecular probes). The cell-permeant dye is non-fluorescent in a reduced state, and exhibits bright fluorescence upon oxidation by ROS. The cells were pre-incubated for 15 min with 100 µM of peptides and then incubated with 400 mM Sorbitol during 1 h. The cells were incubated with 5 µM CellROX Deep Red for 30 min before recording and then were washed with phosphate-buffered saline buffer. The excitation wavelength was set at 640 nm, and the emission was detected at 665 nm (27). The fluorescence intensity of the cells was measured with a Tecan Infinite 200 Spectrophotometer.

SEEDLINGS CULTURE

Arabidopsis thaliana L. seedlings were grown in an environmentally controlled chamber (8 h photoperiod, under 100 µmol photons m^{-2} s^{-1} at the leaf level, 24 ± 2°C) and plants were weekly watered.

PREPARATION OF EPIDERMAL STRIPS

Arabidopsis thaliana leaves from 4 to 6 weeks old plants were harvested 1 h after the beginning of the light period. Epidermal strips were carefully prepared from abaxial epidermis then placed cuticle side-down on microscope slides covered with medical adhesive (Dow Corning 355, Peters surgical) and immediately floated in 10 mM MES pH 6.1, 50 mM KCl, 1 mM $CaCl_2$ (opening buffer) under white light (40 µmol photons m^{-2} s^{-1}), or in 10 mM MES pH 6.1, 10 mM KCl, 1 mM $CaCl_2$ (closing buffer) in dark, for 3 h before future treatments.

STOMATAL APERTURE MEASUREMENTS

Epidermal strips were analyzed with a Laborlux S (Leica, Germany) microscope (×400). For quantifying, microscope fields were digitalized with a Kappa CF11DSP (Nikon, Japan) digital camera. The width of the stomatal aperture was measured using the image analysis software Metreo Kappa Image Base (Kappa, Germany). The pore width from at least 65 stomata from 2 leaves was measured per treatment and pooled together for statistical analysis. Data are expressed as micrometer and are means ± SE.

CHEMICALS

Synthetic peptides (purity >95%) were purchased from Proteogenix (Oberhausbergen, France) and diluted in water.

IN SILICO ANALYSIS

Putative plant FLP precursors were detected using a blastp search against the protein sequence database at the NCBI and TAIR, using FMRF–FMRF, FLRF–FLRF, or ILRF–ILRF as query. Only sequences showing more than three repeats were considered. Further sequences were obtained using these results as query using blastp against the UniProtKB database. All sequences were also blasted using tblastn against *A. thaliana* ESTs database at the NCBI and TAIR. When possible, the corresponding genes were localized using the EnsemblPlant database. Some representative sequences are presented in **Table 1**.

STATISTICS

Significant differences between treatments were determined by the Mann and Whitney test, and P values <0.05 were considered significant.

RESULTS AND DISCUSSION

FLRFa-INDUCED HYPERPOLARIZATION AND ION CURRENT REGULATIONS IN *ARABIDOPSIS THALIANA* CELLS

FMRFamide (FMRFa) is a cardioexcitatory peptide that was first isolated from the nervous system of the clam, *Macrocallista nimbosa* (28), and is active as a tetrapeptide only in mollusks and annelids. Other active tetrapeptides have been identified in lophotrochozoans; these include FLRFa, YLRFa, or YMRFa. In view of the well-known effects of FLPs on ion channel regulation in metazoan cells, we first checked for the putative effect of FLRFa, a typical metazoan FLP, on plasma membrane polarization and ion channel regulations in cultured cells of the model plant *A. thaliana* by using single electrode voltage clamp (24). In control conditions (in culture medium), the cell plasma membrane potential (V_m) of cells was −33 ± 4 mV (n = 20) – similar to those we observed in previous studies (25, 26, 29). Addition of 100 µM FLRFa induced a hyperpolarization of the cells of −8 ± 1.5 mV (n = 4, **Figure 1A**). These FLRFa-induced hyperpolarizations were correlated with a decrease in inward currents (**Figures 1B,E**) that we previously described as anion currents (25, 26, 30), and an increase in time dependent outward rectifying currents (**Figures 1B,E**), previously described as K^+ outward currents [KORC, (24, 26, 30)]. It is noteworthy that these ion current regulations are the same as those observed in response to a shift in osmolarity (128–330 mOsm induced by addition of 200 mM sorbitol in the cell culture medium; **Figures 1C–E**). Inhibition

Table 1 | Identification of some putative pro-peptides and their genes in *Arabidopsis thaliana*.

Name	Prot id			EnsemblPlants – TAIR10				
	UniProt	gb	gi	Gene localization	EST	Repeats	Transposon	Tandem repeats
F26F24.19	Q9LR27_ARATH	AAF87013.1	9295707	NF	NF	NF	NF	NF
F4N2.6	Q9LQB1_ARATH	AAF27054.1	6730633	1:25976374–25976607	No	Yes	Type II	Yes
F1L3.6	Q9LNR8_ARATH	AAF79458.1	8778450	1:5965755–5966750	TC290437	Yes	Type II	Yes
At2g42050 hypothetical protein	P93742_ARATH	AAB63539.1	1871179	2:17546143–17546559	No	Yes	Type II	Yes
Unnamed protein product (BAB01828)	Q9LS63_ARATH	BAB01828.1	9293925	3:11226216–11226473	No	Yes	Type II	Yes
F22O6_210	Q9SVC3_ARATH	CAB43442.1	4886286	3:19428300–19428917	No	Yes	Type II	Yes
F19F18.60 = At4g37570	Q9SZF1_ARATH	CAB38296.1	4468982	4:17655176–17655730	TC297785	Yes	Type II	Yes
Unnamed protein product (BAB09258)	Q9FGB7_ARATH	BAB09258.1	9758805	5:14153439–14154089	No	Yes	Type I	Yes

The gene coordinates are given regarding TAIR10 genome assembly (accessible at EnsemblPlants http://plants.ensembl.org/) as chromosome:extend. The EST, repeats, transposon, and tandem are the automatic annotations given during the automatic annotation procedure. NF, not found.

of outward anion currents is a process by which ion leakage is decreased, and thus, results in rapid adaptation to hyperosmotic condition by ion accumulation (31–33). The activation of KORC favoring K^+ efflux previously reported in other models (34, 35) is opposite to ion accumulation but could be a part of an initial signaling that could result in osmotic regulation (34). In view of these data, we searched for putative plant gene(s) coding for these peptides in gene and protein databases.

PUTATIVE CANDIDATE GENES FOR FLPs SYNTHESIS IN *ARABIDOPSIS THALIANA*

A FLP can be defined as a peptide that ends in RFa while a FaRP is a peptide homologous to FMRFa in metazoans (36, 37). The number of FLPs identified is increasing with the availability of genome and transcriptome databases and the development of constrained algorithm to search for them (37, 38). The length of these peptides ranges from 4 to 52 amino-acids and 37% of those found do not exceed 10 amino-acids (36). Active peptides are cleaved-out of a pro-peptide; the amino-acids allowing this cleavage are the basic amino-acids K or R, either alone or as a dimer (39) (**Figure 2A**). In FaRPs, the terminal cleavage site (R/K) is preceded by a G, allowing the amidation of the peptide: the XXRFamide form is biologically active, whereas a non-amidated peptide is considered to be inactive. Regarding FLP, the structure $[KR](X)_nRFG[KR]$ appears to be the most common organization (**Figure 2A**). Nevertheless, the amino-acid before the RF ends may vary between peptides and/or organisms. Espinoza et al. (38) have shown that the distribution and the type of amino-acids (the order and the respective position) of each is not random. Although the specificity of each peptide seems to be very high and adapted to each species [see for example (40–42)], the structure and the relationships between structure (composition) and function of the genes, as well as the biochemical characteristic of the couple ligand/receptor, are not clearly established.

By exploring genomic and transcriptomic databases, we found in *A. thaliana*, several putative genes that may allow the production of pro-peptides including repeated FLPs peptide sequences. Some representative sequences are listed in **Table 1** and illustrated in **Figure 2**. As underlined in **Table 1**, most of these sequences are not annotated as ESTs in the automatic genome annotations, probably because of the "repeat masker" step. Instead, they are interpreted to be transposable elements. But several similar sequences were found in *A. thaliana* ESTs database, suggesting that these genes could in fact be expressed (for example: EG509196 and EG509184). It is noteworthy that the ESTs are issued from *Arabidopsis* stressed with several factors, including salinity, an osmotic stress.

The sequences in **Figure 2A** were initially identified using a poly-ILRF query and all of them showed the characteristic properties of pro-peptides: tandem repeats and cleavage sites. Each sequence of the alignment in **Figure 2B** presents 3–12 repeats ending with RF. In these sequences, the IL[RK]F feature is the most abundant. The ILRF sequence is one classical ending identified among the 23 groups of longer metazoan FaRPs (38). Conventional cleavage sites (R or K) are present, suggesting a RENMIL[R,K]FWR peptide sequence. As observed in other genes/species, all the repeats are not followed and/or preceded by a putative cleavage site; these unbreakable repeats are often interpreted as non-functional.

Other repeated peptides with FLPs-like structures have been evidenced. **Figure 2C** illustrates a group of sequences rich in KI[MIST]GLRFWR and also presenting other putative peptides. Each of these five sequences are found on different chromosomes, indicating that they correspond to different genes.

The peptide ILRF (and YLRF) is found in several combinations of metazoan peptides, suggesting that this peptide could be functional, at least in metazoans (37). This observation, combined with biological effect of tetrapeptide in *Arabidopsis* cells shown above,

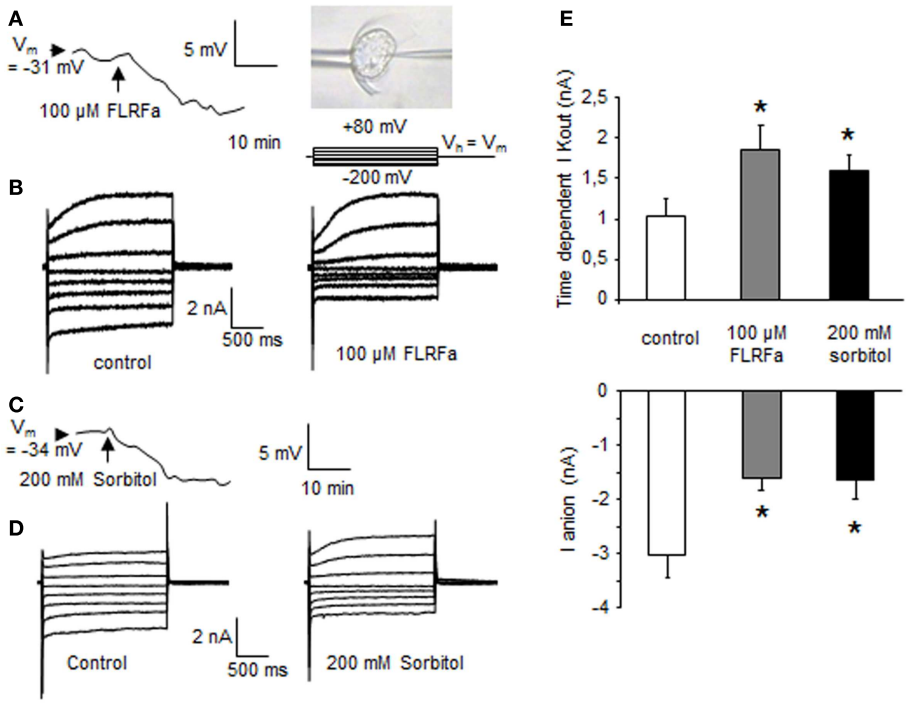

FIGURE 1 | FLRFa-induced hyperpolarization of a cultured cell of *Arabidopsis thaliana* maintained by a microfunnel and impaled by a microelectrode (A). Modulation of *A. thaliana* whole cell currents in response to 100 µM FLRFa **(B)**. Sorbitol induced hyperpolarization **(C)**. Modulation of *A. thaliana* whole cell currents in response to 200 mM sorbitol **(D)**. The protocol was as illustrated, holding potential (V_h) was V_m. Mean values of anion currents recorded at −200 mV after 1.8 s and of time dependent K^+ outward currents at +80 mV after 1.8 s **(E)**. The data correspond to means of four independent experiments and error bars correspond to SD. *Significantly different from controls, $P < 0.05$.

strongly suggests that functional bioactive FLPs could be synthesized in *Arabidopsis*, as in metazoans. Surprisingly, practically none of the putative peptides observed in *Arabidopsis* ended with a G, but with a W instead. This suggests that in *Arabidopsis*, amidation does not occur when the peptide is generated. This raises the question of the activity of these molecules in *Arabidopsis*, as amidation appears to be necessary to their bioactivity in metazoans.

The putative presence of RF-amide peptides in plants is, at the moment, based on genomic and transcriptomic databases. Future work should aim at characterizing the presence of translated peptides. However, because of the similarity with the metazoan peptides observed in the peptide ILRF found and whatever the differences around the cleavage site, we have explored their activity in *Arabidopsis* cells.

PUTATIVE FLPs FROM *A. THALIANA* COULD REGULATE SORBITOL-INDUCED PCD AND ROS GENERATION

As in animal cells, plant cell hyperosmotic stress may result in the induction of signaling events that leads to programed cell death (PCD) (2, 43–47), an active cellular process that facilitates the removal of unwanted or damaged cells and is essential for cellular differentiation and tissue homeostasis. We recently showed that hyperosmotic stresses-induced ion channel regulations participate in pathways leading to PCD in plant cultured cells (2). Using synthetic peptides, we tested the effect of putative plant FLPs (ILRF and ILKF, 10 µM each) on sorbitol-induced PCD in *A. thaliana* suspension cells. The shifts in osmolality induced by addition of 400 mM sorbitol (from 128 to 524 mOsm) led to the death of about half of the cell population after 6 h (**Figures 3A,B**) when FLPs did not induce a significant increase in cell death (**Figure 3A**). Pretreatments of *A. thaliana* cells with 10 µM ILRF or ILKF 15 min before addition of 400 mM sorbitol decreased the extent of the sorbitol-induced cell death (**Figure 3A**). Due to the lack of terminal G, which was systematically replaced by a W in plant sequence, the putative plant FLP could not be amidated (cf data from **Figure 2**). Thus, we further investigated the putative role of the terminal W by testing the same peptides augmented with a terminal W (ILKFW and ILRFW, 10 µM each). These treatments decreased the extent of the sorbitol-induced cell death in the same range (**Figure 3A**), suggesting no specific role for the terminal W in this response. It is noteworthy that the terminal amidation did not increase the bioactivity, since the decrease in sorbitol-induced cell death by pretreatment with amidated peptides, ILRFa, ILKFa, and FLRFa (100 µM each), were not drastically modified even with a 10 times higher concentration (**Figure 3C**).

A delayed $O_2^{\cdot-}$ generation from NADPH-oxidase activity was also shown to play a central role in the hyperosmotic stress-induced PCD in plant cells (2). We thus evaluated the impact of putative plant FLPs (ILRF, ILKF, ILKFW, and ILRFW, 100 µM each) on sorbitol-induced ROS generation. As observed for sorbitol-induced cell death, pretreatments of *A. thaliana* cells with these FLPs 15 min before addition of 400 mM sorbitol did

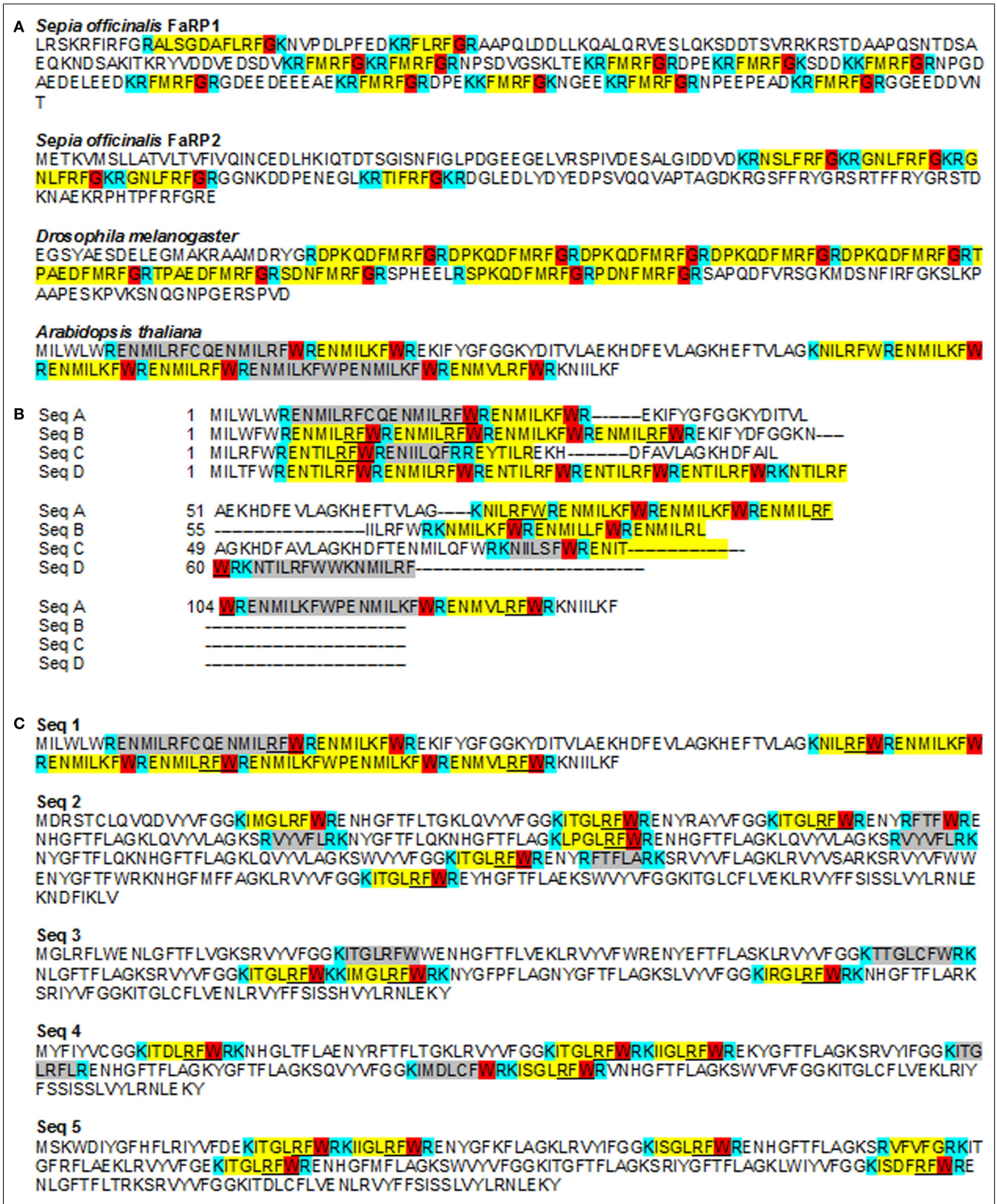

FIGURE 2 | FMRFamide-like peptides sequences in metazoans and *Arabidopsis thaliana*. **(A)** Partial sequences of FLPs from *Sepia officinalis* (Mollusca), *Drosophila melanogaster* (Arthropoda), and *Arabidopsis thaliana* showing the diversity of FaRP between species, in the same species and in the same gene. For example, two different genes coding for FaRPs were

(Continued)

FIGURE 2 | Continued
characterized in *Sepia officinalis* with different composition and length. All peptides characterized in metazoans end with a G allowing amidation of the peptide after cleavage (38). The sequence of *A. thaliana* shows similar repetitions ending with RF but with a W instead of a G. Acc number: *S. officinalis* FaRP1: P91889; *S. officinalis* FaRP2: D8WXV2; *D. melanogaster*: AY070639; *A. thaliana*: P93742. **(B)** Different putative pro-peptides in A. *thaliana* genome detected using a poly-ILRF query. Sequences were manually aligned in Jalview. Acc. Numbers: SeqA At2g42050 (P93742_ARATH).1-139; SeqB: BAB01828 (Q9LS63_ARATH)/1-86; SeqC: F26F24.19 (Q9LR27_ARATH_F26F24.19)/1-86; SeqD: F4N2.6_(Q9LQB1_ARATH)/1-77. **(C)** Examples of putative small peptides from *A. thaliana* including a RFW end-sequence (underlined) or terminated by another sequence (highlighted in gray). These sequences are chosen to illustrate the presence of putative pro-peptide genes on each chromosome. Each sequence has been detected in mRNA sequencing (as shown by a tblastn against *A. thaliana* ESTs at the NCBI site) but are not recognized as ESTs in *A. thaliana* EnsemblPlants genomic automatic annotation due to their repeat structure. Instead, all are flagged as transposable elements. Acc. numbers: *A. thaliana*: seq1: P93742_ARATH; seq2: Q9LNR8_ARATH; seq.3: Q9SVC3_ARATH; seq.4: Q9SZF1_ARATH; seq.5: Q9FGB7_ARATH. The putative cleavage sites (mono or dibasic) are indicated in blue, the "transitional peptide" in red, the putative functional peptide in yellow. The sequence highlighted in gray in *Arabidopsis* sequences is a putative peptide.

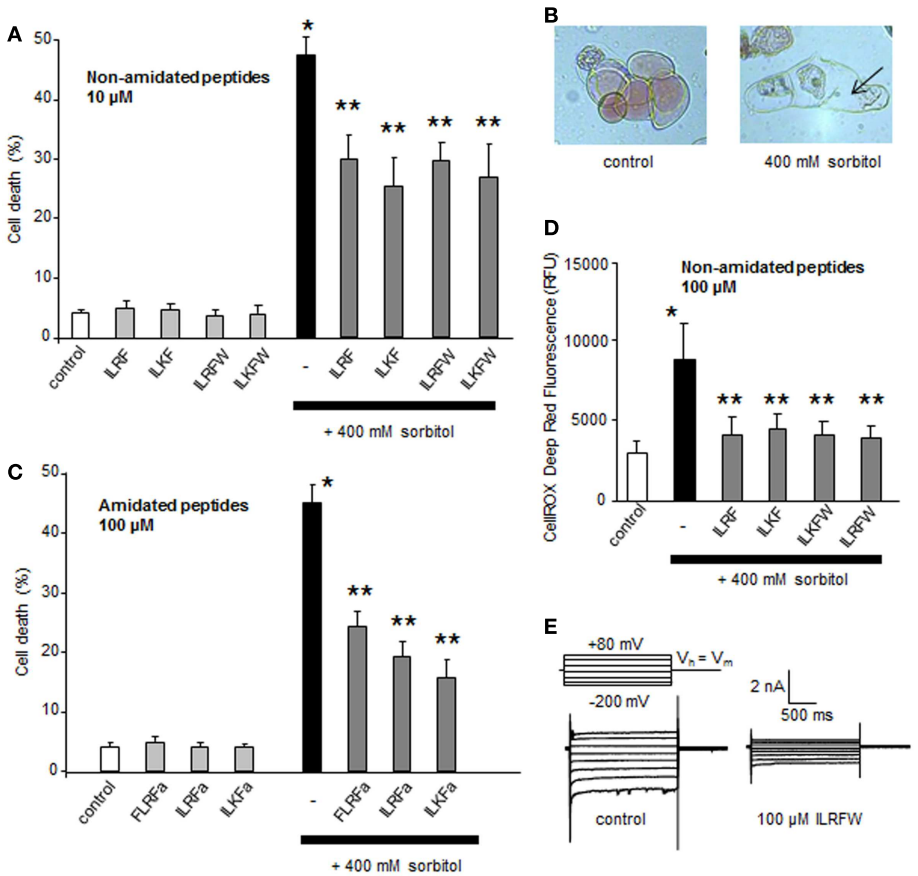

FIGURE 3 | Effect of different putative plant FLPs on sorbitol-induced cell death in *Arabidopsis thaliana* cells. **(A)** Effect of non-amidated synthetic FLPs (ILRF, ILKF, ILRFW, and ILKFW, 10 µM each) on sorbitol-induced cell death extent. **(B)** Light micrographs of *A. thaliana* cultured cells stained with Neutral Red 6 h after incubation with 400 mM sorbitol (right) compared to living control cells maintained in their medium (left). Arrows indicate the cell shrinkage. **(C)** Effect of various amidated synthetic FLPs (ILRFa, ILKFa, and FLRFa, 100 µM each) on sorbitol-induced cell death extent. **(D)** Effect of various non-amidated synthetic FLPs (ILRF, ILKF, ILRFW, and ILKFW, 100 µM each) on sorbitol-induced ROS generation. Each data point and error bar reflect the mean and SD, respectively, of at least three independent replicates. *Significantly different from controls, $P < 0.05$ and **significantly different from the sorbitol treated cells, $P < 0.05$. **(E)** Inhibition of *A. thaliana* anion current in response to 100 µM ILRFW.

not cause sorbitol-induced ROS generation (**Figure 3D**). Moreover, the peptide ILRFW could reduce anion channel activity (**Figure 3E**) and induce a hyperpolarization of the cells of about −10 mV (not shown) as does FLRFa (**Figure 1A**). It is noteworthy that non-amidated putative plant FLPs were efficient in decreasing sorbitol-induced ROS generation and anion currents, indicating that the peptide amidation was not necessary for their activities.

From these data it seems that, like in animal cells, FLPs could participate in osmoregulation through regulation of different events as ROS and PCD. The fact that the early FLP-induced ion current regulations in plant cells are the same as the one

observed in response to a hyperosmotic stress (**Figures 1** and **3E**) suggests that FLPs could participate to early induced process to maintain ion homeostasis and/or signalization, allowing to limit hyperosmotic stress-induced-PCD progress (**Figure 3A**).

PUTATIVE FLPs FROM *A. THALIANA* COULD REGULATE STOMATAL OPENING

Since putative plant FLPs could inhibit ROS generation (**Figure 3D**) and anion channel activity (**Figures 1B** and **3E**) – both of which are known to regulate the stomatal aperture (48, 49) – we checked for the effect of a putative plant FLP (ILRF) on stomatal regulation. Stomata are pores in the plant epidermis that allow gas exchange between the intercellular spaces to the external environment. Two guard cells surround the stomatal pore, and changes in their turgor pressure regulate the size of the pore aperture allowing the CO_2 assimilation and limit excessive water loss by optimizing the aperture in response to the external environment. Epidermal strips from *A. thaliana* leaves were thus floated 2 h in two different conditions to either optimize the opening (opening buffer) or the closure of the stomata (closing buffer) (29) before addition of 100 µM ILRF. No effect could be observed on open stomata in response to addition of ILRF (**Figure 4**), while the same treatment induced an increase in the aperture of stomata from the epidermal strips placed in the closing buffer (**Figure 4**).

This ILRF-induced stomatal opening could appear counterintuitive, since upon drought stress, the stomatal closing is thought to be an important primary defense against tissue dehydration (48, 50). However, if such response is part of a fundamental response to severe drought stress, upon mild drought stress, prolonged closed stomata will stop growth by depriving the plant of CO_2 for photosynthesis. Adaptive plant growth in sporadic water availability will thus depend on the optimal tradeoff between stomatal closing and rapid re-opening capability. Thus, plants should adapt to mild soil water deficit by mechanisms that are distinct from those of severe dehydration. This was highlighted by a recent report in which acetylated 1,3-diaminopropane counteracted the canonical abscisic acid-induced stomatal closing (48) upon mild drought stress, but not upon severe drought stress (51).

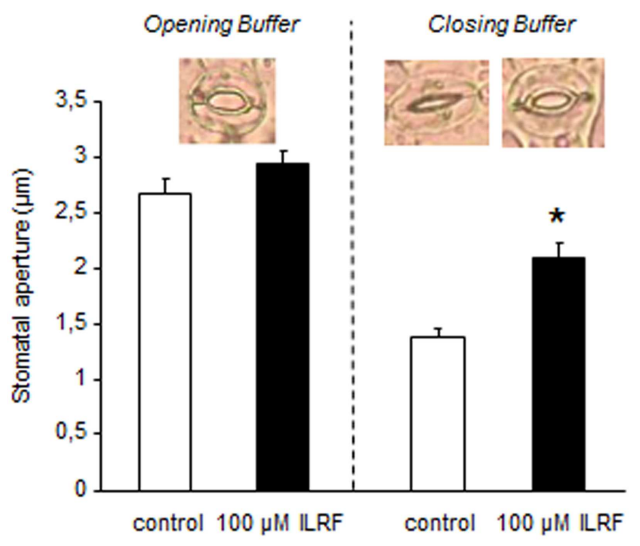

FIGURE 4 | Changes in *Arabidopsis* stomatal apertures of epidermal strips maintained in opening buffer or closing buffer upon treatment with 100 µM ILRF. Means ± SE ($n = 65$–75 stomata for each treatment). *Significant difference ($P < 0.05$) in stomatal aperture when compared to the control was found after treatment with ILRF in closing buffer, but not opening buffer.

CONCLUSION

Numerous cellular features are conserved in eukaryotic cells (18, 19), and many discoveries with direct relevance to animal biology and even human health and disease have been elaborated using plants (17). In this context, conservation of mechanisms of response to hyperosmotic stress, a stress that most of the living organisms have to face, appears logical. In a recent paper, Aalen (10) mentioned that plant peptide research is coming of age and that plant peptide signaling is of crucial importance for all aspects of plant growth and development. Recent works effectively implicate several families of small signaling peptides in various developmental processes in plants (11). However, the families of characterized peptides in plants represent <10% of the estimated number of secreted peptide ligands. Our preliminary results showing that some putative FLPs genes are present in *A. thaliana* genome and that putative plant FLPs could induce physiological responses involved in hyperosmotic stress responses warrant further studies on this topic. Furthermore, we cannot dismiss the possibility that other genes could be responsible for synthesis of others putative FLPs in plants. Drought frequency may increase by more than 20% in some regions of the globe by the end of the twenty-first century, with reductions in crop yields due to decreased water availability. Thus, understanding the putative role of FLPs in plants as regulators that mediate environmental influences on plant development and fitness is particularly relevant for plant biology. Moreover, this topic could also be relevant for metazoan biology since it could bring new insight in FLPs structures, functions, and evolution.

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