Pituitary Actions of RFamide Peptides: A Critique of the Evidence

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Abstract: RFamides are a relatively recently-discovered class of peptides characterised by an arginine-phenylalanine-amide at their C terminus. In mammals, all of the five known RFamide families (neuropeptide FFs, prolactin-releasing peptides, RFamide-related peptides, kisspeptins and pyroglutamylated RFs) were originally discovered as neuropeptides influencing central nervous system functions such as reproduction, feeding behavior and nociception; however presence of their G-protein-coupled receptors in the pituitary gland has led to speculation that they also influence the function of this gland by either autocrine/paracrine or neuroendocrine mechanisms. In support of such actions, prolactin-releasing peptide is produced locally within the pituitary gland, and pulses of neuropeptide FF are secreted into blood from nerve terminals in the posterior pituitary lobe originating from the hypothalamus. In contrast, it is argued that the balance of experimental evidence does not yet support such roles for RFamide-related peptides, kisspeptins and pyroglutamylated RF peptides due to their absence or scarcity in the neurosecretory zone of the median eminence, blood and pituitary tissue. In some cases, assignment of functional nomenclature has been based on inconclusive evidence of the proposed function, causing confusion in the field. To resolve these issues it will be important to compare the peptide concentrations required to elicit pituitary effects with those found in portal and peripheral blood and in pituitary tissue. To date this has only been systematically done for kisspeptin, for which the portal blood concentration was found to be several orders of magnitude lower than that which stimulated gonadotrophin release from primary pituitary cell cultures.

INTRODUCTION

RFamides form a class of neuropeptides characterized by an arginine-phenylalanine-amide motif at their C terminus. They were first identified in 1977 in molluscan cerebral ganglia [1], but almost another decade passed before they were purified and chemically characterised in any mammalian species [2]. There are now five RFamide families known to exist in the mammalian central nervous system, where their widespread fibre distribution implies varied roles including regulation of energy balance, behaviour and reproduction [3, 4]. In particular, at least four of the five known mammalian genes encoding RFamide peptides have been shown to influence food intake [5]. Grouped by the precursor peptides that encompass the mature RFamide peptides and listed in order of their date of isolation in mammals, these families are: neuropeptide FF (NPFF) precursor, prolactin-releasing peptide (PrRP) precursor, RFamide-related peptide (RFRP) precursor, kisspeptin precursor and pyroglutamylated RFamide peptide (QRFP) precursor (see Table **1** for details).

In addition to their diverse roles within the central nervous system, direct pituitary actions have been proposed for all mammalian RFamides, either via autocrine/paracrine or hypophysiotrophic (secreted by nerve terminals in the

external zone of the hypothalamic median eminence and carried to the anterior pituitary gland by the portal blood system) mechanisms. RFamide peptides have been implicated in the regulation of almost all of the major pituitary hormones, including prolactin, luteinising hormone (LH), follicle stimulating hormone (FSH), growth hormone, adrenocorticotrophic hormone (ACTH), oxytocin and arginine vasopressin (AVP), although some of these are likely to be regulated indirectly via other hypophysiotrophic regulatory hormones. Thus, their sphere of influence on endocrine functions is likely to include maternal physiology, reproduction, growth, stress responses, diuresis and blood pressure regulation. In two cases where the evidence for hypophysiotrophic effects is not compelling (PrRP and RFRP, sometimes known as gonadotrophin-inhibitory hormone), the peptide nomenclature nevertheless reflects this putative mode of action. At present there is a need for a unified and formalised nomenclature for RFamide peptides, since some are named after the function initially assigned to them, others have nomenclature that reflects their molecular structure, and most have more than one name in common usage.

All mammalian RFamide peptides have been found to be ligands of formerly 'orphan' G protein-coupled receptors. In some cases there is considerable overlap in the binding affinity between different RFamides and their receptors, since the receptor selectivity appears to be determined largely by the few relatively conserved amino acids at the C terminus [6]. In particular, a number of RFamides show considerable affinity and agonistic activity to the preferred

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Table 1. Details of the Known Mammalian RFamides, their Precursor Peptides and G Protein-Coupled Receptors (Alternative Names to those Used in this Manuscript are Shown in Brackets)

RFamide Peptide	Precursor Peptide Gene	Produced by Pituitary Gland	Receptor (Highest Affinity First)	Pituitary Receptor Levels	Reference to Initial Discovery
Neuropeptide FF (FMRFamide-related peptide) Neuropeptide AF	*Npff*	No, but secreted in posterior lobe by hypophysiotropic neurons	GPR74 (NPFF2 receptor; HLWAR77) EC_{50}=2-28 nM [7, 27] GPR147 (NPFF1 receptor; OT7T022) EC_{50}=9-10 nM [9, 27]	+ +	[2]
Prolactin-releasing peptide 20 Prolactin-releasing peptide 31	*Prrp (Prh)*	Yes	GPR10 (hGR3; UHR-1) EC_{50}=2 nM [7] GPR74 (NPFF2 receptor; HLWAR77) EC_{50}=170-240 nM [7]	++ +	[54]
RFamide-related peptide-1 (neuropeptide SF) RFamide-related peptide-3 (neuropeptide VF; gonadotrophin-inhibitory hormone)	*Rfrp (Npvf)*	No	GPR147 (NPFF1 receptor; OT7T022) EC_{50}=1-3 nM [7] GPR74 (NPFF2 receptor; HLWAR77) EC_{50}=160-1000 nM [7, 27]	+ +	[9]
Kisspeptin 54	*Kiss1*	Barely detectable mRNA; but immunoreactivity detected	GPR54 (OT7T175; AXOR12) EC_{50}=1-7 nM [119]	+	[119-121]
Pyroglutamylated RFamide peptide 43 (43RFa) 26RFa (P518; QRFP26)	*Qrfp (26RFa, P518)*	Barely detectable mRNA	GPR103 (AQ27; SP9155) EC_{50}=7 nM	+	[15, 160]

receptors for NPFF family members, namely GPR147 and GPR74 [7]. The promiscuity of these two receptors may not be limited to RFamide peptides, since they also interact moderately with neuropeptide Y (an RYamide) and possibly orexin and cholecystokinin peptides, whose function of food intake regulation overlaps with that of RFamides [7, 8]. Because of such potential interactions, the location of the secreted peptides must be considered in relation to that of the receptors to which they have agonist activity when attempting to determine their effects at the pituitary level and at other peripheral sites. RT-PCR measurements concur that all of the identified RFamide receptor genes are expressed in the pituitary gland; albeit usually at low levels [8-15]. The most commonly utilised approach to determining if RFamide peptides act on the pituitary gland has been to assess their effects on pituitary hormone secretion in primary pituitary cell cultures. In this review I advocate caution in making conclusions about *in vivo* function based solely on this approach, if for no other reason than the fact that receptors known to be involved in RFamide peptide signalling [16] may be expressed in primary cell cultures at levels considerably greater than those normally seen *in vivo* [17]. Furthermore, RFamide effects observed in *in vitro*

experiments only become meaningful if the minimum effective dose of RFamide peptide is lower than the concentration measured within the pituitary gland or in portal blood; a direct comparison that has been reported for kisspeptin [18] but not other RFamide peptides. Where hypophysiotrophic mechanisms are suggested, these need to be substantiated by testing for uptake by the putative hypophysiotrophic neurones of a retrograde tract tracing dye from the pituitary gland or systemic circulation [19-21].

This purpose of this review is to critique and discuss the merits of the proposed direct pituitary actions for each RFamide family. The scope is generally limited to the mammalian RFamide peptides; however reference to mechanisms of action in other vertebrate classes is made where the comparison is of interest.

NEUROPEPTIDE FF (NPFF) AND NEUROPEPTIDE AF (NPAF)

The octapeptide NPFF and the structurally related octadecapeptide NPAF were originally isolated in 1985 from bovine brain tissue using an antibody raised against the molluscan peptide FMRFamide [2] and named after their N

and C terminal amino acids. They share a common precursor protein containing a single copy of both peptides in mouse, bovine and human brain [22, 23], although NPAF equates to NPSF in rodents [22, 23]. Two G protein-coupled receptors (GPR74, also called NPFF2 receptor or HLWAR77, and GPR147, also called NPFF1 receptor or OT7T022) have been identified for NPFF and NPAF [8, 9, 24, 25], with the former binding to the peptides with the greatest affinity [26]. NPFF and NPAF act on GPR74 and GPR147 through coupling to the $G_{\alpha i/o}$ pathway [9, 24, 25, 27]. Their principal role within the central nervous system appears to be associated with the modulation of painful stimuli [2, 28-31]. NPFF is a modulator of the opioid system, having both opioid and anti-opioid-like effects depending whether it is administered intrathecally or intraventricularly [2, 32]. Intracerebroventricular injection of NPFF precipitates opioid withdrawal symptoms [33, 34], and causes reduced food intake by an antiopioid mechanism [35, 36]. Central administration of exogenous NPAF has been shown to stimulate the hypathalamo-pituitary-adrenal axis via corticotrophin-releasing hormone [37].

GPR74 mRNA has been shown to be present at low levels in both the pituitary gland and brain of rats [8] and humans [8, 25], being most abundant in the rat spinal cord and human thymus and placenta. GPR147 mRNA is present in the pituitary gland of rats [8-10], Syrian hamsters [38] and human [8, 39] although the level of expression is very low compared to that seen in the brain [8-10]. In rats, NPFF and NPAF are not themselves produced by the pituitary gland, since their mRNA expression is only seen in the lungs and spleen among peripheral organs examined [11, 40]. Nevertheless, radioimmunoassay detection showed that the NPFF peptide is present at considerably higher levels in the neurointermediate pituitary lobe (500-1000 fmol per mg protein) than in the brain or spinal cord, and NPFF was undetectable in the anterior pituitary lobe [41, 42]. Immunohistochemistry confirmed that the peptide was absent in both the anterior and intermediate lobes and confined to nerve terminal-like structures in the neural lobe [41, 43, 44]. The latter observation suggested that the hypothalamus might be the source of neural lobe NPFF. In confirmation of this, pituitary stalk transection almost completely abolished neurohypophyseal NPFF [43, 45] and NPAF [19] content. The same researchers went on to show that neurohypophyseal NPFF originates at least in part from the supraoptic nucleus, since electrolytic lesions or injections of an anterograde tract tracing dye in this region caused a 50% reduction in pituitary NPFF content and labelling of NPFF-immunopositive fibres in the neurohypophysis, respectively [42]. In agreement with these findings, injection of the retrograde tracer Fluoro-Gold into the neurointermediate lobe of rats led to uptake by NPAF-immunoreactive neuronal cell bodies in the supraoptic and paraventricular nuclei (Fig. **1A** and **B**) [19]. NPFF [42, 44] and NPAF [19] are coexpressed in AVP and possibly oxytocin magnocellular neurones of the paraventricular and supraoptic nuclei of rats. Under conditions of hyperosmotic stimuli [45] or genetic AVP deficiency [45] in this species, reductions in pituitary NPFF content closely parallel those of AVP, suggesting a functional connection between the two peptides. This may include a role for AVP in NPFF packaging and transport or release by the pituitary gland [46]. In contrast to the rat, no NPFF and AVP-immunostained cells were observed in the human supraoptic nuclei in a recent study [47], but this difference may reflect the fact that pretreatment with colchicine has been used to enhance NPFF/AF cell body immunoreactivity in rodent studies.

Surprisingly, there is little known concerning NPFF/-NPAF effects in the pituitary gland. In primary cultures of rat pituitary cells, NPFF had no effect at less than μM doses on cAMP formation [48]. NPFF-immunoreactive nerve terminals have been shown to make apparent synaptic contacts with pituicytes [44], suggesting a possible modulatory of these glia in NPFF actions. Because NPFF terminal immunoreactivity is also frequently associated with blood vessels [43], it is likely that it plays endocrine roles in addition to any local effects. In this regard, NPFF is readily detectable in human blood [49], with basal concentrations of ~2 pg/ml and pulses of ~15 pg/ml occurring at approximately hourly intervals [50]. This circulating NPFF is likely to exert a number of peripheral functions, including the regulation of cardiovascular function [51], gene expression in adipose tissue [52] and proliferation of the T lymphocytes [53].

PROLACTIN-RELEASING PEPTIDE (PRRP)

First purified in 1998 by reverse pharmacology from a bovine hypothalamic extract as the endogenous ligand of the orphan receptor GPR10 (also known as hGR3 or UHR-1), the PrRP precursor encompasses a single 20 amino acid RFamide peptide (PrRP20, although an N terminally-extended form, PrRP31, has also been isolated) [54]. PrRP also shows considerable affinity to GPR74, the formerly identified NPFF and NPAF receptor which is characterised by seemingly promiscuous binding to various RFamide peptides [7]. In contrast, NPFF is not efficacious in binding to GPR10 [7]. GPR10 has been shown to couple to $G_{\alpha q}$ and $G_{\alpha i/o}$ but not to G_s [54, 55]. Throughout the central nervous system and periphery its gene expression is seen most abundantly in the anterior pituitary lobe of rats [11-14] and humans [12, 56], as well as in regions of the thalamus, hypothalamus, brainstem, and adrenal medulla of rats [13]. The central distribution of the peptide and its receptor indicate that the PrRP system acts in many regions unrelated to prolactin regulation [14, 57-59], pointing instead to a diverse range of functions for the peptide. Central roles that have been proposed for PrRP, based on the effects of intracerebroventricular injections, include regulation of corticotrophin releasing hormone [60-63], oxytocin [64], somatostatin [65], gonadotrophins and their preovulatory surge [66-68], food intake [69] and blood pressure [70]. In mice, transgenic mutation of the genes that produce GPR10 [71, 72] or PrRP [73] leads to obesity and lower energy expenditure, emphasising a role of endogenous PrRP in the control of food intake and metabolism.

The naming of PrRP was based on the initial finding that it caused prolactin release from female rat primary pituitary cultures at nM doses [54]. While this finding has been generally verified in several other studies in rats [74-76], sheep [77] and humans [78], it is noteworthy that the potency of PrRP is generally slightly less than that of thyrotropin-releasing hormone [74, 75, 77]. PrRP was shown in one study to markedly increase the prolactin

responsiveness to thyrotropin-releasing hormone [79]. Others, however, have reported no effects in primary pituitary culture experiments at lower than µM doses in either sex of rats [14, 63, 74]. The efficacy of PrRP in inducing prolactin release appears to be greatest in pituitary cells from lactating females and least in cells from males [74, 75], although the low PrRP mRNA levels and peptide immunoreactivity in the brains of lactating versus virgin rats [69, 80] and the lack of effect of lactation on GPR10 mRNA levels in the anterior pituitary gland do not support a stimulatory role for PrRP during lactation. *In vivo*, intravenous injection of PrRP stimulated sex and cycle stage-dependant prolactin release in rats (greatest in proestrous or estrogen-treated females, least in males) [81, 82]. Intravenous PrRP was without effect on prolactin secretion in ovariectomized ewes [77] and male and lactating female rats [14]. Thus, it is possible that high circulating concentrations of estrogens are required in order for effects of exogenous PrRP to be observed.

PrRP was originally postulated to act as a classical hypophysiotrophic releasing hormone [54]; however subsequent immunohistochemical studies in the rat showed no evidence for PrRP nerve terminals in the median eminence, particularly its external layer [20, 58, 59, 83] where hypophysiotrophic hormones are released around the portal blood vessels. Moreover, the concentration of PrRP in this region did not appear to change in push-pull perfusates during physiological prolactin surges [68]. One study did, however, report a very small number of PrRP nerve fibres in the neural lobe of the pituitary gland of rats [59]. Following intravenous injections of the retrograde tract tracing dye fast blue, less than 1% of the PrRP neurones in the rat brain were observed to be colocalised with this dye, indicating negligible projections to the median eminence neurosecretory zone or posterior pituitary gland (Fig. **1C** and **D**) [20]. In contrast, PrRP orthologues may act via hypophysiotrophic mechanisms in some non-mammalian species. Immunoreactive fibres of the PrRP orthologue C-RFa have been shown to project to the anterior pituitary gland in rainbow trout, and C-RFa elicits prolactin secretion in this species *in vitro* at pM doses and systemically at pg/kg doses [84].

The foregoing considerations suggest that mammalian PrRP was inappropriately named. A robust expression of PrRP in the anterior pituitary gland, however, could mean that the name carries a sensible connotation as an autocrine/paracrine regulator of prolactin secretion. Indeed, one group reported moderate PrRP mRNA levels in the anterior pituitary lobe using *in situ* hybrisation, starting at embryonic day 18 when the lactotropes are first observed [11, 40]. This is in agreement with RT-PCR studies on human pituitary glands [56], and in a teleost fish species pituitary PrRP mRNA levels closely correlate with prolactin mRNA changes [85]. A quantitative RT-PCR study of rat tissue showed the pituitary PrRP mRNA levels to be very low relative to that seen in the brainstem [12], whereas measurement of the peptide by enzyme immunoassay showed pituitary levels to be similar to that in the brain [86]. PrRP was also shown to be present at very low but detectable levels in peripheral blood [86]. Taking into consideration the generally higher level of receptor expression for PrRP compared other RFamides in the pituitary gland, it seems likely that PrRP may function in an autocrine/paracrine manner to regulate prolactin secretion directly at this level especially in the presence of elevated circulating estrogens. However the functional significance of this remains to be determined.

RFAMIDE-RELATED PEPTIDE-1 AND -3 (RFRP-1 AND -3)

A third mammalian RFamide precursor protein was discovered in 2000 by the same group that isolated PrRP, using a bioinformatics approach [9]. The precursor was predicted to give rise to two mature RF amide peptides (designated RFRP-1 and -3; also occasionally referred to as NPSF and NPVF or gonadotrophin-inhibitory hormones) and one similar peptide with an RSamide C terminus (RFRP-2), although subsequent biochemical characterisation revealed that only RFRP-1 and -3 are produced *in vivo* [6, 87, 88]. They bind with high affinity to the putative NPFF and NPAF receptor GPR147 and to a lesser extent GPR74 (in fact, their affinity to GPR147 is higher than NPFF and NPAF) [7, 9, 27], since they share the four C terminal amino acid residues required for binding with these receptors [6]. GPR147 mRNA has been shown to be present in the pituitary gland of rats [9, 10], Syrian hamsters [38] and humans [39], with a very low level of expression relative to that seen in the brain [9, 10]. GPR74 mRNA is present at low levels in both the pituitary gland and brain of humans [25].

In the rat brain, RFRP-1 has been shown to elicit prolactin release by suppression of hypothalamic periventricular dopamine neurones [9, 63] which express GPR147 mRNA [9]. In contrast, RFRP-3 acts to inhibit (and in a very few cases stimulate) the firing rate of rodent gonadotrophin-releasing hormone (GnRH) neurones [89, 90], presumably by direct action since immunoreactive RFRP-3 neurones project to GnRH cell bodies in all species examined [39, 91-94] and about 25% of GnRH neurones express GPR147 mRNA in mice (MZ Rizwan and GM Anderson, unpublished data). The physiological reproductive roles that may be modulated centrally by RFRP-3 include the timing of the preovulatory surge of GnRH and LH that drives ovulation [38, 95], seasonal breeding cycles and photoperiodism [93, 96-98], and stress-induced reproductive suppression [99]. Non-reproductive actions of RFRP-3 include stimulation of food intake [91, 100] and the somatotropic axis [91, 101] in rats. The divergent effects of RFRP-1 and RFRP-3 are in agreement with a recept study by Pineda *et al.* [102] which showed that intracerebroventricular RFRP-3 but not RFRP-1 caused an inhibition of serum LH concentration. The reason for this is unclear since both peptides are produced in the same neurones [103] and act on the same receptors [7, 9, 27], although one study showed that RFRP-1 had a higher agonistic activity for GPR74 than RFRP-3 [7]. Curiously, the RFRP-1 sequence aligns with its avian orthologue gonadotrophin-inhibitory hormone; yet the latter peptide has been used in some mammalian studies as a surrogate for RFRP-3 with apparently similar responses [92]. Other authors, however, have recently demonstrated that RFRP-1 and -3 act similarly to induce oxytocin neuronal activity, stress hormone (oxytocin and ACTH) release and anxiety-related behaviours [104].

It is currently controversial as to whether RFRPs exert a hypophysiotrophic role in mammals, as attested by the titles of recent publications [21, 100, 105]. Immunohistochemical descriptions of mammalian RFRP-1 and -3 prior to 2008 consistently reported a marked absence of fibres innervating the external zone of the hypothalamic median eminence of rats, mice and hamsters [21, 91, 92, 103, 106], using a variety of primary antibodies. Two recent papers reported similar findings in rats [21] and rhesus macaques [107]. The identification, characterisation and naming of avian RFRP orthologues, gonadotrophin inhibitory hormone (GnIH) and GnIH-related peptides [108], sparked interest in the possibility that mammalian RFRPs might also act as direct gonadotrophin inhibitory homones via hypophysiotrophic release, as extensive GnIH fibre immunoreactivity is present in the neurosecretory zone of avian species [108-112]. Three groups very recently reported presence of fibre-like immunoreactivity in this zone in hamsters [38], sheep [105], rhesus macaques [94] and humans [39]. The fibre density is sparse compared to that seen in birds [113], with an immunohistochemical amplification protocol being required in hamsters [38]. Because of the important implication of this issue in regard to the possible hypophysiotrophic nature of RFRP-1 and -3 and the possibility of glial cells in the median eminence giving false positive staining [114], we sought to confirm our own anatomical observation of neurosecretory fibre absence [21] by testing whether RFRP-1/3 neurones were able to take up the retrograde tract tracer Fluoro-Gold from the systemic circulation. Whereas ~95% of GnRH neurones (known to be hypophysiotrophic) were found to contain Fluoro-Gold after intraperitoneal administration (Fig. **1G** and **H**) , only 1% (3 of 234) of RFRP-1/3 neurones expressed Fluoro-Gold (Fig. **1E** and **F**) [21]. RFRP-1/3 neurones were, however, able to take up Fluoro-Gold from other brain regions to which they are known to project [21]. This makes it very unlikely, at least in the rat, that hypothalamic RFRP-1/3 neurones are able to regulate gonadotrophin secretion at the level of the anterior pituitary gland.

There are also recent reports of direct inhibitory effects of RFRP-3 at pM doses on GnRH-induced LH (and occasionally FSH, although the longer biological half-life of this hormone makes suppressive effects difficult to detect) secretion from primary cultured pituitary cells using rat [100, 102], sheep [105, 115] and bovine [116] donors. This effect was blocked by RF9 in rats [102], a receptor antagonist that blocks GPR147 and GPR74 (RFRP and NPFF receptors) but not GPR10, GPR54 or GPR103 (PrRP, kisspeptin and QRFP receptors) [117]. RFRP-3 also inhibited GnRH-stimulated mobilisation of intracellular calcium in cultured ovine gonadotrophs [105]. In contrast our group observed no effect of RFRP-3 on LH secretion from rat pituitary cell cultures at doses of up to 10^{-7} M, either with or without GnRH co-treatment [95]. The hypophysiotrophic relevance of such effects depends entirely on whether the peptides are secreted into portal blood at concentrations sufficient to elicit a pituitary response. To date this has not been measured for RFRP-1 or -3 as it has been for kisspeptin (see below), although RFRP-1 is undetectable in peripheral blood [87]. For this reason as well as the fact that it implies gonadotrophin antagonism, the use of the name gonadotrophin-inhibitory hormone seems inappropriate. In order to resolve the question of whether RFRP-1 or -3 act as direct gonadotrophin regulating hormones *in vivo*, whole animal experimental models will be required. In this regard, inhibition of circulating LH concentration by peripherally-administered RFRP-3 has also been noted in hamsters [92], sheep [105], cattle [116] and rather variably in rats [95, 100, 102]. However such effects could well occur indirectly via a hypothalamic inhibition of GnRH neuronal activity [89]. A more useful approach will be to test for LH responses to systemically-injected RFRP antagonists [118], but this will have to await the development of antagonists that do not cross the blood-brain barrier in order to isolate non-hypothalamic responses.

Even in the absence of secretion into portal blood, RFamide peptides could exert autocrine or paracrine effects if produced locally. To address this possibility, we [10] and others [9] measured RFRP mRNA levels in the rat pituitary gland under high and low circulating estrogen states as an indicator of potential peptide production by this tissue. Unlike PrRP, the data for RFRP concur that there is no possibility of a paracrine role in the pituitary gland under any of the endocrine situations tested, since its gene expression is undetectable. RFRP-1 peptide has also been shown to be undetectable in the rat pituitary gland [87]. Overall, the reported effects of systemic or *in vitro* RFRP-3 treatments on gonadotrophins remain to be reconciled with the conspicuous absence of endogenous RFRP-3 in the pituitary gland.

KISSPEPTINS

The fourth mammalian RFamide peptide family, isolated from the human placenta on the basis of its high affinity for the orphan receptor GPR54 (occasionally called OT7T175 or AXOR12), is termed kisspeptin (formerly and sometimes still referred to as metastin, since it was originally isolated as a metastasis-suppressing factor) [119-121]. A search of the human genome revealed that kisspeptins are the mature product of the previously-identified *KISS1* gene [121, 122]. GPR54 appears to exclusively transduce kisspeptin signalling, utilising the $G_{\alpha q}$ pathway [119-121, 123]. The receptor has a high amino acid sequence identity to the receptor for galanin, but this peptide is unable to bind to or activate GPR54 [123]. It is also similar to the PrRP receptor GPR10 [124], but ligand-receptor interactions between these RFamide families have not been reported to date. In mammals, N terminally truncated variants of kisspeptin-54 (kisspeptin-52 in rats and mice) may include kisspeptin-14, kisspeptin-13 and kisspeptin-10 [119, 125], with the C terminally-amidated sequence from Tyr 45 to Phe 54 (kisspeptin-10) possessing the essential sequence required for full receptor interaction [121]. However, no obvious cleavage sites in the prohormone can explain the generation of shorter fragments, and it still remains the possibility that these peptides are the result of degradation of the longer form during mass spectrometry analysis [126]. While a number of differences in human/rodent kisspeptin 54/52 exist, including a tentative Cys–Cys disulfide bridge in rodent positions 4 and 16, kisspeptin-10 is highly conserved with only a single phenylalanine - tyrosine amino acid substitution between humans and other mammals. Nevertheless the C terminal location of this substitution renders the non-human form an RYamide rather than RFamide peptide [127].
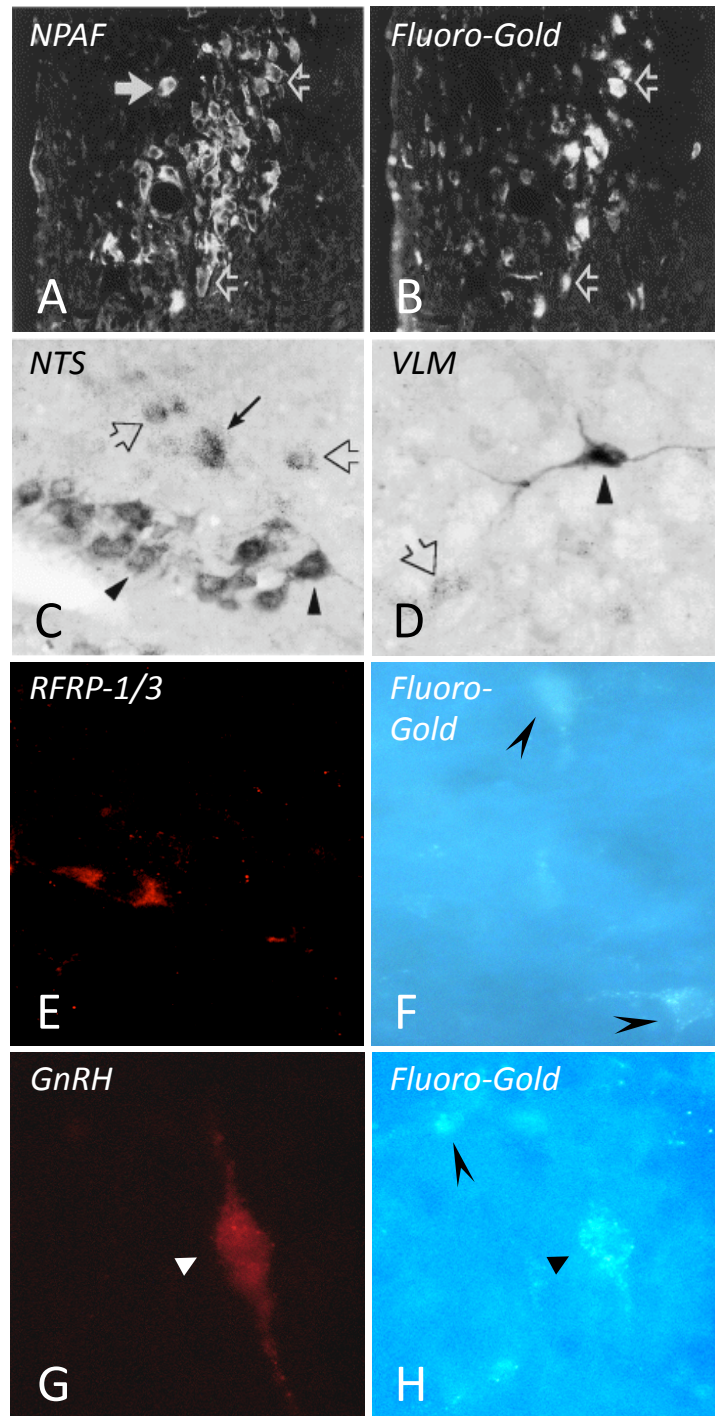
Fig. (1). Use of retrograde tracer uptake from the systemic circulation or pituitary gland to test for RFamide neuronal projections outside the blood-brain barrier (e.g. to the median eminence external zone or pituitary neural lobe) in rats. A and B: NPAF immunoreactive (A) and Fluoro-Gold-filled (B) cell bodies in the paraventricular nucleus following injection of Fluoro-Gold into the neural lobe. Extensive colocalisation (open arrows) confirmed that many of these neurons project to the pituitary gland. Filled arrows depict NPAF-immunoreactive neurones devoid of Fluoro-Gold. Reproduced with permission from [19]. C and D: Following systemic injection of fast blue, cells expressing PrRP mRNA (silver grain *in situ* hybridisation signal; open arrows) are seen in close proximity to retrogradely-labelled cells (dark stain; arrowheads) in the nucleus tractus solitarius (NTS; C) and ventrolateral medulla (VLM; D). However less than 1% of all PrRP-expressing neurones were colocalised (black arrows) with fast blue, indicating negligible projections to the median eminence neurosecretory zone or posterior pituitary gland. Reproduced with permission from [20]. E-H: RFRP-1/3-immunoreactive cells (E) in the dorsomedial hypothalamus also virtually never took up Fluoro-Gold (F) following an intraperitoneal injection, indicating that they do not project beyond the blood-brain barrier. In contrast, ~95% of GnRH neurones (G), which are well known to be hypophysiotrophic, coexpressed Fluoro-Gold (H). Open arrows indicate RFRP-1/3 neurons devoid of Fluoro-Gold; filled arrows indicate GnRH neurons colocalised with Fluoro-Gold; arrowheads indicate Fluoro-Gold in nearby unidentified cells. Reproduced with permission from [21].

In the hypothalamus, kisspeptin neurones potently stimulate GnRH neurones and hence drive gonadotrophin secretion and gonadal functions [128-131] (although it should be noted that in experiments where kisspeptin-10 is administered peripherally no activation of GnRH soma is observed, leading to speculation that it can also act on GnRH terminals in the median eminence) [126]. Through this centrally-mediated stimulation of the reproductive axis, kisspeptin has been shown in numerous recent studies to play critical roles in the timing of puberty [132], the positive estrogenic feedback that drives the preovulatory GnRH/LH surge [133], and metabolic [134] and photoperiodic [135, 136] gating of fertility. *Kiss1* or *Gpr54* null mice fail to undergo sexual maturation and cannot generate a preovulatory surge even when stimulated with high concentrations of estrogens [137, 138]; furthemore *GPR54* mutations have been shown to lead to hypogonadotrophic hypogonadism in humans [137, 139, 140]. Aside from hypothalamo-pituitary-gonadal axis regulation, kisspeptin has also recently been shown to stimulate prolactin release via central inhibition of hypothalamic dopamine neurones in proestrous or estradiol-treated rats, albeit at doses 10-fold higher than those which elicits an LH response [141]. Kisspeptin inhibits metastasis in various tumours and represses trophoblast invasion during pregnancy, and is highly expressed in the placenta during the first trimester when invasiveness peaks and regulation of this is of central importance [142].

While the hypothalamus represents the primary site of action for kisspeptin regulation of reproduction, the direct effect of kisspeptins at the pituitary level is still unclear [143]. GPR54 protein immunoreactivity has been reported in pituitary tissue of rats [144], and gene expression for the receptor is observed in pituitary glands of humans [119, 120], sheep [18] and rats [10, 144, 145], albeit at levels that are an order of magnitude lower than those seen in hypothalamic regions [10]. The receptor may be present in gonadotrophs as well as in somatotrophs and lactotrophs [18, 144]. However there is inconsistent evidence that kisspeptin is able to affect pituitary hormone release directly. In some primary cell culture studies, kisspeptin-10 in the nM dose range has been able to elicit modest releases of LH from rat [145] and sheep [18] cells, and growth hormone from rat [145] cells. Others, however, have reported no effects of lower than μm doses of kisspeptin-10 on gonadotrophin release from rat [131, 146, 147], bovine [148] and porcine [148] pituitary cells and of growth hormone [149] and prolactin [141, 149] from rat cells. There is no clear pattern of sex or steroid treatments to reconcile these findings, although Smith *et al* noted that an LH response to kisspeptin-10 was limited to pituitary cultures collected from ewes during the follicular phase of the estrous cycle [18].

It is very unlikely that kisspeptin reaches the pituitary gland at physiological concentrations via a hypophysiotrophic mechanism. Kisspeptin immunoreactive fibres are scarce or non-existent in the external zone of the mouse [150], rat [151, 152] and rhesus monkey [153] median eminence. In the latter species a pulsatile pattern of kisspeptin release in microdialysis samples from the stalk-median eminence has been reported [154], but the tendency of these pulses to slightly precede GnRH pulses suggests a primary central role rather than one at the pituitary level. In the

sheep, kisspeptin immunoreactive fibres may be more abundant in the median eminence [155]. Importantly in this species however, the levels of kisspeptin measured in hypophyseal portal blood, while detectable, did not fluctuate during the period of an estrogen-induced GnRH/LH surge and only attained concentrations that were several orders of magnitude lower than that required to elicit an LH release in primary pituitary cell cultures [18]. These results strongly suggest that a hypophysiotrophic action on the pituitary gland is not of major importance in terms of LH release. They do not, however, preclude the possibility that kisspeptin has an autocrine or paracrine pituitary action. *Kiss1* gene expression has been reported in whole pituitary extracts from rat [144] and goldfish [156] using RT-PCR, but others including ourselves have reported undetectable or barely detectable pituitary *KISS1* gene expression in humans [120] and rats [10] under high and low circulating estrogen states. Immunoreactivity for kisspeptin peptide has also been observed in pituitary tissue; predominantly in gonadotrophs in the rat [144] whereas in the monkey it appears to be most abundant in the intermediate lobe [157].

The physiological relevance of pituitary kisspeptin production is open to debate, but insofar as gonadotrophin regulation is concerned it is telling that systemically-administered kisspeptin cannot stimulate LH secretion in various species where GnRH action is prevented by pretreatment with GnRH antagonists [131, 158] surgical hypothalamo-pituitary-disconnection [18] or transgenic mutation of the gene encoding GnRH [159]. These unequivocal findings strongly imply that the regulation of gonadotrophin secretion by kisspeptin occurs almost exclusively at the level of the hypothalamic GnRH neurone.

PYROGLUTAMYLATED RFAMIDE PEPTIDE 43 (QRFP43) AND 26RFA

As with the *RFRP* gene, the human *QRFP* gene was identified by a DNA database search by two different groups independently [15, 160]. This gene was shown to encode a preproprotein that can potentially be processed into several peptides, including a 43 amino acid RF-amide peptide with pyroglutamic acid at the N terminus (QRFP43; also termed 43RFa) [15] and an N terminally-truncated 26 amino acid (26RFa; also termed P518 or QRFP26) [160]. QRFP43 [161, 162] and 26FRa [162] were subsequently purified from the central nervous systems of rats and humans, respectively. Both peptides were shown to have a high affinity for the orphan G protein-coupled receptor GPR103 (also referred to as AQ27 and SP9155) utilising the $G_{\alpha i/o}$ and $G_{\alpha q}$ pathways [15, 160, 163], but QRFP43 exhibits more potent agonistic activity [15]. GPR103 shares high homology with NPFF/-NPAF/RFRP-1/3, orexin, and cholecystokinin receptors [15, 160, 164], and it has been suggested that 26RFa may interact with the NPFF receptor GPR74 in the rat central nervous system [165]. However NPFF cannot displace 26RFa from its receptor [165]. PrRP, RFRP-1 and RFRP-3 are also unable to activate GPR103 [15]. In the mouse [161] and rat [166], two homologues of human GPR103 with similar affinities for QRFP43 have been identified and termed GPR103A and GPR103B. These two variants of the receptor appear to be differentially distributed in multiple regions of the brain [161, 166].
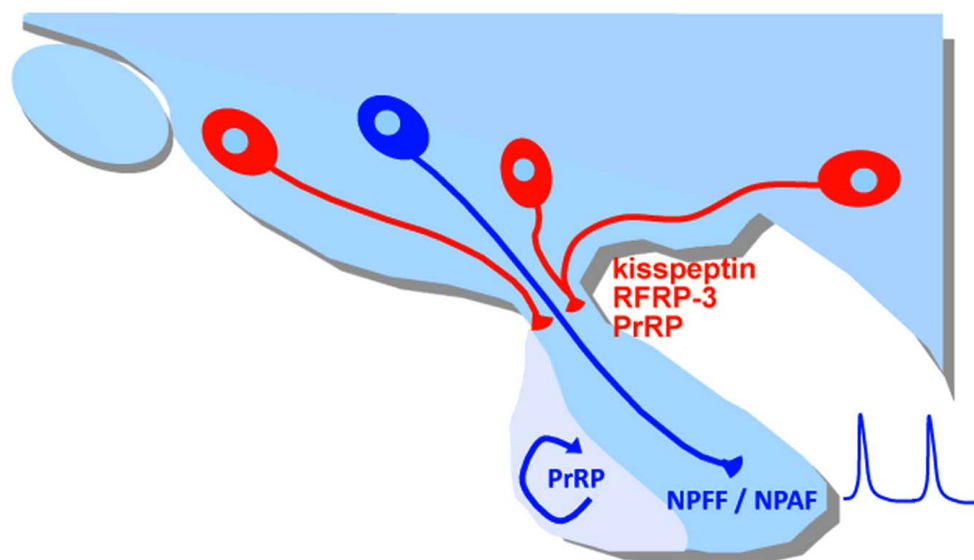
Fig. (2). Schematic representation of the ways by which it has been proposed that RFamide peptides act on the pituitary gland. Blue lines represent mechanisms that are supported by measurement of the endogenous peptide and/or uptake of retrograde tracers; red lines represent proposed mechanisms that are not supported by these techniques and are based on the observed effects of exogenous RFamide treatments. NPFF and NPAF have been shown to be secreted in the posterior pituitary lobe by magnocellular neurons; these neurohormones are released into the systemic circulation in a pulsatile pattern but their roles in the pituitary gland are unknown. Kisspeptin, RFRP-3 and PrRP have been proposed to act as hypophysiotrophic neurohormones but the scarcity of their fibre terminals in the median eminence external zone makes this unlikely. However PrRP appears to be produced by the anterior pituitary lobe, where it may stimulate prolactin release by autocrine or paracrine mechanisms.

GPR103 mRNA and 26RFa binding activity is observed throughout the central nervous system [15, 160, 164, 165], with high binding in the preoptic area and anterior hypothalamic area (implying a modulatory role in the gonadotrophic axis) as well as the dorsal horn of spinal cord (implying a role in pain modulation) [165]. Consistent with this, centrally-administered QRFP43 and 26RFa have been shown to stimulate gonadotrophin secretion in male and female rats via GnRH [167, 168], while intracerebroventricular [169] and intrathecal [170] injections of 26RFa produce antinociceptive effects in rats. In addition, 26RFa stimulates food intake in rats [171] and mice [48, 161, 172], and *Qrfp* gene expression is increased in fasted or leptin-deficient mice [161] suggesting that QRFP may be regulated by leptin.

GPR103 gene expression has been documented in the pituitary glands of humans [160, 164], rats [15, 160, 168] and mice [160], but as with most other RFamide receptors the level of expression is very low in comparison to the central nervous system, as well as to the adrenal gland [15, 160]. An immunohistochemical study of 26RFa neurones in the human hypothalamus did not report any immunoreactive fibres in the median eminence [162], and there is no other evidence to date to suggest that QRFP or 26RFa are released in a manner consistent with a hypophysiotrophic mode of action.

Pituitary effects of QRFP peptides have, nevertheless, been documented *in vitro*. At concentrations above the nM - μM range, 26RFa stimulated cAMP production by rat pituitary cell cultures [48], and both QRF43 and 26RFa enhanced basal and GnRH-stimulated LH secretion from male and female rat pituitary cells [168]. *In vivo*, systemic administration of QRFP [167] or 26RFa [168] evoked an increase in serum LH levels in female but not male rats. However there

is relatively little evidence to date to suggest that the pituitary gland generates sufficient QRFP peptides to cause autocrine or paracrine effects. While the gene encoding the QRFP precursor is expressed in the pituitary gland of rats [15, 160, 168], mice [160] and humans [160], the levels of expression are very low when compared to the brain [15, 160] where it is exclusively expressed in the periventricular hypothalamus (mice) [161], ventromedial hypothalamus (rats) [48] and lateral hypothalamic area (mice and rats) [48, 161]. Thus the situation for QRFP peptides regarding potential direct pituitary actions is similar to that described above for RFRP and kisspeptin peptides, in that the effects seen using exogenous peptide treatments are inconsistent with the near absence of endogenous peptide reaching or produced in the pituitary gland.

CONCLUSIONS

At various stages during their characterisation, direct actions on the pituitary gland via autocrine/paracrine or neuroendocrine mechanisms have been proposed for all mammalian RFamide peptides. As summarised in Fig. (**2**), the available data most strongly support such actions for NPFF/NPAF and PrRP. NPFF/NPAF peptides are neither released into the portal blood from the median eminence nor produced within the pituitary gland itself, but rather appear to be produced by magnocellular neurones in the supraoptic and paraventricular nuclei and secreted by nerve terminals in the neural lobe along with AVP. Uptake of a retrograde tracer following its injection into the neurointermediate lobe has been used to confirm this. While the pulsatile presence of NPFF in peripheral blood implies endocrine functions, any role(s) within the pituitary gland itself remain to be identified. In contrast PrRP was named on the assumption that it acted on the anterior pituitary gland in a

hypophysiotrophic manner, but the lack of immunoreactive nerve terminals in the median eminence external zone and the negligible uptake of a systemically-injected retrograde tracer have showed this not to be the case. However the relatively high levels of PrRP mRNA and peptide expression in the anterior pituitary gland mean that autocrine/paracrine effects, in particular stimulation of prolactin release in the presence of elevated circulating estrogen levels, could occur at this site. Determining the functional significance of this may require the use of more specific *in vivo* approaches, such as PrRP antagonists that do not cross the blood-brain barrier or cell-specific conditional knockouts of the gene encoding PrRP.

In contrast, the evidence for direct actions of RFRP-1/3, kisspeptins and QRFP43/26RFa are limited to variable results from experiments involving treatment with exogenous RFamide peptide, with little consideration given to the possible source of endogenous peptide in the pituitary gland. The pituitary mRNA levels of the precursor peptides of all three of these RFamide groups are low to undetectable, suggesting negligible peptide production locally. Immunoreactive fibre terminals within the median eminence external zone are mostly reported to be scarce or non-existent, and in the case of RFRP-1/3 lack of retrograde tracer uptake from the systemic circulation has been used to confirmed the absence of hypophysiotrophic peptide secretion in rats. Even in cases where fibre presence in the neurosecretory zone has been reported, it should be noted that this often does not correlate with peptide presence in portal blood [173]. In the case of kisspeptin, portal blood sampling has clearly showed that the levels reaching the pituitary gland by this route are insufficient to stimulate release of LH. Thus, until such time as the endogenous presence of these peptides in the pituitary gland in sufficient concentrations to elicit physiological responses is demonstrated, their direct pituitary actions remain questionable.

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