RF313, an orally bioavailable neuropeptide FF receptor antagonist, opposes effects of RF-amide-related peptide-3 and opioid-induced hyperalgesia in rodents

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Abbreviations :

AUC, area-under-the-curve; CHO, chinese hamster ovary; FSH, follicle stimulating hormone; HI, hyperalgesia index; i.c.v., intracerebroventricular; i.p., intraperitoneal; i.t., intrathecal; Kp, kisspeptin; LH, luteinizing hormone; NPFF, neuropeptide FF; OIH, opioid-induced hyperalgesia; p.o., per os; PrRP, prolactin releasing peptide; RFRP, RF-amide related peptide; s.c., subcutaneous.

ABSTRACT

Although opiates represent the most effective analgesics, their use in chronic treatments is associated with numerous side effects including the development of pain hypersensitivity and analgesic tolerance. We recently identified a novel orally active neuropeptide FF (NPFF) receptor antagonist, RF313, which efficiently prevents the development of fentanyl-induced hyperalgesia in rats. In this study, we investigated the properties of this compound into more details. We show that RF313 exhibited a pronounced selectivity for NPFF receptors, antagonist activity at NPFF1 receptor (NPFF1R) subtype both *in vitro* and *in vivo* and no major side effects when administered in mice up to 30 mg/kg. When co-administered with opiates in rats and mice, it improved their analgesic efficacy and prevented the development of long lasting opioid-induced hyperalgesia. Moreover, and in marked contrast with the dipeptidic NPFF receptor antagonist RF9, RF313 displayed negligible affinity and no agonist activity (up to 100 µM) toward the kisspeptin receptor. Finally, in male hamster, RF313 had no effect when administered alone but fully blocked the increase in LH induced by RFRP-3, while RF9 *per se* induced a significant increase in LH levels which is consistent with its ability to activate kisspeptin receptors. Altogether, our data indicate that RF313 represents an interesting compound for the development of therapeutic tools aiming at improving analgesic action of opiates and reducing adverse side effects associated with their chronic administration. Moreover, its lack of agonist activity at the kisspeptin receptor indicates that RF313 might be considered a better pharmacological tool, when compared to RF9, to examine the regulatory roles of RF-amide-related peptides and NPFF1R in reproduction.

1. Introduction

Neuropeptide FF (NPFF) receptors belong to a subfamily of G protein-coupled receptors (GPCRs), called RF-amide receptors, that encompasses Neuropeptide FF1 (NPFF1R alias GPR147), Neuropeptide FF2 (NPFF2R alias GPR74), prolactin-releasing peptide (PrRPR alias GPR10), Kisspeptin (Kiss1R alias GPR54) and QRFP (QRFPR alias GPR103) receptors (Quillet et al., 2016). All of them bind endogenous neuropeptides that display a conserved Arg-Phe-NH_2 (RF-amide) signature at their carboxyl-terminal end. This sequence has been shown to be mandatory for the affinity and the activity of these peptides towards their receptors. Although both NPFF receptor subtypes have been shown to display promiscuous binding properties for all mammalian RF-amide peptides (Elhabazi et al., 2013; Roumeas et al., 2015), RF-amide-related peptide-1 and -3 (RFRPs), and NPFF and neuropeptide AF (NPAF), are considered as the endogenous ligands of NPFF1R and NPFF2R respectively (Ayachi and Simonin, 2014).

NPFF receptors and their endogenous ligands have been shown to modulate several functions including feeding behavior (Johnson et al., 2007; Kovacs et al., 2014; Nicklous and Simansky, 2003), reproduction (Murakami et al., 2008; Pineda et al., 2010a), arterial blood pressure (Prokai et al., 2006; Roth et al., 1987) and nociception (Ayachi and Simonin, 2014; Roumy and Zajac, 1998). The pain modulating properties of NPFF and its relationship with the opioid system were revealed very early by Yang and coworkers (Yang et al., 1985) who showed the capacity of this peptide to reverse morphine-induced analgesia. Since then, accumulating evidence support the hypothesis that NPFF belongs to an anti-opioid pronociceptive system involved in the homeostatic regulation of opioid antinociceptive activity (Ayachi and Simonin, 2014). More recently, RFRP-1 and RFRP-3 were also shown to display hyperalgesic activity on their own or to prevent the development of morphine analgesia (Elhabazi et al., 2013; Fang et al., 2011; Liu et al., 2001). In contrast, other findings

support that NPFF and NPFF related peptides have a pro-opioid effect. Indeed, when these peptides are administered intrathecally they produce antinociception and enhance morphine analgesia (Gouarderes et al., 1993; Jhamandas et al., 2006; Kontinen and Kalso, 1995; Xu et al., 1999). Altogether, these data indicate that both NPFF1 and NPFF2 receptor subtypes and their respective peptides are involved in the modulation of nociception and pain. However, their precise roles of in this function still needs further investigation. In the last decade, RFRP-3 and its receptor NPFFR1 have also been shown to regulate the hypothalamo-pituitary-gonadal axis although with different effects according to sex and species (Henningsen et al., 2016; Leon and Tena-Sempere, 2015).

The adamantane dipeptide derivative RF9 has been identified ten years ago as a selective NPFF receptor antagonist, with similar nanomolar affinity at NPFF1R and NPFF2R (Simonin et al., 2006). Although this compound cannot discriminate between both receptor subtypes, it proved a very useful tool to establish the roles of NPFF receptors in acute or chronic opioid-induced hyperalgesia, analgesic tolerance, dependence and hypothermia (Elhabazi et al., 2012; Simonin et al., 2006; Wang et al., 2008). The role of endogenous RFRPs in the control of the reproductive axis in mammals has also been evaluated by using the RF9 compound. Unfortunately, data were obscured by its potent gonadotropin-releasing activity *in vivo* (Pineda et al., 2010b; Quillet et al., 2016; Rizwan et al., 2012), which was later shown to rely on its agonist activity at Kiss1R (Kim et al., 2015; Min et al., 2015). Thus, the design of novel NPFF1R- or NPFF2R- selective antagonists, devoid of secondary RF-amide binding components, is of prime importance to address the various physiological functions mediated by RF-amide receptors.

Previous SAR study conducted on NPFF receptors revealed important residues involved in ligand recognition and receptor activation. Among these residues, positions 5.27 and 6.59, have a strong impact on receptor activation and were suggested to form an acidic

negatively binding pocket in both NPFF receptor subtypes, while the position 7.35 was shown to play an important role in functional selectivity (Findeisen et al., 2012). On the other hand, we explored in a previous work the impact (in terms of affinity, selectivity and antagonistic nature at NPFF1 and NPFF2 receptors) of modifications introduced at the N-terminus (Gealageas et al., 2012) or at the carboxy-amidated parts of Arg-Phe-NH$_2$ dipeptides as well as of substitutions of either Arg or Phe residues for other aminoacids (Bihel et al., 2015). Replacement of arginine with a non-natural ornithine derivative (Schneider et al., 2015) bearing a piperidine moiety on its side chain led to the obtention of the RF313 compound, a novel antagonist of NPFF receptors referred to as compound **12e** in (Bihel et al., 2015).

In this study, we examined the pharmacological properties of RF313 into more details. We first performed *in vitro* binding experiments to examine its affinity and selectivity profile toward the five RF-amide receptor subtypes. Then, the ability of RF313 to antagonize the effects of RFRP-3 was evaluated both *in vitro* and *in vivo.* We further addressed the potential of RF313 to improve the analgesic efficacy of opiates and to prevent the development of long-lasting hyperalgesia induced by acute fentanyl or chronic morphine injections, both in rats and mice. Examination of its anti-hyperalgesic property according to the mode of administration (subcutaneous or oral route) clearly pointed out RF313 as an orally-active compound. Finally, the comparison of the effects of RF9 and RF313 on gonadotropin release in male hamster indicated that RF313, given its lack of activity at Kiss1R, is a more suitable pharmacological tool to examine the regulatory roles of RFRPs and NPFF1R in reproduction.

2. Materials and methods

2.1. Materials

Fentanyl citrate, naloxone hydrochloride, forskolin, 3-isobutyl-1-methylxanthine (IBMX) and probenecid were from Sigma-Aldrich (Saint Quentin Fallavier, France). Morphine hydrochloride was from Francopia (Paris, France). Fluo-4 acetoxymethyl ester was from Molecular Probes (Invitrogen, Cergy Pontoise, France). N-adamantane-1-carbonyl-Arg-Phe-NH$_2$ trifluoroacetate (RF9) was synthesized as reported (Simonin et al., 2006). Human RF-amide peptides were from Polypeptide (Strasbourg, France; Kp-10, NPFF, PrRP-20), and Tebu-Bio (Le Perray-en-Yvelines, France ; QRFP26 or 26RFa, RFRP-3). Mouse RFRP-3 was from Genecust (Luxembourg).

[^{3}H]-FFRF-amide (13.6 Ci/mmol) was from the CEA (Saclay, France). [^{3}H]-PrRP-20 (150 Ci/mmol) and [^{35}S]Guanosine 5'-O-[γ-thio] triphosphate ([^{35}S]GTPγS; 1000 Ci/mmol) were from Hartmann Analytic (Braunschweig, Germany). [^{125}I]-Kp-10 (2200 Ci/mmol) and [^{125}I]-QRFP43 (2200 Ci/mmol) were purchased from Perkin Elmer Life and Analytical Sciences (Courtaboeuf, France).

2.2. RF313 synthesis and physico-chemical properties

RF313 (Fig. 1) was synthesized in seven steps, starting from commercially available Fmoc-L-Glu (OtBu)-OH with an overall yield of 40% (Bihel et al., 2015). Purity was established as superior to 98% by reversed-phase high-performance liquid chromatography (RP-HPLC). RF313 showed a good solubility in aqueous solution with a thermodynamic solubility in water greater than 20 mM.

2.3. Receptor cDNA constructs, cell expression and membrane preparations

Human NPFF1R, NPFF2R and Kiss1R cDNAs were subcloned into the pCDNA3.1 expression vector (Invitrogen, Cergy Pontoise, France) and transfected into CHO cells before

selection for stable expression, as reported (Elhabazi et al., 2013). CHO cells expressing human PrRPR and QRFPR were a gift from M. Parmentier (IRIBHM, Brussels, Belgium).

Membranes from CHO cells expressing RF-amide receptors were prepared as described (Elhabazi et al., 2013) and stored at −80°C as aliquots (1 mg prot./mL) until use.

2.4. Radioligand binding assays

Binding assay conditions were essentially as described in (Elhabazi et al., 2013). Briefly, NPFF1R- or NPFF2R-containing membranes were incubated with 10 nM or 3 nM [^{3}H]-FFRF-NH$_2$, respectively. When [^{125}I]-(D-Tyr1, N-Me-Phe3)-Neuropeptide FF was used as a radiotracer, both NPFF1R- or NPFF2R-containing membranes were incubated with 0.015 nM of this radioligand. Non-specific binding levels were determined in the presence of 1 μM RFRP-3 (NPFF1R) or 1 μM NPFF (NPFF2R). Membranes of PrRPR-expressing CHO cells were incubated with 0.3 nM [^{3}H]-PrRP-20, in the absence or presence of 1 μM PrRP-20 to define total and non specific binding levels, respectively. Kiss1R-containing membranes were incubated with 0.02 nM [^{125}I]-Kp-10 using 1 μM Kp-10 to determine non specific radioligand binding. QRFPR membranes were incubated with 0.03 nM [^{125}I]-43RFa, using 1 μM 26RFa for non specific binding measurement.

Competition-type experiments were performed at 25°C, under binding equilibrium conditions (30 min, 0.5 mL final volume for NPFF1R and NPFF2R; 1 h, 0.25 mL final volume for the three other receptor subtypes), in the presence of increasing concentrations of unlabelled peptides or compounds to be tested for their binding affinity.

Membrane-bound radioactivity was separated from free radioligand by rapid filtration through a 96-well GF/B unifilter apparatus (Perkin Elmer Life and Analytical Sciences, Courtaboeuf, France) and quantified using a TopCount scintillation counter (Perkin Elmer).

2.5. In vitro functional experiments

2.5.1. [^{35}S]-GTPγS binding

Stimulation by endogenous RF-amide peptides of [^{35}S]-GTPγS binding to membranes from CHO cells expressing each individual RF-amide receptor, and its inhibition by RF9 or RF313 compounds, were examined as reported (Simonin et al., 2006).

2.5.2. cAMP accumulation

Variations in the overall cAMP content of CHO cells expressing NPFF1 or NPFF2 receptors were monitored according to (Elhabazi et al., 2013). Cells were pre-incubated with 1 mM IBMX, then challenged for 10 min at 37°C with various concentrations of compounds or peptides to be tested, in the absence or the presence of 10 μM forskolin. The reaction was stopped by the addition of ice-cold 0.5 M HCl and cell freezing for 1 h at -80°C. After centrifugation at 2,000 g for 15 min, cell supernatants were stored at −20°C until quantitation of cAMP levels by radioimmunoassay.

2.5.3. Calcium mobilization

Kiss1R-expressing CHO cells were loaded with 2.5 μM Fluo-4 AM in the presence of 2.5 mM probenecid, as described (Elhabazi et al., 2013).
Compounds were serial diluted in assay buffer (in mM: 10 HEPES, 0.4 NaH$_2$PO$_4$, 137.5 NaCl, 1.25 MgCl$_2$, 1.25 CaCl$_2$, 6 KCl, 10 glucose and 1 mg/mL BSA, pH 7.4) and antagonists were allowed to pre-incubate with cells for 10 min before agonist challenge. Agonist-evoked increases in intracellular calcium were recorded over time (5 sec intervals over 220 sec) at 37 °C through fluorescence emission at 520 nm (excitation at 485 nm). Peak response amplitudes were normalized to basal and maximal (cells permeabilized with 20 μM digitonin) fluorescence levels.

2.6. In vivo pharmacological studies

All experiments were carried out in strict accordance with the European guidelines for the care of laboratory animals (European Communities Council Directive 2010/63/EU) and

approved by the local ethical committee. All efforts were made to minimize animal discomfort and to reduce the number of animals used.

2.6.1. Animals

Nociception tests were performed on male, awake, unrestrained Sprague-Dawley rats (250-350 g weight; Charles River Laboratories, L'Arbresle, France) and on C57BL/6N male mice (25-30 g weight; Taconic, Denmark). Animals were housed in groups of three to five per cage and kept under a 12 h/12 h light/dark cycle at $21 \pm 1°C$ with *ad libitum* access to food and water. Experiments were performed during the light-on phase of the cycle. Rats and mice were habituated to the testing room and equipment and handled for 1-2 weeks before starting behavioral experiments. Control and treated group assignment as well as pain responses measurements were performed in a blinded manner. Every animal was used only once. At the end of experiments, animals were euthanized with sodium pentobarbital (120 mg/kg, i.p.) or CO_2 (fill rate of 20% of the chamber volume per minute).

Adult male Syrian hamsters were bred in-house and maintained under a 14h /10 h light/dark cycle at $22 \pm 2°C$ with free access to food and water.

All experiments were carried out in accordance with the european directive for the care of laboratory animals (2010/63/EU) and approved by the local commitee (C.R.E.M.E.A.S)

2.6.2. Drug administration

All drugs were dissolved in physiological saline (0.9%) and administered subcutaneously (s.c.) or orally (p.o.) at 1 mL/kg or 10 mL/kg (vol/body weight) for rats and mice, respectively.

Intracerebroventricular (i.c.v., left lateral ventricle, 5 µL total volume) injections in mice were performed using a modified Hamilton syringe as previously described (Haley and McCormick, 1957).

Male hamsters were prepared for i.c.v. injections as reported (Ancel et al., 2012). A stainless steel 30-gauge cannula was placed in the lateral ventricle and animals were allowed to recover from surgery for one week.

2.6.3. Measurement of the nociceptive threshold in rats and mice

The nociceptive mechanical response of rats was measured using the paw-pressure vocalization test adapted from the Randall–Selitto method (Elhabazi et al., 2014; Kayser et al., 1990). Uniformly increasing pressure (in g) was applied to the hind paw until the rat squeaked. The Basile analgesimeter (Apelex, Massy, France; 1 mm stylus tip diameter) was used and a 600 g cut-off value was set to prevent tissue damage.

The nociceptive thermal threshold of mice was determined using the tail immersion test as previously described (Elhabazi et al., 2014; Simonin et al., 1998). Briefly, mice were restrained in a grid pocket and their tail was immersed in a thermostated water bath. The latency (in sec) for tail withdrawal from hot water (52 ± 0.5 °C or 48 ± 0.5 °C) was taken as a measure of the nociceptive response. In the absence of any nociceptive reaction, a cut-off value of 15 sec (52°C) and of 25 sec (48°C) was set to avoid tissue damage.

2.6.4. Effect of RF313 on fentanyl-induced analgesia and hyperalgesia

Experiments were designed according to a protocol enabling the visualization of an early analgesic effect of opiates and of a delayed hyperalgesic state lasting for several days, both in rats and mice (Celerier et al., 2000; Elhabazi et al., 2012).

The impact of the dose and route (subcutaneous or oral) of administration of RF313 on the short-term analgesic response of rats to four consecutive injections (4x80 µg/kg, s.c., 15 min interval) of fentanyl was evaluated using the paw pressure test. RF313 was administered subcutaneously (0.05, 0.1 and 0.5 mg/kg) or orally (0.3, 1 and 3 mg/kg) 30 min before fentanyl or saline. Nociceptive responses were measured before (time 0) and every 2 h after the first fentanyl injection until return to baseline values.

Very similar experiments were performed on mice with few modifications : animals received RF313 (5 mg/kg, s.c.) 20 min before fentanyl (4x60 µg/kg, s.c.) and nociceptive thresholds were measured every 1 h after the last fentanyl injection using the tail immersion test (48°C).

The impact of RF313 on long-term changes in nociceptive sensitivity induced by fentanyl injection at day 0 was evaluated once daily in rats (paw pressure test) and mice (tail immersion test, 48°C) over a 4- to 6-days period, until recovery of the pre-drug baseline value.

2.6.5. Effect of RF313 on morphine-induced analgesia and hyperalgesia in mice

On day 0, RF313 (5mg/kg) or saline were administered (s.c.) 20 min before morphine (5 mg/kg, s.c.) or saline. Variations of nociceptive responses were measured using the tail immersion test (at 52°C), at 30 min-intervals, over a 0 to 180 min period following morphine or saline injections. The same treatment was then repeated once-daily over the next 7 days. During this period, the nociceptive threshold of the animals was measured every day, 60 min before morphine injection, using the tail immersion test (at 48°C). On day 8, analgesia time-course experiments were performed as described for day 0.

2.6.6. Effect of RFRP-3, RF9 and RF313 on LH secretion in the male Syrian hamster

I.c.v. injections of RFRP-3 or drugs (1.5 µg in 3 µL/animal infused at a flow rate of 1 µL/min), alone or in combination, were given in the morning under light anaesthesia with isoflurane vapour. Thirty min after injection, hamsters were deeply anesthetized with CO_2 vapor and the blood was taken by intracardial puncture for subsequent LH dosage using a validated radioimmunoassay (Tena-Sempere et al., 1993).

2.7. Data and statistical analyses

Binding and functional data were analyzed using Prism 4.0 (GraphPad Software, San Diego, CA, USA) and Kaleidagraph 4.0 (Synergy Software, Reading, PA, USA).

Data for nociceptive tests are expressed as mean values ± S.E.M. for 5 to 15 animals depending on the group. Analgesia was quantified as the area-under-the-curve (AUC) calculated by the trapezoidal method (Celerier et al., 2000). Hyperalgesia was quantified either as the AUC or the hyperalgesia index (HI) in the fentanyl induced-hyperalgesia experiment, which represents the area above the curve expressed as a mean percentage relative to the reference values for the control group. Both types of data were analyzed using one-way or two-way analysis of variance (ANOVA). Post-hoc analyses were performed with Fisher's PLSD test. The level of significance was set at $p < 0.05$. All statistical analyses were carried out using the StatView software.

LH levels are mean values ± S.E.M. for 6 to 7 hamsters. Data were analyzed by one-way ANOVA, followed by Fisher's PLSD test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. In vitro characterization of RF313 pharmacological properties

In a recent study, we have identified an orally active peptidomimetic antagonist of NPFF receptors that prevents the development of fentanyl-induced hyperalgesia in rats (Bihel et al., 2015). This compound, that we called here RF313 and referred to as compound **12e** in (Bihel et al., 2015), is built on a single non-natural amino acid. As shown in Figure 1, RF313 is a derivative of the NPFF receptor dipeptide antagonist RF9 (Simonin et al., 2006), in which the C-terminal amide function was removed, the arginine residue replaced with a non-natural ornithine derivative bearing a piperidine moiety on its side chain and the adamantine ring replaced with a biphenyl moiety.

3.1.1. Binding affinity properties of RF313

In order to further characterize RF313 properties, we performed competition experiments with cell membranes expressing each of the five RF-amide receptors (NPFF1R, NPFF2R, PrRPR, Kiss1R and QRFPR). RF313 was tested in parallel with endogenous RF-amide peptides to verify the receptor specificity of the binding assays and with RF9 for a comparison. As shown in Table 1, RF313 exhibited a marked preference for NPFF receptors together with a moderate selectivity towards the NPFF1R subtype, while its interaction with the other RF-amide receptors was marginal ($K_i > 20$ µM for PrRPR and QRFPR, $K_i > 100$ µM for Kiss1R). As the radioligand used in this study for binding experiments with NPFF receptors ([^{3}H]-FFRF-NH$_2$) have not been extensively characterized yet, we further evaluated the affinity of RF313 for NPFF receptors in binding experiments with [^{125}I]-(D-Tyr1, N-Me-Phe3)-Neuropeptide FF, which is more routinely used, and found similar Ki values for NPFF1R and NPFF2R (245 ± 41 nM and 2238 ± 418 nM, respectively). RF313 binding

properties resembled those of RF9 although this later compound displayed a higher affinity

for both NPFF receptors and a low but significant affinity for Kiss1R (Ki = 3500 ± 740 nM).

Further pharmacological profiling of RF313 at numerous receptors, channels or transporters,

selected for their implication in pain modulation, indicated no significant interaction of RF313

(tested at 1 µM) with these targets (Bihel et al., 2015).

3.1.2. Examination of in vitro functional activities of RF313

We next addressed the agonist or antagonist nature of RF313 at NPFF1R through two

different *in vitro* functional assays. When tested alone (up to 50 µM), RF313 did not modify

basal [^{35}S]-GTPγS binding to membranes from CHO cells expressing hNPFF1R. However, as

illustrated in Figure 2A, RF313 (at 10 µM) was able to promote a rightward shift in the

RFRP-3 dose-response curve indicating that this compound displayed antagonist activity at

hNPFFR1. The lack of agonistic activity of RF313 at hNPFF1R was also confirmed by its

incapacity (at 10 µM) to inhibit forskolin-stimulated production of cAMP in hNPFF1R

expressing cells. In contrast, RF313 clearly opposed RFRP-3-mediated inhibition of this

cellular response (Fig. 2B), with a pA$_2$ of 977 ± 145 nM. For a comparison, a pA$_2$ value of

340 ± 44 nM was obtained for RF9 under the same experimental conditions. These data

clearly demonstrate that RF313 displays antagonist activity at hNPFF1R *in vitro*. Although

this compound also showed a significant affinity for the hNPFF2 receptor subtype, we did not

detect any agonist or antagonist activity in hNPFF2R-expressing CHO cells (cAMP assay).

As RF9 has recently been shown to display agonist activity at Kiss1R (Liu and Herbison,

2014), we further examined the activity of both RF313 and RF9 in calcium mobilization

experiments on Kiss1R expressing cells. As shown in Figure 2C, the endogenous Kiss1R

ligand Kp10 potently stimulated calcium release from those cells at subnanomolar

concentrations (EC$_{50}$ = 0.1 ± 0.025 nM). As expected, RF9 exhibited full agonism at Kiss1R

but at micromolar concentration (EC_{50} 1.3 ± 0.5 µM). RF313 (up to 100 µM) did not display any agonist activity at this receptor (Figure 2C), which is in agreement with the absence of affinity of this compound for Kiss1R observed in this study (Table 1).

3.2. In vivo evaluation of RF313

3.2.1. Evaluation of toxic and adverse effects of RF313

In a first series of experiements we examined in mice unexpected adverse effects of RF313 treatment susceptible to introduce bias in nociception measurements or to impede further use of the compound. To this end, C57BL/6N male mice received s.c. injections of RF313 (up to 30 mg/kg) and were checked for general health parameters (modified SHIRPA parameters, weight and temperature), for sensory-motor responses (muscular strength, proprioceptive, vestibular and fine-tuned motor abilities) as well as for anxiety- or depression-like behaviors. This preliminary phenotyping analysis showed no significant adverse side-effects for RF313 except a slight decrease at 10 mg/kg of the immobility time, which could reveal a potential anti-depressive effect of RF313 at this dose as evidenced from the tail suspension test (Suppl. Figure S1).

3.2.2. RF313 prevents RFRP-3-induced hyperalgesia in mice

We then investigated in mice whether RF313 can antagonize the effect of RFRP-3, the endogenous ligand of NPFF1 receptors (Figs. 3A and 3B). As expected (Elhabazi et al., 2013), RFRP-3 significantly reduced the basal nociceptive threshold of the animals when injected (10 nmol, i.c.v.) alone (AUC: - 181 ± 33 for RFRP-3 vs 2.2 ± 26 for saline, $F_{(3, 33)}$ = 6.3 ; $p < 0.01$, one way ANOVA followed by Fisher's PLSD test $p < 0.01$, Fig. 3B). RF313 (10 nmol, i.c.v.) had no significant effect on the basal nociceptive threshold of the animals when administered alone but it fully prevented RFRP-3-induced hyperalgesia (AUC: 53 ± 54

for RFRP-3 + RF313 vs - 181 ± 33 for RFRP-3. $F_{(1, 33)} = 3.3$; $p < 0.05$, two way ANOVA followed by Fisher's PLSD test $p < 0.001$, Fig. 3B) indicating that this compound well behaves as a NPFF1R antagonist *in vivo*.

3.2.3. RF313 prevents secondary hyperalgesia induced by fentanyl in rodents when administered by subcutaneous or oral routes.

We next examined the consequences of NPFF receptors blockade with RF313 on fentanyl-induced analgesia and secondary hyperalgesia in mice and rats as previously described (Celerier et al., 2000; Elhabazi et al., 2012).

As shown in Figure 4A, fentanyl (4x60 µg/ kg, s.c.) promoted a short lasting analgesic response in mice, which returned to basal within 3 h after the last injection of fentanyl (AUC: 807 ± 211 for fentanyl vs - 6 ± 44 for saline, $F_{(3, 34)} = 19.8$; $p < 0.001$, one way ANOVA followed by Fisher's PLSD test $p < 0.001$), and a delayed hyperalgesic response that lasted for at least two days (HI: 100 ± 21 for fentanyl vs 21 ± 12 for saline, $F_{(3, 34)} = 4.4$; $p < 0.01$, one way ANOVA followed by Fisher's PLSD test $p < 0.01$, Fig. 4B). When administered 20 min before fentanyl (Fig. 4A), RF313 (3 mg/kg, s.c.) significantly potentiated the amplitude and duration of fentanyl analgesia (AUC: 1578 ± 165 for RF313+fentanyl vs 807 ± 211 for fentanyl, $F_{(1, 34)} = 4.3$; $p < 0.05$, two- way ANOVA followed by Fisher's PLSD test $p < 0.01$) and significantly prevented the development of hyperalgesia (HI: 36 ± 20 for RF313+fentanyl vs 100 ± 21 for fentanyl, $F_{(1, 34)} = 4.5$; $p < 0.05$, two- way ANOVA followed by Fisher's PLSD test $p < 0.01$, Fig. 4B). RF313 alone did not modify the basal nociceptive threshold of the animals. Interestingly, once the hyperalgesic state was already initiated, subsequent administration of RF313 (Fig. 4A, dashed arrow) was ineffective in restoring normal nociceptive baseline values. These observations suggest that NPFF receptors

are involved in the onset of opioid-induced hyperalgesia but probably not in the maintenance of the hyperalgesic state.

We next examined the effect of RF313 on fentanyl-induced analgesia and hyperalgesia in rats according to its dosage and mode of administration, either subcutaneously (Figs. 4C and 4D) or orally (Figs. 4E and 4F). Higher RF313 doses were chosen for oral administration in order to account for bioavailability limitations. Whatever the route or the dose, RF313 (administered 30 min before fentanyl) did not modify the amplitude of the analgesic response after 2 hours, probably because fentanyl effect was already maximal (paw pressure test, cut-off 600 g). However, we can notice that 6 hours after fentanyl administration, only rats orally treated with RF313 still displayed an analgesic response. Analyses of hyperalgesia index data with one- way ANOVA showed that the development of fentanyl-induced hyperalgesia was dose-dependently prevented by RF313 when it was administered by subcutaneous ($F_{(3, 30)} = 45$; $p < 0.001$) or oral ($F_{(3, 35)} = 50$; $p < 0.001$) routes (fig. 4D and 4F, respectively). Altogether, these results indicate that NPFF receptors blockade by RF313 (s.c. or p.o.) efficiently prevents the development of hyperalgesia induced by acute fentanyl administration in rodents.

3.2.4. RF313 improves acute morphine analgesia and reduces hyperalgesia associated with chronic morphine administration in mice.

We further examined the impact of RF313 (2.5 and 5 mg/kg, s.c.) on acute morphine (5 mg/kg, s.c.) analgesia in mice (Fig. 5A). RF313 alone did not significantly modify the basal nociceptive threshold of the animals. As expected, morphine induced a strong analgesic effect as shown by the significant increase in tail immersion latencies compared with control animals (AUC: 725 ± 68 for morphine vs 40 ± 18 for saline, $F_{(5, 54)} = 33$; $p < 0.001$, one way ANOVA followed by Fisher's PLSD test $p < 0.001$; Fig. 5B). When injected 20 min

before morphine, RF313 (5 mg/kg) significantly enhanced morphine-induced analgesia (AUC: 1071 ± 91 for morphine + RF313 vs 725 ± 68 for morphine, $F_{(1, 54)} = 4.7$; $p < 0.05$, two way ANOVA followed by Fisher's PLSD test $p < 0.001$; Fig. 5B).

In a subsequent experiment, we performed daily injections of morphine (5 mg/kg, s.c.) for 8 consecutive days and the thermal nociceptive response of mice was measured every day before morphine administration. Such a daily morphine treatment led to a progressive decrease of basal nociceptive reaction latency (Fig. 6A), indicative for the development of a robust hyperalgesic state in mice (AUC: - 45.2 ± 4.8 for morphine vs - 12.5 ± 4.1 for saline, $F_{(3, 28)} = 8.6$; $p < 0.001$, one way ANOVA followed by Fisher's PLSD test $p < 0.001$; Fig. 6B). When injected before daily morphine administration (Fig. 6A), RF313 (5 mg/kg, s.c.) significantly attenuated hyperalgesia (AUC: - 20.3 ± 4.3 for RF313 + morphine vs - 45.2 ± 4.8 for morphine, $F_{(1, 28)} = 4.2$; $p < 0.05$, two- way ANOVA followed by Fisher's PLSD test $p < 0.01$; Fig. 6B).

We next addressed the question whether hyperalgesia was accompanied with the development of analgesic tolerance to acute morphine and again, we considered the impact of RF313. To this end, morphine (5 mg/kg, s.c.) analgesic effect was measured in time-course experiments at day 0 (Fig. 6C, naïve mice) and at day 8 (Fig. 6D, hyperalgesic mice) on mice daily pre-treated with either saline or RF313 (5 mg/kg, s.c.). At day 0, as expected from previous experiments (Fig. 5), RF313 potentiated and prolonged the analgesic response of mice to morphine (AUC: 800 ± 62 for RF313+morphine vs 594 ± 69 for morphine, $F_{(3, 36)} = 12.4$; $p < 0.001$, one way ANOVA followed by Fisher's PLSD test, $p < 0.01$; Figs. 6C and 6E). After 8 days of treatment , the analgesic effect of morphine alone was strongly reduced (AUC: 330 ± 43 at day 8 vs 594 ± 69 at day 0, $p < 0.01$, Fisher's PLSD test; Figs. 6D and 6E) indicating that tolerance did develop in these animals. In mice treated with RF313 and morphine, analgesic tolerance still developed (AUC: 503 ± 39 at day 8 vs 800 ± 62 at day 0, p

< 0.001, Fisher's PLSD test ; Figs. 6D and 6E) but to a lower extent than in animals treated

with morphine alone (AUC: 503 ± 39 for RF313 + morphine vs 330 ± 43 for morphine, $p <$

0.01, Fisher's PLSD test). Altogether, these results indicate that RF313 can both improve

acute morphine analgesic effect and attenuate the development of hyperalgesia as well as of

analgesic tolerance following chronic morphine administration.

3.2.5. RF313, but not RF9, antagonizes RFRP-3 stimulation of LH secretion in the hamster.

Two RF-amide receptors, Kiss1R and NPFF1R (also termed GPR54 and GPR147,

respectively), play essential functions in the control of reproduction in seasonal mammals

(Simonneaux et al., 2013). Conversely to other species, in male hamster RFRP-3 has been

shown to display stimulatory action on LH release (Ancel et al., 2012; Simonneaux et al.,

2013). This species is therefore particularly well suited to study the antagonist action of

RF313 on NPFF1R. As our *in vitro* experiments showed that RF9 (but not RF313) displays

micromolar affinity and agonist activity at Kiss1R (Table 1 and Fig. 2C), we decided to

compare the effects of these two NPFF receptor antagonists on RFRP-3-induced LH release in

the male Syrian hamster. Data analyses with one way ANOVA highlighted significant

differences between the tested groups ($F_{(4, 27)} = 49$; $p < 0.001$). Post-hoc analyses with

Fisher's PLSD test (Fig. 7) revealed that i.c.v. administration of the endogenous NPFF1R

agonist RFRP-3 resulted in an increase in LH plasma levels (6.4 ng/ml $\pm$ 0.4 for saline vs 15.2

ng/ml $\pm$ 1.9 for RFRP-3, $p < 0.001$). RF313 did not affect basal LH levels (6.4 ng/ml $\pm$ 0.4 for

RF313 vs 6.4 ng/ml $\pm$ 0.4 for saline, $p > 0.05$) but fully antagonized the stimulatory effect of

RFRP-3 on LH secretion (15.2 ng/ml $\pm$ 1.9 for RFRP-3 vs 5.4 ng/ml $\pm$ 0.7 for RF313 +

RFRP-3, $p < 0.001$). Conversely, RF9 exhibited *per se* a strong gonadotropin-releasing

activity *in vivo* (6.4 ng/ml $\pm$ 0.4 for saline vs 27.8 ng/ml $\pm$ 1.2 for RF9, $p < 0.001$). Altogether,

these results confirm previous observations linking RF9 gonadotropin-releasing activity to a

possible agonist activity toward Kiss1R (Garcia-Galiano et al., 2012; Glanowska et al., 2014; Liu and Herbison, 2014; Sahin et al., 2015) and point out RF313 as a safer pharmacological tool to dissect properly the role of NPFF1R in the central control of gonadotropin release in mammals.

4. Discussion

In this paper, we provide a detailed analysis of the *in vitro* and *in vivo* pharmacological properties of RF313, a compound (referred to as **12e** in (Bihel et al., 2015)) we selected in a drug discovery program aiming at identifying orally-bioavailable antagonists of NPFF receptors. This non-natural ornithine derivative displays a high solubility in aqueous buffer, a low toxicity, a high selectivity, as well as an antagonist activity at NPFF1R both *in vitro* and *in vivo*. Moreover, it potentiates opioid analgesic effects in mice both by subcutaneous and oral routes. It completely blocks fentanyl-induced hyperalgesia and attenuates morphine-induced hyperalgesia as well and analgesic tolerance. By comparison with our previous studies conducted with RF9 (Elhabazi et al., 2013; Elhabazi et al., 2012; Simonin et al., 2006), RF313 appeared to block the hyperalgesia induced by RFRP-3 and fentanyl at similar doses than RF9 but was slighly less efficient at preventing the development of hyperalgesia and analgesic tolerance induced by chronic morphine administration. Our results confirm that NPFF receptor blockade might represent an interesting strategy to improve opiates analgesic action while limiting the adverse effects associated with their chronic administration. Altogether, the data described in this study point out RF313 as an interesting lead compound for further drug development.

Despite the good *in vivo* activity displayed by RF313 at relatively low doses and by oral route, *in vitro* binding experiments showed that its affinity for NPFF1R and NPFF2R was lower than that of the dipeptide RF9 for the same receptors (Simonin et al., 2006). As molecular rigidification has been described as a standard approach for the development of GPCR antagonists, a lead-optimization process with particular focus on a decrease in the flexibility of the RF313 backbone could represent an alternative strategy to improve its affinity for NPFF receptors.

Binding experiments also indicated that RF313 displayed a slight preference (3-fold selectivity) for the NPFF1R subtype compared to NPFF2R. When testing *in vitro* the nature of the interaction of RF313 at NPFF receptors, we were not able to detect any agonist or antagonist activity of this compound toward NPFF2R while it clearly blocked NPFF1R-mediated cellular responses to RFRP-3. These data suggest that the functional selectivity of RF313 toward NPFF1R is probably greater than that afforded from binding experiments. Although one cannot completely rule out a role of NPFF2R, our *in vivo* data point out NPFF1R as an important actor in the development of secondary hyperalgesia induced by opiates. These results are in agreement with previous observations made with NPFF1R/NPFF2R compounds with varying degrees of functional activity, which suggest that these two receptors display opposing pro- and anti-nociceptive roles, respectively, in various models of pain (Lameh et al., 2010). However, several studies have shown that neuropeptide FF, the endogenous ligand of NPFF2R, can display both pro- or anti-nociceptive actions depending on its site of administration i.t. (Gouarderes et al., 1993) versus i.c.v. (Journigan et al., 2014; Oberling et al., 1993). Thus, despite the recent design of several peptidic and non peptidic NPFF receptors ligands (Gaubert et al., 2009; Journigan et al., 2014; Lameh et al., 2010; Mazarguil et al., 2012), the development of highly selective ligands still represents an important challenge that should greatly help clarifying the respective contribution of NPFF1/2 receptor subtypes in the modulation of nociception.

In this study, we also compared the *in vitro* binding and activity profiles of RF313 and RF9, a dipeptidic compound we identified previously as a NPFF receptor antagonist (Simonin et al., 2006). We observed that RF9 displayed a low but detectable affinity for KISS1R, as well as full agonist activity, while RF313 neither binds nor activates this receptor at concentrations up to 100 μM. Kiss1R has been shown to play a key role in reproduction in numerous species including rodents and human (Goodman and Lehman, 2012). It is expressed in GnRH neurons of the hypothalamus and its activation by kisspeptin has been shown to

strongly stimulate GnRH release, which controls the release of sexual hormones . More recently, NPFF1R and its endogenous ligands RFRP-1 and RFRP-3 have been proposed to play an inhibitory role on GnRH release, thus counteracting the stimulatory role of the kisspeptin/Kiss1R system (Kriegsfeld, 2010). This hypothesis was based in part on results showing that RF9 (when administered i.c.v. in rodents or ewes) is a potent stimulator of LH and FSH release (Caraty et al., 2012; Pineda et al., 2010b; Rizwan et al., 2012) suggesting the existence of an endogenous tone of RFRPs that exerts a negative feedback on GnRH neurons (Pineda et al., 2010a, b). However, several studies also suspected RF9 impact on gonadotropin release to rely on an off-target action on kisspeptin receptors (Garcia-Galiano et al., 2012; Glanowska et al., 2014; Liu and Herbison, 2014; Sahin et al., 2015), an issue that was later elucidated with the demonstration of RF9 agonist activity at Kiss1R, both *in vitro* and *in vivo* (Kim et al., 2015; Min et al., 2015). As RF9 effect was absent in Kiss1R KO mice but well preserved in NPFF1R KOs (Kim et al., 2015; Liu and Herbison, 2014; Min et al., 2015), one may conclude that this compound modulates gonadotropin release primarily through Kiss1R activation rather than via blockade of NPFF1R. We further extended this observation in male hamsters, in which RF9 strongly increased LH levels while RF313 had no activity *per se* but efficiently blocked RFRP-3 stimulation of LH release.

Altogether, these results indicate that RF313, despite its lower affinity at NPFF1R than RF9, may be regarded as a more suitable pharmacological tool to study the involvement of NPFF1R in the central control of reproduction, owing to its lack of Kiss1R agonist activity. Moreover, our data also indicate that an important clue to developing high affinity NPFF1R antagonists will be the careful investigation of their selectivity toward other RF-amide receptors and particularly Kiss1R.

5 . Conclusion

In conclusion, we characterized RF313 as a novel antagonist of the NPFF1 receptor. It displays many characteristics for a lead compound amenable to further therapeutic development in order to improve analgesic action of opiates and reduce adverse side effects associated with their chronic administration. Moreover, and in contrast with RF9, it is devoid of agonist activity at Kiss1R and may therefore be used as a safer pharmacological tool to study the role of NPFF1R in the central control of reproduction. The development of highly selective compounds for both NPFF1 and NPFF2 receptors will be mandatory in the next future to dissect their own roles in the modulation of essential physiological functions such as nociception, feeding and reproduction.

Disclosures

The authors declare no conflict of interest.

References

Ancel, C., Bentsen, A. H., Sebert, M. E., Tena-Sempere, M., Mikkelsen, J. D., Simonneaux, V., 2012. Stimulatory effect of RFRP-3 on the gonadotrophic axis in the male Syrian hamster: the exception proves the rule. Endocrinology 153, 1352-1363.

Ayachi, S., Simonin, F., 2014. Involvement of Mammalian RF-Amide Peptides and Their Receptors in the Modulation of Nociception in Rodents. Front Endocrinol (Lausanne) 5, 158.

Bihel, F., Humbert, J. P., Schneider, S., Bertin, I., Wagner, P., Schmitt, M., Laboureyras, E., Petit-Demouliere, B., Schneider, E., Mollereau, C., Simonnet, G., Simonin, F., Bourguignon, J. J., 2015. Development of a peptidomimetic antagonist of neuropeptide FF receptors for the prevention of opioid-induced hyperalgesia. ACS Chem Neurosci 6, 438-445.

Caraty, A., Blomenrohr, M., Vogel, G. M., Lomet, D., Briant, C., Beltramo, M., 2012. RF9 powerfully stimulates gonadotrophin secretion in the ewe: evidence for a seasonal threshold of sensitivity. J Neuroendocrinol 24, 725-736.

Celerier, E., Rivat, C., Jun, Y., Laulin, J. P., Larcher, A., Reynier, P., Simonnet, G., 2000. Long-lasting hyperalgesia induced by fentanyl in rats: preventive effect of ketamine. Anesthesiology 92, 465-472.

Elhabazi, K., Ayachi, S., Ilien, B., Simonin, F., 2014. Assessment of morphine-induced hyperalgesia and analgesic tolerance in mice using thermal and mechanical nociceptive modalities. J Vis Exp, e51264.

Elhabazi, K., Humbert, J. P., Bertin, I., Schmitt, M., Bihel, F., Bourguignon, J. J., Bucher, B., Becker, J. A., Sorg, T., Meziane, H., Petit-Demouliere, B., Ilien, B., Simonin, F., 2013. Endogenous mammalian RF-amide peptides, including PrRP, kisspeptin and 26RFa, modulate nociception and morphine analgesia via NPFF receptors. Neuropharmacology 75C, 164-171.

Elhabazi, K., Trigo, J. M., Mollereau, C., Mouledous, L., Zajac, J. M., Bihel, F., Schmitt, M., Bourguignon, J. J., Meziane, H., Petit-demouliere, B., Bockel, F., Maldonado, R., Simonin, F., 2012. Involvement of neuropeptide FF receptors in neuroadaptive responses to acute and chronic opiate treatments. Br J Pharmacol 165, 424-435.

Fang, Q., Jiang, T. N., Li, N., Han, Z. L., Wang, R., 2011. Central administration of neuropeptide FF and related peptides attenuate systemic morphine analgesia in mice. Protein Pept Lett 18, 403-409.

Findeisen, M., Wurker, C., Rathmann, D., Meier, R., Meiler, J., Olsson, R., Beck-Sickinger, A. G., 2012. Selective mode of action of guanidine-containing non-peptides at human NPFF receptors. J Med Chem 55, 6124-6136.

Garcia-Galiano, D., van Ingen Schenau, D., Leon, S., Krajnc-Franken, M. A., Manfredi-Lozano, M., Romero-Ruiz, A., Navarro, V. M., Gaytan, F., van Noort, P. I., Pinilla, L., Blomenrohr, M., Tena-Sempere, M., 2012. Kisspeptin signaling is indispensable for neurokinin B, but not glutamate, stimulation of gonadotropin secretion in mice. Endocrinology 153, 316-328.

Gaubert, G., Bertozzi, F., Kelly, N. M., Pawlas, J., Scully, A. L., Nash, N. R., Gardell, L. R., Lameh, J., Olsson, R., 2009. Discovery of Selective Nonpeptidergic Neuropeptide FF2 Receptor Agonists. J Med Chem 52, 6511-6514.

Gealageas, R., Schneider, S., Humbert, J.-P., Bertin, I., Schmitt, M., Laboureyras, E., Dugave, C., Mollereau, C., Simonnet, G., Bourguignon, J.-J., Simonin, F., Bihel, F., 2012. Development of sub-nanomolar dipeptidic ligands of neuropeptide FF receptors. Bioorg Med Chem Lett 22, 7471-7474.

Glanowska, K. M., Burger, L. L., Moenter, S. M., 2014. Development of gonadotropin-releasing hormone secretion and pituitary response. J Neurosci 34, 15060-15069.

Goodman, R. L., Lehman, M. N., 2012. Kisspeptin neurons from mice to men: similarities and differences. Endocrinology 153, 5105-5118.

Gouarderes, C., Sutak, M., Zajac, J. M., Jhamandas, K., 1993. Antinociceptive effects of intrathecally administered F8Famide and FMRFamide in the rat. Eur J Pharmacol 237, 73-81.

Haley, T., McCormick, W., 1957. Pharmacological effects produced by intracerebroventricular injections of drug in conscious mice. Br. J. Pharmacol 12, 12-16.

Henningsen, J. B., Gauer, F., Simonneaux, V., 2016. RFRP Neurons - The Doorway to Understanding Seasonal Reproduction in Mammals. Front Endocrinol (Lausanne) 7, 36.

Jhamandas, K., Milne, B., Sutak, M., Gouarderes, C., Zajac, J. M., Yang, H. Y., 2006. Facilitation of spinal morphine analgesia in normal and morphine tolerant animals by neuropeptide SF and related peptides. Peptides 27, 953-963.

Johnson, M. A., Tsutsui, K., Fraley, G. S., 2007. Rat RFamide-related peptide-3 stimulates GH secretion, inhibits LH secretion, and has variable effects on sex behavior in the adult male rat. Horm Behav 51, 171-180.

Journigan, V. B., Mésangeau, C., Vyas, N., Eans, S. O., Cutler, S. J., McLaughlin, J. P., Mollereau, C., McCurdy, C. R., 2014. Nonpeptide Small Molecule Agonist and Antagonist Original Leads for Neuropeptide FF1 and FF2 Receptors. J Med Chem.

Kayser, V., Basbaum, A. I., Guilbaud, G., 1990. Deafferentation in the rat increases mechanical nociceptive threshold in the innervated limbs. Brain Res 508, 329-332.

Kim, J. S., Brownjohn, P. W., Dyer, B. S., Beltramo, M., Walker, C. S., Hay, D. L., Painter, G. F., Tyndall, J. D., Anderson, G. M., 2015. Anxiogenic and Stressor Effects of the Hypothalamic Neuropeptide RFRP-3 Are Overcome by the NPFFR Antagonist GJ14. Endocrinology 156, 4152-4162.

Kontinen, V. K., Kalso, E. A., 1995. Differential modulation of alpha 2-adrenergic and mu-opioid spinal antinociception by neuropeptide FF. Peptides 16, 973-977.

Kovacs, A., Laszlo, K., Galosi, R., Ollmann, T., Peczely, L., Zagoracz, O., Bencze, N., Lenard, L., 2014. Intraamygdaloid microinjection of RFamide-related peptide-3 decreases food intake in rats. Brain Res Bull 107, 61-68.

Kriegsfeld, L. J., 2010. Overcoming sexual inhibition: exploring the role of RFamide-related peptide using a novel receptor antagonist. Endocrinology 151, 1387-1389.

Lameh, J., Bertozzi, F., Kelly, N., Jacobi, P. M., Nguyen, D., Bajpai, A., Gaubert, G., Olsson, R., Gardell, L. R., 2010. Neuropeptide FF receptors have opposing modulatory effects on nociception. J Pharmacol Exp Ther 334, 244-254.

Leon, S., Tena-Sempere, M., 2015. Dissecting the Roles of Gonadotropin-Inhibitory Hormone in Mammals: Studies Using Pharmacological Tools and Genetically Modified Mouse Models. Front Endocrinol (Lausanne) 6, 189.

Liu, Q., Guan, X. M., Martin, W. J., McDonald, T. P., Clements, M. K., Jiang, Q., Zeng, Z., Jacobson, M., Williams, D. L., Jr., Yu, H., Bomford, D., Figueroa, D., Mallee, J., Wang, R., Evans, J., Gould, R., Austin, C. P., 2001. Identification and characterization of novel mammalian neuropeptide ff- like peptides that attenuate morphine-induced antinociception. J Biol Chem 276, 36961-36969.

Liu, X., Herbison, A. E., 2014. RF9 Excitation of GnRH Neurons Is Dependent Upon Kiss1r in the Adult Male and Female Mouse. Endocrinology 155, 4915-4924.

Mazarguil, H., Mollereau, C., Czaplicki, G., Zajac, J. M., 2012. Study of the N-terminal part of peptidic selective NPFF2 agonists. Peptides 37, 157-160.

Min, L., Leon, S., Li, H., Pinilla, L., Carroll, R. S., Tena-Sempere, M., Kaiser, U. B., 2015. RF9 Acts as a KISS1R Agonist In Vivo and In Vitro. Endocrinology 156, 4639-4648.

Murakami, M., Matsuzaki, T., Iwasa, T., Yasui, T., Irahara, M., Osugi, T., Tsutsui, K., 2008. Hypophysiotropic role of RFamide-related peptide-3 in the inhibition of LH secretion in female rats. J Endocrinol 199, 105-112.

Nicklous, D. M., Simansky, K. J., 2003. Neuropeptide FF exerts pro- and anti-opioid actions in the parabrachial nucleus to modulate food intake. Am J Physiol Regul Integr Comp Physiol 285, R1046-1054.

Oberling, P., Stinus, L., Le Moal, M., Simonnet, G., 1993. Biphasic effect on nociception and antiopiate activity of the neuropeptide FF (FLFQPQRFamide) in the rat. Peptides 14, 919-924.

Pineda, R., Garcia-Galiano, D., Sanchez-Garrido, M. A., Romero, M., Ruiz-Pino, F., Aguilar, E., Dijcks, F. A., Blomenrohr, M., Pinilla, L., van Noort, P. I., Tena-Sempere, M., 2010a. Characterization of the inhibitory roles of RFRP3, the mammalian ortholog of GnIH, in

the control of gonadotropin secretion in the rat: in vivo and in vitro studies. Am J Physiol Endocrinol Metab 299, E39-46.

Pineda, R., Garcia-Galiano, D., Sanchez-Garrido, M. A., Romero, M., Ruiz-Pino, F., Aguilar, E., Dijcks, F. A., Blomenrohr, M., Pinilla, L., van Noort, P. I., Tena-Sempere, M., 2010b. Characterization of the potent gonadotropin-releasing activity of RF9, a selective antagonist of RF-amide-related peptides and neuropeptide FF receptors: physiological and pharmacological implications. Endocrinology 151, 1902-1913.

Prokai, L., Zharikova, A. D., Juhasz, A., Prokai-Tatrai, K., 2006. Cardiovascular effects of neuropeptide FF antagonists. Peptides 27, 1015-1019.

Quillet, R., Ayachi, S., Bihel, F., Elhabazi, K., Ilien, B., Simonin, F., 2016. RF-amide neuropeptides and their receptors in Mammals: Pharmacological properties, drug development and main physiological functions. Pharmacol Ther 160, 84-132.

Rizwan, M. Z., Poling, M. C., Corr, M., Cornes, P. A., Augustine, R. A., Quennell, J. H., Kauffman, A. S., Anderson, G. M., 2012. RFamide-related peptide-3 receptor gene expression in GnRH and kisspeptin neurons and GnRH-dependent mechanism of action. Endocrinology 153, 3770-3779.

Roth, B. L., Disimone, J., Majane, E. A., Yang, H. Y., 1987. Elevation of arterial pressure in rats by two new vertebrate peptides FLFQPQRF-NH2 and AGEGLSSPFWSLAAPQRF-NH2 which are immunoreactive to FMRF-NH2 antiserum. Neuropeptides 10, 37-42.

Roumeas, L., Humbert, J. P., Schneider, S., Doebelin, C., Bertin, I., Schmitt, M., Bourguignon, J. J., Simonin, F., Bihel, F., 2015. Effects of systematic N-terminus deletions and benzoylations of endogenous RF-amide peptides on NPFF1R, NPFF2R, GPR10, GPR54 and GPR103. Peptides 71, 156-161.

Roumy, M., Zajac, J. M., 1998. Neuropeptide FF, pain and analgesia. Eur J Pharmacol 345, 1-11.

Sahin, Z., Canpolat, S., Ozcan, M., Ozgocer, T., Kelestimur, H., 2015. Kisspeptin antagonist prevents RF9-induced reproductive changes in female rats. Reproduction 149, 465-473.

Schneider, S., Ftouni, H., Niu, S., Schmitt, M., Simonin, F., Bihel, F., 2015. Rapid and scalable synthesis of innovative unnatural alpha,beta or gamma-amino acids functionalized with tertiary amines on their side-chains. Org Biomol Chem 13, 7020-7026.

Simonin, F., Schmitt, M., Laulin, J.-P., Laboureyras, E., Jhamandas, J. H., MacTavish, D., Matifas, A., Mollereau, C., Laurent, P., Parmentier, M., Kieffer, B. L., Bourguignon, J.-J., Simonnet, G., 2006. RF9, a potent and selective neuropeptide FF receptor antagonist, prevents opioid-induced tolerance associated with hyperalgesia. Proc. Natl. Acad. Sci. USA 103, 466-471.

Simonin, F., Valverde, O., Smadja , S., Slowe, S., Kitchen, I., Dierich, A., Le Meur, M., Roques, B. P., Maldonado, R., Kieffer, B. L., 1998. Disruption of the k-opioid receptor gene in mice enhances sensitivity to chemical visceral pain, impairs pharmacological actions of the selective k-agonist U-50,488H and attenuates morphine withdrawal. EMBO J. 17, 886-897.

Simonneaux, V., Ancel, C., Poirel, V. J., Gauer, F., 2013. Kisspeptins and RFRP-3 Act in Concert to Synchronize Rodent Reproduction with Seasons. Front Neurosci 7, 22.

Tena-Sempere, M., Pinilla, L., Aguilar, E., 1993. Follicle-stimulating hormone and luteinizing hormone secretion in male rats orchidectomized or injected with ethylene dimethane sulfonate. Endocrinology 133, 1173-1181.

Wang, Y. Q., Guo, J., Wang, S. B., Fang, Q., He, F., Wang, R., 2008. Neuropeptide FF receptors antagonist, RF9, attenuates opioid-evoked hypothermia in mice. Peptides 29, 1183-1190.

Xu, M., Kontinen, V. K., Panula, P., Kalso, E., 1999. Effects of (1DMe)NPYF, a synthetic neuropeptide FF analogue, in different pain models. Peptides 20, 1071-1077.

Yang, H. Y., Fratta, W., Majane, E. A., Costa, E., 1985. Isolation, sequencing, synthesis, and pharmacological characterization of two brain neuropeptides that modulate the action of morphine. Proc Natl Acad Sci U S A 82, 7757-7761.

Appendix A. Supplementary information

Supplementary information accompanies the paper on the Neuropharmacology website.

Figure S1 : Preliminary phenotyping analyses of mice receiving RF313 injections.

Figure legends

Figure 1: Chemical structures of RF313 and RF9 compounds.

Figure 2: Assessment of *in vitro* pharmacological properties of RF313 and RF9 on NPFF1R and Kiss1R.

A: Stimulation of $[^{35}S]$-GTPγS binding by RFRP-3 on membranes from CHO cells expressing human NPFF1 receptors. RF313 (10 µM) shifted the EC_{50} values for RFRP-3 from 98 ± 10 to 695 ± 95 nM. Data are expressed as percentages of maximal $[^{35}S]$-GTPγS binding and are mean $\pm$ S.E. values for two separate experiments in triplicate.

B: Reversal of RFRP-3-induced inhibition of forskolin-induced cAMP accumulation in CHO-hNPFF1R cells. RF313 (10 µM) shifted the EC_{50} values for RFRP-3 from 6.9 ± 1.1 to 47 ± 12 nM. Data are expressed as percentages of maximal cAMP levels and represent mean $\pm$ S.E.M values for four separate experiments in triplicate.

C: Effect of Kp-10, RF9 and RF313 on calcium mobilization in Kiss1R expressing cells. Peak response amplitudes were normalized to basal and maximal (digitonin-permeabilized cells) fluorescence levels. Data points are from a representative experiment performed in duplicate. $EC_{50} \pm$ S.E. values for Kp-10 (0.1 ± 0.025 nM) and RF9 (1.3 ± 0.5 nM) are from two experiments performed in triplicate.

Figure 3: RF313 blockade of RFRP-3-induced hyperalgesia in mice.

A: Intracerebroventricular injections of RFRP-3 (10 nmol), alone or in combination with RF313 (10 nmol), were performed as described under Materials and methods. Nociceptive thresholds were measured every 15 min post-injection using the tail immersion test at 48°C.

B: Comparison between groups of area-under-the-curve (AUC values) over the 0-90 min time course.

Data are expressed as mean ± S.E.M, *n* = 6–11. **p < 0.01 by Fisher's test as compared with the saline group. [###]p < 0.001 by Fisher's test as compared with the RFRP-3-treated group.

Figure 4: Effect of RF313 on analgesic and hyperalgesic responses of mice and rats to fentanyl.

A, B: On day 0 (grey area), a single dose of RF313 (5 mg/kg; s.c.) or saline was administrated to mice 20 min before fentanyl injections (4x60µg/kg; 15 min interval; s.c.). Nociceptive responses were measured using the tail immersion test (48°C) every 1 h after the last fentanyl injection until return to baseline and were repeated once daily from d1 to d4 Panel A). In a parallel experiment, RF313 (5 mg/kg; s.c.) was injected once to fentanyl-treated mice on day 1 (arrow), 30 min before daily measurement of the nociceptive threshold (dashed line, panel A). Hyperalgesia indexes (HI) were calculated as described in the Data and statistical analyses section (Panel B).

C, D, E, F: On day 0 (grey area), increasing doses of RF313 or saline were administrated to rats either subcutaneously (0.05, 0.1 and 0.5 mg/kg; panels **C**, **D**) or orally (0.3, 1 and 3 mg/kg; panels **E**, **F**). 30 min later, animals received four consecutive fentanyl injections (80 µg/kg; 15 min interval; s.c.). Nociceptive thresholds were measured using the paw pressure test : once daily before experiment start (d-2, d-1, d0) to verify baseline stability, every 2 h after the first fentanyl injection (d0) to monitor analgesia amplitude, and once daily (d1 to d6) to follow the development of hyperalgesia. Hyperalgesia indexes (HI) are presented as histograms in panels **D** and **F**.

Data are expressed as mean ± S.E.M, n = 6-11. **p < 0.01, ***p < 0.001 by Fisher's test as compared with the saline group. ##p < 0.01, ###p < 0.001 by Fisher's test as compared with the fentanyl-pretreated group.

Figure 5: Effect of RF313 on basal nociception and morphine analgesia in mice.

A: RF313 (2.5 or 5 mg/kg, s.c.) or saline was injected to mice 20 min before morphine (5 mg/kg, s.c.) or saline. Tail withdrawal latencies were measured at 52°C, at 30 min intervals, over a 150 min period after morphine injection.

B: Comparison between groups of area-under-the-curve (AUC values) over the 0-150 min time course. Data are expressed as mean ± S.E.M, n = 7–15. ***p < 0.01 by Fisher's test as compared with the saline group. ###p < 0.001 by Fisher's test as compared with the morphine-pretreated group.

Figure 6: Effect of RF313 on morphine-induced hyperalgesia and analgesic tolerance.

A, B: From d0 to d7, mice received daily injections of RF313 (5 mg/kg; s.c.) 20 min prior to morphine (5 mg/kg; s.c.), as reported under Materials and methods. Saline replaced either RF313 or morphine, according to groups. Basal nociceptive values were measured once daily before experiment start (d-1) and every day before treatment (d1 to d7), using the tail immersion test (48°C). **B**: Comparison between tested groups of AUC values calculated from d-1 to d7.

C: On day 0, the analgesic effect of the first morphine injection (5 mg/kg; s.c.) was monitored every 30 min over a 3 h period. Mice that received RF313 (5 mg/kg; s.c.) 20 min prior to morphine were similarly examined for their nociceptive responses, using the tail immersion test at 52°C.

D: On day 8, 24 h after the end of the chronic RF313/morphine treatment, mice received either saline or RF313 (5 mg/kg; s.c.) 20 min prior to saline or morphine (5 mg/kg; s.c.) injections. Nociceptive responses were determined using the tail immersion test (set at 52°C), according the time-course paradigm already defined in **C**.

E:Comparison between groups of calculated AUC values from C and D.

Data are expressed as mean ± S.E.M, $n = 5–10$. ***$p < 0.001$ by Fisher's test as compared with the saline group. [##]$p < 0.01$ by Fisher's test as compared with the morphine-pretreated group. [°°]$p < 0.01$, [°°°] $p < 0.001$ by Fisher's test as compared with the AUC value of the same group at d0.

Figure 7: Effects of central injection of RFRP-3 and of NPFF antagonists on LH secretion in the male Syrian hamster.

Drugs (1.5 µg) were injected i.c.v., alone or in combination, in a 2 µl final volume. 30 min later, the blood was sampled and assayed for LH content (ng/ml plasma) as described under Materials and methods. Mean ± S.E.M. values for 6 to 7 animals are presented as histograms. ***$p < 0.001$ by Fisher's test as compared with the saline group. [###]$p < 0.001$ by Fisher's test as compared with RFRP-3 treated group.

Table 1

Binding affinity properties of endogenous RF-amide peptides, RF313 and RF9 at the five RF-amide receptors.

Binding affinity constants (K_i values in nM) of drugs are from competition experiments performed as described under Materials and methods.

Mean values ± S.E.M. for at least 3 independent experiments performed in duplicate are reported.

Compound	NPFF1R	NPFF2R	PrRPR [a]	Kiss1R [a]	QRFPR [a]
			Binding affinity constants (nM)		
RFRP-3	0.17 ± 0.06	4.8 ± 1.8	> 20,000	> 20,000	> 20,000
NPFF	0.39 ± 0.15	0.14 ± 0.06	> 20,000	> 20,000	> 20,000
PrRP-20	4.7 ± 0.3 [a]	0.7 ± 0.1 [a]	0.06 ± 0.01	> 20,000	> 20,000
Kp-10	0.5 ± 0.1 [a]	1.6 ± 0.3 [a]	9,200 ± 1,200	0.51 ± 0.04	8,000 ± 120
26RFa	24 ± 1 [a]	3 ± 1 [a]	>10,000	> 20,000	21 ± 1
RF313	172 ± 13	560 ± 160	> 20,000	> 100,000	> 20,000
RF9	17 ± 8	9.3 ± 2.1	> 20,000	3,500 ± 740	> 20,000

[a] K_i values for agonists are from (Elhabazi et al., 2013)