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Review Article

Neuropeptide FF receptors as therapeutic targets

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CONTENTS

Abstract

Neuropeptide FF (NPFF) receptors are G-protein-coupled receptors that belong to the subfamily of RF-amide receptors. Endogenous ligands for these receptors are peptides that display a conserved Arg-Phe-NH_2 (RF-amide) sequence at their carboxyl-terminal end. Several physiological functions have been proposed to be modulated by these receptors, mainly based on in vivo effects of their endogenous peptides or related molecules. These include the modulation of pain, feeding and cardiac function, and the control of insulin release. A wide body of evidence indicates a close relationship between NPFF and opioid systems. In particular, the NPFF system has been shown to display anti-opioid properties, which has led to the hypothesis that functional blockade of NPFF receptors could be a means to avoid the development of pain hypersensitivity and tolerance associated with chronic opioid treatment. This hypothesis was recently confirmed using a potent and selective antagonist of these receptors in a model of discontinuous opioid administration in rats. These results strongly suggest that NPFF receptors might represent a novel therapeutic target for improving the efficacy of opioid treatment of chronic pain.

Introduction

G-protein-coupled receptors (GPCRs) form one of the largest protein family encoded by the human genome (1). A recent estimation indicates that, except for the odorant receptor subfamily which by itself contains several hundred members, the repertoire of GPCRs responding to endogenous ligands consists of 367 receptors in humans (2). Interestingly, almost all of these receptors have a clearly defined orthologue in rodents and the expression profile of most of them is unique (2). GPCRs are activated by a large variety of endogenous ligands, including metals, biogenic amines, fatty acids, Krebs cycle intermediates, peptides, proteins, amino acids, nucleotides and steroids (3). They are involved in numerous physiological processes, including the regulation of neuronal excitability, metabolism, reproduction, development, hormonal homeostasis and behavior. They represent major targets for pharmaceutical drugs, with more than 30% of marketed small-molecule therapeutics acting on this receptor family (4). However, the majority of GPCRs targeted by drugs are biogenic amines, which leaves an enormous reservoir of receptor targets to be explored, including orphan receptors, which comprise more than 100 family members (5), as well as receptors for which an endogenous ligand has already been described but whose function has been poorly defined.

NPFF receptors and RF-amide receptor subfamily

Neuropeptide FF (NPFF) receptors belong to the RF-amide receptor subfamily. Endogenous ligands for receptors from this family are peptides of variable length which all share an Arg-Phe-NH_2 sequence at their COOH termini. The first RF-amide peptide was isolated and sequenced 25 years ago by Price and Greenberg from the mollusk *Marcollista nimbosa* (6). It is a tetrapeptide amidated at its carboxyl-terminal end (FMRF-amide) that displays cardioactive properties. Since then, a large family of related peptides have been found in the nervous system of invertebrates. For example, in the nematode *Caenorhabditis elegans,* 22 genes encode a predicted total of 59 distinct RF-amide peptides (7). In mammals, the family is comparatively small, with only 5 genes encoding 10 RF-amide peptides having been identified. These peptides act through five GPCRs.

NPFF and neuropeptide AF (NPAF) were the first two mammalian RF-amide peptides isolated from bovine brain using an antiserum against FMRF-amide and reverse-phase HPLC (8). They are derived from the same precursor, the gene for which was cloned less than 10 years ago (9). They display high affinity for two GPCRs which have been cloned recently and are referred to as NPFF1R and NPFF2R (10, 11). Concomitantly, the

characterization of a novel gene that encodes two neuropeptides very similar to NPFF was reported (12, 13). These two peptides, named NPSF and NPVF (alias RFRP-1 and RFRP-3), have been shown to preferentially activate the NPFF1 receptor subtype, while NPFF and NPAF display better activity at the NPFF2 subtype (13). These results, together with the distribution of mRNAs coding for those peptides and their receptors in rat brain (10, 12, 13), suggest that the physiologically relevant ligands for the NPFF1R and NPFF2R receptor subtypes could be NPSF/NPVF and NPFF/NPAF, respectively.

Although the main actions of NPFF and related peptides are mediated through GPCRs, it is noteworthy that these peptides have also been shown to potentiate H^+-gated currents of heterologously expressed ASIC channels (14). Whether or not this effect has a physiological significance remains to be demonstrated.

During the same period, three other receptors of RF-amide peptides and their endogenous ligands were identified. Hinuma *et al.* (15) identified two novel mammalian RF-amide peptides that bind to the orphan GPCR GPR10 and named them prolactin-releasing peptides due to their capacity to stimulate the release of prolactin from rat anterior pituitary cells *in vitro*. These two peptides, prolactin-releasing peptide-20 (PrRP-20) and prolactin-releasing peptide-31 (PrRP-31), originate from the proteolytic cleavage of the same precursor, the prepro-prolactin-releasing peptide. Deorphanization of another GPCR, GPR54, led to the identification of three other RF-amide peptides of 54, 14 and 13 amino acids (16, 17). All three derive from the same precursor, which is encoded by the metastasis suppressor gene KiSS-1, and they were therefore named kisspeptin-54, -14 and -13 (alias metastin).

More recently, three groups have independently identified a new member of the RF-amide peptide family, named 26RFa (alias QRFP and P518), as the endogenous ligand of GPR103 (18-20). Mammalian RF-amide receptors together with their endogenous ligands are presented in Table I.

NPFF receptor distribution

Initial studies focused on the distribution of NPFF using IgG from NPFF antiserum (21). Cloning of cDNA encoding the NPFF1 and NPFF2 receptor subtypes and their endogenous ligands NPFF/NPAF and NPVF/NPSF has allowed the distribution of mRNAs encoding these receptors and their ligands to be studied more precisely, both by *in situ* hybridization and RT-PCR in rats and humans (10-13, 22). In addition, novel radiolabeled ligands derived from both NPFF/NPAF and NPVF/NPSF have been shown to display high affinity and good selectivity for NPFF2R and NPFF1R, respectively (12, 23). These ligands have therefore been used to study extensively the distribution of both NPFF receptor subtypes in several mammalian species, including rodents and primates (24, 25).

Although there are some discrepancies among the different studies, both NPFF receptor subtypes have been shown to be expressed mainly in the central nervous system (CNS). In humans, high levels of expression of NPFF2R mRNA have also been detected in placenta (10, 11), indicating a possible role for this receptor subtype in gestational regulation. The NPFF2R subtype seems to be much more abundant than the NPFF1R subtype in the CNS of all species studied, except *Octodon degus* (degu) (24-26).

Within the CNS, NPFF1R and NPFF2R mRNAs have been shown to be broadly expressed both in humans and rodents (10, 11, 26), except in one study where a much more restricted pattern of expression was shown for NPFF2R compared to NPFF1R (13). Conversely, at the receptor level, quantitative autoradiographic mapping studies have shown a wider distribution of NPFF2R compared to NPFF1R in mouse, rat and monkey CNS (27). Although the broad distribution in the CNS of both NPFF receptor subtypes suggests a regulatory role for these receptors in multiple functions, the constant presence of NPFF1R mRNA and binding sites in the hypothalamus of all species studied indicates a modulatory role for this subtype in neuroendocrine function. In addition, the distribution of NPFF2R mRNA and binding sites in the diencephalon, spinal cord, spinal trigeminal and dorsal root ganglia supports a modulatory role for this receptor subtype in the control of nociception. It should be noted that, in humans, NPFF1R mRNA is more abundant in the spinal cord than NPFF2R mRNA (10, 27). This suggests that the NPFF1R subtype might play a more prominent role in the modulation of sensory inputs in humans than in other species. In rats, the distribution of mRNAs coding for NPFF1R and NPFF2R endogenous peptides is in agreement with the role described above for their receptors. Indeed, NPVF/NPSF mRNA has been observed

Table I: Mammalian RF-amide receptors and their endogenous ligands.

RF-amide receptors	Putative endogenous peptide ligands	Ref.
NPFF receptors		
- NPFF1R (OT7T022)	NPSF (RFRP-1), NPVF (RFRP-3)	10, 12, 13
- NPFF2R (HLWAR77)	NPFF, NPAF	9-11, 28
Prolactin-releasing peptide receptor (GPR10, hGR3, UHR-1)	PrRP	15
Kisspeptin receptor (GPR54, OT7T175, AXOR12)	Kisspeptins (metastin)	16, 17
GPR103 (AQ27, SP9155)	26Fa (P518, QRFP(43))	18-20

mainly in rat hypothalamus (12, 13), whereas NPFF/NPAF mRNA has been detected in several brain regions, including some hypothalamic areas, the nucleus of the solitary tract and the superficial layers in the dorsal horn of the spinal cord (28).

NPFF receptors and pain

The management of pain is a major health problem worldwide. In the U.S. alone, the cost of pain has recently been estimated to be 62.2 billion dollars per year (29). Although in recent years great advances have been made in the understanding of the mechanisms that underlie pain, systemic administration of opiate analgesics such as morphine remains the most effective means of alleviating severe pain across a wide range of conditions that includes acute, persistent inflammatory and neuropathic pain states. However, opioid treatments are associated with several side effects, including the development of tolerance to the analgesic effect that emerges following repeated exposure. It has been proposed that adaptative modifications in cellular responsiveness, and particularly desensitization and downregulation of opioid receptors, are at the origin of this phenomenon (30).

A challenging hypothesis is that stimulation of opioid receptors triggers activation of anti-opioid systems, which in turn produces hyperalgesia, thus diminishing the net analgesic effect of the opioid agonist (31-33). This phenomenon, so-called opioid-induced hyperalgesia (OIH), has been observed *in vivo* in rats, where both acute and prolonged opioid treatments induce a long-lasting hyperalgesia that persists for several days after the last opioid administration (34-37). In man, several reports indicate that acute opioid administration, as used in surgery, and chronic opioid treatments can be associated with paradoxical hyperalgesia and/or allodynia (38, 39), and enhancement of pain sensitivity has been reported in heroin addicts (40). Chronic and cancer-related pain, which is often unresponsive to opioids, is also frequently associated with pain hypersensitivity, suggesting that anti-opioid systems could be critically implicated in the development of this type of pain. Therefore, anti-opioid receptor antagonists could represent a promising strategy for opposing the pain hypersensitivity component associated with both exaggerated postoperative pain and chronic pain, especially in the case of opioid treatment (32, 33, 39).

Several neuromodulating systems have been shown to display anti-opioid properties, including the NPFF system (31, 41-44). For example, intracerebroventricular (i.c.v.) administration of NPFF is able to produce a transient hyperalgesia in rats (45, 46), while opioid administration triggers the release of NPFF-like immunoreactive material from rat spinal cord both *in vitro* and *in vivo* (47, 48). Moreover, the administration of an antibody against NPFF partially abrogates the tolerance to the analgesic effect of opioids (45, 49). However, contradictory results have been obtained when NPFF was co-administered with opioids in animal models of pain, depending on the route of administration. Thus, i.c.v. administration of NPFF reverses morphine-induced analgesia in rats (59), whereas intrathecal (i.t.) administration produces a long-lasting opioid-induced analgesia and prolongs morphine-induced analgesia (51). The origin of this difference is presently unknown.

The lack of NPFF receptor antagonists with good metabolic stability together with CNS bioavailability after systemic administration has severely limited the study of the opioid-modulating properties of the NPFF system *in vivo*. Very recently, we described the generation of a chemical library of Arg-Phe-NH_2 derivatives and the successful identification of the first small, potent and selective NPFF receptor antagonist from this library (52). We have shown that this compound, named RF9, when chronically co-administered with heroin by systemic injection, completely blocks the delayed and long-lasting paradoxical opioid-induced hyperalgesia and prevents the development of associated tolerance. Although this compound cannot discriminate between NPFF1R and NPFF2R subtypes, these results clearly indicate that NPFF receptors are part of a *bona fide* anti-opioid system.

Interestingly, the NPFF system has also been implicated in chronic pain. In particular, a strong increase in NPFF mRNA together with a moderate increase in NPFF2R mRNA has been observed during inflammatory hyperalgesia in rats either in the early phase (53) or in the late phase (27) of carrageenan-induced inflammation of the hind paw. Moreover, NPFF and a stable analogue of NPFF have been shown to attenuate allodynia both in inflammatory and neuropathic pain models after i.t. or i.c.v. injections (54-56). In contrast to the anti-opioid properties of the NPFF system described above, these results suggest that stimulation of NPFF receptors attenuates pain sensitization which occurs in chronic pain models. However, the implication of NPFF receptors in this phenomenon remains to be demonstrated.

NPFF receptors and other functions

Several other functions have been proposed to be modulated by NPFF receptors. Besides their role in the modulation of opioid-induced analgesia, NPFF receptors have also been implicated in other aspects of opioid function, and particularly opioid dependence. Very early, Malin *et al*. had shown that NPFF and NPFF analogues precipitated an opiate abstinence syndrome, while IgG from NPFF antiserum attenuated the development of naloxone-precipitated withdrawal signs in dependent rats (50, 57, 58). In agreement with these data, Gelot *et al.* (59) have further shown that antisense oligonucleotides complementary to the sequence of human NPFF significantly attenuated the naloxone-precipitated withdrawal syndrome in morphine-treated mice. More recently it has been shown that a putative NPFF antagonist produced conditioned place preference, which was completely blocked by naloxone, suggesting a close relationship between NPFF and opioid systems in drug reward (60).

Altogether, these data indicate that NPFF receptors may be involved in opioid dependence.

In a recent review, Dockray proposed that mammalian RF-amide receptors, like in invertebrates, could be involved in the control of feeding behavior (61). When injected i.c.v. in rats, NPFF has been shown to reduce food intake and to stimulate water consumption (62, 63). Conversely, when injected at similar doses directly into the parabrachial nucleus, where NPFF-like immunoreactivity has been observed, NPFF stimulates food intake (64). In both cases, these effects of NPFF on food intake might involve an interaction with the opioid system. Whether these effects are mediated or not by NPFF receptors remains to be determined. Indeed, with regard to the high local concentrations of NPFF that can be reached following i.c.v. or intraparabrachial nucleus injections, it cannot be excluded that this peptide could act via other peptide receptors, and particularly other mammalian RF-amide receptors. This issue is particularly important when we consider that two other mammalian RF-amide peptides (prolactin-releasing peptide and 26RFa) have already been shown to affect feeding (61).

In an early report, Roth *et al.* (65) demonstrated that peripheral administration of NPFF and NPAF elevates mean arterial blood pressure in conscious rats. This was further confirmed by Allard *et al*. (66). These results correlate well with the high level of NPFF2R mRNA detected in rat heart (10). Interestingly, neither NPFF1R nor NPFF2R mRNA was detected in human heart, suggesting that NPFF receptors are not implicated in the peripheral modulation of cardiovascular function in humans (10). In rats, central administration of NPFF can also cause a rise in arterial blood pressure that is accompanied by a significant increase in heart rate (52, 67, 68). When co-administered with NPFF, our recently identified NPFF receptor antagonist prevents these effects, indicating that they are mediated by NPFF receptors (52). Moreover, using whole-cell patch clamp recordings in brain slices, we have recently shown that NPFF and NPVF have a disinhibitory role in the hypothalamic paraventricular nucleus via an attenuation of GABAergic inhibitory input to parvocellular neurons of this nucleus (Jhamandas *et al*., in preparation). This action could explain the central cardiovascular effects of this peptide on arterial blood pressure. This effect is fully prevented by our NPFF receptor antagonist, indicating that it is mediated by NPFF receptors.

NPFF and NPAF have also been shown to inhibit glucose- and arginine-stimulated insulin and somatostatin release from perfused pancreas and from isolated rat islets (69, 70). However, the expression of NPFF1R and NPFF2R mRNA has not been observed in pancreas in rats or humans (10, 12). This effect of NPFF and NPAF could therefore be mediated by another peptide receptor. In line with this, significant levels of expression of GPR54 mRNA have been detected in human pancreas (16, 17, 71). Again, our selective NPFF receptor antagonist would be a valuable tool to investigate this issue.

NPFF receptor ligands

One severe limitation to the evaluation of the potential of NPFF receptors as therapeutic targets is the lack of pharmacological tools (both agonists and antagonists) showing high affinity and good selectivity for these receptors. Structure-activity relationship studies of NPFF-related peptides have shown that the carboxyl-terminal Arg-Phe-NH_2 is critical for NPFF receptor activation and affinity, while the NH_2-terminal sequence is involved in binding (72-74). Based on these observations, several groups have identified NPFF derivatives as novel ligands for NPFF receptors (75). Most of these ligands display agonist activity at NPFF receptors, but whether they can discriminate between NPFF receptor subtypes is rarely known and their selectivity towards other mammalian RF-amide receptors has not been evaluated. Moreover, they display poor CNS penetration after systemic administration and must therefore be administered using i.t. or i.c.v. injections. Some NPFF derivatives and small molecules have also been reported to display NPFF receptor-antagonist activity, including BIBP-3226 derivatives (the prototypical neuropeptide Y [NPY] Y_1 receptor antagonist), and some other recently patented compounds (75). However, none of these ligands was shown to meet the criteria of a good antagonist (high affinity, good selectivity and bioavailability).

As mentioned above, we recently reported the successful identification of the first small, potent and selective NPFF receptor antagonist. This compound, RF9, is a derivative of the Arg-Phe-NH_2 dipeptide with an adamantine substitution on its *N*-acyl extremity (see Fig. 1). We have shown that this compound displays good affinity for both NPFF receptor subtypes, can efficiently antagonize NPFF action *in vitro* and *in vivo,* and displays good selectivity towards a subset of GPCRs, including other mammalian RF-amide, opioid and orphanin FQ receptors, and the NPY Y_1 receptor subtype (52). RF9 allowed us to confirm the role played by NPFF receptors in the central modulation of arterial blood pressure and in opioid-induced hyperalgesia and associated tolerance. This compound should also be useful to study the involvement of NPFF receptors in the different functions that have been shown to be modulated by NPFF and NPFF-related peptides. In the future, the development of RF9 derivatives with better selectivity for NPFF1R or NPFF2R will greatly help us to elucidate the respective roles of these two subtypes.

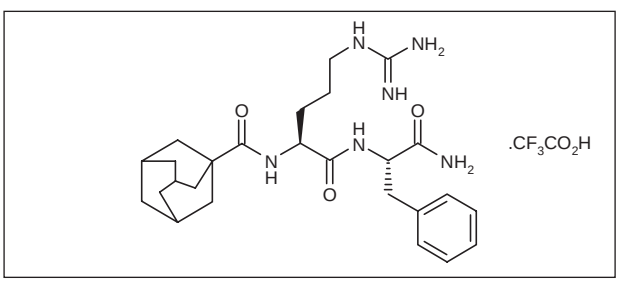

Fig. 1. Structure of the selective NPFF receptor antagonist RF9.

Conclusions

NPFF and NPFF-related peptides have been shown to modulate multiple physiological functions, suggesting that NPFF receptors could represent attractive novel therapeutic targets. However, in most cases, it remains to be demonstrated whether NPFF receptors mediate these effects and which receptor subtype (NPFF1R or NPFF2R) is involved. A large body of evidence has suggested that NPFF and NPFF receptors play important roles in the control of pain and analgesia through their interaction with the opioid system. Using an antagonist, we have been able to show that blockade of NPFF receptors can prevent opioid-induced hyperalgesia and associated tolerance. These results strongly suggest that selective antagonists of these receptors could represent a promising strategy for improving the efficacy of opioid therapy in chronic pain. In the future, the identification of both small agonists and antagonists that can discriminate between NPFF receptor subtypes will undoubtedly allow us to identify other therapeutic applications for these targets, particularly in the treatment of pain hypersensitivity associated with chronic pain and opioid dependence.

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