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## Identification of an N -acylated- D Arg-Leu-NH<sub>2</sub> Dipeptide as a Highly Selective Neuropeptide FF1 Receptor Antagonist That Potently Prevents Opioid-Induced Hyperalgesia

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Raphaëlle Quillet, Séverine Schneider, Valérie Utard, Armand Drieu La Rochelle, Khadija Elhabazi, et al.. Identification of an N -acylated- D Arg-Leu-NH<sub>2</sub> Dipeptide as a Highly Selective Neuropeptide FF1 Receptor Antagonist That Potently Prevents Opioid-Induced Hyperalgesia. *Journal of Medicinal Chemistry*, 2021, 64 (11), pp.7555-7564. 10.1021/acs.jmedchem.1c00256 . hal-03363456

**HAL Id: hal-03363456**

**<https://hal.science/hal-03363456v1>**

Submitted on 18 Nov 2021

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**Identification of a *N*-acylated-<sup>D</sup>Arg-Leu-NH<sub>2</sub> dipeptide as a highly selective Neuropeptide FF1 receptor antagonist that potently prevents opioid-induced hyperalgesia.**

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## **Abstract**

RFamide-related peptide-3 (RFRP-3) and neuropeptide FF (NPFF) target two different receptor subtypes called Neuropeptide FF1 (NPFF1R) and Neuropeptide FF2 (NPFF2R) that modulate several functions. However, the study of their respective role is severely limited by the absence of selective blockers. We describe here the design of a highly selective NPFF1R antagonist called **RF3286**, which potently blocks RFRP3-induced hyperalgesia in mice and luteinizing hormone release in hamster. We then showed that pharmacological blockade of NPFF1R in mice prevents the development of fentanyl-induced hyperalgesia while preserving its analgesic effect. Altogether, our data indicate that RF3286 represent a useful pharmacological tool to study the involvement of NPFF1R/RFRP-3 system in different functions and different species. Thanks to this compound, we showed that this system is critically involved in the development of opioid-induced hyperalgesia suggesting that NPFF1R antagonists might represent promising therapeutic tools to improve the use of opioids in the treatment of chronic pain.

## **Introduction**

Chronic pain is a major health problem and a socio-economic burden. Despite the increasing diversity of available therapeutic approaches, opiates and their derivatives still represent the most effective means of treating severe pain across a wide range of conditions. However, repeated administration of opioids exacerbates adverse effects, such as pain hypersensitivity also known as opioid-induced hyperalgesia (OIH), which in turn impairs the analgesic efficacy (tolerance) and triggers the need for dose-escalation. High opioid consumption increases the risks of respiratory depression, addiction potential and constipation, which considerably affect the patient's quality of life and can lead to fatal consequences <sup>1</sup>. This is particularly true in the USA where almost half a million Americans have died from drug overdose (mainly opiates) since 2000 <sup>2</sup>. There is therefore a strong need to characterize novel therapeutic targets involved in the modulation of nociception.

RFamide-related peptide-3 (RFRP-3) and Neuropeptide FF (NPFF) belong to the family of so-called RFamide peptides, which display a typical Arg-Phe-NH<sub>2</sub> C-terminal sequence. They both display nanomolar affinities for two G protein-coupled receptor subtypes called NPFF1 and NPFF2 receptors <sup>3-6</sup>, and RFRP-3 is considered the cognate ligand for NPFF1R, while NPFF displays preferential binding affinity for and activity at NPFF2R <sup>6</sup>. Several lines of evidence suggest that both receptors and their endogenous ligands play a role in the modulation of nociception and opioid analgesia <sup>7,8</sup>, but their respective involvement in these functions is still unclear. NPFF was shown to display opposite effects (pro-nociceptive and anti-morphine versus anti-nociceptive) depending on the route of administration <sup>9</sup> and few studies reported that RFRP-3 leads to a decrease of the nociceptive threshold whereas its effect on morphine analgesia remains controversial <sup>10-12</sup>, highlighting the need to develop highly selective antagonists for NPFF1 and NPFF2 receptor subtypes, respectively.

In 2006, the dipeptide RF9 was introduced as the first antagonist endowed with a high affinity for both NPPF receptor subtypes<sup>13</sup>. Numerous experiments proved its efficacy in opposing opioid-induced hyperalgesia and in reducing the nociceptive response to RFamide peptides in various *in vivo* pain models in rodents<sup>10, 11, 13, 14</sup>. Nevertheless, RF9 exhibited no NPPF1/NPPF2 receptor selectivity and turned also to be a Kiss1R agonist, albeit with moderate affinity and efficacy<sup>6, 15, 16</sup>. Several studies have previously described peptidic or heterocyclic molecules that display good affinity for, and antagonist activity at NPPF receptors (for review see<sup>6</sup>). However, none of these compounds displayed a level of selectivity towards NPPF1R or NPPF2R that was good enough ( $\geq 100$ ) to allow to efficiently discriminating between both receptor subtypes *in vivo*. Thus, the discovery of highly selective antagonists is mandatory to examine under safe conditions the contribution of NPPF1R/RFRP and NPPF2R/NPPF systems not only in the modulation of pain but also in the regulation of the hypothalamic-pituitary-gonadal axis and their relationship with the kisspeptin system<sup>6, 17-22</sup>. Our expertise in RF9 Structure-Activity Relationship (SAR) studies led us to consider the importance of the dipeptide Arg-Phe-NH<sub>2</sub> to modulate both selectivity and antagonistic nature at NPPF1 and NPPF2 receptors<sup>23</sup>. Based on this rational approach, we describe here the identification and characterization of the first highly selective NPPF1R antagonist. We further used this compound to study the role RFRP-3/NPPF1R in the modulation of nociception and opioid analgesia.

## Results

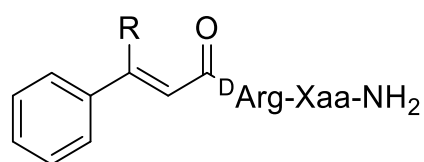
### ***Identification and characterization of the first highly selective antagonist for NPPF1R***

In this study we sought to identify small and highly selective compounds for NPPF1R in order to study the function of this receptor *in vivo*. To this goal, we started from two previously described Arg-Phe-NH<sub>2</sub> dipeptide derivatives (cpd 37 and cpd 38 in<sup>23</sup>), both

containing a D-arginine as well as a cinnamoyl moiety at the N-terminus part, and that already displayed good affinity and some selectivity for hNPFF1R. As shown in Table 1, these two compounds (cp37 and cp38), showed a selectivity of 24 fold and 29 fold respectively for hNPFF1R vs hNPFF2R when measured in competition experiments with [<sup>125</sup>I]-1DMeNPFF as a radioligand <sup>24</sup> in recombinant CHO cells. The addition of a second aromatic ring at the beta position of the cinnamoyl moiety led to two novel compounds (**1** and **2**, Table 1) with enhanced affinity and selectivity for recombinant human NPFF1 or NPFF2 receptors. Both compounds displayed nanomolar affinity for hNPFF1R and a 50 to 100 fold lower affinity for hNPFF2R. By comparison, in the same experiments **RF9** displayed no selectivity and **GJ14**, a previously reported selective NPFF1R compound <sup>15</sup>, showed only a 4 fold lower affinity for NPFF2R (Table 1). To further improve the selectivity of these compounds, we synthesized a series of dipeptides in which Phe was replaced by different L- or D-amino acids (Table 1). The substitution of Phe at the C-terminus by another aromatic amino acid (**3-8**) maintains a good affinity for NPFF1R in the 1 to 30 nM range but tends to decrease selectivity vs. NPFF2R (selectivity ratio <20). D-Trp (**6**) and hPh (**3**) derivatives exhibited the best selectivity profile with ratios of 42 and 84, respectively, but still remained below **2**. The replacement of Phe by polar amino acids such as Gln (**15**) or Asn (**16**) showed less affinity toward NPFF1R with Ki values of 58 nM and 132 nM, respectively. Eventually, the replacement of Phe by aliphatic amino acids (**9-14**, **RF3286** and **RF3287**) led to the most interesting molecules in terms of selectivity between NPFF1R & NPFF2R. Among them, the presence of a glycine (**RF3287**) or a L-leucine (**RF3286**) at the N-terminus led to a significant improvement in comparison with initial phenylalanine. Indeed, **RF3287** and **RF3286** exhibited high affinity for NPFF1R with Ki of 11.2 ± 1.3 nM and 4.7 ± 0.7 nM, respectively, while presenting micromolar affinities for NPFF2R (Table 1, and Figure S3 in the Supporting Information). The selectivity ratio in favor of NPFF1R was estimated at 150 for **RF3287** and

471 for **RF3286**, making them the most selective ligands of NPFF1R ever described. Interestingly, **RF3286** and **RF3287** display a leucine and a glycine residue at their C-terminus, respectively, which have never been identified in SAR studies on NPFF peptide.

**Table 1: binding affinity constants at human NPFF1 and NPFF2 receptors of the different compounds designed and characterized in this study**



Cpd	R	Xaa	Binding affinity constant		Selectivity
			(Ki, nM)		Ki R2 / Ki R1
<b>Cpd37<sup>a</sup></b>	H	<sup>l</sup> Phe	7.2 ± 2.8	175 ± 45	24
<b>Cpd38<sup>a</sup></b>	H	<sup>D</sup> Phe	73 ± 15	2,150 ± 154	29
<b>1</b>	Ph	<sup>l</sup> Phe	4.3 ± 0.3	205 ± 55	48
<b>2</b>	Ph	<sup>D</sup> Phe	7.2 ± 1.0	727 ± 130	101
<b>3</b>	Ph	<sup>h</sup> Ph	6.6 ± 1.0	557 ± 220	84
<b>4</b>	Ph	<sup>l</sup> Phe(2Br)	0.73 ± 0.03	3.3 ± 0.9	5
<b>5</b>	Ph	<sup>D</sup> Phe(2Br)	10 ± 1	84 ± 11	8
<b>6</b>	Ph	<sup>D</sup> Trp	6.3 ± 0.6	267 ± 88	42
<b>7</b>	Ph	<sup>l</sup> Trp	21 ± 3	153 ± 187	7
<b>8</b>	Ph	<sup>l</sup> Tyr	32 ± 8	512 ± 80	16
<b>RF3287</b>	Ph	Gly	11.2 ± 1.3	1,685 ± 257	150
<b>9</b>	Ph	<sup>l</sup> Ala	79 ± 5	6,035 ± 3,735	76
<b>10</b>	Ph	<sup>D</sup> Ala	16 ± 10	557 ± 122	35
<b>11</b>	Ph	<sup>l</sup> Val	68 ± 42	4,775 ± 1,562	70

<b>RF3286</b>	Ph	<sup>1</sup> Leu	4.7 ± 0.7	2,213 ± 163	471
<b>12</b>	Ph	<sup>0</sup> Leu	523 ± 672	3,045 ± 1963	6
<b>13</b>	Ph	<sup>1</sup> Ile	39 ± 9	1,492 ± 338	38
<b>14</b>	Ph	<sup>1</sup> Cha	591 ± 664	1,113 ± 674	2
<b>15</b>	Ph	<sup>1</sup> Gln	58 ± 15	1,742 ± 380	30
<b>16</b>	Ph	<sup>1</sup> Asn	132 ± 46	4,838 ± 1,125	37
<b>RF9</b>			14	18	1.3
<b>GJ14</b>			58 ± 2	255 ± 5	4
<b>RFRP-3</b>			0.01	0.9	90
<b>NPFF</b>			0.06	0.02	0.3

Binding affinity constants (K<sub>i</sub>) are from competition experiments performed with [<sup>125</sup>I]-1DMeNPFF according to material and methods. K<sub>i</sub> values are means ± SEM for of least 2 independent experiments performed in duplicate. hPh : Homophenylalanine

We further confirmed the high affinity and high selectivity of the three most promising ligands **2**, **RF3286** and **RF3287** on recombinant mouse mNPFF1 versus mNPFF2 receptors (Table 2 and Figure S3 in the Supporting Information).

**Table 2: Binding affinity constants of 2, RF3286 and RF3287 for mouse NPFF1 and NPFF2 receptors**

<b>Compound</b>	<b>mNPFF1R</b>	<b>mNPFF2R</b>	<b>Selectivity ratio</b>
	<b>Binding affinity constant (K<sub>i</sub>, nM)</b>		<b>Ki R2 / Ki R1</b>
<b>2</b>	22 ± 2	1,450 ± 150	166



<b>RF3286</b>	8 ± 1	4,300 ± 100	538
<b>RF3287</b>	45 ± 6	2,750 ± 50	61
<b>GJ14</b>	78 ± 18	824 ± 77	11
<b>RFRP-3</b>	0.19 ± 0.03	nd	
<b>NPFF</b>	nd	1.1 ± 0.3	

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Binding affinity constants ( $K_i$ ) are from competition experiments performed with [ $^{125}$ I]-1DMeNPFF according to material and methods. Data were obtained from at least two independent experiments performed in duplicates.  $K_i$  values are mean ± S.E.M. Nd: not determined

We next evaluated the selectivity of these compounds toward the other human RFamide receptors, prolactin releasing peptide receptor (PrRPR or GPR10), Kiss1R (GPR54) and pyroglutamylated RFamide peptide receptor (QRFPR or GPR103) as well as the opioid receptors, MOPr, DOPr, KOPr and NOPr. All the binding affinity studies were performed using specific radioligands in competition tests as described in the experimental section. As shown in Table 3, they displayed almost no affinity for the three RFamide receptors ( $K_i > 5$ -50  $\mu$ M). **RF3286** and **RF3287** also displayed a very low affinity for the four opioid receptors ( $K_i > 5$ -50  $\mu$ M) while **2** showed a detectable affinity for MOPr, KOPr and NOPr in the micromolar range ( $K_i > 1 \mu$ M).

**Table 3: Binding affinity constants of 2, RF3286 and RF3287 at the three other human RFamide receptors and the four human opioid receptors.**

Compound	RFamide receptors			Opioid receptors			
	PrRPR	Kiss1R	QRFPR	MOPr	DOPr	KOPr	NOPr
	Binding affinity constants (K <sub>i</sub> , nM)			Binding affinity constants (K <sub>i</sub> , nM)			
<b>PrRP-20</b>	2.1 ± 0.4 <sup>a</sup>	Nd	Nd	Nd	Nd	Nd	Nd
<b>Kp-10</b>	Nd	0.062 ± 0.009 <sup>a</sup>	Nd	Nd	Nd	Nd	Nd
<b>26RFa</b>	Nd	Nd	2.04 ± 0.58 <sup>a</sup>	Nd	Nd	Nd	Nd
<b>DAMGO</b>	Nd	Nd	Nd	14.6 ± 3.8 <sup>a</sup>	Nd	Nd	Nd
<b>DPDPE</b>	Nd	Nd	Nd	Nd	57.1 ± 18.8 <sup>a</sup>	Nd	Nd
<b>Naloxone</b>	Nd	Nd	Nd	Nd	Nd	8.4 ± 4.3 <sup>a</sup>	Nd
<b>Nociceptin</b>	Nd	Nd	Nd	Nd	Nd	Nd	0.0013 ± 0.0002 <sup>a</sup>
<b>2</b>	> 50,000	> 50,000	> 5,000	> 1,000	> 5,000	>1,000	> 1,000
<b>RF3286</b>	> 50,000	> 50,000	> 50,000	> 5,000	> 50,000	> 10,000	> 5,000
<b>RF3287</b>	> 50,000	> 50,000	> 50,000	> 5,000	> 50,000	> 20,000	> 50,000

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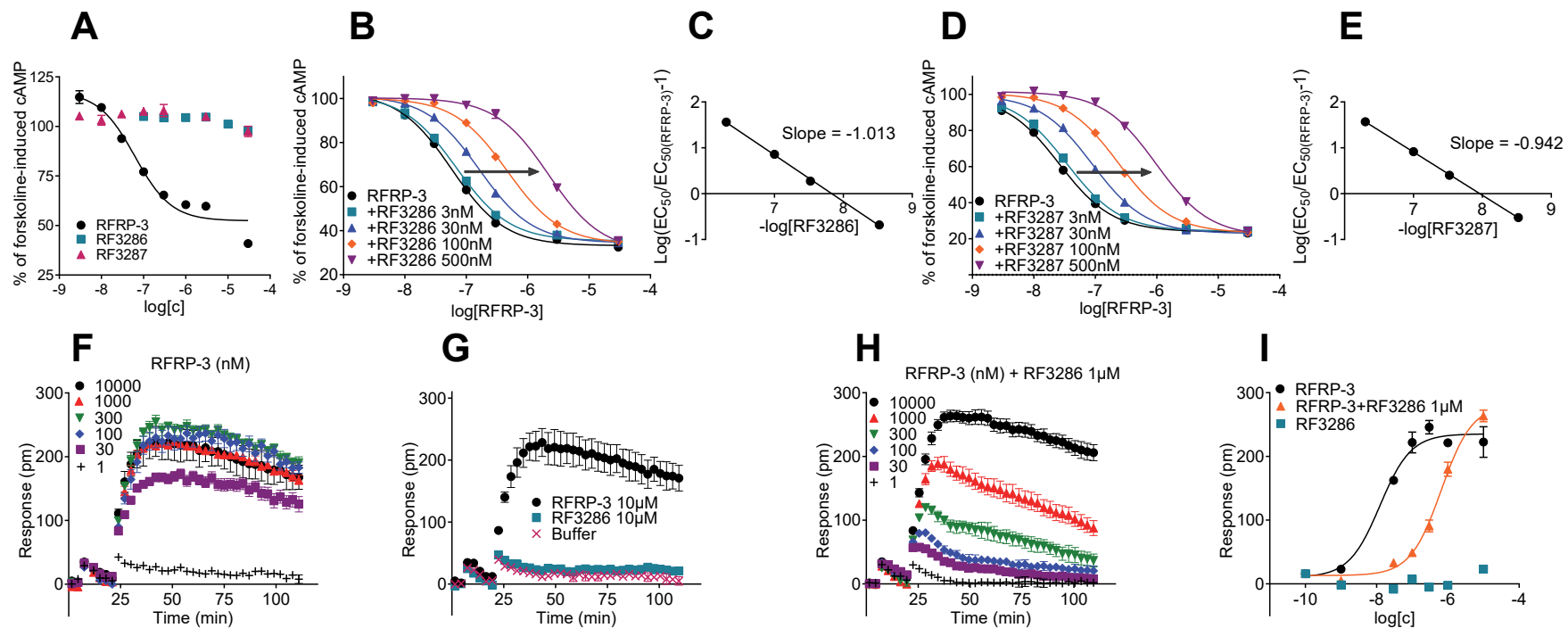
<b>GJ14</b>	ND	>5,000	ND	>10,000	>30,000	>1,000	>30,000
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Binding affinity constants (K<sub>i</sub>) are from competition experiments performed with specific radiolabelled ligands as described in material and methods. Data are means ± S.E.M. of at least 2 independent experiments performed in duplicate. <sup>a</sup>from <sup>25</sup>.

Nd: not determined

Based on these results we selected **RF3286** and **RF3287** for further evaluations in different functional assays with HEK293 cells stably expressing human NPFF1R. We first evaluated whether these compounds displayed agonist activity toward Gi protein by measuring their capacity to decrease the forskolin-stimulated cAMP level in those cells. As shown in Figure 1A, the NPFF1R endogenous agonist RFRP-3 efficiently decreased cAMP level with an EC<sub>50</sub> around 50 nM while **RF3286** and **RF3287** had no effect up to 10 μM. We further showed that both compounds induced a rightward shift of the dose-response curve of RFRP-3 in a potent and dose-dependent manner, displaying a K<sub>b</sub> of 16 ± 2 nM (pA<sub>2</sub> = 7.8, **RF3286** Figures 1B and 1C) and 20 ± 5 nM (pA<sub>2</sub> = 7.7, **RF3287** Figures 1D and 1E). For both compounds the slope of the schild plot was close to -1 indicating that they are both competitive antagonists (Figures 1C and 1E). We also confirmed the antagonist activity of **RF3286** in a GTPγS assay with membranes from CHO cells stably expressing hNPFF1R and found a K<sub>b</sub> value of 32 nM (pA<sub>2</sub> = 7.5, Figure S4 in the Supporting Information). We then studied the activity of **RF3286** on hNPFF1R HEK293 cells in a label-free dynamic mass redistribution assay (DMR). As shown in Figure 1F, the agonist RFRP-3 induced a dose-dependent response with an EC<sub>50</sub> around 30 nM. **RF3286** induced no signal in this assay up to 10 μM (Figure 1G) and the activity of RFRP3 was clearly attenuated in presence of 1 μM **RF3286** with a rightward shift of 53 fold in the dose-response curve (Figures 1H and 1I).



**Figure 1: *In vitro* functional characterization of RF3286 and RF3287.**

**A:** Inhibition of forskolin-induced cAMP accumulation in HEK293-Glo hNPFF1R cells induced by RFRP-3 or **RF3286** and **RF3287** alone.

**B-E:** Inhibition of forskolin-induced cAMP accumulation in HEK293-Glo hNPFF1R cells induced by RFRP-3 alone or in presence of different concentrations of **RF3286** (**B**) or **RF3287** (**D**). Schild plot for **RF3286** and **RF3287** are shown in **C** and **E**, respectively.

**F-I:** Experiments of dynamic mass redistribution (DMR) induced by increasing dose of RFRP-3 alone (**F**), **RF3286** alone (**G**) and RFRP-3 with **RF3286** 1 $\mu$ M (**H**, **I**) in HEK293-hNPFF1R cells.

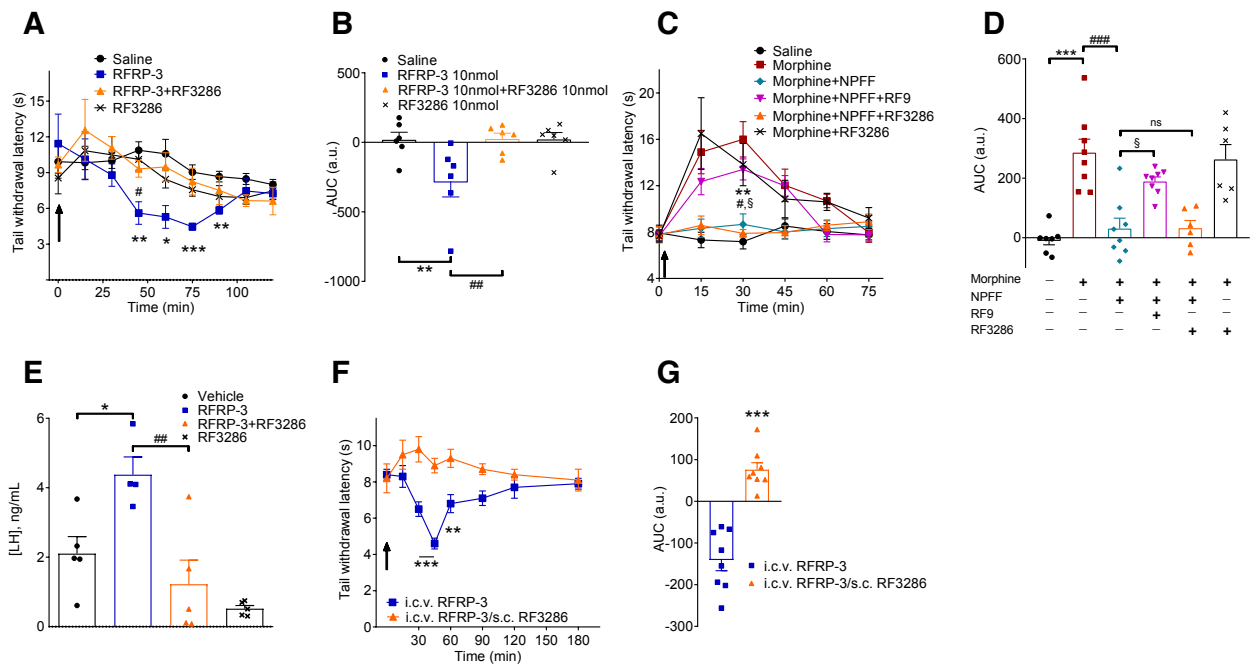
All experiments were performed at least two times in duplicates.

We also investigated the activity of **RF3286** in HEK-293 cells expressing human NPPF2R. As expected from binding experiments this compound displayed neither agonist nor antagonist activity on those cells up to 10  $\mu$ M (Figure S5A in the Supporting Information). To complete the selectivity profile of these two compounds, we tested them in functional calcium assays with recombinant CHO cells expressing Kiss1, PrRP or QRFP receptors that are coupled to Gq protein. As expected from binding experiments, **RF3286** and **RF3287** displayed no or very low activity at these 3 receptors (Figure S5B-5D, in the Supporting Information). Overall, our *in vitro* data show that we have identified two highly potent and highly selective NPPF1R antagonists. As **RF3286** displayed a better selectivity than **RF3287** toward human and mouse NPPF2R and a very good selectivity toward the other RFamide and opioid receptors, we selected this compound for further *in vivo* characterization.

#### ***RF3286 blocks the effect of RFRP-3 but not of NPPF in mice***

We first evaluated the capacity of **RF3286** to antagonize RFRP-3-induced hyperalgesia in mice following i.c.v. administration. As shown in Figures 2A and 2B, when administered alone, **RF3286** (10 nmol, i.c.v.) had no effect on the nociceptive threshold while RFRP-3 (10 nmol, i.c.v.) induced a strong and significant decrease of tail withdrawal latency of mice. When co-administered with RFRP-3 at the same dose, **RF3286** completely prevented the decrease of the basal nociceptive threshold (Figures 2A and 2B), showing that this compound displays antagonist activity at NPPF1R *in vivo*. In order to test the *in vivo* selectivity of **RF3286**, we next studied its effect on the anti-opioid analgesia induced by the endogenous NPPF2R agonist NPPF in mice. When administered i.c.v., morphine (1.5 nmol) caused analgesia that was significantly inhibited by NPPF (Figures 2C and 2D). As expected, nonselective NPPF1/2R antagonist RF9 (10 nmol, i.c.v.) efficiently reversed the effect of NPPF on morphine analgesia<sup>10</sup> while **RF3286** (10 nmol, i.c.v.) did not (Figs. 2C and 2D).

These data suggest that NPFF action on morphine analgesia is mediated by NPFF2R and not through NPFF1R and confirm that **RF3286** is selective for NPFF1R vs NPFF2R.



**Figure 2: *In vivo* selectivity of RF3286.**

**A:** Intracerebroventricular injections of RFRP-3 (10 nmol) and **RF3286** (10 nmol) alone or in combination were performed in mice, and nociceptive threshold of the animals was measured every 15 minutes post-injection using the tail immersion test. Data are expressed as mean  $\pm$  SEM,  $n = 6$ . \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$  between Saline and RFRP-3 groups and # $p < 0.05$  between RFRP-3 and RFRP-3 + **RF3286** groups by two-way ANOVA test followed by a Bonferroni *post-hoc* test.

**B:** Comparison of AUC between the different groups shown in **A** over the total time-course. Data are expressed as mean  $\pm$  SEM,  $n = 6$ . \*\* $p < 0.01$  between Saline and RFRP-3 groups and ### $p < 0.01$  between RFRP-3 and RFRP-3 + **RF3286** groups by one-way ANOVA test followed by a Bonferroni *post-hoc* test.

**C:** Intracerebroventricular injection of morphine (1.5 nmol) and blockade of the morphine analgesia by the co-administration of NPFF (10 nmol). Effect of RF9 (10 nmol, i.c.v.) and



**RF3286** (10 nmol, i.c.v.) on the prevention of morphine analgesia by NPFF. Data are expressed as mean  $\pm$  SEM, n = 6-8. \*\*p < 0.01 between Morphine and Saline groups, #p < 0.05 between Morphine and Morphine + NPFF groups and §p < 0.05 between Morphine + NPFF and Morphine + NPFF + RF9 by two-way ANOVA test followed by a Bonferroni *post-hoc* test.

**D:** Comparison of AUC between the different groups shown in **C** over the total time-course. Data are expressed as mean  $\pm$  SEM, n = 6-8. \*\*\*p < 0.001 between Morphine and Saline groups, ###p < 0.001 between Morphine and Morphine + NPFF groups and §p < 0.05 between Morphine + NPFF and Morphine + NPFF + RF9 by one-way ANOVA test followed by a Bonferroni *post-hoc* test.

**E:** Effects of central injection of RFRP-3 and **RF3286** on LH secretion in the male Syrian hamster. RFRP-3 and **RF3286** (10 nmol) were injected i.c.v., alone or in combination, in a 2  $\mu$ L final volume. 30 min later, the blood was sampled and assayed for LH content (ng/mL plasma) as described in Materials and methods. Data are expressed as mean  $\pm$  SEM, n = 4-5. \*p < 0.05 between RFRP-3 and Vehicle groups and ##p < 0.01 between RFRP-3 and RFRP-3 + **RF3286** groups by one-way ANOVA followed by Bonferroni *post-hoc* test.

**F:** Intracerebroventricular injections of RFRP-3 (10 nmol), alone or in combination with subcutaneous administration of **RF3286** (5 mg/kg), were performed in mice and nociceptive threshold of the animals was measured at different time intervals during 3h using the tail immersion test. Data are expressed as mean  $\pm$  SEM, n = 8. \*\*\*p < 0.001 and \*\*p < 0.01 between RFRP-3 and RFRP-3 + **RF3286** groups by two-way ANOVA test followed by a Bonferroni *post-hoc* test (**F**).

**G:** Comparison of AUC between the two groups shown in **F** over the total time-course. Data are expressed as mean  $\pm$  SEM, n = 8. \*\*\*p < 0.001 by one-way ANOVA test followed by a Bonferroni *post-hoc* test.

### ***RF3286 blocks the effect of RFRP-3 in male Syrian hamster***

In order to fully confirm the antagonist activity of **RF3286** toward NPFF1R *in vivo*, we sought to investigate its capacity to reverse a RFRP-3 action that is not related to nociception and in a non-murine mammalian species. As several studies have shown that RFRP-3 is able to provoke luteinizing hormone (LH) release in male Syrian hamster<sup>26,27</sup>, we studied **RF3286** activity in this model. We first evaluated the consequences of the administration of **RF3286** alone on LH release. As shown in Figure 2E, **RF3286** (10 nmol, i.c.v.) did not increase LH blood levels as compared to vehicle treated animals while we have shown previously that RF9 strongly increased LH blood levels due to its Kiss1R agonist activity<sup>28</sup>. This result clearly shows that conversely to RF9, **RF3286** at the dose used did not display any Kiss1R agonist activity. As expected, acute central injection of RFRP-3 (10 nmol, i.c.v.) stimulated the blood release of LH and **RF3286** fully blocked this effect (Figure 2E). Altogether, these data highlight the involvement of RFRP-3/NPFF1R in the control of LH blood levels in male Syrian hamster and further confirm that **RF3286** is a highly selective NPFF1R receptor antagonist that will be useful to study the different functions that are modulated by the RFRP-3/NPFF1R system in different species.

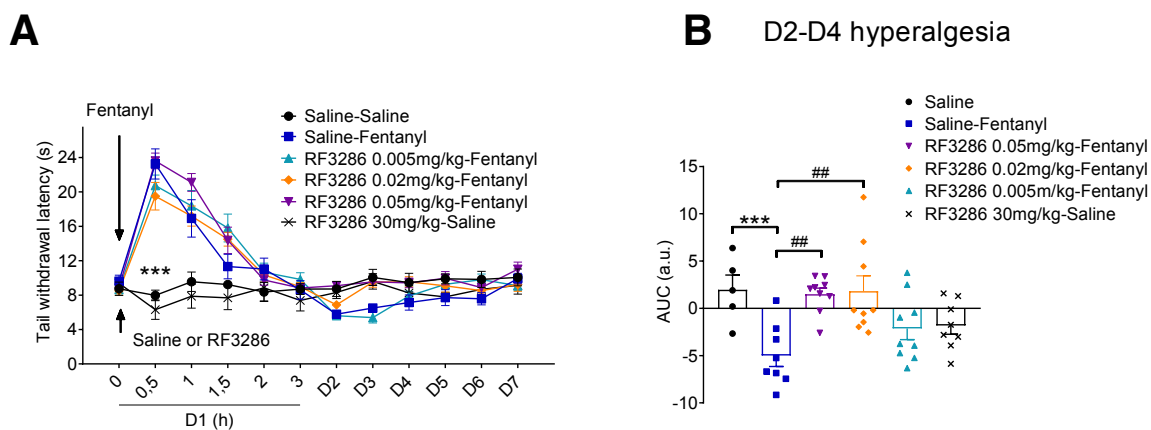
### ***Peripheral administration of RF3286 blocks the effect of central administration of RFRP3***

As shown in Figures 2F and 2G, subcutaneous administration of **RF3286** (5 mg/kg) efficiently prevented hyperalgesia induced by the central administration of 10 nmol of RFRP-3 indicating that this compound can cross the blood- brain-barrier to block central action of RFRP-3. We further evaluated the pharmacokinetic parameters of **RF3286** in mouse plasma. When injected subcutaneously in mice (5 mg/kg, s.c.), we observed a peak concentration of

the compound 10 minutes after its administration, with a half-life of about 30 minutes (Figure S6 in the Supporting Information).

### ***Pharmacological blockade of NPFF1R prevents opioid-induced hyperalgesia***

We next investigated the consequences of NPFF1 receptor blockade with **RF3286** on acute fentanyl-induced analgesia and secondary hyperalgesia. As shown previously<sup>28</sup>, when injected subcutaneously in mice, fentanyl (4 x 60 µg/kg) induced a short lasting analgesia (Figure 3A) that was followed on days 2 and 3 by a significant decrease of the nociceptive threshold of the animals indicating that they displayed pain hypersensitivity (Figures 3A and 3B). When administered alone at a high dose (30 mg/kg, s.c.), **RF3286** had no effect on the nociceptive threshold of the animals. When co-administered at low doses (from 0.005 to 0.05 mg/kg) with fentanyl, **RF3286** did not modify fentanyl-induced analgesia but very efficiently prevented the secondary hyperalgesia induced by fentanyl with an ED<sub>50</sub> of approximately 0.01mg/kg (Figures 3A and 3B).



**Figure 3: Effect of RF3286 on acute and chronic administration of opiates.**

**A:** Effect of **RF3286** on analgesic and hyperalgesic responses of mice to fentanyl. On day 1, different doses of **RF3286** (0.005 to 0.05 mg/kg, s.c.) were administered to mice 20 minutes before fentanyl injections (4 x 60 µg/kg; 15 min interval; s.c.). Nociceptive response of the

animals was measured using the tail immersion test every 30 min after fentanyl injection until return to baseline and then once daily from D2 to D7. Data are expressed as mean  $\pm$  SEM, n = 8-9. \*\*\*p < 0.001 between Fentanyl and Saline groups by two-way ANOVA followed by a Bonferroni *post-hoc* test.

**B:** Comparison of AUC for each group shown in **A** were calculated from D2 to D4. Data are expressed as mean  $\pm$  SEM, n = 8-9. \*\*\*p < 0.001 between Fentanyl and Saline groups and ##p < 0.01 between Fentanyl and Fentanyl + **RF3286** (0.02 mg/kg or 0.05 mg/kg) groups by one-way ANOVA followed by a Bonferroni *post-hoc* test.

## Discussion and Conclusions

We describe here the discovery and characterization of **RF3286**, the first high affinity antagonist for NPFF1R that displays a high selectivity *in vitro* and *in vivo* towards other RFamide receptors and opioid receptors. We showed that **RF3286** displays a selectivity of about 500 fold for human and mouse NPFF1R versus NPFF2R. Moreover, we showed that in mice **RF3286** efficiently reversed RFRP-3 induced hyperalgesia while it had no effect on NPFF-induced reversion of morphine analgesia. These results clearly demonstrate that **RF3286** can efficiently block NPFF1R both *in vitro* and *in vivo* without blocking NPFF2R and is therefore a useful pharmacological tool to delineate the role of NPFF1R in the modulation of different functions, particularly those in which NPFF1/2 receptor subtypes have been involved such as the modulation of nociception and opioid analgesia.

Another issue to consider when developing a novel NPFF1R antagonist is the selectivity, particularly towards the kisspeptin receptor. Indeed, previous studies aiming at understanding the role of NPFF1R in the modulation of reproduction have used the nonselective NPFF1/2R antagonist RF9<sup>13</sup> in *in vivo* experiments<sup>29-31</sup>. In these studies, RF9

was shown to stimulate LH release suggesting that NPF1R receptors and most likely NPF1R subtype, which is expressed in Gonadotropin-releasing hormone neurons, tonically inhibits LH release and that blocking this tonic inhibition leads to an increase of LH. This conclusion was later questioned by several studies reporting some agonist activity of RF9 at the kisspeptin receptor<sup>15, 16</sup>. Although this activity was observed at micromolar concentrations, Min and collaborators clearly demonstrated that *in vivo*, RF9 stimulation of LH release was mainly due to its agonist activity at Kiss1R<sup>16</sup>. In this study, we therefore examined the affinity of **RF3286** at the PrRP, the QRFP and the kisspeptin receptors and observed no or very low (> 50  $\mu$ M) affinity for these three RFamide receptors. We further examined its activity at these receptors and detected no Gq signaling on both PrRP and QRFP receptors while we observed a slight agonist activity at kisspeptin receptor at 10  $\mu$ M. In order to address this issue *in vivo*, we evaluated the activity of **RF3286** in hamsters in conditions in which RF9 was previously shown to strongly stimulate LH release<sup>28</sup>. No stimulatory effect of **RF3286** was observed (see Fig. 3E) indicating that it did not display any agonist activity at the kisspeptin receptor under these conditions. Moreover, we observed that **RF3286** efficiently blocked the activity of RFRP-3 on LH release showing that **RF3286** is a useful pharmacological tool to study the involvement of NPF1R in the modulation of reproduction without interfering with the kisspeptin receptor function.

We have used **RF3286** to study the role of the RFRP-3/NPF1R system in the modulation of nociception and opioid analgesia. Previous studies suggested that both RFRP-3/NPF1R and NPF/NPF2R systems are involved in the modulation of these functions but the absence of selective antagonists did not allow to clearly delineate their respective contribution (see<sup>7</sup>). In agreement with our previous observations<sup>10</sup>, we show in this study that i.c.v. administration of RFRP-3 in mice induced a strong decrease of the nociceptive threshold, which was fully blocked by **RF3286** indicating that this effect is mediated by

NPFF1R. We confirmed the pronociceptive action of RFRP-3/NPFF1R system in an *in vivo* experiment in which hyperalgesia was induced by acute opioid administration (fentanyl), and we observed that NPFF1R receptor blockade with **RF3286** fully prevented the development of opioid-induced hyperalgesia.

In conclusion, we describe in this study the design of a highly selective NPFF1R antagonist that allowed us to identify the RFRP-3/NPFF1R system as an important player in the neuroadaptations that occur upon exogenous opioid administration particularly in the nociceptive pathways. Our data further point to selective NPFF1R antagonists as potential therapeutic tools to improve the effect of opiates for the treatment of chronic pain.

## 5. Experimental section

### *Materials*

Human RFamide peptides were from Polypeptide (Strasbourg, France; Kp-10), GeneCust (Boynes, France; NPFF, RFRP-3, PrRP-20), Peptanova (Sandhausen, Germany ; 43RFa) and Tebu-Bio (Le Perray-en-Yvelines, France ; 26RFa and RFRP-3). Mouse RFRP-3 was from Genecust (Luxembourg). Orphanin FQ (nociceptin), fentanyl citrate, naltrexone hydrochloride, forskolin, 3-isobutyl-1-methylxanthine (IBMX), probenecid and Complete Freund's Adjuvant (CFA) were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Fluo-4 acetoxymethyl ester was from Molecular Probes (Invitrogen, Cergy Pontoise, France). D-luciferin was purchased from Synchem (Felsberg-Altenburg, Germany). N-adamantanecarbonyl-Arg-Phe-NH<sub>2</sub> trifluoroacetate (RF9) was synthesized as reported<sup>13</sup>. [<sup>3</sup>H]-PrRP-20 (150 Ci/mmol) was from American Radiolabeled Chemicals (Saint Louis, MO, USA). [<sup>35</sup>S]Guanosine 5'-O-[g-thio] triphosphate ([<sup>35</sup>S]GTPγS; 1250 Ci/mmol) and [<sup>125</sup>I]-1DMe-NPFF (2200 Ci/mmol), were from Hartmann Analytic (Braunschweig, Germany). [<sup>125</sup>I]-Kp-10 (2200 Ci/mmol), [Leucyl-<sup>3</sup>H]-nociceptin (42 Ci/mmol) and [<sup>3</sup>H]-Diprenorphine (42 Ci/mmol) were purchased from Perkin Elmer Life and Analytical Sciences (Courtaboeuf, France). [<sup>125</sup>I]-QRFP43 (2200 Ci/mmol) was from Phoenix Pharmaceuticals (Strasbourg, France).

### *General solid-phase peptide synthesis procedures*

Fmoc-protected natural amino acids were purchased from commercial suppliers. All chemicals and solvents were used without further purification. All peptides were synthesized by manual solid-phase synthesis by using a Fmoc strategy with a Rink-amide resin SS (Advanced ChemTech, 0.70 mmol/g). The side-chain protective groups included t-butyl (tBu) for tyrosine, t-butoxycarbonyl (Boc) for lysine and tryptophane, Trityl (Trt) for histidine, asparagine and glutamine, and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for

arginine. Fmoc deprotection was performed by using a solution of 2% piperidine and 2% DBU in DMF at rt during 40 min. With natural amino acids, coupling reactions were performed by the addition of N,N-diisopropylethylamine (DIEA, 6 equiv), Fmoc-protected amino acid or carboxylic acid (3 equiv), and 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU, 2.9 equiv) in DMF, and the mixture was agitated for 1 h at rt. All coupling reactions were monitored by Kaiser test. Resin cleavage was performed twice by using a TFA/thioanisol/triisopropylsilane (92:5:3) solution at rt during 90 min. The mixture was filtered, and the peptide solution was concentrated in vacuo, and precipitated by adding cold diethyl ether. The mixture was centrifuged, and the resulting crude peptide was purified by flash chromatography (Simply connect C18 column (50g, 40 – 60  $\mu$ m) from AIT; Phase A: 0.05% TFA in water; B : MeOH; eluent : phase B : from 10 to 100% in 30 min). Final compounds were recovered with purity > 95% by HPLC.

#### ***Pharmacokinetic study of RF3286 in mice***

5mg/kg of RF3286 was injected subcutaneously (s.c.) in C57BL/6N mice and blood was drawn by intracardiac puncture at different time points (n = 3 per point), from 5 minutes to 2 hours. Blood samples were then placed into EDTA-coated tubes and centrifuged at 12,000g (4°C), during 10 minutes and the supernatant were collected and immediately placed at -80°C. For the detection of RF3286, 40  $\mu$ L of each plasma sample was mixed with 100  $\mu$ L of acetonitrile to precipitate and extract the compound. Samples were stirred for 3 minutes, sonicated for 3 minutes and then centrifuged at 15,000g (4°C) during 5 minutes to sediment the proteins. Supernatants were transferred in a microplate before LC-MS/MS analysis.

#### ***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 chinese hamster ovary (CHO) cells before selection for stable expression, as reported<sup>10</sup>. CHO cells expressing human prolactin releasing peptide receptor (PrRPR) and pyroglutamylated RFamide peptide receptor (QFRPR) were a gift from M. Parmentier (IRIBHM, Brussels, Belgium).

cDNAs encoding human NPFF1R, NPFF2R or each of the four opioid receptors ( $\mu$ , MOPr; delta, DOPr; kappa, KOPr and Nociceptin, NOPr) were further transfected into human embryonic kidney (HEK293) cells stably expressing the cyclic adenosine monophosphate (cAMP) GloSensor<sup>TM</sup>-20F before G418 selection for stable receptor expression. Membranes from CHO cells expressing RFamide receptors were prepared as reported<sup>10</sup>. Membranes from HEK293 cells with transient or stable expression of human  $\mu$ , delta or kappa opioid receptors were obtained as previously described<sup>13,32</sup>. All these membrane preparations were stored at  $-80^{\circ}\text{C}$  as aliquots (1 mg prot./mL) until use in radioligand binding experiments.

### ***Radioligand binding assays***

#### ***- RFamide receptors***

Competition experiments were conducted essentially as described<sup>10</sup>. Briefly, 5 to 10  $\mu\text{g}$  of membranes from cells expressing the different receptors were incubated at  $25^{\circ}\text{C}$  in 96-well plates with the appropriate radioligand and increasing concentrations of unlabelled peptides or test compounds under binding equilibrium conditions. [<sup>125</sup>I]-1DMe-NPFF (0.015nM) was used to label NPFF1 and NPFF2 receptors. [<sup>3</sup>H]-PrRP-20 (0.3 nM), [<sup>125</sup>I]-Kp-10 (0.02 nM) and [<sup>125</sup>I]-43Rfa (0.03 nM) were used to label PrRP, Kiss1 and QRFP receptors, respectively. Dilution and binding buffers for [<sup>125</sup>I]-1DMe-NPFF, [<sup>3</sup>H]-PrRP-20, [<sup>125</sup>I]-Kp-10, and [<sup>125</sup>I]-43Rfa were composed of HEPES pH 7.4 50mM, MgCl<sub>2</sub> 1mM, CaCl<sub>2</sub> 1mM, BSA 0.10% ; HEPES pH 7.4 50mM, MgCl<sub>2</sub> 5mM, CaCl<sub>2</sub>1mM, BSA 0.50% ; Tris-HCl pH 7.4 50mM, MgCl<sub>2</sub>10mM, EDTA 1mM, BSA 0.50% and Tris-HCl pH 7.4 50mM,

Bacitracine 100µg/mL, Saponine 10µg/mL, BSA 0.50%, respectively. Washing buffers for [<sup>125</sup>I]-1DMe-NPFF, [<sup>3</sup>H]-PrRP-20, [<sup>125</sup>I]-Kp-10, and [<sup>125</sup>I]-43RFa were composed of Hepes pH 7.4 25mM, MgCl<sub>2</sub> 1mM and CaCl<sub>2</sub> 1mM; Hepes pH 7.4 25mM, MgCl<sub>2</sub> 5mM, CaCl<sub>2</sub> 2mM and NaCl 0.5M ; Tris-HCl pH 7.4 50mM and Tris-HCl pH 7.4 50mM, NaCl 0.5M, respectively. Membrane-bound radioactivity was separated from free radioligand by rapid filtration through GF/B filtermats presoaked in 0.5% PEI (polyethylenimine) for [<sup>125</sup>I]-1DMe-NPFF ; 0.1% PEI for [<sup>3</sup>H]-PrRP-20 and [<sup>125</sup>I]-Kp-10 ; and Tris-HCl pH 7.4 50mM, BSA 0.50% and PEI 0.10% - diluted in washing buffer - using a cell harvester (Perkin Elmer Life and Analytical Sciences, Courtaboeuf, France). Filter-bound radioactivity was quantified using a TopCount scintillation counter (Perkin Elmer).

#### ***- Opioid receptors***

Opioid receptor-rich membranes were incubated at 25°C with [<sup>3</sup>H]-Diprenorphine (0.6 nM; MOPr, DOPr and KOPr) or [<sup>3</sup>H]-Nociceptin (0.15 nM; NOPr) and increasing concentrations of compounds to be tested. Dilution and binding buffers for [<sup>3</sup>H]-Diprenorphine and [<sup>3</sup>H]-Nociceptin were respectively composed of Tris-HCl pH 7.4 50mM, EDTA 1mM and Tris-HCl pH 7.4 50mM, BSA 0.10%. Washing buffer (Tris-HCl pH 7.4 50mM) and saturation buffer (PEI 1% diluted in washing buffer) are common for both radioligands. Non specific binding was defined in the presence of 10 µM naloxone (MOPr, DOPr and KOPr) or Nociceptin (NOPr). Membrane-bound radioactivity was determined by scintillation counting as described above.

#### ***[<sup>35</sup>S]GTPγS binding***

Stimulation by endogenous RFamide peptides of [<sup>35</sup>S]GTPγS binding to membranes from CHO cells expressing NPFF1R and its inhibition by test compounds was examined as previously reported<sup>13</sup>.

### ***cAMP accumulation***

Variations in the overall cAMP content of HEK cells expressing NPFF1 and NPFF2 receptors were monitored using the GloSensor™-20F cAMP assay according to manufacturer recommendations (Promega, Madison WI, USA) with a few modifications. HEK cells were suspended ( $10^6$  cells per mL) in physiological HEPES buffer (10 mM HEPES, 0.4 mM  $\text{NaH}_2\text{PO}_4$ , 137.5 mM NaCl, 1.25 mM  $\text{MgCl}_2$ , 1.25 mM  $\text{CaCl}_2$ , 6 mM KCl, 5.6 mM glucose and 1 mg/mL BSA, pH 7.4) supplemented with 1 mM D-Luciferin. After equilibration for 2 h at 25°C, cells were recorded in real time for their luminescence levels in 96-well plates using a FlexStation<sup>R</sup> 3 (Molecular Devices, Sunnyvale CA, USA). The agonist/antagonist nature of test compounds was determined according to two distinct experimental paradigms. In the agonist mode, compounds were injected at various concentrations 15 min before forskolin (0.4  $\mu\text{M}$  final concentration) and readings were pursued for 90 min. In the antagonist mode, compounds were allowed to pre-incubate with cells for 15 min before the addition of prototypical agonists for NPFF1R and NPFF2R (RFRP-3, NPFF). Experiments were conducted at 25°C in the presence of 0.1 mM IBMX to prevent the degradation of cAMP by phosphodiesterases. According to their preferential coupling to  $G_{i/o}$  proteins, the stimulation of NPFF1 and NPFF2 receptors by agonists was monitored as a dose-dependent reduction in steady-state luminescence levels, reflecting the inhibition of forskolin-induced cAMP accumulation.

### ***Calcium mobilization***

CHO cells stably expressing  $G_q$ -coupled receptors (PrRPR, Kiss1R and QFRPR) were loaded with 2  $\mu\text{M}$  Fluo-4 AM in the presence of 2.5 mM probenecid, as described<sup>10</sup>. Compounds were serially diluted in physiological HEPES buffer and antagonists were pre-incubated with cells for 10 min before agonist challenge. Agonist-evoked increases in intracellular calcium were recorded over time at 37 °C through fluorescence emission at 520

nm using a FlexStation 3 microplate reader. Peak response amplitudes were normalized to basal and maximal (cells permeabilized with 20  $\mu$ M digitonin) fluorescence levels.

### ***Label-Free dynamic mass redistribution assay***

HEK293 cells expressing NPFF1 receptor were seeded at 10,000 per well in Perkin Elmer 6057408 plate pre-coated during 45 minutes with 15  $\mu$ L of collagen 60 $\mu$ g/mL and containing 10  $\mu$ L of DMEM medium. After 24h of culture in a cell culture incubator at 37°C with 5% CO<sub>2</sub>, cells were washed 4 times in PBS with Robot Biomek FXP and plate were incubated 2h in Enspire reader to equilibrate the temperature. Agonists and antagonists were dissolved in HEPES/BSA buffer and deposited in Greiner 784201 plate. After a baseline of 6 minutes, cells were incubated 15 minutes with antagonist and then 90 minutes with agonist, while (Dynamic Mass Redistribution assay) measurements were recorded.

### ***Animals***

Male or female C57BL/6N mice (25-30 g weight; Janvier Labs, Le Genest St Isle, 53941 Saint Berthevin Cedex, France) were housed in groups of five per cage and kept under a 12h/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. 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 pentobarbital sodium (120 mg/kg, i.p.) or CO<sub>2</sub> (fill rate of 20% of the chamber volume per minute). Adult male Syrian hamsters were bred in-house and maintained under a 14h/10h light/dark cycle at 22  $\pm$  2°C with free access to food and water. 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.

### ***Drug administration***

All drugs were dissolved in physiological saline (0.9%) and administered subcutaneously (10mL/kg mice bodyweight), or intracerebroventricularly as previously described<sup>33</sup>. **RF3286** (10 nmol), morphine (1.5 nmol), RFRP-3 (10 nmol), NPFF (10 nmol) were administered (alone or in combination; 5  $\mu$ L total volume) in the left lateral ventricle using a modified Hamilton syringe. Proper injection site was verified in pilot experiments through administration and localization of methylene blue dye. Male hamsters were prepared for i.c.v. injections as reported<sup>26</sup>. A stainless steel 30-gauge cannula was placed in the lateral ventricle and animals were allowed to recover from surgery for one week, prior to further i.c.v. injections.

### ***Measurement of nociception in mice***

The nociceptive threshold of naive and treated mice was assessed in the tail immersion test<sup>14,34</sup> by measuring the latency time to withdraw their tail from a thermostated ( $47 \pm 0.5$  °C) water bath. In the absence of any nociceptive reaction, a cut-off of 25 sec was set to prevent tissue damage.

### ***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<sup>14,35</sup>. The impact of the dose of **RF3286** on the short-term analgesia to four consecutive injections of fentanyl (4 x 60  $\mu$ g/kg, s.c., 15 minutes interval) was evaluated using the tail immersion test. **RF3286** (0.005, 0.02 and 0.05 mg/kg) was administered subcutaneously 20 minutes before fentanyl and nociceptive threshold were measured every 1h after the last

fentanyl injection, during 3h. The impact of **RF3286** on long-term changes in nociceptive sensitivity induced by fentanyl injection on day 1 was evaluated using the tail immersion test once daily over a 6-days period, until recovery of the pre-drug baseline value.

***Measurement of the effect of drugs on luteinizing hormone (LH) secretion in the male Syrian hamster***

I.c.v. injections of drugs, alone or in combination, were performed (2  $\mu\text{L}/\text{animal}$ ; flow rate 0.15  $\mu\text{L}/\text{h}^{-1}$ ) in the morning under light anesthesia with isoflurane vapour. 30 min after injection, hamsters were deeply anesthetized with  $\text{CO}_2$  vapor and the blood was taken by intracardiac puncture for subsequent LH dosage using a validated radioimmunoassay<sup>36</sup>.

***Data and statistical analyses***

Binding and functional data were analyzed using Prism 5.02 (GraphPad Software, San Diego, CA, USA). Occupancy curves were fit to a four-parameter logistic equation to derive top and bottom plateau values, the drug concentration producing 50% of the maximal effect ( $\text{EC}_{50}$  value) or inhibiting 50% of specific binding or actual response ( $\text{IC}_{50}$  value) and the midpoint Hill slope. When the slope was not significantly different from unity, it was constrained as such.  $\text{IC}_{50}$  values of drugs derived from binding competition experiments were converted into equilibrium dissociation constants ( $K_i$  values) using the Cheng and Prusoff relationship. Antagonist-induced rightward shifts of agonist dose-response curves were globally fitted to the Schild equation to derive the  $\text{pA}_2$  value as well as Hill and Schild slopes. If not significantly deviating from 1, the slope parameters were constrained to 1 enabling the assimilation of the  $\text{pA}_2$  value for a competitive antagonist to  $\text{pK}_B$ , the negative log of its equilibrium dissociation constant for the receptor. Data for nociceptive tests are expressed as mean values  $\pm$  SEM for 6 to 12 animals depending on the group. Analgesia and hyperalgesia were quantified as the area under the curve (AUC) calculated by the trapezoidal method<sup>35</sup>. Data were analyzed using one-way or two-way ANOVA. *Post-hoc* analyses were performed

with Bonferonni's *post-hoc* test after having ensured that F achieved the level of significance of  $P < 0.05$ . All statistical analyses were carried out using the GraphPad Prism software.

## **Ancillary Information**

### *Supporting information*

- Analytical data and characterization of all synthesized compounds (Supplementary Table S1 and Figures S1 and S2)
- Molecular formula strings of all final compounds (CSV)
- Supplementary Figures S3, S4, S5 and S6

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F.B. and F.S. contributed equally. The article was written through contributions of all the authors. All the authors have given approval to the final version of the article.

#### *Note*



The authors declare no competing financial interest.

### *Acknowledgement*

We thank Dr M. Parmentier (IRIBHM, Brussels, Belgium) for the gift of CHO cells expressing human PrRPR and QRFPR. HEK293 cells stably expressing the cAMP GloSensor™ were kindly donated by J. Hanson (Molecular Pharmacology GIGA-Signal Transduction & Medicinal Chemistry Drug Research Centre, Liege, Belgium). This work also benefited from the efficient support of the Integrative Biological Chemistry Platform (UMS 3286 CNRS, Illkirch, France). We thank the staff from INCI for technical assistance and advices. Frédéric Simonin's laboratory, V.S and J.B.H were supported by the CNRS, Université de Strasbourg and Agence Nationale de la Recherche (ANR-13-BSV1-0001). M. S., F. B. and F. S. were supported by Conseil Régional d'Alsace (Pharmadol), Communauté Urbaine de Strasbourg (Pharmadol), ICFRC (Pharmadol), OSEO (Pharmadol), Direction Générale des Entreprises (Pharmadol). This work has been published within the LABEX Medalis and Simonin's laboratory and S. S., M. S., P. G., F.B. received financial support from the French government managed by "Agence Nationale de la Recherche" under "Programme d'investissement d'avenir" (ANR-10-LABX-0034 and ANR-17-EURE-0022, EURIDOL graduate school of pain).

### *Abbreviations used*

[<sup>125</sup>I]-1DMeNPFF, [<sup>125</sup>I]-<sup>D</sup>Tyr-Leu-(*N*-meth-yl)Phe-Gln-Pro-Glu-Arg-Phe-NH<sub>2</sub> - cAMP, cyclic adenosine monophosphate - CHO, Chinese hamster ovary - DOPr, delta-opioid receptor - DMR, dynamic mass redistribution - HEK, Human embryonic kidney - i.c.v., intracerebroventricular - Kiss1R, kisspeptin1 receptor - KOPr, kappa-opioid receptor - LH, luteinizing hormone - MOPr, mu-opioid receptor- NOPr, Nociceptin/OFQ receptor - NPFF,

neuropeptide FF - NPPF1R, Neuropeptide FF1 receptor - NPPF2R, Neuropeptide FF2 receptor - OIH, opioid-induced hyperalgesia - PrRPR, prolactin releasing peptide receptor - QRFPR, pyroglutamylated RFamide peptide receptor - RFRP-3, RFamide-related peptide-3 - s.c., subcutaneous

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## Table of Contents graphic

