Corrections

GENETICS. For the article “A Sanger/pyrosequencing hybrid approach for the generation of high-quality draft assemblies of marine microbial genomes,” by Susanne M. D. Goldberg, Justin Johnson, Dana Busam, Tamara Feldblyum, Steve Ferriera, Robert Friedman, Aaron Halpern, Hoda Khouri, Saul A. Kravitz, Federico M. Lauro, Kelvin Li, Yu-Hui Rogers, Robert Straussberg, Granger Sutton, Luke Tallon, Torsten Thomas, Eli Venter, Marvin Frazier, and J. Craig Venter, which appeared in issue 30, July 25, 2006, of Proc Natl Acad Sci USA (103:11240–11245; first published July 13, 2006; 10.1073/pnas.0604351103), the authors note that in Fig. 3, the text in the lower green box, “Reference Genome Unavailable,” should read “Reference Genome Available.” The corrected figure and its legend appear below. This error does not effect the conclusions of the article.

![Decision tree for hybrid sequencing strategy.](https://www.pnas.org/content/103/43/16057/F3.large.jpg)

**Fig. 3.** Decision tree for hybrid sequencing strategy. For organisms with a small genome size (<3 Mb) and/or a small number of gaps and/or high levels of repetitive structure inducing physical ends, we found 8× Sanger sequencing to be the most cost-effective approach. For organisms with a large genome size, many sequencing gaps, and/or hard stops, we found initial sequencing of 5.3× Sanger data followed by the addition of two 454 runs to be the most cost-effective approach.

www.pnas.org/cgi/doi/10.1073/pnas.0607197103
PHARMACOLOGY. For the article “RF9, a potent and selective neuropeptide FF receptor antagonist, prevents opioid-induced tolerance associated with hyperalgesia,” by Frédéric Simonin, Martine Schmitt, Jean-Paul Laulin, Emilie Laboureyras, Jack H. Jhamandas, David MacTavish, Audrey Matias, Catherine Mollereau, Patrick Laurent, Marc Parmentier, Brigitte L. Kieffer, Jean-Jacques Bourguignon, and Guy Simonnet, which appeared in issue 2, January 10, 2006, of Proc Natl Acad Sci USA (103:466–471; first published January 3, 2006; 10.1073/pnas.0502090103), the authors note that in Fig. 1B, the position of the bond between the adamantane system and the rest of the RF9 compound is incorrect. The corrected figure and its legend appear below. This error does not affect the conclusions of the article.

NEUROSCIENCE. For the article “Target cell-specific modulation of neuronal activity by astrocytes,” by A. S. Kozlov, M. C. Angulo, E. Audinat, and S. Charpak, which appeared in issue 26, June 27, 2006, of Proc Natl Acad Sci USA (103:10058–10063; first published June 16, 2006; 10.1073/pnas.0603741103), the authors note that some affiliation and correspondence information was incorrect or incomplete as given. The correct addresses for the authors’ laboratories are as follows: Institut National de la Santé et de la Recherche Médicale, U603, Paris, F-75006 France; Centre National de la Recherche Scientifique, FRE2500, Paris, F-75006 France; Laboratory of Neurophysiology, Ecole Supérieure de Physique et de Chimie Industrielles, Paris, F-75005 France; and Laboratory of Neurophysiology and New Microcopies, Université Paris Descartes, Paris, F-75006 France. In addition, S. Charpak should have been listed as one of the corresponding authors. E-mail: serge.charpak@univ-paris5.fr.

www.pnas.org/cgi/doi/10.1073/pnas.0608114103

Fig. 1. Screening of RFamide derivatives on hNPFF2R. (A) hNPFF2R membranes were labeled with [125I]Tyr-NPFF, and three concentrations of RFamide derivatives were tested in competition experiments. Each concentration was tested in duplicate. Results for the reference and the six most active compounds are shown. Arrowheads indicate compounds that were selected for further characterization. (B) Structures of RF2, RF9, RF48, RF49, and BIBP3226.
RF9, a potent and selective neuropeptide FF receptor antagonist, prevents opioid-induced tolerance associated with hyperalgesia


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Edited by Tomas Hökfelt, Karolinska Institutet, Stockholm, Sweden, and approved November 18, 2005 (received for review March 15, 2005)

Neuropeptide FF (NPFF) has been proposed to play a role in pain modulation, opioid tolerance, and several other physiological processes. However, pharmacological agents that would help define physiological roles for this peptide are still missing. Here we report the discovery of a potent and selective NPFF receptor antagonist, RF9, that can be administered systemically. This compound does not show any effects by itself but can block efficiently the increase in blood pressure and heart rate evoked by NPFF. When chronically coadministered with heroin, RF9 completely blocks the delayed and long-lasting paradoxical opioid-induced hyperalgesia and prevents the development of associated tolerance. Our data indicate that NPFF receptors are part of a bona fide antiopioid system and that selective antagonists of these receptors could represent useful therapeutic agents for improving the efficacy of opioids in chronic pain treatment.

Conflict of interest statement: No conflicts declared.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: AUC, area under the curve; CHO, Chinese hamster ovary; Dn, day; i.c.v., intracerebroventricular; MAP, mean arterial blood pressure; MPE, maximal possible effect; NPFF, neuropeptide FF; hNPFF, human NPFF.

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J.J.B. and G.S. contributed equally to this work.

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Branes were labeled with \[^{125}\text{I}\]Tyr-NPFF, and three concentrations of RFamide hNPFF2R and \[^{125}\text{I}\]YVP for hNPFF1R.

*Represents the K

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†Value from ref. 42.

To identify NPFF receptor ligands, we focused our interest in the search of small peptides (dipeptides). In a preliminary experiment, we had shown that benzoyl-RFamide derivatives from the screening to inhibit forskolin-stimulated cAMP accumulation, whereas RF2, RF9, RF48, and RF49 were inactive up (Fig. 1). We prepared \(\sim 100\) derivatives by substitution of the phenyl ring of N-benzoyl RFamide or replacement by other lipophilic groups (tertiobutyl, cyclohexyl, or adamantane). Three concentrations of each compound were tested (0.01, 0.1, and 1 \(\mu\)M) for their capacity to inhibit the binding of \[^{125}\text{I}\]Tyr-NPFF to membrane homogenates of COS-1 cells expressing recombinant hNPFF2R (data not shown). As expected, our reference, RF2, showed significant competition activity at 1 \(\mu\)M, whereas six compounds displayed a better competition activity at hNPFF2R than RF2 (Fig. 1A). We selected RF9, RF48, and RF49 (Fig. 1B) for further pharmacological characterization.

**Pharmacological Characterization of NPFF Receptor Ligands in Vitro.**

In a first step, we determined the \(K_i\) values of the selected compounds and of reference ligands RF2, NPFF, and BIBP3226 (Table 1) for recombinant hNPFF1R and for hNPFF2R. BIBP3226, the prototypical neuropeptide Y receptor subtype Y1 antagonist, displays structural similarities to RFamide derivatives (Fig. 1B) and has recently been shown to bind to the two NPFF receptor subtypes (27, 28). RF2 displayed submicromolar affinity for both receptors (526 and 756 nM, respectively). As expected, the three ligands selected from the screening exhibited better affinity for hNPFF2R and hNPFF1R than did RF2. RF48 and RF49 displayed the best affinity for hNPFF2R (27 and 38 nM) and a slight selectivity for this receptor \([K_i, (hNPFF2R)/K_i (hNPFF1R)] \text{ ratio of 1.6 and 1.4, respectively}\), whereas RF9 displayed an equally good affinity for both receptor subtypes. Because these compounds are structurally related to BIBP3226, we investigated further the affinity of RF9 for human neuropeptide Y receptor subtype Y1 and for a subset of related G protein-coupled receptors, including the three other RFamide receptors (GPR10, GPR54, and GPR103), opioid (\(\mu, \delta, \kappa\)) and ORL-1 receptors. No competition activity was observed for RF9 on these receptors at doses up to 10 \(\mu\)M, except on \(\mu\) and \(\kappa\), for which we observed a slight competition at this concentration (see Fig. 5, which is published as supporting information on the PNAS web site). Altogether, these results indicate a good selectivity of RF9 for NPFF receptors.

We further characterized these compounds in a functional assay consisting of agonist-promoted stimulation of the \[^{35}\text{S}\]GTP\(\gamma\)S binding to hNPFF2R cell membranes. To this purpose, COS cells were transiently transfected with a construct encoding hNPFF2R fused to a signal sequence to increase its expression level (29). NPFF stimulated \[^{35}\text{S}\]GTP\(\gamma\)S binding to COS-signal peptide/hNPFF2R membranes with an EC\(_{50}\) of 22.5 \(\pm\) 1 nM and a maximal activity corresponding to 205 \(\pm\) 5% that of the basal level of \[^{35}\text{S}\]GTP\(\gamma\)S binding (Fig. 2A and Table 2). From the four tested compounds, RF2, RF48, and RF49 slightly stimulated \[^{35}\text{S}\]GTP\(\gamma\)S binding, with EC\(_{50}\) values of \(>10,000\), 1,950 \(\pm\) 450 and 694 \(\pm\) 103 nM, respectively, and maximal activity ranging from 122\% to 145\% (Table 2). These results indicate that these compounds had partial agonist activity at high concentrations. In contrast, RF9 had no effect on \[^{35}\text{S}\]GTP\(\gamma\)S binding at concentrations up to 100 \(\mu\)M. To further confirm the antagonist activity of this compound, we performed concentration–effect curves of NPFF in the presence of a high concentration of RF9 corresponding to \(100 \times K_i\) of this compound for hNPFF2R (Fig. 2B). RF9 (7.5 \(\mu\)M) shifted the concentration–effect curve of NPFF to the right by \(160\)-fold \((K_i = 45 \pm 5\) nM). This result confirmed that RF9 displays potent antagonist activity at hNPFF2R in vitro.

We next examined the capacity of compounds selected from the screening to inhibit forskolin-stimulated cAMP accumulation in Chinese hamster ovary (CHO) cells expressing hNPFF1R (28). In this test, NPVF (0.1 \(\mu\)M), the endogenous ligand of NPFF1R, strongly inhibited forskolin-stimulated cAMP accumulation, whereas RF2, RF9, RF48, and RF49 were inactive up

**Results**

**Identification of NPFF Receptor Ligands by Screening of RFamide Derivatives.** To identify NPFF receptor ligands, we focused our interest in the search of small peptides (dipeptides). In a preliminary experiment, we had shown that benzoyl-RFamide dipeptide RF2 (Fig. 1B) displayed reasonable affinity for NPFF receptors from rat spinal cord (data not shown) and recombinant hNPFF2R (Table 1). We therefore screened derivatives from the RFamide dipeptide by competition on recombinant hNPFF2R

Table 1. Binding affinities for hNPFF2R and hNPFF1R of ligands selected from the screening

<table>
<thead>
<tr>
<th>Ligands</th>
<th>hNPFF2R (K_i) nM</th>
<th>hNPFF1R (K_i) nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPFF</td>
<td>0.2 (\pm) 0.05</td>
<td>9.8 (\pm) 0.8</td>
</tr>
<tr>
<td>BIBP 3226</td>
<td>461 (\pm) 107</td>
<td>121 (\pm) 1</td>
</tr>
<tr>
<td>RF2</td>
<td>526 (\pm) 51</td>
<td>756 (\pm) 91</td>
</tr>
<tr>
<td>RF9</td>
<td>75 (\pm) 9</td>
<td>58 (\pm) 5</td>
</tr>
<tr>
<td>RF48</td>
<td>27 (\pm) 3</td>
<td>169 (\pm) 11</td>
</tr>
<tr>
<td>RF49</td>
<td>38 (\pm) 2</td>
<td>153 (\pm) 6</td>
</tr>
</tbody>
</table>

Values are mean \(\pm\) SEM from three or more separate experiments performed in duplicate. \(K_i\) values were determined by using \[^{125}\text{I}\]Tyr-NPFF for hNPFF2R and \[^{125}\text{I}\]YVP for hNPFF1R.

*Represents the \(K_a\) value for \[^{125}\text{I}\]Tyr-NPFF.

†Value from ref. 42.
We then showed that RF9 dose-dependently reverses the inhibitory effect of NPVF with an EC$_{50}$ of 4.7 ± 1 nM (Fig. 2C), thus confirming that this compound displays antagonist activity at hNPFF1R as well.

RF9 Prevents Increase in Blood Pressure and Heart Rate Elicited by NPFF in Vivo. We next wanted to investigate whether RF9 can directly antagonize NPFF effects in vivo. Because i.c.v. administration of NPFF in rats has been shown to cause a rise in arterial blood pressure (MAP) that occurred within 1 min of onset of i.c.v. infusion and was sustained for ~25 min before returning to normal levels (Fig. 3A). The increase in MAP was also accompanied by a significant increase in heart rate over saline-infused rats (Fig. 3B). RF9 (10 µg) infused alone did not result in a significant alteration of MAP or heart rate. Conversely, MAP and heart rate increases evoked by NPFF were significantly blocked when NPFF was applied in conjunction with RF9 (Fig. 3). This result demonstrates the capacity of RF9 for antagonizing NPFF action in vivo and confirmed that this activity is mediated by NPFF receptors.

Coadministration of RF9 with Heroin Prevents Delayed Heroin-Induced Hyperalgesia and Associated Tolerance. Because NPFF had been shown previously to display antiopioid and pronociceptive properties, we decided to investigate whether RF9 could oppose to delayed heroin-induced hyperalgesia and associated tolerance. Therefore, we used a model of discontinuous opioid administration at low doses in rats because it better mimics opioid use in chronic pain patients or heroin addicts. We measured the basal nociceptive threshold of animals after coadministration of

<table>
<thead>
<tr>
<th>Ligands</th>
<th>EC$_{50}$, nM</th>
<th>Maximal activation, %</th>
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<tbody>
<tr>
<td>NPFF</td>
<td>22.5 ± 1</td>
<td>205 ± 5</td>
</tr>
<tr>
<td>RF2</td>
<td>&gt;10,000</td>
<td>133 ± 7</td>
</tr>
<tr>
<td>RF9</td>
<td>&gt;100,000</td>
<td>—</td>
</tr>
<tr>
<td>RF 48</td>
<td>1950 ± 450</td>
<td>145 ± 10.5</td>
</tr>
<tr>
<td>RF 49</td>
<td>694 ± 103</td>
<td>122 ± 6.6</td>
</tr>
</tbody>
</table>

Maximal activation is expressed as the percentage of basal [35S]GTP-S binding, and values represent mean ± SEM from at least two separate experiments performed in triplicate. —, not determined.

Fig. 2. In vitro characterization of compounds selected from the screening. (A) Stimulation of [35S]GTP$_S$ binding to hNPFF2R by NPFF (■), RF2 (▲), RF48 (●) and RF49 (●). (B) Stimulation of [35S]GTP$_S$ binding to hNPFF2R by NPFF alone (■) or NPFF in presence of 7.5 µM RF9 (▲). RF9 shifted the concentration–effect curve of NPFF to the right by ~160-fold. Data are expressed as percentage of basal [35S]GTP$_S$ binding and represent mean ± SE from at least two separate experiments in triplicate. (C) RF9 (black bars) reversed the inhibition of forskolin-induced cAMP by NPVF in CHO-hNPFF1R cells. RF9 alone was inactive, whereas NPVF (white bar) inhibited ~60% of the stimulated cAMP. Error bars represent the mean ± SEM of data from three experiments performed in duplicate.

Fig. 3. RF9 blocks blood pressure effects of NPFF. (A) Changes in MAP [expressed in mmHg (1 mmHg = 133 Pa)] in rats receiving either i.c.v. saline (●), NPFF (■), RF9 (▲), or NPFF and RF9 applied together (▲). Time 0 indicates the injection point for i.c.v. saline or drug applications. (B) Heart rate changes that accompany MAP alterations in A. Pooled MAP and heart rate data are from five animals. *, significant difference in MAP or heart rate compared with control ($P < 0.05$).
RF9 with heroin by using the paw-pressure vocalization test. The basal nociceptive threshold values were measured every day 1 h before drug administration (see Materials and Methods). As described in ref. 13, when injected alone for 2 weeks, once-daily doses of 0.3 mg/kg heroin induced a gradual lowering of the basal nociceptive threshold value (P < 0.05, Dunnett’s test) (Fig. 4A) that returned to the predrug value on day 25 (D25), i.e., 11 days after cessation of heroin treatment (P > 0.05, Dunnett’s test). In this paradigm, 0.1 mg/kg saline or RF9 (s.c.) alone did not alter the basal nociceptive threshold of the animals (P > 0.05, one-way ANOVA). When injected 30 min before each daily heroin administration, RF9 completely prevented the progressive decrease of the nociceptive threshold (P > 0.05, one-way ANOVA). As expected, when we measured the analgesic effect of the first (D1) and the last (D14) heroin administrations (Fig. 4B), we observed a strong decrease of the maximum analgesic effect of heroin in rats that were treated by the opioid for 2 weeks [peak at 279 g corresponding to 29.6% maximal possible effect (MPE)] compared with the first heroin injection on D1 (peak at 392 g corresponding to 40% MPE). These data demonstrate that tolerance did develop in these animals. However, we observed no change in both the time course and area under the curve (AUC) related to the analgesic effect of heroin between D1 and D14 (Fig. 4B), suggesting that this change in MPE reflects a shift of basal nociceptive threshold rather than a lack of pharmacological effect of the opioid. When RF9 was coadministered with heroin, both the basal nociceptive threshold and AUC were identical at D1 and D14 (P > 0.05, two-way ANOVA), indicating that neither tolerance nor hyperalgesia developed in these animals. When injected in animals that had recovered their predrug nociceptive threshold after 14 once-daily heroin administrations, a 0.3 mg/kg heroin injection (D28) produced the same analgesic effect than at D1 (P > 0.05, two-way ANOVA) (Fig. 4B). As we described in ref. 13, analgesia was followed by an exaggerated decrease of the nociceptive threshold for several days (P < 0.05, Dunnett’s test) (Fig. 4A) compared with the initial heroin administration on D1. Such a hyperalgesia was not observed in rats that received prior 14 once-daily coadministrations of RF9 and heroin (P > 0.05, one-way ANOVA). Altogether, these results demonstrate that blocking NPFF receptor can prevent efficiently heroin-induced hyperalgesia and associated tolerance.

**Discussion**

Although in recent years great advances have been made in the understanding of mechanisms that underlie pain, opioids are still the most powerful analgesics. However, their use is limited by tolerance that accrues after repeated exposure. Our discovery of a potent selective antagonist of NPFF receptors that can be administered systemically has enabled us to establish that blockade of NPFF receptors prevents the development of sustained hyperalgesia and consequently opposes the associated decrease in maximum heroin analgesic effect. This result strengthens the hypothesis that development of tolerance to opioids is not only due to a decrease in cellular responsiveness but may also originate from the secondary up-regulation of antinociceptive systems with pronociceptive properties leading to long-lasting enhancement in pain sensitivity (9–11, 15, 20).

In previous reports, several substances, including N-methyl-D-aspartate receptor antagonists have been shown to prolong opioid analgesic effect and prevent long-lasting hyperalgesia (9, 10, 13, 15). However, these substances act on neurotransmitter systems that are critical to normal brain function; therefore, their use is limited by serious side effects (32). Antagonists of antinociceptive peptide receptors could be a new and safer strategy because their side effects are often more limited because of lower receptor density in the CNS. Although we have not yet investigated all of the functions that are considered modulated by the NPFF system, we show here that RF9 can be systemically administered and does not display any effects on the nociceptive threshold and on the blood pressure or the heart rate when administered alone. These results suggest that the basal level of activation of this system is low under normal conditions. In keeping with this notion, it has been shown that NPFF levels in the spinal fluid of morphine-treated rats are elevated significantly (33) and that antisense oligonucleotides to human SQA-NPFF attenuate tolerance to analgesic effect of morphine in mice (34). Our data showing that coadministration of the NPFF receptor antagonist RF9 with heroin prevents the development of tolerance are in good agreement with these results and suggest that the NPFF system is triggered by activation of the opioid system as observed in in vitro studies (23). Overall, our results and data from the literature indicate that RF9 should display limited adverse side effects.

A large body of evidence already suggests that NPFF plays important roles in the control of pain and analgesia through its...
interaction with the opioid system (1, 6). However, it has been shown that NPFF displays both antinociceptive and proopioid actions in animal models of pain, depending on the route of administration. Thus, i.v. administration of NPFF reverses morphine-induced analgesia in rats (33), whereas intrathecal administration induces a long-lasting opioid-induced analgesia and prolongs morphine-induced analgesia (35). Recently, a novel family of G protein-coupled receptors specifically expressed in neurons of trigeminal and dorsal root ganglia was identified (36). Some of these receptors display a good affinity for NPFF and are most likely involved in pain processing (37). In addition to the NPFF2R receptor subtype that is expressed in the dorsal horn of spinal cord in rodents (38), these receptors may also be activated after local intrathecal injection of a high concentration of NPFF, thus mediating spinal analgesic activity of this peptide. Our results of systemic administration of RF9 on opioid-induced hyperalgesia clearly define NPFF and its receptors as a bona fide antinociceptoid system.

In rats that have been chronically treated with heroin, we observed that a single heroin challenge, administered once the animals had recovered their initial basal nociceptive threshold, induced analgesia followed by a marked hyperalgesia. This finding indicates that these animals have developed a latent process of pain sensitization, which has been proposed to be due to a long-lasting up-regulation of pronociceptive systems (9). The fact that we did not observe the development of hyperalgesia in RF9-treated rats both during the chronic heroin treatment and after the single heroin challenge strongly suggests that the NPFF system is involved in pain sensitization. In the same heroin model, we have previously reported that naloxone precipitates a marked hyperalgesia 2 months after stopping heroin administrations (13). Collectively, these data suggest that return to predrug nociceptive thresholds after cessation of heroin administrations is not due to a deactivation of pronociceptive systems but would rather result from a progressive opposition by an active opioid-dependent, counter-adaptation system leading to a new equilibrium (allostasis) associated with a high-level balance between antinociceptive and pronociceptive systems.

The precise role of both NPFF receptor subtypes has not been clarified yet. Distribution of NPFF1R and NPFF2R in several mammalian species (3, 38, 39) indicates that NPFF2R is localized to pain-processing regions, whereas NPFF1R would most likely participate in neuroendocrine function. A notable exception is human spinal cord, where NPFF1R expression has been observed while NPFF2R is present in the rodent, rabbit, and monkey (3, 38, 39). This exception suggests that in humans NPFF1R could play the same role in pain processing as does NPFF2R in other mammals. We show here that RF9 compound displays both the same affinity and antagonist activity at NPFF1R and NPFF2R subtypes. Therefore, it is likely that RF9 will display comparable activity in man and in rats independently of the NPFF receptor subtype involved in pain processing.

In conclusion, although the precise mechanism by which NPFF acts to block opioid-induced hyperalgesia and associated tolerance is currently unknown, we provide convincing evidence that NPFF system acts as an antinociceptoid system with pronociceptive properties. We describe the discovery of a potent and selective NPFF receptor antagonist and demonstrate that blocking this system in vivo represents an innovative strategy for improving the efficacy of opioids as therapy in chronic pain.

Materials and Methods

Synthesis of RFamide Derivatives. Boc-Arg(Pmc)-OH and H-Phe-NH2 (Bachem, Germany) were reacted overnight in dimethylformamide in presence of hexafluorophosphate of N-acyl tris(dimethylamino)phosphonium benzotriazole and N-methylmorpholine at room temperature yielding the dipeptide (86%), which was submitted to quantitative acid hydrolysis (2.4 M HCl/AcOEt) at 20°C overnight. The free NH2 intermediate was then N-acylated in the presence of the corresponding carboxylic acid, affording the N-acyl Arg(Pmc)-Phe-NH2. After a last step of deprotection in the presence of trifluoroacetic acid/dichloromethane (50:50), the trifluoroacetate of RF-amide was obtained and purified by HPLC (218TP C18 column; i.d., 22 mm; length, 250 mm; pore size, 10-15 μm gradient. H2O plus 0.1% trifluoroacetic acid/MeOH: (100/0-100), 40 min, 10 ml/min; Dionex).

Cloning of hNPFF2R. The cDNA encoding hNPFF2R was subcloned into the pcDNA3 expression vector (Invitrogen) (see Supporting Materials and Methods, which is published as supporting information on the PNAS web site). In a second construct, a sequence encoding a signal sequence and a Flag epitope was fused to the 5′ coding sequence of hNPFF2R cDNA, resulting in a SF-hNPFF2R construct.

Cell Membrane Preparations and Receptor Binding Assays. COS-1 cells were grown and electroporated with pcDNA3/hNPFF2R or SF-hNPFF2R, and membranes were prepared as described in ref. 40. Membranes from CHO cells stably expressing hNPFF1R were prepared as described in ref. 28. hNPFF2R membranes were incubated in a final volume of 0.2 ml containing 50 mM Hepes (pH 7.4), 10 mM CaCl2, 10 mM MgCl2, 0.1% BSA, 0.1 mM phenylmethylsulfonyl fluoride, 0.2 nM [125I]Tyr-NPFF (specific activity, 2,175 Ci/mmol (1 Ci = 37 GBq); Amersham Biosciences, which is now GE Healthcare), and the ligands to be tested as described in ref. 41. Nonspecific binding was determined in the presence of 1 μM NPFF. Typical total and nonspecific binding were 2,500 and 500 cpm, respectively. hNPFF1R membranes were incubated in a final volume of 0.5 ml containing 50 mM Tris-HCl (pH 7.4), 60 mM NaCl, 25 μM bestatin (Sigma-Aldrich), 0.1% BSA, 0.05 nM [125I]YVP (specific activity, 2,175 Ci/mmol), and the ligands to be tested as described in ref. 42. Nonspecific binding was determined in the presence of 1 μM YVPNLPQRFa. Typical total and nonspecific binding were 2,800 and 300 cpm, respectively. Competition experiments with neuropeptide Y subtype Y1, opioid, and RFamide receptors are described in Supporting Materials and Methods.

Cellular Assays. For [35S]GTPγS binding experiments, SF-hNPFF2R membrane proteins (5 μg) were incubated for 30 min at 30°C in 20 mM Hepes, pH 7.4/100 mM NaCl/3 mM MgCl2/3 μM GDP/10 mg/ml saponin/0.1 nM [35S]GTPγS and ligands. Nonspecific binding was determined in the presence of 10 μM GTPγS. Incubation mixtures were rapidly filtered and washed with 20 mM Hepes, pH 7.4/100 mM NaCl/3 mM MgCl2 on Whatman GF/B filters. Bound radioactivity was determined by scintillation counting. Antagonist K0 value was calculated by using the following formula: K0 = [antagonist]/(dose ratio) - 1. For intracellular cAMP experiments, CHO cells stably expressing hNPFF1R were assayed as described in ref. 28.

Blood Pressure and Heart Rate Measurements. Experimental details are given in Supporting Materials and Methods. Rats (n = 5) were catheterized for measurement of arterial blood pressure and received an in-dwelling cannula into the lateral cerebral ventricle as described in ref. 31. Arterial blood pressure and heart rate were continuously monitored. NPFF (10 μg in 10 μl of saline) was injected into the lateral ventricle over 15–20 sec. Control injections of saline (10 μl) were carried out in the same animals. Upon return of blood pressure to baseline, RF9 (10 μg) was injected i.c.v. followed by another i.c.v. injection of NPFF with RF9. After 1.5 h, another i.c.v. infusion of NPFF was repeated. Data from each animal were pooled to obtain blood pressure and
heart rate changes with i.c.v. saline, NPFF, RF9, and RF9 with NPFF.

**Measurement of Nociceptive Mechanical Threshold.** Experimental details are given in Supporting Materials and Methods. Nociceptive mechanical thresholds in rats (n = 8) were determined by using the paw-pressure vocalization test. Heroin (0.3 mg/kg; Francopia, Gentilly, France) was injected once-daily for 14 days. Basal nociceptive threshold was measured once-daily 60 min before heroin administration and after the cessation of heroin injections until the end of the experiment. Changes in nociceptive threshold were also measured every 30 min after heroin injection on D1, D14, and D28 until the end of the pharmacological effect. RF9 (0.1 mg/kg) was injected once-daily for 14 days. Basal nociceptive threshold was measured once-daily 60 min before heroin injection or saline injection. Four experimental groups were constituted: saline/saline, RF9/saline, saline/heroin, and RF9/heroin.

**Analysis of the Data.** All data are expressed as means ± SEM. For in vitro experiments, nonlinear regression analysis of the data were performed with PRISM 2.0 (GraphPad, San Diego). For blood pressure and heart rate data, Student’s paired t test was used for determining the significance of effect between the different treatment groups. For nociceptive threshold data, one-way and two-way ANOVA with repeated measurements followed by Dunnett’s test were performed. AUC for analgesic effects were calculated by using the trapezoidal method. The MPE was calculated as 100 × (paw pressure value after injection − baseline paw pressure value)/600 − baseline paw pressure value. A Newman–Keuls test was used for multiple comparisons between groups. P < 0.05 was considered significant.

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