REVIEW

The role of RFamide peptides in feeding

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Abstract

In the three decades since FMRFamide was isolated from the clam *Macrocallista nimbosa*, the list of RFamide peptides has been steadily growing. These peptides occur widely across the animal kingdom, including five groups of RFamide peptides identified in mammals. Although there is tremendous diversity in structure and biological activity in the RFamides, the involvement of these peptides in the regulation of energy balance and feeding behaviour appears consistently through

evolution. Even so, questions remain as to whether feeding-related actions represent a primary function of the RFamides, especially within mammals. However, as we will discuss here, the study of RFamide function is rapidly expanding and with it so is our understanding of how these peptides can influence food intake directly as well as related aspects of feeding behaviour and energy expenditure.

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Introduction

The first recognised member of the RFamide neuropeptide family was the cardioexcitatory peptide, FMRFamide, isolated from ganglia of the clam *Macrocallista nimbosa* (Price & Greenberg 1977). Since then a large number of these peptides, defined by their carboxy-terminal arginine (R) and amidated phenylalanine (F) residues (hence RFamide), have been identified in the nervous systems of animals within all major phyla. Vertebrates and more especially invertebrates can each express an array of RFamide peptides, owing to the fact that multiple genes encoding RFamides are often present in a single species, and multiple mature RFamide peptides can be generated by a single polypeptide precursor. The prevalence of RFamides in invertebrates is illustrated by the nematode *Caenorhabditis elegans*, in which 22 known genes encoding RFamide peptides (referred to as *flp* genes) and 59 distinct RFamide peptides have been identified (Li *et al.* 1999). Impressively, *flp* gene products are expressed in almost half of the 302 neurons found in the adult nematode (Li *et al.* 1999, Kim & Li 2004).

RFamide peptides show a remarkable diversity in N-terminal sequence, and as a likely consequence, a broad pattern of biological activities. Pharmacological studies have implicated RFamide peptides in roles that include cardiovascular function, modulation of muscle contraction, control of locomotor activity, water balance, neuroendocrine and neuromodulatory activities (Dockray 2004, Sun *et al.* 2005, Fukusumi *et al.* 2006). As with most neuropeptides, the RFamides are often

co-localised with classical neurotransmitters, including acetylcholine, serotonin and gamma-amino butyric acid (GABA).

Although a role for RFamides in feeding behaviour was first suggested over 20 years ago, when FMRFamide was shown to be anorexigenic in mice (Kavaliers *et al.* 1985), the question of whether regulating food intake represents a primary function of RFamide signalling remains. One convincing piece of evidence is that RFamide involvement in feeding behaviour has been demonstrated across a wide range of animal classes, including coelenterates, molluscs, amphibians, birds and mammals (see below), suggesting that this function has been conserved through evolution (Dockray 2004). Here, we review the evidence that explores the ability of RFamide peptides to influence feeding behaviour in both mammalian and non-mammalian species.

Mammalian RFamide peptides

Immunoreactive staining of vertebrate tissues with antibodies raised against the molluscan FMRFamide peptide first suggested that the RFamide peptides were to be found in higher animal groups (Boer *et al.* 1980, Dockray *et al.* 1981, Weber *et al.* 1981). Soon after, LPLRFamide was purified from chicken brain (Dockray *et al.* 1983) and since then the list of vertebrate RFamides has grown steadily, including five genes encoding RFamide peptides identified in mammals (Table 1; Fig. 1). In addition to the increasing number of peptides, several G-protein-coupled receptors (GPRs) are now recognised as receptors for the mammalian RFamides.

Table 1 Primary sequences of RFamide peptides

	Species	Sequence
RFamide peptide		
FMRFamide	Clam	FMRF-NH ₂
NPFF	Human	FLFQPQRF-NH ₂
NPAF	Human	AGEGLSPFWSLAÄPQRF-NH ₂
PrRP20	Human	TPDINPAWYASRGIRPVGRF-NH ₂
PrRP31	Human	SRTHRHSMEIRTPDINPAWYASRGIRPVGRF-NH ₂
C-RFa	Carp	SPEIDPFWYVGRGVRPIGRF-NH ₂
LPLRFamide	Chicken	LPLRF-NH ₂
RFRP-1	Bovine	SLTFEEVKDWAPKIKMNKPVVNMPPSAANLPLRF-NH ₂
RFRP-3	Bovine	AMAHPLRLGKNREDSLSRWVPNLPQRF-NH ₂
GnIH	Quail	SIKPSAYLPLRF-NH ₂
Metastin	Human	GTSLSPPPESSGSRQQPGLSAPHSRQIPAPQGAVLVQREKDLPNYNWNSFGLRF-NH ₂
QFRP	Human	<EDEGSEATGFLPAAGEKTSGLPLGNLAEELNGYSRKKGGFSRF-NH ₂
26RFa	Frog	VTGALGSLAEELNGYNRRKGGFSRF-NH ₂

Pyroglutamic acid is shown as <E.

NPFF family

Neuropeptide FF (NPFF, shortened from F8Famide) and neuropeptide AF (NPAF, from A18F) were isolated originally from bovine brain by affinity purification using antibodies

raised against FMRFamide (Yang *et al.* 1985). The two peptides are derived from the same gene and precursor peptide, and their expression has been demonstrated in the brains of several mammalian species (Perry *et al.* 1997, Vilim

Figure 1 Unrooted phylogenetic tree of the identified and the putative RFamide peptides in mammals and other vertebrates. The neighbour-joining method was used to construct this phylogenetic tree. Data were re-sampled by 1000 bootstrap replicates to determine the confidence indices within the phylogenetic tree. Scale bar refers to a phylogenetic distance of 0.1 amino acid substitutions per site. Frog 26RFa does not appear on the map and non-mammalian homologues of kisspeptin have not yet been identified. However, receptors related to GPR54 and GPR103 have been predicted from the zebrafish and chicken genomes respectively. Figure reprinted with minor modification from Osugi *et al.* (2006), *FEBS Journal* 273; copyright, with permission from Blackwell-Synergy.

et al. 1999, and references cited below). The best-documented function of NPFF is its ability to modulate opioid-induced analgesia (Yang *et al.* 1985, Panula *et al.* 1999, Dong *et al.* 2001), although cardiovascular (Allard *et al.* 1995) and feeding (Murase *et al.* 1996, Sunter *et al.* 2001, Bechtold & Luckman 2006) effects have also clearly been demonstrated.

Two GPRs have been identified as putative receptors for NPFF and consequently designated NPFF1 and NPFF2 (Bonini *et al.* 2000). Subsequent studies have shown that NPFF1 exhibits a higher affinity for RFRP than for NPFF, and NPFF displays a higher potency for NPFF2 (Liu *et al.* 2001, Yoshida *et al.* 2003). Therefore, NPFF2 is now generally considered the endogenous receptor for NPFF, with NPFF1 as the RFRP receptor. However, NPFF2 appears to be fairly promiscuous, showing relatively high affinities for NPFF, RFRPs as well as prolactin-releasing peptide (PrRP; Engstrom *et al.* 2003). Human NPFF1 and NPFF2 share 46% sequence identity and exhibit 31–37% identities with the receptors for orexin, neuropeptide Y, CCK₁ and PrRP.

The major population of NPFF-expressing neurons in the rat brain lies within the nucleus of the tractus solitarius (NTS; Kivipelto *et al.* 1989, Boersma *et al.* 1993, Lee *et al.* 1993, Vilim *et al.* 1999) and projects to the ventral lateral medulla (VLM), lateral parabrachial nucleus (IPBN) and paraventricular nucleus of the hypothalamus (PVN; Jhamandas *et al.* 2001, Goncharuk *et al.* 2006). NPFF-expressing neurons have also been reported in the vicinity of the ventromedial (VMN) and dorsomedial (DMN) nuclei of the hypothalamus. However, the lack of mRNA expression in this region (Vilim *et al.* 1999) suggests that the immunostaining may be due to cross-reactivity of antibodies with other related RFamide peptides (such as RFRP) which have been reported in the region (Hinuma *et al.* 2000). While NPFF immunoreactive fibres have a fairly wide distribution in the rat brain, major targets include the PVN, the IPBN and the VLM, as all the three areas exhibit dense NPFF-containing nerve fibres, NPFF receptor expression and NPFF-binding activity (Kivipelto *et al.* 1989, Boersma *et al.* 1993, Vilim *et al.* 1999, Hinuma *et al.* 2000, Liu *et al.* 2001, Gouarderes *et al.* 2002, 2004a, Goncharuk *et al.* 2006). PVN neurons have also been shown to be electrically responsive to NPFF (Jhamandas *et al.* 2006) and express the immediate early gene, *c-fos*, following intracerebroventricular (ICV) injection of the peptide (Jhamandas & MacTavish 2003). In line with its role in nociception, NPFF and NPFF2 are expressed in dorsal horn of the spinal cord (Panula *et al.* 1996, Vilim *et al.* 1999, Gouarderes *et al.* 2002). It is important to point out that pronounced inter-species and inter-strain differences have been reported for the localisation of NPFF and its receptors (Boersma *et al.* 1993, Gouarderes *et al.* 2002, 2004b).

The ability of NPFF to influence feeding behaviour has been demonstrated in rats and mice. Specifically, NPFF injected into the cerebral ventricles inhibits short-term food intake in rats (Murase *et al.* 1996, Sunter *et al.* 2001) and in both pre-fasted and *ad libitum* fed mice (Bechtold & Luckman

2006). Sunter *et al.* (2001) have reported that the attenuation of feeding in rats by NPFF is accompanied by an acute stimulation of water intake; we have not observed this effect in mice (unpublished results). As described above, both NPFF and its receptors are localised in the hypothalamus, consistent with a direct action of NPFF on hypothalamic neurons regulating food intake. NPFF immunoreactive fibres densely innervate another area implicated in feeding behaviour, the PBN. Administration of NPFF into the PBN in relatively low doses (<10 nmol) can inhibit the stimulation of food intake in response to the μ -opioid receptor agonist, [D-Ala, N-Me-Phe, Gly-oL]-enkephalin (DAMGO; Nicklous & Simansky 2003). Interestingly, higher doses of NPFF caused an increase in food intake which could be blocked by naloxone, an opioid receptor antagonist. Combined with the well-documented ability of NPFF to modulate opioid-induced analgesia (Panula *et al.* 1999), these findings suggest that the modulation of feeding behaviour by NPFF and opioids involve similar pathways (Murase *et al.* 1996). NPFF has been shown to depress excitatory glutamatergic synaptic transmission in PBN neurons, evidently acting via pre-synaptic δ -opioid receptors (Chen *et al.* 2000). However, NPFF shows no affinity for opioid receptors (Raffa *et al.* 1994, Gouarderes *et al.* 1998), but may indirectly modify opioid receptor activity. For example, *in vivo* intrathecal infusion of an NPFF analogue enhances met-enkephalin release from the rat spinal cord (Ballet *et al.* 1999).

Since most of the studies relating to the feeding effects of NPFF are pharmacologically based (i.e., injection of exogenous peptide), it is difficult to establish whether NPFF or its receptors play a physiological role in regulating feeding behaviour. Unpublished results from our laboratory suggest that NPFF is unlikely to act as an endogenous satiety signal. ICV administration of NPFF to pre-fasted mice leads to a disruption of the behavioural satiety sequence, especially at the onset of re-feeding, rather than shortening the crossover point from feeding to rest (taken as a measure of satiety), suggesting that NPFF inhibits feeding rather than enhances satiety. Furthermore, NPFF neurons within the NTS are not activated by the endogenous satiety factor, cholecystokinin (CCK). The expression of NPFF2 mRNA is not altered by diet restriction or in diet-induced obese rats (Laemmle *et al.* 2003). However, ICV administration of NPFF has been shown to activate oxytocin neurons in the PVN (Jhamandas & MacTavish 2003). Therefore, the anorexic effects of ICV NPFF may involve oxytocin, as this peptide can also reduce feeding when administered centrally (Arletti *et al.* 1990, Olson *et al.* 1991).

PrRP family

Two PrRP peptides (PrRP-20 and PrRP-31) were isolated originally from bovine hypothalamus and so named because of their ability to induce prolactin release from dissociated rat pituitary cells (Hinuma *et al.* 1998). It now appears unlikely

that PrRP stimulates prolactin release *in vivo* (Taylor & Samson 2001), despite there being evidence that PrRP is involved in regulating the secretion of other hypothalamo-pituitary factors, including corticotrophin-releasing hormone, follicle-stimulating hormone (FSH) and luteinising hormone (LH), and oxytocin (Maruyama *et al.* 1999b, Hizume *et al.* 2000, Matsumoto *et al.* 2000, Samson *et al.* 2003).

A number of observations by our group and others strongly implicate PrRP in the homeostatic regulation of feeding and energy balance. In the rat brain, PrRP mRNA is down-regulated in states of negative energy balance (fasting and lactation; Lawrence *et al.* 2000), and central administration of PrRP decreases feeding and body weight gain in rats and mice (Lawrence *et al.* 2000, 2002, Bechtold & Luckman 2006). The anorexic actions of PrRP appear to be related to satiety, as administration of the peptide does not invoke a conditioned taste aversion, or disrupt the normal behavioural satiety sequence (Lawrence *et al.* 2002). The anorexic actions of PrRP are attenuated in mice (Bechtold & Luckman 2006) and rats (Watanabe *et al.* 2005) that lack functional expression of GPR10, the putative receptor for PrRP. The significance of endogenous PrRP–GPR10 signalling in the regulation of feeding behaviour is also demonstrated by the fact that GPR10-knockout mice become heavier than congenetic wild types, due primarily to an increased accumulation of fat stores (Gu *et al.* 2004). The recent demonstration that a *GPR10* gene mutation is the primary cause of the obese phenotype in the Otsuka Long-Evans Tokushima Fatty rat further iterates the significance of this signalling pathway in energy homeostasis (Okuno *et al.* 2001, Watanabe *et al.* 2004).

In the rat, the majority of PrRP-expressing neurons reside in the brainstem within the NTS and the VLM (Roland *et al.* 1999, Ibata *et al.* 2000, Lee *et al.* 2000). A smaller population of PrRP-expressing neurons also reside in the hypothalamic DMN. The NTS and VLM are regions that receive extensive gastrointestinal and autonomic vagal inputs, and it is established that gut-produced CCK inhibits food intake via activation of vagal afferent neurons that terminate in the NTS (Saper 2004). Central administration of PrRP elicits a similar pattern of neuronal *c-fos* expression as that observed following the peripheral administration of CCK (Lawrence *et al.* 2002, Bechtold & Luckman 2006). The PrRP neurons localised to the NTS and the VLM are also activated by CCK, suggesting that PrRP signalling may be important in relaying peripheral satiety signals, such as CCK, to brain feeding centres (Luckman & Lawrence 2003).

GPR10 mRNA and PrRP-binding activity are present within the dorsal vagal complex of the medulla (Roland *et al.*

1999, Ibata *et al.* 2000, Lee *et al.* 2000, Ellacott *et al.* 2005) and so PrRP may act to regulate vago-vagal reflexes. Direct injection of PrRP into the dorsal motor nucleus of the vagus can alter gastric motor function by the presynaptic modulation of glutamatergic neurones (Grabauskas *et al.* 2004). However, PrRP-immunoreactive fibres and GPR10 mRNA expression have been demonstrated also in a number of hypothalamic nuclei (Fujii *et al.* 1999, Roland *et al.* 1999, Maruyama *et al.* 1999a, Ibata *et al.* 2000, Lee *et al.* 2000). The suggestion that the brainstem PrRP neurons serve to relay satiety signals is supported by the finding that GPR10-knockout mice do not reduce feeding in response to peripherally administered CCK (Fig. 2; Bechtold & Luckman 2006).

Pair-feeding studies suggest that reduced weight gain in rats treated with PrRP is not accounted for solely by a reduction in food intake (Lawrence *et al.* 2000, 2004). The maintenance of proper energy balance involves regulating both energy intake and energy expenditure. Generally, compounds that inhibit energy intake also cause an increase in energy expenditure and, conversely, stimulation of energy intake is linked to decreased energy expenditure and weight gain (Schwartz *et al.* 2000). Indeed, PrRP administration increases both body temperature and O₂ consumption in rats (Lawrence *et al.* 2004), providing direct evidence for a role of PrRP in modulating not only feeding behaviour, but also energy expenditure. Furthermore, GPR10-knockout mice exhibit a much lower basal metabolic rate, when compared with wild-type mice (our unpublished observations), which likely contributes to the development of obesity in these animals (Gu *et al.* 2004).

Within the VLM and NTS, PrRP co-localises with noradrenaline in the A1 and A2 neuronal populations respectively (Chen *et al.* 1999, Roland *et al.* 1999). These noradrenergic neurons are known to mediate stress-related responses, suggesting a role for PrRP in stress. In line with this possibility, models of emotional stress, including conditioned-fear stimuli and water immersion/restraint stress activate both medullary and hypothalamic PrRP neurons (Maruyama *et al.* 2001). The medullary neurons are also activated by nociceptive stimuli including foot shock (Morales & Sawchenko 2003). Furthermore, the expression of PrRP mRNA in the NTS and VLM is up-regulated by restraint stress or formalin injection (Mera *et al.* 2006). Like PrRP, stressful stimuli evoke increases in blood pressure and alter feeding behaviour, making it tempting to speculate that PrRP neurons may regulate feeding in times of stress. Similar to the actions of NPFF, anti-nociceptive actions of PrRP have been demonstrated. Specifically, injection of PrRP into the NTS

Figure 2 (A) PrRP (2 nmol) significantly reduces food intake in pre-fasted GPR10^{+/+} mice compared with mice treated with vehicle, but had no measurable effect on mice lacking GPR10. (B) NPFF (4 nmol) caused a significant reduction in feeding in both wild-type and knockout mice. (C) Administration of CCK caused a dose-dependent reduction in food intake over the first hour of nocturnal feeding in GPR10^{+/+} mice, but had no significant effect on GPR10^{-/-} mice. **P* < 0.05, †*P* < 0.01 and ‡*P* < 0.001, two-way ANOVA with Bonferroni's *post hoc* test. Figure reproduced with minor modifications from Bechtold & Luckman (2006), *Endocrinology* 147; copyright, with permission from The Endocrine Society.

reduces the sensitivity of normal rats to mechanical pressure (paw pinch; Kalliomaki *et al.* 2004). Furthermore, mice lacking GPR10 exhibit higher nociceptive thresholds and stronger stress-induced analgesia than wild-type mice (Laurent *et al.* 2005).

Fish and chicken orthologues to mammalian PrRP have been identified (Moriyama *et al.* 2002, Seale *et al.* 2002, Tachibana *et al.* 2005). ICV administration of rat PrRP can stimulate feeding and reduce body temperature in chicks (Tachibana *et al.* 2004), the opposite effect to that observed in rats and mice. This contrast between chicks and mammals has also been reported for ghrelin and growth hormone-releasing hormone, both of which inhibit feeding in chicks, yet stimulate it in rats (Vaccarino *et al.* 1985, Furuse *et al.* 2001, Nakazato *et al.* 2001). In goldfish, hypothalamic PrRP mRNA expression is modified by feeding status, and either intraperitoneal or ICV administration of fish PrRP peptide has been shown to suppress food intake (Kelly & Peter 2006). It is therefore important to keep in mind that while an evolutionarily conserved role of RFamides in the regulation of feeding behaviour seems clear, the specific actions can vary greatly between class and species.

LPXRFamide family

The mammalian members of the LPXRFamide peptide family are RFRP-1 and -3 (from RFamide Related Pptides; Hinuma *et al.* 2000, Fukusumi *et al.* 2001, Yoshida *et al.* 2003). RFRP-1 and -3 share a similar C-terminal sequence with NPFF (Table 1) and as discussed above RFRP analogues bind with relatively high affinity to the NPFF1 and NPFF2 receptors (Hinuma *et al.* 2000, Liu *et al.* 2001, Engstrom *et al.* 2003, Yoshida *et al.* 2003), first identified as cognate receptors for NPFF (Bonini *et al.* 2000). NPFF1 exhibits a higher affinity for RFRP-1 and -3 than for NPFF and is now considered to be the endogenous RFRP receptor (Hinuma *et al.* 2000, Engstrom *et al.* 2003).

In the mouse brain, the major populations of RFRP-expressing neurons have been demonstrated in the DMN, the lateral superior olive and the NTS, with dense RFRP-immunoreactive fibres observed in the PVN, the IPBN, the NTS and the lateral reticular nucleus, as well as lamina I and II in the dorsal horn of the spinal cord (Ukena & Tsutsui 2001). Studies of the rat brain imply that neurons expressing RFRP-1 and -3 are limited to the caudal portion of the hypothalamus, namely the periventricular nucleus (PeVN), and an area between the VMN and the DMN (Hinuma *et al.* 2000, Fukusumi *et al.* 2001, Yano *et al.* 2003), although it is unclear from these reports whether RFRP-expressing neurons can be found elsewhere. As with the mouse, RFRP immunoreactive nerve fibres are widespread in the rat brain, but notably include the hypothalamic PVN, the supraoptic nucleus (SON) and the external layer of the dorsal horn (Fukusumi *et al.* 2001, Yano *et al.* 2003). ICV administration of the RFRPs induce the expression of *c-fos* in a number of areas, including the anterior NTS, the locus

coeruleus, SON, PeVN and the arcuate nucleus of the hypothalamus (Yano *et al.* 2003). A prominent hypothalamic expression of LPXRFamide peptides has also been demonstrated in birds and other vertebrates. LPXRFamide neurons are localised to the PVN in the quail and sparrow (Tsutsui *et al.* 2000, Bentley *et al.* 2003, Osugi *et al.* 2004), the suprachiasmatic nucleus in the frog (Chartrel *et al.* 2002, Koda *et al.* 2002, Sawada *et al.* 2002a) and the nucleus posterior periventricularis in goldfish (Sawada *et al.* 2002b). The conserved expression of LPXRFamide peptides in hypothalamic neurons (and especially in periventricular regions) suggests an important role of these peptides in neuroendocrine function.

To date, there have been no published reports on the effects of RFRP peptides on feeding, although a lack of effect seems unlikely given the ability of these peptides to bind to the NPFF receptors. Our own studies suggest that both RFRP-1 and -3 have the ability to modify feeding in rats and mice when administered centrally (unpublished observations). ICV administration of RFRP-1 to rats has also been shown to raise circulating levels of prolactin in a concentration-dependent manner, without an observable effect on circulating growth hormone (GH), LH, FSH, thyrotrophin- and adrenocorticotrophin-releasing hormone levels (Hinuma *et al.* 2000, Samson *et al.* 2003). However, RFRP-1 was not able to elicit the release of prolactin from dispersed anterior pituitary cells, and its ability to increase prolactin *in vivo* was attenuated in rats pre-treated with the dopamine D2 receptor blocker, domperidone (Samson *et al.* 2003). These results suggest that RFRP-1 does not stimulate the release of prolactin directly, but may alter dopamine release into the hypothalamo-pituitary portal system.

Like NPFF and PrRP, RFRP-1 may be involved in the perception of nociceptive stimuli, as ICV administration of the peptide to rats can block morphine-induced analgesia in acute and inflammatory models of pain (Liu *et al.* 2001). The occurrence of *Rana* RFamide-containing fibres in the dorsal horn of the frog spinal cord (Chartrel *et al.* 2002) suggests that the frog neuropeptide may also be involved in pain modulation. Similarly, an LPXRFamide peptide (nociception-related peptide) in the newt was found to exert an anti-nociceptive effect that was reversed by the opioid-receptor antagonist naloxone (Kanetoh *et al.* 2003).

Kisspeptin family

The first of several products of the *KiSS* gene (referred to collectively as kisspeptins) was purified from tissue extracts of human placenta as a ligand of the orphan receptor, GPR54. *KiSS-1* was identified as a tumour metastasis suppressor gene and the 54 amino acid peptide product was named metastin by virtue of its ability to inhibit migration in different cancer cell types (Lee *et al.* 1996, Ohtaki *et al.* 2001).

In the rat brain, three main regions containing metastin-immunoreactive neurons have been identified in the DMN, NTS and the caudal VLM (Brailoiu *et al.* 2005), although

immunoreactive fibres exhibit a wide distribution. GPR54, originally cloned from rat brain, shares 45% identity with galanin receptors, and is widely expressed in the rat central nervous system, including hypothalamus, midbrain, pons, medulla, hippocampus and amygdala (Lee *et al.* 1999).

Kisspeptin peptides do not appear to alter food intake in either *ad libitum*-fed or pre-fasted rats (Thompson *et al.* 2004, Castellano *et al.* 2005). However, kisspeptin and its putative receptor GPR54 have been shown to play a pivotal role in the control of gonadotrophin secretion and puberty (de Roux *et al.* 2003, Seminara *et al.* 2003). For example, mice in which GPR54 has been deleted exhibit impaired development of both male and female reproductive systems (Funes *et al.* 2003). The onset of puberty and sexual maturity depends heavily on sufficient body energy stores, and conditions of negative energy balance can delay puberty and cause reproductive failure. Interestingly, the expression of both KiSS-1 and GPR54 are responsive to feeding status in rats. Specifically, food deprivation of pre-pubertal rats is accompanied by reduced KiSS-1 expression in the hypothalamus and a generalised increase in GPR54 expression (Castellano *et al.* 2005). In addition, chronic treatment with kisspeptin is able to restore vaginal opening (a sign of reproductive development) and to elicit gonadotrophin and oestrogen responses in undernourished rats (Castellano *et al.* 2005). Therefore, the responsiveness of hypothalamic KiSS-1 expression to nutritional status might contribute to the suppression of reproductive function in states of negative energy balance.

Non-mammalian homologues of kisspeptin have not yet been identified, although receptors related to GPR103 have been predicted from the chicken genome.

26RFa and QRFP

26RFa and QRFP (from pyroglutamylated RFamide peptide) constitute the most recently identified group of RFamide peptides, having been described by three separate groups in 2003. 26RFa was isolated from brain extracts of the frog *Rana esculenta* on the basis of its cross-reactivity with an antiserum raised against mammalian NPFF (Chartrel *et al.* 2003). This peptide was also identified as a high-affinity ligand for the orphan receptor GPR103 in humans and mice (Fukusumi *et al.* 2003, Jiang *et al.* 2003). In addition to the 26-amino acid residue, 26RFa, a 43-amino acid residue (QRFP) peptide product was also identified (Fukusumi *et al.* 2003). Although both 26RFa and QRFP are derived from the same precursor peptide, and both have been shown to be potent ligands of GPR103 (Fukusumi *et al.* 2003), mass spectrum analyses of brain extracts suggest that QRFP may be the predominant ligand for GPR103, at least in the rat (Takayasu *et al.* 2006). GPR103 shares significant sequence identity with the NPFF2, neuropeptide Y-Y2 and galanin-R1 receptors (Bonini *et al.* 2000, Lee *et al.* 2001).

26RFa QRFP and GPR103 are expressed in the VMN and the lateral hypothalamus, areas of the brain critically associated

with the control of food intake (Chartrel *et al.* 2003, Fukusumi *et al.* 2003, Baribault *et al.* 2006). In fact, in mice, the expression of the peptides is limited almost exclusively to neurons in these two hypothalamic nuclei (Chartrel *et al.* 2003). In humans, the peptides can be found in PVN and VMN neurons, as well as in the dorsal and the lateral horns of the spinal cord (Bruzzzone *et al.* 2006). Two mouse orthologues of the human GPR103 have now been identified (GPR103A and B) which respond similarly to QRFP, yet exhibit distinct expression patterns within the mouse brain (Takayasu *et al.* 2006). Specifically, a particularly pronounced expression of GPR103A is localised to the NTS and VMN, while GPR103B is strongly expressed in the PVN, lateral hypothalamus and triangular septal nuclei. Functional differences between the two receptors are not yet known.

In line with its expression in the hypothalamus, acute ICV administration of 26RFa or QRFP in mice stimulates food intake (Chartrel *et al.* 2003, Moriya *et al.* 2006, Takayasu *et al.* 2006), increases locomotor activity (Chartrel *et al.* 2006, Takayasu *et al.* 2006) and metabolic rate (Takayasu *et al.* 2006). Chronic infusion of QRFP over a 2-week period caused mice to become hyperphagic, and experience significant gains in body weight and fat mass, which was exacerbated when the animals were fed a moderately high-fat diet (Moriya *et al.* 2006). Interestingly, pair-fed, QRFP-infused mice did not exhibit the increase in body weight but still showed a significant increase in fat mass. In contrast to the elevation in metabolic rate following QRFP administration reported by Moriya *et al.* (2006), Takayasu *et al.* (2006) report that both *ad libitum*- and pair-fed QRFP-infused mice exhibit a significant decrease in rectal temperature and reduced expression of brown adipose tissue uncoupling protein-1 mRNA. Nevertheless, these findings demonstrate that QRFP can modulate both appetite and energy expenditure over a period of chronic exposure.

The orexigenic effect of QRFP does not appear to be diminished in orexin-knockout mice, but is attenuated by the neuropeptide Y-Y1 receptor antagonist BIBP3226, suggesting that QRFP-induced feeding might involve neuropeptide Y signalling (Takayasu *et al.* 2006). However, it has been proposed that BIBP3226 mimics the RF residues of NPFF-related peptides (neuropeptide Y terminates in -RYamide) and binding of the compound to RFamide receptors has been demonstrated previously (Bonini *et al.* 2000, Gouarderes *et al.* 2002, Fang *et al.* 2006, Vyas *et al.* 2006). In addition to pharmacological evidence showing a role for QRFP and 26RFa in food intake, the expression of mRNA for the QRFP/26RFa precursor, measured using real-time reverse transcription (RT)-PCR of dissected tissues, is elevated in the 'hypothalamic' region of fasted mice, as well as in obese *ob/ob* and *db/db* mice (Takayasu *et al.* 2006). Importantly, a study looking at bone formation in GPR103-knockout mice reported that body weights are similar between wild-type and knockout littermates, although no data were shown and it was not made clear how the animals were housed or fed (Baribault *et al.* 2006).

QRFP has also been reported to have direct effects on aldosterone secretion from rat adrenal gland (Fukusumi *et al.* 2003). GPR103, but not QRFP, is abundantly expressed in adrenal gland, suggesting a possible hormonal role for QRFP in releasing this hormone. ICV or systemic injection of 26RFa, or ICV injection of QRFP, increases serum LH levels in cycling and ovariectomised female rats, but not in adult males (Castellano *et al.* 2005), suggesting a potential involvement for the peptides in energy balance and reproduction in females.

RFamides and invertebrate feeding

The mechanisms controlling feeding behaviour in mammals and invertebrates obviously differ, nevertheless, the involvement of RFamide peptides in invertebrate feeding provide some interesting parallels. The continuity of feeding-related actions of RFamides between vertebrate and invertebrate suggests either an evolutionary conservation or a convergence of neuropeptide function. It must be acknowledged that RFamides are widely represented in invertebrates and have numerous functions aside from feeding behaviour. In this section, we present a few examples of how RFamide peptides contribute to feeding-related behaviours in invertebrates.

Gastropods

In snails, feeding requires rhythmic motor patterns to drive the movements of the mouth and pharynx (Chase 2002). On the other hand, escape from aversive stimuli involves withdrawal of the whole body into the shell, which is accomplished by a group of the retractor muscles, collectively known as the columellar muscle (CM; Chase 2002). Interestingly, the contraction of the CM is strongly influenced by the degree of satiation of the animal (Balaban 2002). Feeding-induced arousal and food intake in snails are regulated to a large extent by serotonergic 5-hydroxytryptamine (5-HT) and dopaminergic neurons (Murphy 2001, Elliott & Susswein 2002). FMRFamide and FMRFamide-containing pleural neurons both inhibit feeding and facilitate withdrawal behaviour in various gastropod species (Kyriakides & McCrohan 1989, Murphy 1990, Alania *et al.* 2004). This is achieved by FMRFamide rapidly attenuating the rhythmic firing of buccal and cerebral ganglion neurons responsible for initiating and maintaining the repetitive motor outputs required for feeding (Alania *et al.* 2004). In addition, FMRFamide acts directly on muscle groups, such as the pharyngeal retractor muscle (PRM) to antagonise 5-HT signalling and strengthen and prolong contraction (which facilitates withdrawal; Hernadi *et al.* 2005). A model has been proposed in which pro-feeding signals stimulate repetitive contraction of the PRM in response to 5-HT release, but that when a noxious stimulus is encountered FMRFamide is liberated contributing to a sustained contraction of the PRM and body withdrawal (Hernadi *et al.* 2005). The balance between FMRFamide and 5-HT responses seems to be partly dependent of the energy status of the animal. Similarly,

cholinergic motoneurons innervating the accessory radula closer muscle, are both excited by 5-HT and inhibited by FMRFamide (Sossin *et al.* 1987).

RFamides within the land snail *Cepaea nemoralis* also interact with opioid systems to modify nociceptive responsiveness (Tang *et al.* 1984, Kavaliers *et al.* 1985, Kavaliers & Yang 1989). For example, FMRFamide acts antagonistically to endogenous opioids (likely involving downstream serotonergic pathways) in the neuronally controlled switch between passive and active avoidance behaviour (Dyakonova *et al.* 1995).

Nematodes

As mentioned above, *C. elegans* exhibits a large variety of RFamide peptides, with more than 20 RFamide encoding genes (designated *flp-1* to *flp-22*), and in excess of 50 different peptide products (Li *et al.* 1999). In addition, a number of GPRs have been identified in the nematode that serve as RFamide receptors, including the neuropeptide receptor-1 (NPR-1), the putative receptor for the peptides FLP-21 and the FMRFamide-like peptide (FLP-18; Kubiak *et al.* 2003, Rogers *et al.* 2003). The role of NPR-1 and its ligands in *C. elegans* behaviour is quite striking, since a single amino acid polymorphism at position 215 of NPR-1 dictates whether the nematodes engage in aggregate or solitary feeding (de Bono & Bargmann 1998, Rogers *et al.* 2003). Specifically, a Phe residue at position 215 (215F) on NPR-1 is associated with social feeding, in which animals group together. A Val at this position (215V) is associated with solitary feeding, in which the animals spread out over the food source (Fig. 3; de Bono & Bargmann 1998). Aggregating strains also tend to prefer areas of high food density and show higher rates of locomotion over the food. Activation of NPR-1 inhibits a set of body cavity neurons that themselves normally serve to repress solitary behaviour when food is not present. The sensitivity of NPR-1 to FLP-21 and FLP-18 is higher in the 215V variant leading to heightened receptor activity and solitary behaviour in these nematodes (Rogers *et al.* 2003, Davies *et al.* 2004).

The manifestation of aggregating behaviour involves multiple pathways, including the nociceptive ASH and ADL neurons. Ablation of these neurons attenuates aggregation of the nematodes, implying that aggregation may be a response to noxious stimuli (de Bono *et al.* 2002). The induction of solitary behaviour by NPR-1 signalling suggests that its actions may antagonise responses of ASH and ADL to stressful stimuli. FLP-21 is expressed in ASH and ADL neurons (Rogers *et al.* 2003), suggesting that these neurons can also induce solitary behaviour under certain conditions. Interestingly, the NPR-1 receptor was originally described as a nematode homologue of the vertebrate neuropeptide Y receptors (de Bono & Bargmann 1998). Neuropeptide Y is a well-described orexigenic peptide in mammals (Stanley & Leibowitz 1985), but it has also been linked to nociception (Naveilhan *et al.* 2001, Li *et al.* 2002), suggesting that synergistic pathways between feeding behaviour and nociception/stress might have been conserved through natural selection.

Figure 3 A neurogenetic model of food-related behaviours in *C. elegans*. Arrows represent inductive effects, whereas blunt-ended lines indicate repression. In natural strains the NPR-1 215F isoform is less active and permits aggregation, whereas the NPR-1 215V isoform is more active and represses aggregation. FLP-18 and FLP-21 (FMRFamide-like neuropeptides) are ligands for both isoforms of NPR-1. (a) The chemosensory neurons ASH and ADL mediate nociception and aggregation during feeding. The *ocr-2* and *ocr-9* genes are required for aggregation; they encode transient receptor potential cation channel sub family V (TRPV) cation channel subunits that form a transduction channel linking sensory input to neuronal activity. In solitary animals, NPR-1 215V signalling suppresses this circuit, possibly by inhibiting sensitivity to nociceptive stimuli (not shown). (b and c) The AQR, PQR and URX body cavity neurons mediate food-dependent aggregation. In solitary animals, this pathway is inhibited by food-dependent signalling through NPR-1 215V (c). (d) Depolarisation of AQR, PQR and URX neurons is also required for locomotory slowing in response to low oxygen levels in aggregating worms, which is also suppressed in solitary animals by NPR-1 215V signalling (not shown). (e) Since food-dependent slowing also occurs in solitary animals, an unidentified, oxygen-independent neural circuit involving the NPR-1 215V form of NPR-1 operates to suppress locomotion in response to food. Figure reproduced from Douglas SJ, Dawson-Scully K & Sokolowski MB (2005) The neurogenetics and evolution of food-related behaviour. *Trends in Neurosciences* **28** 644–652. *Trends in Neuroscience* **28**; copyright, reproduced with permission from Elsevier.

Insects

Like other animal species, *Drosophila melanogaster* contains several genes that encode FMRFamide-related peptides (Nichols 2003). FMRFamide immunostaining reveals an extensive network of immunoreactive fibres covering the surface of the crop (Duttlinger *et al.* 2002) and one of the *Drosophila* RFamide peptides, dromyosuppression (DMS) has been shown to attenuate crop contractions. In addition, four protein sequences have been identified from the fly as putative neuropeptide Y-like receptors (Hewes & Taghert 2001), one of which is a receptor for the RFamide, neuropeptide F (NPF-A1; Garczynski *et al.* 2002). Changes in the expression level of NPF-A1 are correlated with gustatory exposure to sugar (Shen

& Cai 2001). Similarly, both the feeding state (fasted or post meal) and the diet composition influences FMRFamide-like immunoreactivity in the gut tissues of *Locusta migratoria* (Hill & Orchard 2005), suggesting that RFamides play a role in maintaining balanced nutrient content in insects.

Conclusions

The RFamides represent one of the largest and most widespread groups of peptides, having been identified in animals across most classes of the animal kingdom. Further, in a great number of species, RFamides are involved in the regulation of

feeding behaviour demonstrating a level of conservation through evolution which is rare in neuropeptides. Strangely, the question of whether regulating food intake represents a primary function of RFamide signalling remains. This issue is complicated by the size and diversity of the RFamide family. However, for at least some peptides (such as PrRP in rodents and FLP18/FLP21 in *C. elegans*) their influence on feeding seems irrefutable. In addition to altering food intake, many RFamides also act on metabolic rate, the other factor in body energy homeostasis. As discussed above, the expression of many RFamides are altered by energy status and therefore may serve to link this status with other physiological (such as reproduction in the case of the kisspeptins) and endocrine systems.

One intriguing evolutionary continuity is the relationship between the RFamide peptides and the nociception and opioid systems. Opioids have a strong influence on feeding and a great deal of evidence links the RFamide peptides to opioid signalling. Under natural conditions, animals have to decide whether to engage in feeding-related behaviours when presented with an attractive food source, a decision made more complicated when the food is available in an environment containing aversive or noxious stimuli. Both physical and emotional stress can have profound effects on feeding, and it is tempting to speculate that RFamide and opioid systems interact to integrate feeding with stress.

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