British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2011.01563.x
www.brjpharmacol.org

RESEARCH PAPER

Involvement of neuropeptide FF receptors in neuroadaptive responses to acute and chronic opiate treatments

K Elhabazi[1], JM Trigo[2], C Mollereau[3], L Moulédous[3], J-M Zajac[3], F Bihel[4], M Schmitt[4], JJ Bourguignon[4], H Meziane[5], B Petit-demoulière[5], F Bockel[6], R Maldonado[2] and F Simonin[1]

[1]*Institut de Recherche de l'ESBS, Université de Strasbourg-CNRS, Illkirch, France,* [2]*Laboratori de Neurofarmacologia, Universitat Pompeu Fabra, Barcelona, Spain,* [3]*Institut de Pharmacologie et de Biologie Structurale, Université de Toulouse-CNRS, Toulouse, France,* [4]*Laboratoire d'Innovation Thérapeutique, Université de Strasbourg-CNRS, Illkirch, France,* [5]*Institut Clinique de la Souris, IGBMC, Université de Strasbourg-CNRS-INSERM, Illkirch, France, and* [6]*Phenopro, Illkirch, France*

Correspondence
Dr Frédéric Simonin, IREBS-UMR7242, Ecole Supérieure de Biotechnologie de Strasbourg, Boulevard Sébastien Brant, 67412 Illkirch Cedex, France. E-mail: simonin@unistra.fr

Keywords
RF9; NPFF receptors; anti-opioid system; RFamide; opioid-induced hyperalgesia; morphine tolerance; reward; withdrawal dependence

Received
23 December 2010
Revised
26 May 2011
Accepted
7 June 2011

BACKGROUND AND PURPOSE

Opiates remain the most effective compounds for alleviating severe pain across a wide range of conditions. However, their use is associated with significant side effects. Neuropeptide FF (NPFF) receptors have been implicated in several opiate-induced neuroadaptive changes including the development of tolerance. In this study, we investigated the consequences of NPFF receptor blockade on acute and chronic stimulation of opioid receptors in mice by using RF9, a potent and selective antagonist of NPFF receptors that can be administered systemically.

EXPERIMENTAL APPROACH

The effects of RF9 were investigated on opioid pharmacological responses including locomotor activity, antinociception, opioid-induced hyperalgesia, rewarding properties and physical dependence.

KEY RESULTS

RF9 had no effect on morphine-induced horizontal hyperlocomotion and slightly attenuated the decrease induced in vertical activity. Furthermore, RF9 dose-dependently blocked the long-lasting hyperalgesia produced by either acute fentanyl or chronic morphine administration. RF9 also potentiated opiate early analgesic effects and prevented the development of morphine tolerance. Finally, RF9 increased morphine-induced conditioned place preference without producing any rewarding effect by itself and decreased naltrexone-precipitated withdrawal syndrome following chronic morphine treatment.

CONCLUSION AND IMPLICATIONS

The NPFF system is involved in the development of two major undesirable effects: tolerance and dependence, which are clinically associated with prolonged exposure to opiates. Our findings suggest that NPFF receptors are interesting therapeutic targets to improve the analgesic efficacy of opiates by limiting the development of tolerance, and for the treatment of opioid dependence.

Abbreviations

CPP, conditioned place preference; HMA, horizontal motor activity; NPFF, neuropeptide FF; OIH, opioid-induced hyperalgesia; VMA, vertical motor activity

© 2011 The Authors
British Journal of Pharmacology © 2011 The British Pharmacological Society

Introduction

Management of pain is a major worldwide health problem with a financial burden of 1 trillion dollars per year in developed countries (Max and Stewart, 2008). Systemic administration of opiate analgesics such as morphine remains the most effective treatment for alleviating severe pain across a range of conditions including acute pain, cancer-related pain and neuropathic pain states. However, their use is often limited by the development of several undesirable effects including tolerance and dependence. These side effects have been proposed to be a result of adaptive modifications in cellular responsiveness, and particularly desensitization and down-regulation of opioid receptors (Kieffer and Evans, 2002). A challenging hypothesis proposes that stimulation of opioid receptors triggers activation of anti-opioid systems that counteract opioid receptor stimulation by producing opposite effects (Rothman, 1992; Mollereau *et al.*, 2005a). Indeed, numerous experimental and clinical data have shown that opiates also activate anti-opioid systems that produce opposite and long-lasting effects leading to opioid-induced hyperalgesia (OIH), which reduces analgesic effects (tolerance) (Rothman, 1992; Mao *et al.*, 1995; Celerier *et al.*, 2000; Simonnet and Rivat, 2003). Although the molecular mechanisms underlying OIH are poorly understood, this phenomenon is associated with the sensitization of pronociceptive pathways in response to opioid treatment. In particular, abnormal activation of NMDA receptors in the CNS has been proposed to play a crucial role (Mao, 1999). Indeed, abrupt opioid withdrawal can induce long-term potentiation at synapses between nociceptive C fibres and neurons in superficial dorsal horn *via* NMDA receptor-dependent mechanisms, which could represent a mechanism of OIH (Drdla *et al.*, 2009). High doses of opiates in humans, particularly during surgery, are often associated with increased pain and post-operative demand for analgesics (Guignard *et al.*, 2000). Furthermore, the development of tolerance following chronic treatment with opiates requires consumption of escalating doses, which in turn renders the patient more hypersensitive to pain (Wilson and Reisfield, 2003). Activation of anti-opioid systems may also be involved in other adverse effects related to chronic opiate treatments including dependence and abstinence syndrome (Rothman, 1992; Ueda, 2004). Therefore, drugs blocking the activation of anti-opioid systems may prevent the development of tolerance to opioid analgesic effects and limit other adverse effects related to chronic opiate administration.

Several neuromodulator systems have been shown to display anti-opioid properties including NMDA, cholecystokinin (CCK), nociceptin/orphanin FQ (OFQ) and neuropeptide FF (NPFF) systems (Rothman, 1992; McNally, 1999; Mollereau *et al.*, 2005a; Ueda and Ueda, 2009). The NPFF system is composed of two receptor subtypes, NPFF1 and NPFF2, which bind to and are activated by four different peptides (Simonin, 2006). NPFF and neuropeptide AF arise from the same precursor protein and have greater affinity for NPFF2, whereas neuropeptide SF and neuropeptide VF (alias RFamide-related peptide-1 and RFamide-related peptide-3, respectively) arise from another precursor and preferentially activate NPFF1. Several lines of evidence support the role of NPFF as anti-opioid system: i.c.v. injections of NPFF or NPFF-related peptides produce transient hyperalgesia in rats and attenuate the analgesic effects of morphine (Yang *et al.*, 1985), while i.c.v. injections of IgG from NPFF antiserum restore sensitivity to morphine and the analgesic response in tolerant rats (Lake *et al.*, 1991; 1992). Furthermore, pretreatment with IgG prepared from NPFF antiserum prevents naloxone-precipitated abstinence syndrome in morphine-dependent rats (Malin *et al.*, 1990), whereas NPFF significantly potentiates the overall morphine withdrawal syndrome (Tan *et al.*, 1999) and inhibits morphine-induced conditioned place preference (CPP) (Kotlinska *et al.*, 2007). In mice, NPFF attenuated the development of morphine-induced CPP (Marchand *et al.*, 2006) and antisense nucleotides against NPFF decreased morphine tolerance and dependence (Gelot *et al.*, 1998). Altogether, these findings suggest that concomitant with activating opioid receptors opiates also trigger NPFF release, which counteracts excess activation of the opioid system leading to the development of tolerance and dependence. In agreement with this hypothesis, we have shown that RF9, a potent antagonist of both NPFF receptor subtypes, completely blocks long-lasting OIH and prevents the development of tolerance in rats undergoing chronic heroin treatment (Simonin *et al.*, 2006).

In the present study, we investigated the consequences of NPFF receptor blockade on acute and chronic stimulation of opioid receptors in mice. RF9 did not modify spontaneous locomotion and had no effect on morphine-induced horizontal hyperlocomotion, while it slightly reversed the decrease in vertical locomotor activity produced by morphine injection. Interestingly, RF9 potentiated the analgesic effects of opiates and completely prevented OIH and associated tolerance in two different paradigms and two different strains of mice. Furthermore, RF9 was found to increase morphine reward even though it had no rewarding properties by itself. Finally, blockade of NPFF receptors with RF9 decreased the severity of naltrexone-precipitated withdrawal syndrome. These data confirm the hypothesis that opioid receptor stimulation leads to long-lasting homeostatic adaptations and NPFF receptors play an essential role in these adaptations.

Methods

Animals

Experiments were performed on adult male mice C57BL6/N (Taconic, Ry, Denmark) weighing 25–30 g and Swiss mice (Dépré, St. Doulchard, France) weighing 20–25 g. Animals were housed in groups of five per cage under a 12 h/12 h light/dark cycle at a constant temperature of 21 ± 1°C with free access to food and water. They were habituated to the experimental room and handled for 1 week before starting experiments. All animal care and experimental procedures complied with the European guidelines for the care of laboratory animals (European Communities Directive 86/609/ECC) and were approved by the local ethical committees.

Drugs

Morphine hydrochloride was obtained from Francopia (Paris, France). Fentanyl citrate and naltrexone hydrochloride were

purchased from Sigma-Aldrich (Lyon, France). Trifluoroacetate of n-adamantanecarbonyl-Arg-Phe-NH_2 (RF9) was prepared as previously described (Simonin *et al.*, 2006). Compounds were dissolved in physiological saline (0.9%) and administered in a volume of 10 mL·kg^{-1}.

Actimetry assay

Locomotor activity was measured using automated actimetry cages (Imetronic, Pessac, France) equipped with infrared captors to measure horizontal and vertical activity. Before testing, mice were habituated to the cages for 30 min. Then they received a s.c. injection of either RF9 (5 mg·kg^{-1}) or saline. Fifteen minutes later, animals were treated with either morphine (5 mg·kg^{-1}, s.c.) or saline and then replaced in the cages. Locomotor activity was recorded for 2 h under a light intensity of 24 lux in a soundproof room.

Nociceptive tests

Tail immersion test. The tail immersion test was conducted as previously described by Simonin *et al.* (1998). Briefly, mice were restrained in a cylinder and their tail was immersed in a thermostated water bath. The nociceptive threshold was assessed by measuring the latency to withdraw the tail from the hot water, which was maintained at 52 ± 0.5°C. In the hyperalgesia experiments, the water temperature was maintained at 48 ± 0.5°C. At this temperature, the basal withdrawal latencies are higher (around 12 s) than at 52°C (around 2 s), which allows detection of a significant decrease in the baselines (hyperalgesia). In the absence of any nociceptive reaction, cut-offs of 15 s at 52°C and 25 s at 48°C were used to prevent tissue damage.

Hot plate test. In this test, animals were placed in a glass cylinder on a heated metal plate maintained at 52 ± 1°C (Columbus Instruments, Columbus, OH, USA). The latency of nociceptive responses such as licking or shaking one of the paws or jumping was recorded as the nociceptive threshold (Trigo *et al.*, 2009). In the absence of any nociceptive reaction, a 240 s cut-off was used to prevent tissue damage.

Tail flick test. In this test the mice tail was exposed to radiant heat using LE7106 Tail-flick Meter (Bioseb, Paris, France). When the animal feels pain, it reacts with a sudden movement of the tail (tail flick), which automatically stops the stimulation and the timer. Two consecutive trials with an interval of about 1 min were performed at two different sites on the tail. The first recording was performed near the peak of the mouse tail and the second was done near the base of the tail and the mean latency of both measures was calculated. A cut-off time of 15 s was used to avoid tissue damage.

Tail pressure test. This test is based on the method originally described in rats by Randall and Selitto (1957) and determines the response threshold to painful stimuli through pressure on the rat's paw. A constant increased pressure was applied to the tail of the mice using the analgesimeter LE 7306 (Panlab, Barcelona, Spain; tip of diameter of the stylus: 1 mm) until the first nociceptive reaction of struggling or squeaking. The pressure was applied on the distal, median and proximal part of the tail. The nociceptive threshold expressed in g was considered as the mean of these three experimental values (Celerier *et al.*, 2004). A 600 g cut-off value was used to avoid tissue damage.

Experimental protocols for studies on nociception

Before the effects of RF9 on OIH were investigated, experiments were conducted in mice to assess the effect of RF9 (5 mg·kg^{-1}, s.c.) on nociception and morphine analgesia. RF9 was administered 20 min before morphine (5 mg·kg^{-1}, s.c.) or saline injection. The nociceptive threshold was measured at 15 min after morphine or saline injection in the tail immersion test and 1 min later in the hot plate test as previously described (Trigo *et al.*, 2009).

The effect of RF9 on the OIH and tolerance was evaluated in two different models of OIH. In the first model, hyperalgesia induced by fentanyl was evaluated as previously described (Celerier *et al.*, 2000). A dose of fentanyl was injected four times (4 × 60 µg·kg^{-1}, s.c.) at 15 min intervals, which mimics its clinical use in surgery. RF9 was administered s.c. at 5 mg·kg^{-1}, 20 min before fentanyl or saline injections. The effect of RF9 on the short-lasting analgesic effect of fentanyl was evaluated by tail immersion test (52°C) on the day of fentanyl injection. The nociceptive threshold was measured 60 min after the final dose of fentanyl and then every 60 min over a period of 300 min. The effect of RF9 on long-lasting hyperalgesia induced by fentanyl was tested the day after fentanyl injections (day1; 24 h) and on days: day2 (48 h), day3 (72 h) and day4 (96 h). For this purpose, the nociceptive threshold was determined using tail immersion (48°C) and tail pressure tests. In a separate experiment, three additional doses of RF9 (2.5, 0.5 and 0.1 mg·kg^{-1}) were also used to establish the dose–response effect on the fentanyl-induced hyperalgesia using the same experimental procedure. The nociceptive threshold was measured in the tail immersion test (48°C) 1 day before fentanyl injections (day–1) and in the next days following fentanyl injections (day1, day2, day3 and day4). To study the effect of the four tested doses of RF9 (5, 2.5, 0.5 and 0.1 mg·kg^{-1}) on the overall long-lasting hyperalgesia of fentanyl (day1–day4), an algesia index was calculated for each group using the formula: [(nociceptive threshold values at dayn) – basal threshold value × n], where n represents the number of days (Celerier *et al.*, 2000). In order to better compare the amplitude of OIH at the different RF9 doses, we have presented, in Figure 3D, an OIH index that was calculated using the formula [algesia index of the tested group × 100/algesia index of the saline – fentanyl group)].

In the second model, hyperalgesia and tolerance were induced by daily administration of morphine at a high dose (20 mg·kg^{-1}, s.c.) for 7 days (day0 to day6) in the first experiment or at a moderate dose (5 mg·kg^{-1}, s.c.) for 10 days (day0 to day9) in the second experiment. RF9 (5 mg·kg^{-1}, s.c. or 2 mg·kg^{-1}, i.p., respectively) was administered 20 min before s.c. administration of morphine or saline. To evaluate the effects of RF9 on morphine analgesia and development of tolerance, tail-flick latencies were measured 30 min after morphine injection (5 mg·kg^{-1}, s.c.) on day0 and day7 in the first experiment and on days0, 4, 7 and 9 in the second experiment. The effect of RF9 on the hyperalgesia induced by morphine in the first experiment was evaluated by once-daily measurements of the basal nociceptive threshold in the tail pressure test 60 min before morphine administration

(20 mg·kg^{-1}, s.c.). Finally, to assess the effects of RF9 on the expression of morphine tolerance in the second experiment, RF9 (2 mg·kg^{-1}, i.p.) or saline was injected only on day9, 30 min before morphine (5 mg·kg^{-1}, s.c.) in tolerant mice and tail-flick latencies were measured 30 min later.

Conditioned place preference

This test was used to investigate the effect of RF9 on morphine-rewarding properties. The CPP apparatus consists of an acrylic plastic box divided into three compartments, two conditioning squares of identical size separated by a narrower area set in between. The walls and the ground of the conditioning compartments were different, one having striated walls and smooth ground and the other with dotted walls and a rough ground. The three compartments were connected by removable guillotine doors.

The CPP procedure (unbiased method) was similar to the method previously described by Maldonado *et al*. (1997) with slight modifications. It consists of the pre-conditioning phase (1 day), the conditioning phase (8 days) and the testing phase (1 day). During the pre-conditioning phase, each mouse was placed in the middle of the central division and had free access to the other compartments for 15 min. During the conditioning phase, mice were treated with alternate injections of morphine (3 or 5 mg·kg^{-1}, s.c.) and saline. Mice were confined to the corresponding compartment immediately after injection for 20 min. Treatments were counterbalanced between the two compartments to use an unbiased procedure. For the groups receiving RF9, a dose of 5 mg·kg^{-1} (s.c.) was administered 20 min before morphine or saline on days 1, 3, 5 and 7 and only saline was administered on days 2, 4, 6 and 8. Control animals received only saline each day.

After each conditioning trial, the whole box was cleaned thoroughly to prevent any interference due to the smell of faeces and urine. On the testing day, the experimental conditions were similar to the pre-conditioning phase and animals had free access to the compartments. A score was calculated for each mouse reflecting the difference between test and pre-conditioning time spent in the drug-paired compartment.

Morphine dependence and withdrawal

Morphine physical dependence was induced by an intermittent and escalating procedure of morphine treatment. Briefly, mice were treated with two s.c. injections of morphine each day (9:00 h and 17:00 h) at increasing doses. On the first day, mice received doses of 10 mg·kg^{-1} at 9:00 h and 20 mg·kg^{-1} at 17:00 h. On the second day, mice received doses of 20 mg·kg^{-1} at 9:00 h and 30 mg·kg^{-1} at 17:00 h. On day 3, mice received doses of 30 and 40 mg·kg^{-1} and then 40 and 50 mg·kg^{-1} on day 4. On day 5, mice received only one dose of 50 mg·kg^{-1} of morphine. Two hours after the last injection of morphine, the withdrawal syndrome was precipitated by injection of naltrexone (5 mg·kg^{-1}, s.c.) and evaluated over 30 min starting immediately after naltrexone injection. Jumping and paw tremors were counted over 30 min. The presence of diarrhoea was checked for 30 min with one point given for any signs of it during each 5 min period (maximum score: 6). Body weight was also measured before naltrexone injection and at the end of the observation period.

To study the effect of RF9 on morphine dependence, RF9 5 mg·kg^{-1} was administered s.c. before each morphine injection. Groups receiving RF9 or saline and saline instead of morphine were also included.

Statistical analyses

For the actimetry, thermal nociception, CPP and withdrawal experiments, data were analysed using two-way ANOVA. For the OIH and morphine tolerance experiments, data were analysed using two-way repeated-measures ANOVA. *Post hoc* analyses were performed with Bonferroni's test. In cases where mean of data and SEM were null (OIH index and jumping data), statistical analysis was performed by Kruskal–Wallis test followed by Mann–Whitney *U*-test. The level of significance was set at $P < 0.05$. All statistical analyses were carried out using the software STAT view.

Results

The RF9 doses used in this study were chosen because they completely prevented the development of fentanyl-induced hyperalgesia (see Figure 3D). Moreover, RF9 (5 mg·kg^{-1}, s.c. or 10 nmol, i.c.v) completely prevented the anti-morphine analgesia induced by 10 nmol (i.c.v.) NPFF, demonstrating that, at the doses used in this study, RF9 acts as an antagonist of NPFF receptors (see Figure S1).

Modulation of horizontal (HMA) and vertical (VMA) motor activity in mice

In these experiments the effects of RF9 on spontaneous motor activity were investigated and whether it modulated morphine locomotor effects. As shown in Figure 1, when administered with saline, RF9 (5 mg·kg^{-1}, s.c.) did not show any significant effect on horizontal (HMA) or vertical (VMA) motor activity. As expected, morphine (5 mg·kg^{-1}, s.c.) significantly increased HMA ($F_{1,51} = 54.07$, $P < 0.01$) but decreased significantly the VMA ($F_{1,51} = 18.99$, $P < 0.001$). Co-administration of RF9 (5 mg·kg^{-1}, s.c.) with morphine did not change the hyperlocomotion induced by morphine and partially reversed the effect of morphine on VMA ($F_{1,51} = 5.36$, $P < 0.05$).

Thermal nociception

In these experiments the effects of RF9 on acute morphine analgesia were assessed in two different tests of thermal nociception: tail immersion and hot plate (Figure 2). In the tail immersion test (Figure 2A), RF9 (5 mg·kg^{-1}, s.c.) had no significant effect on the basal nociceptive threshold of the animals, whereas morphine 5 mg·kg^{-1} (s.c.) had a significant analgesic effect ($F_{1,43} = 57.68$, $P < 0.001$). When administered s.c. 20 min before morphine, RF9 significantly potentiated this analgesic effect ($F_{1,43} = 5.07$, $P < 0.05$). In the hot plate test (Figure 2B,C), morphine (5 mg·kg^{-1}, s.c.) significantly increased the latency for licking ($F_{1,44} = 20.27$, $P < 0.001$) and jumping in mice ($F_{1,44} = 91.51$, $P < 0.001$). When administered before morphine or saline, RF9 (5 mg·kg^{-1}, s.c.) had no significant effect on basal nociceptive threshold and morphine analgesia.

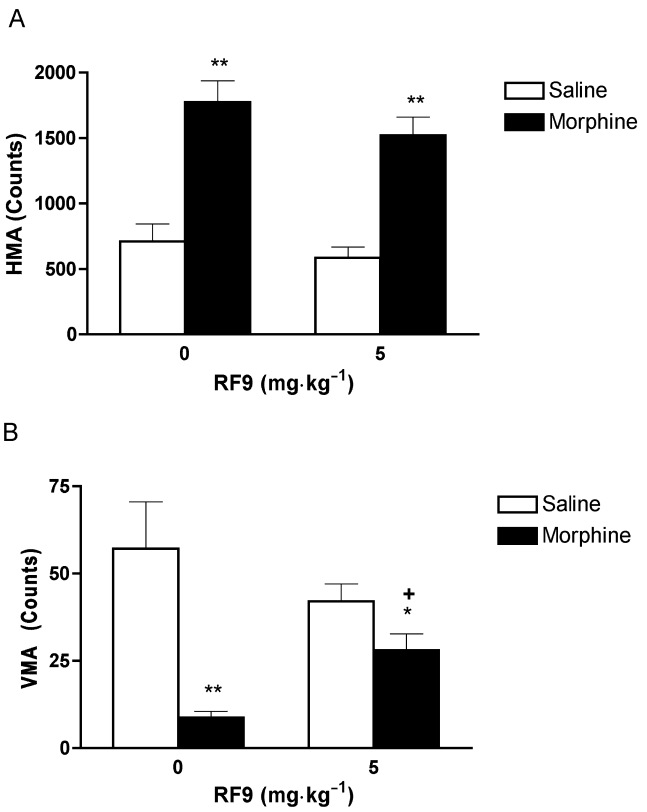

Figure 1

Effect of RF9 on the horizontal (A) and vertical (B) motor activity in mice. RF9 (5 mg·kg^{-1}, s.c.) was administered 20 min before morphine (5 mg·kg^{-1}, s.c.) or saline injection. Data are expressed as mean ± SEM, n = 10–18. *P < 0.05, **P < 0.01, Bonferroni's test as compared with the control group. +P < 0.05, Bonferroni's test as compared to the morphine-pretreated saline group.

Acute opioid-induced hyperalgesia

Fentanyl is a potent μ-opioid receptor agonist, which is used clinically. In rodents, it produces potent analgesia and secondary long-lasting hyperalgesia for several days following acute administration (Celerier *et al.*, 2000; 2004). We investigated whether RF9 modulated these effects in mice.

Following pretreatment with RF9 or saline, the analgesic effects of fentanyl were evaluated in the tail immersion test at 52°C. The data summarized in Figure 3A indicate that treatment with fentanyl (240 μg·kg^{-1}, s.c.) produced a strong analgesic effect for 5 h ($F_{4,144}$ = 52.96, P < 0.001). Pretreatment with RF9 (5 mg·kg^{-1}, s.c.) significantly potentiated the analgesic effect of fentanyl at 3 h ($F_{1,36}$ = 12.88, P < 0.001), and 5 h ($F_{1,36}$ = 7.42, P < 0.01) after fentanyl administration. As already shown in the previous experiment, RF9 at 5 mg·kg^{-1} had no effect on the basal nociceptive threshold.

On the following days, the effects of RF9 on the delayed hyperalgesic effects of fentanyl were evaluated by the tail pressure (Celerier *et al.*, 2004) and tail immersion tests. In the latter, water temperature was decreased to 48°C – compared with 52°C on day 0 – to increase the latency of tail withdrawal and allow a better measurement of the decrease in basal nociceptive threshold. As shown in Figure 3B,C, fentanyl significantly decreased the nociceptive threshold in both tail pressure ($F_{4,140}$ = 3.32, P < 0.01) and tail immersion tests ($F_{4,140}$ = 7.36, P < 0.001), respectively. *Post hoc* analyses revealed that the hyperalgesic effects were significant the first (P < 0.01) and second days (P < 0.01) following fentanyl treatment. In the group pretreated with RF9 at 5 mg·kg^{-1} before fentanyl, the hyperalgesia was completely abolished in both the tail pressure ($F_{4,14}$ = 3.71, P < 0.001) and tail immer-

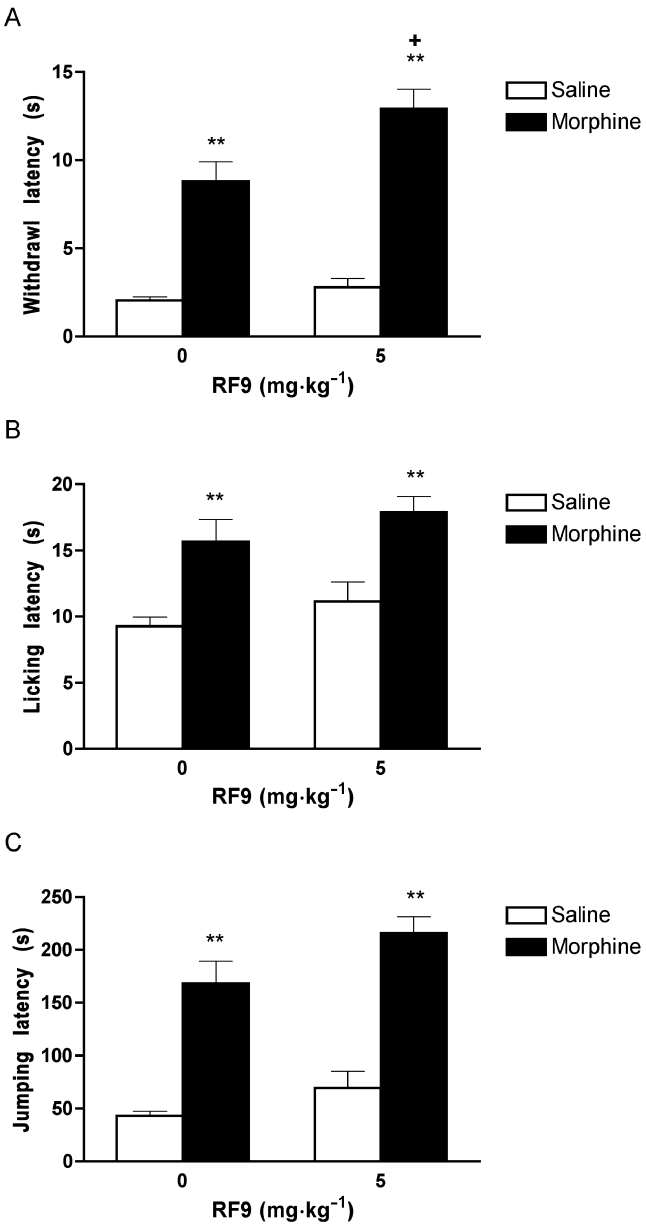

Figure 2

Effect of RF9 on basal nociceptive threshold and morphine analgesia in tail immersion (A) and hot plate (B, C) tests. RF9 (5 mg·kg^{-1}, s.c.) was administered 20 min before morphine (5 mg·kg^{-1}, s.c.) or saline injection. Data are expressed as mean ± SEM, n = 11–12. *P < 0.05, **P < 0.01, Bonferroni's test as compared with the control group. +P < 0.05, Bonferroni's test as compared with the morphine-pretreated saline group.

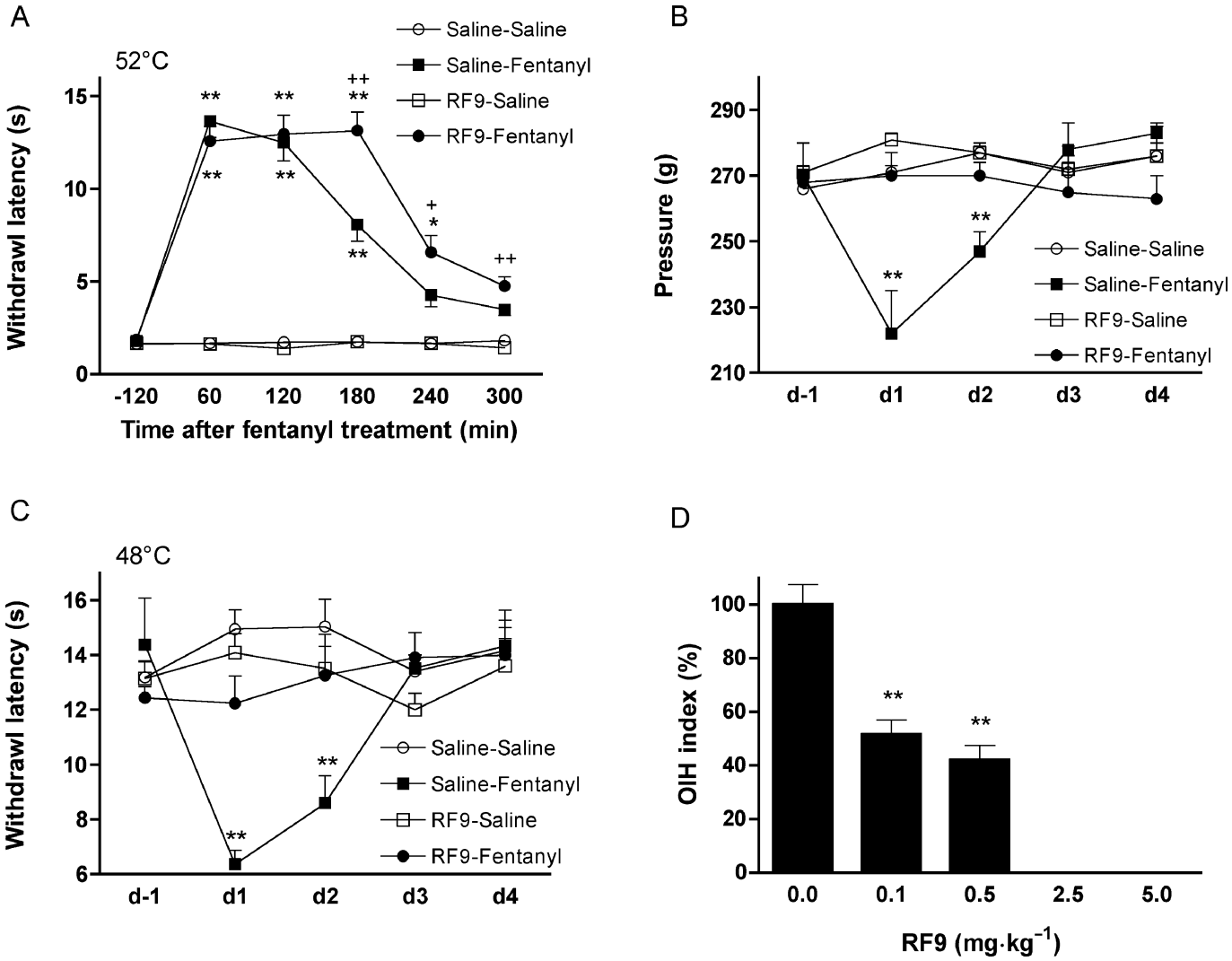

Figure 3

Effect of RF9 on the short-lasting fentanyl analgesia and long-lasting fentanyl-induced hyperalgesia. At day0 (d0), nociceptive threshold was measured after treatment with 4 × 60 μg·kg^{-1} (s.c.) of fentanyl, starting at 60 min after the last dose of fentanyl, using tail immersion test at 52°C (A). The long-lasting effects of fentanyl on the basal nociceptive threshold were determined in both mechanical (tail pressure, panel B) and thermal (tail immersion at 48°C, panel C) tests over the days following fentanyl treatment: day1 (24 h), day2 (48 h), day3 (72 h) and day4 (96 h). RF9 (5 mg·kg^{-1}, s.c.) was administered 20 min before the fentanyl or saline injection. To establish the dose–response effect of RF9 on fentanyl-induced hyperalgesia, four doses of this compound (5, 2.5, 0.5 and 0.1 mg·kg^{-1}, s.c.) were tested in a separate experiment and an OIH index was calculated for each dose as described in Methods section (D). Data are expressed as mean ± SEM, n = 9–10. *P < 0.05, **P < 0.01 Bonferroni's test as compared to the control group. +P < 0.05, ++P < 0.01 Bonferroni's test as compared to fentanyl-pretreated saline group. In (D), analyses were made by Mann–Whitney U-test.

sion tests ($F_{4,144}$ = 3.48, P < 0.001). We further evaluated the anti-hyperalgesic effect of lower doses of RF9 using the tail immersion test. For each dose, an OIH index was calculated (see Methods) and the results are presented in Figure 3D. The anti-hyperalgesic effect of RF9 was observed starting at a dose of 0.1 mg·kg^{-1}, with the maximal effect seen at 2.5 and 5 mg·kg^{-1} (H = 10.04, P < 0.01).

Morphine tolerance

We have previously shown that RF9 prevents the development of hyperalgesia and associated analgesic tolerance produced by repeated heroin administration in rats (Simonin *et al.*, 2006). In the present study, we evaluated the effects of RF9 on delayed hyperalgesia induced by repeated morphine administration in mice using the tail pressure test and on tolerance to morphine analgesia using the tail flick test.

When administered daily at 20 mg·kg^{-1}, morphine produced a significant and progressive lowering of the basal nociceptive threshold compared with control animals ($F_{6,216}$ = 6.06, P < 0.001; Figure 4A). This effect was significant from the fourth day after daily administration of morphine and

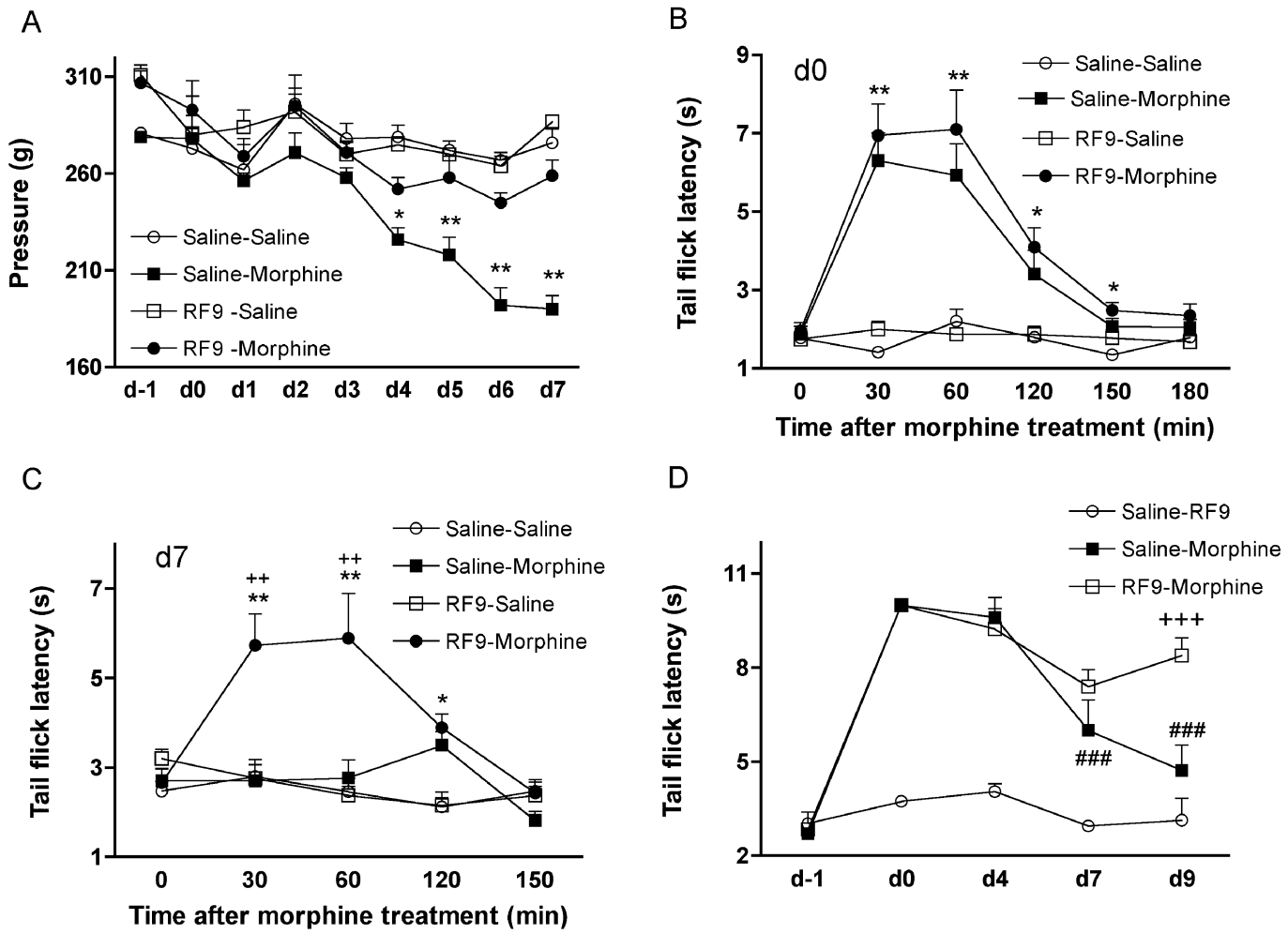

Figure 4

Effect of RF9 on the morphine-induced hyperalgesia (A) and morphine analgesic tolerance (B, C, D). For morphine-induced hyperalgesia, basal nociceptive threshold was measured in the tail pressure test once daily 1 h before morphine treatment (20 mg·kg^{-1}, s.c., day0 to day6). In the same experiment, morphine analgesia was determined after morphine treatment (5 mg·kg^{-1}, s.c.) in the tail flick test at day 0 (B) and day 7 (C). In a separate experiment, morphine was administered daily at a dose of 5 mg·kg^{-1} (s.c.) for10 days and the nociceptive threshold was measured in the tail flick test at days (d) indicated in the figure (D). RF9 was administered either 5 mg·kg^{-1}, s.c. (A, B, C) or 2 mg·kg^{-1}, i.p. (D), 20 min before morphine or saline injection. Data are expressed as mean ± SEM, $n = 9$–10. *$P < 0.05$, **$P < 0.01$ by Bonferroni's test as compared with the control group. +$P < 0.05$, ++$P < 0.01$, Bonferroni's test as compared with morphine-pretreated saline group. ###$P < 0.001$, Bonferroni's test as compared with day4.

persisted until the end of the experiment ($P < 0.01$). The hyperalgesic effect of morphine was completely prevented by co-administration of RF9 (5 mg·kg^{-1}, s.c.) with morphine ($F_{6,216} = 2.17$, $P < 0.05$), while RF9 co-administered with saline had no effect on the basal nociceptive threshold. In the same experiment, we measured the analgesic effect of morphine at a dose of 5 mg·kg^{-1} on days 0 (Figure 4B) and 7 (Figure 4C). On day0, morphine produced analgesia, as shown by the significant increase in tail flick latencies compared with control animals ($F_{5,180} = 22.92$, $P < 0.001$). Morphine displayed a similar analgesic effect in animals that were pretreated with RF9 (5 mg·kg^{-1}, s.c.). On day 7, mice treated with morphine (5 mg·kg^{-1}, s.c.) did not show any significant increase in tail flick latencies ($F_{4,72} = 2.34$, $P = 0.063$), indicating that these animals had developed tolerance to morphine analgesia. Conversely, in mice that were co-administered RF9, morphine displayed an antinociceptive effect on day7 at 30 min and 60 min after morphine treatment ($F_{1,36} = 9.08$, $P < 0.01$; $F_{1,36} = 6.56$, $P < 0.05$, respectively). RF9 *per se* had no effect on the basal nociceptive threshold.

To confirm the robustness of our observations, we further evaluated the development of tolerance with moderate dose of morphine (5 mg·kg^{-1}, s.c., daily administration for 10 days) in mice from a different genetic background (Swiss vs. C57BL6) using a lower dose of RF9 (2 mg·kg^{-1}) and a different route of administration (i.p. vs. s.c). Figure 4D shows that daily injection of morphine (5 mg·kg^{-1}, s.c.) resulted in the development of tolerance as indicated by a significant reduction of the antinociceptive effect of morphine at days 7 and 9 ($F_{4,34} = 27.8$, $P < 0.001$, one-way ANOVA). Again, chronic

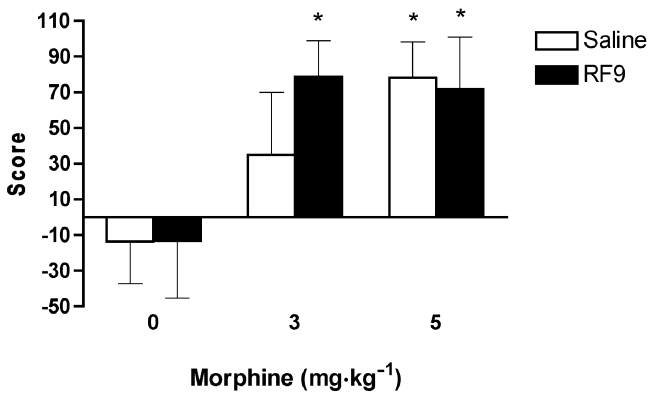

Figure 5

Effect of RF9 on the morphine-induced place preference. Results are represented as the difference between post-conditioning and pre-conditioning time spent in the drug-paired compartment. RF9 (5 mg·kg^{-1}, s.c.) was administered 20 min before saline or morphine injection. Morphine was administered at two doses: 3 and 5 mg·kg^{-1}, s.c. Data are expressed as mean ± SEM, n = 10–12. *P < 0.05, Bonferroni's test as compared with the control group.

administration of RF9 (2 mg·kg^{-1}, i.p.) had no effect on tail flick latencies but significantly reduced tolerance when co-administered with morphine ($F_{1,65}$ = 5.85, P < 0.001). Finally, we conducted experiments to evaluate the effects of acute RF9 treatment on the expression of tolerance to morphine analgesia. Mice treated daily with morphine (5 mg·kg^{-1}, s.c.) were challenged with RF9 (2 mg·kg^{-1}, i.p.) on day9, 30 min before morphine administration. Tail flick latencies of these mice (9.27 ± 0.39 s on day0 and 5.64 ± 0.64 s on day9) were not significantly different from those of mice treated with morphine only (8.90 ± 0.42 s on day0 and 5.93 ± 0.63 s on day9). This shows that once morphine analgesic tolerance is established, it cannot be reversed by a single dose of RF9.

Morphine conditioned place preference

To evaluate the role of NPFF receptors in other effects of chronic treatment with opiates, we performed morphine CPP experiments with or without co-administration of RF9 (Figure 5). Two-way ANOVA showed a significant effect of morphine treatment ($F_{2,64}$ = 5.31, P < 0.01), no significant effect of RF9 treatment and no interaction between RF9 and morphine treatments. RF9 (5 mg·kg^{-1}, s.c.) did not induce any place preference. When it was administered at a dose of 5 mg·kg^{-1}, morphine induced a significant rewarding effect in mice (P < 0.05), while at 3 mg·kg^{-1} it had no significant effect on place preference. Animals that received both morphine (3 mg·kg^{-1}, s.c.) and RF9 (5 mg·kg^{-1}, s.c.) showed a significant preference for the drug-associated compartment (P < 0.05).

Morphine withdrawal symptoms

These experiments were aimed at determining whether RF9 modulates the expression of naltrexone-precipitated withdrawal syndrome after chronic administration of escalating doses of morphine (see Methods for details). Figure 6 shows

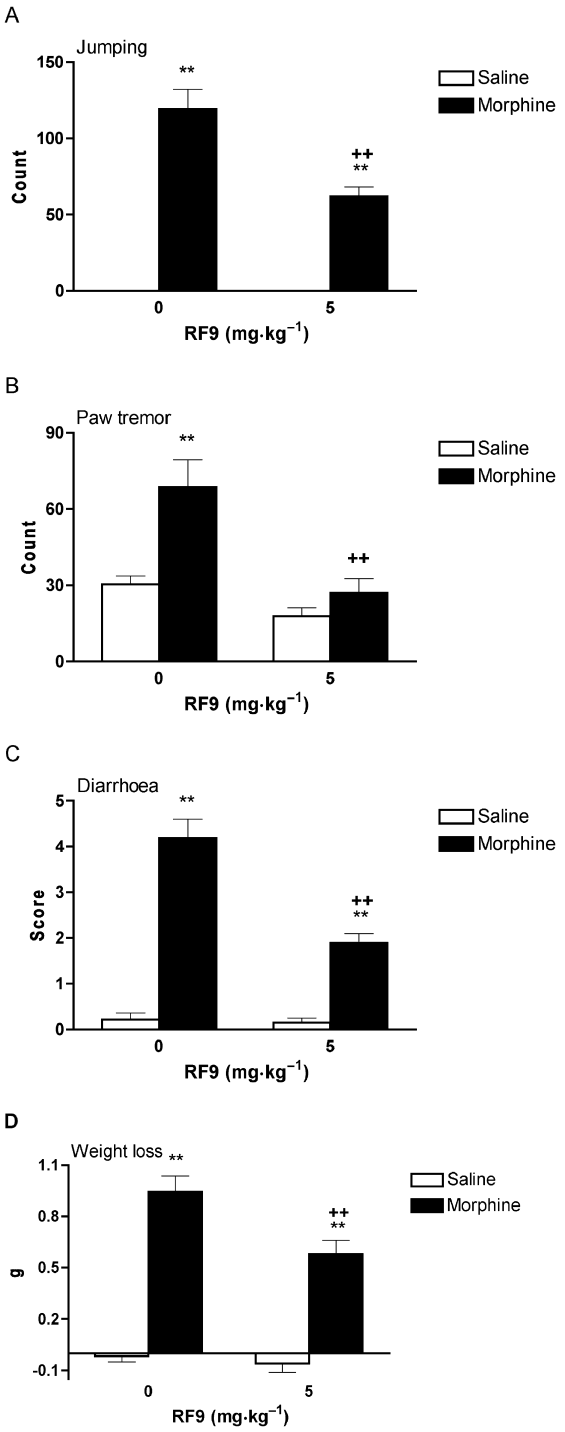

Figure 6

Effect of RF9 on naltrexone-precipitated withdrawal signs in morphine-dependent mice (A–D). The signs of withdrawal were measured over 30 min immediately after naltrexone injection. Morphine was given twice daily at escalating doses (from 10 to 50 mg·kg^{-1}, s.c.) over a period of 5 days. RF9 (5 mg·kg^{-1}, s.c.) was administered 20 min before morphine or saline injection. Data are expressed as mean ± SEM, n = 8–11. *P < 0.05, **P < 0.01, Bonferroni's test as compared with the control group. +P < 0.05, ++P < 0.01, Bonferroni's test as compared with morphine-pretreated saline group. In (A), analyses were made by Mann–Whitney U-test.

that administration of naltrexone 2 h after the last morphine injection induced high scores on several somatic and vegetative signs in the dependent mice compared with controls, including jumping (H = 30.73, $P < 0.001$), paw tremors ($F_{1,34}$ = 10.7, $P < 0.05$), diarrhoea ($F_{1,34}$ = 90.39, $P < 0.001$) and a loss of body weight ($F_{1,34}$ = 105.31, $P < 0.001$). In the group pretreated with RF9 (5 mg·kg^{-1}, s.c.) 20 min before each morphine administration, there was a significant decrease in the number of jumps (H = 30.73, $P < 0.001$) and paw tremors ($F_{1,34}$ = 5.4, $P < 0.05$). RF9 (5 mg·kg^{-1}, s.c.) also significantly decreased the intensity of the diarrhoea ($F_{1,34}$ = 13.11, $P < 0.001$) and prevented loss of body weight induced by injection of naltrexone in dependent mice ($F_{1,34}$ = 6.21, $P < 0.05$). When administered before saline, RF9 did not induce any signs of the withdrawal syndrome following an injection of naltrexone.

Discussion

RF9, a potent small antagonist of NPFF receptors, which can be administered systemically, has been shown to antagonize several different effects of NPFF both *in vivo* and *ex vivo* including increase in blood pressure and heart rate, hypothermia and reduction of bicuculline-sensitive inhibitory postsynaptic currents in parvocellular neurons from hypothalamic paraventricular nucleus (Simonin *et al.*, 2006; Jhamandas *et al.*, 2007; Wang *et al.*, 2008; Mouledous *et al.*, 2010). Using this compound in rats, we have demonstrated that blockade of NPFF receptors completely prevents the development of long-lasting OIH and associated analgesic tolerance (Simonin *et al.*, 2006). In the present study we used RF9 to confirm our previous observations and to further investigate the consequences of NPFF receptor blockade on analgesic responses and other opioid effects in mice. Our data revealed that RF9 has no effect on horizontal hyperlocomotor activity and only slightly attenuates the decrease in vertical activity induced by morphine. Furthermore, we showed that this compound completely and dose-dependently blocks the secondary and long-lasting hyperalgesia produced by either acute fentanyl or chronic morphine administrations. RF9 also potentiated the early analgesic effects of fentanyl and morphine and prevented the development, but not the expression, of morphine tolerance. Finally, NPFF receptor blockade with RF9 potentiated the CPP induced by morphine without inducing any rewarding effect by itself and decreased the naltrexone-precipitated morphine withdrawal syndrome.

NPFF binding sites have been reported to be colocalized with opioid receptors in the ventral tegmental area (Marco *et al.*, 1995). Administration of NPFF in this brain structure inhibited morphine-induced locomotor hyperactivity in rats (Marco *et al.*, 1995) and also reduced the morphine-induced release of dopamine and 5-HT in the nucleus accumbens (Kersanté *et al.*, unpublished observations). The NPFF system may therefore affect the modulation of mesocorticolimbic neurons activity induced by opioids. Our data revealed that RF9 did not affect the spontaneous motor activity and had no major effect on morphine-induced modifications of HMA and VMA. These results indicate that NPFF receptors do not play a major role in the modulation of motor activity in mice. RF9 is therefore a good pharmacological tool to investigate the involvement of NPFF receptors in the different effects of chronic opiate administration.

Two different models were used to investigate the effect of RF9 on OIH and tolerance. In the first model, opiate hyperalgesia was induced following acute administration of fentanyl using a procedure (four consecutive injections with 15 min intervals between each injection) that was designed to mimic its use in human surgery (Celerier *et al.*, 2000; 2004). In this model, we observed significant hyperalgesia in both the thermal and mechanical nociceptive tests over the 2 days following fentanyl administration. Pretreatment with RF9 before fentanyl completely prevented this hypersensitivity to either mechanical or thermal nociceptive stimuli. As previously suggested (Celerier *et al.*, 2000), these data indicate that anti-opioid systems are rapidly activated after the acute administration of a high-affinity full agonist of μ-opioid receptors such as fentanyl and this produces a long-lasting hyperalgesia. They also show that NPFF receptors play an essential role in this phenomenon. Accordingly, we also observed that RF9 – which has no effect on the basal nociceptive threshold in mice – potentiated and prolonged the analgesic effects of acute fentanyl or morphine injections in the tail immersion test. In the second model of OIH, in which we used chronic daily injections of morphine, we observed a progressive lowering of the basal nociceptive threshold that was completely prevented by co-administration of RF9. Tolerance to the analgesic effects of morphine was also prevented by co-administration of RF9 with the morphine in this model. However, a single dose of RF9 failed to restore morphine analgesia in tolerant mice. These results are in agreement with our previous studies in rats (Simonin *et al.*, 2006) and with the data from the first OIH model. Altogether, our results highlight the involvement of NPFF receptors in the development of opioid tolerance, but not in its expression. Opioids have recently been shown to induce synaptic long-term potentiation in the spinal cord (Drdla *et al.*, 2009; Zhou *et al.*, 2010). This represents a mechanism that could account for the development of OIH where NPFF receptors may be implicated. In agreement with this hypothesis, the NPFF2 receptor subtype has been shown to be expressed in the superficial layers of rodent spinal cord (Zeng *et al.*, 2003; Gouarderes *et al.*, 2004). Further studies to evaluate whether RF9 could block opioid-induced long-term potentiation are clearly of interest. The mechanism by which stimulation of NPFF receptors can block the activation of μ-opioid receptors remains unclear at the cellular level. Although these receptors are all coupled to Gi/o proteins, stimulation of both NPFF receptors subtypes – endogenously expressed in primary neurons or heterologously expressed in SH-SY5Y neuroblastoma cells – can antagonize the effect of μ agonists (Rebeyrolles *et al.*, 1996; Roumy and Zajac, 1999; Roumy *et al.*, 2003; Mollereau *et al.*, 2005b; Kersante *et al.*, 2006). However, the molecular mechanisms underlying these effects are yet to be determined.

Several studies have suggested that NPFF may play a role in opioid physical dependence and rewarding effects. NPFF and its stable derivative 1Dme respectively inhibit the expression and acquisition of morphine-induced CPP in rats (Marchand *et al.*, 2006; Kotlinska *et al.*, 2007). Pretreatment with IgG prepared from NPFF antiserum prevented

naloxone-precipitated withdrawal syndrome in morphine-dependent rats (Malin *et al.*, 1990). Furthermore, s.c. injection of dansyl-PQRamide, a putative antagonist of NPFF, attenuated the manifestations of naloxone-precipitated morphine abstinence in dependent rats (Malin *et al.*, 1995; Tan *et al.*, 1999). Our results indicate that blocking NPFF receptors using RF9 increased the expression of morphine CPP, but did not induce any rewarding effect *per see*. In addition, we showed that RF9, chronically administered with morphine, significantly reduced the expression and the severity of both the behavioural and somatic symptoms of morphine withdrawal. The reduction of the expression of four different withdrawal signs by RF9 suggests that this agent reverses the state of dependence in animals rather than the expression of a particular sign. Altogether, these data support an important role of the NPFF system in opioid rewarding effects and the development of opioid physical dependence. They clearly show that the interactions between NPFF and opioid systems are not limited to the nociceptive mechanisms and also extend to other responses related to opioid abuse liability, such as rewarding effects and physical dependence. Similar results were obtained by blocking other anti-opioid systems including nociceptin/OFQ and CCK systems. Pharmacological and genetic blockade of the endogenous nociceptin/OFQ system signalling has been shown to antagonize the anti-morphine action of nociceptin, reverse morphine tolerance and potentiate the rewarding effect of morphine in rats (Calo *et al.*, 2000; Scoto *et al.*, 2010; Rutten *et al.*, 2011). Similarly, a CCK_2 receptor antagonist has been found to enhance morphine analgesia and prevent morphine tolerance in rats (Dourish *et al.*, 1990; Idanpaan-Heikkila *et al.*, 1997). The data from these studies along with our work strongly support the hypothesis that activation of opioid receptors triggers the release of anti-opioid neuropeptides, including NPFF. These act as part of a homeostatic system to attenuate the effects of opioids and stopping opioid administration produces an excess of anti-opioids, which is partly responsible for the withdrawal syndrome. In line with this, i.c.v. injections of NPFF have been reported to reduce the expression of cocaine-induced CPP and sensitization to cocaine hyperlocomotor effects in rats, suggesting that the NPFF system could also be activated following chronic administration of other addictive drugs (Kotlinska *et al.*, 2008).

In conclusion, our data provide new evidence that the NPFF system is involved in the development of tolerance and dependence, two major undesirable effects clinically associated with prolonged exposure to opiates. Our findings support the hypothesis that these adaptations originate from an altered balance between the opioid and anti-opioid systems, particularly NPFF. We highlight NPFF receptors as interesting therapeutic targets to improve the analgesic efficacy of opioids, by limiting the development of tolerance and physical dependence. Our results should also lead to the development of new strategies to prevent abuse liability with opioids. In the future, the development of selective antagonists of each NPFF receptor subtype together with knockout animals for both NPFF1 and NPFF2 receptors will be helpful to confirm the role of these receptors and clarify their respective contribution to the modulation of opioid responses.

Acknowledgements

We gratefully acknowledge Jean Paul Humbert and Claire Gavériaux for technical assistance. This work was supported by grants from Agence National de la Recherche (ANR-06-Neuro-041-01), Direction Générale des Entreprises, Association de la Recherche pour le Cancer (4955). This work was also supported by the Spanish 'Ministerio de Ciencia e Innovación' (#SAF2007-64062), 'Instituto de Salud Carlos III' (#RD06/001/001), the Catalan Government (SGR2009-00131), the ICREA Foundation (ICREA Academia-2008). JMT was supported by 'becas para estancias cortas de especialización e investigación en psiquiatría y psicología de la infancia y adolescencia o en neurociencias', Fundación Alicia Koplowitz.

Conflicts of interest

F B is an employee of Phenopro. There are no other conflicts to declare.

References

Calo G, Guerrini R, Bigoni R, Rizzi A, Marzola G, Okawa H *et al.* (2000). Characterization of [Nphe(1)]nociceptin(1-13)NH(2), a new selective nociceptin receptor antagonist. Br J Pharmacol 129: 1183–1193.

Celerier E, Rivat C, Jun Y, Laulin JP, Larcher A, Reynier P *et al.* (2000). Long-lasting hyperalgesia induced by fentanyl in rats: preventive effect of ketamine. Anesthesiology 92: 465–472.

Celerier E, Simonnet G, Maldonado R (2004). Prevention of fentanyl-induced delayed pronociceptive effects in mice lacking the protein kinase Cgamma gene. Neuropharmacology 46: 264–272.

Dourish CT, O'Neill MF, Coughlan J, Kitchener SJ, Hawley D, Iversen SD (1990). The selective CCK-B receptor antagonist L-365,260 enhances morphine analgesia and prevents morphine tolerance in the rat. Eur J Pharmacol 176: 35–44.

Drdla R, Gassner M, Gingl E, Sandkuhler J (2009). Induction of synaptic long-term potentiation after opioid withdrawal. Science 325: 207–210.

Gelot A, Frances B, Gicquel S, Zajac JM (1998). Antisense oligonucleotides to human SQA-neuropeptide FF decrease morphine tolerance and dependence in mice. Eur J Pharmacol 358: 203–206.

Gouarderes C, Puget A, Zajac JM (2004). Detailed distribution of neuropeptide FF receptors (NPFF1 and NPFF2) in the rat, mouse, octodon, rabbit, guinea pig, and marmoset monkey brains: a comparative autoradiographic study. Synapse 51: 249–269.

Guignard B, Bossard AE, Coste C, Sessler DI, Lebrault C, Alfonsi P *et al.* (2000). Acute opioid tolerance: intraoperative remifentanil increases postoperative pain and morphine requirement. Anesthesiology 93: 409–417.

Idanpaan-Heikkila JJ, Guilbaud G, Kayser V (1997). Prevention of tolerance to the antinociceptive effects of systemic morphine by a selective cholecystokinin-B receptor antagonist in a rat model of peripheral neuropathy. J Pharmacol Exp Ther 282: 1366–1372.

Jhamandas JH, Simonin F, Bourguignon JJ, Harris KH (2007). Neuropeptide FF and neuropeptide VF inhibit GABAergic neurotransmission in parvocellular neurons of the rat hypothalamic paraventricular nucleus. Am J Physiol Regul Integr Comp Physiol 292: R1872–R1880.

Kersante F, Mollereau C, Zajac JM, Roumy M (2006). Anti-opioid activities of NPFF1 receptors in a SH-SY5Y model. Peptides 27: 980–989.

Kieffer BL, Evans CJ (2002). Opioid tolerance – In search of the holy grail. Cell 108: 587–590.

Kotlinska J, Pachuta A, Dylag T, Silberring J (2007). Neuropeptide FF (NPFF) reduces the expression of morphine – but not of ethanol-induced conditioned place preference in rats. Peptides 28: 2235–2242.

Kotlinska J, Pachuta A, Silberring J (2008). Neuropeptide FF (NPFF) reduces the expression of cocaine-induced conditioned place preference and cocaine-induced sensitization in animals. Peptides 29: 933–939.

Lake JR, Hammond MV, Shaddox RC, Hunsicker LM, Yang HY, Malin DH (1991). IgG from neuropeptide FF antiserum reverses morphine tolerance in the rat. Neurosci Lett 132: 29–32.

Lake JR, Hebert KM, Payza K, Deshotel KD, Hausam DD, Witherspoon WE *et al*. (1992). Analog of neuropeptide FF attenuates morphine tolerance. Neurosci Lett 146: 203–206.

Maldonado R, Saiardi A, Valverde O, Samad TA, Roques BP, Borrelli E (1997). Absence of opiate rewarding effects in mice lacking dopamine D2 receptors. Nature 388: 586–589.

Malin DH, Lake JR, Hammond MV, Fowler DE, Rogillio RB, Brown SL *et al*. (1990). FMRF-NH2-like mammalian octapeptide: possible role in opiate dependence and abstinence. Peptides 11: 969–972.

Malin DH, Lake JR, Smith DA, Jones JA, Morel J, Claunch AE *et al*. (1995). Subcutaneous injection of an analog of neuropeptide FF prevents naloxone-precipitated morphine abstinence syndrome. Drug Alcohol Depend 40: 37–42.

Mao J (1999). NMDA and opioid receptors: their interactions in antinociception, tolerance and neuroplasticity. Brain Res Rev 30: 289–304.

Mao J, Price DD, Mayer DJ (1995). Mechanisms of hyperalgesia and morphine tolerance: a current view of their possible interactions. Pain 62: 259–274.

Marchand S, Betourne A, Marty V, Daumas S, Halley H, Lassalle JM *et al*. (2006). A neuropeptide FF agonist blocks the acquisition of conditioned place preference to morphine in C57Bl/6J mice. Peptides 27: 964–972.

Marco N, Stinus L, Allard M, Le Moal M, Simonnet G (1995). Neuropeptide FLFQRFamide receptors within the ventral mesenchephalon and dopaminergic terminal areas: localization and functional antiopioid involvement. Neuroscience 64: 1035–1044.

Max MB, Stewart WF (2008). The molecular epidemiology of pain: a new discipline for drug discovery. Nat Rev Drug Discov 7: 647–658.

McNally GP (1999). Pain facilitatory circuits in the mammalian central nervous system: their behavioral significance and role in morphine analgesic tolerance. Neurosci Biobehav Rev 23: 1059–1078.

Mollereau C, Roumy M, Zajac JM (2005a). Opioid-modulating peptides: mechanisms of action. Curr Top Med Chem 5: 341–355.

Mollereau C, Mazarguil H, Zajac JM, Roumy M (2005b). Neuropeptide FF (NPFF) analogs functionally antagonize opioid activities in NPFF2 receptor-transfected SH-SY5Y neuroblastoma cells. Mol Pharmacol 67: 965–975.

Mouledous L, Mollereau C, Zajac JM (2010). Opioid-modulating properties of the neuropeptide FF system. Biofactors 36: 423–429.

Randall LO, Selitto JJ (1957). A method for measurement of analgesic activity on inflamed tissue. Arch Int Pharmacodyn Ther 111: 409–419.

Rebeyrolles S, Zajac JM, Roumy M (1996). Neuropeptide FF reverses the effect of mu-opioid on Ca2+ channels in rat spinal ganglion neurones. Neuroreport 7: 2979–2981.

Rothman RB (1992). A review of the role of anti-opioid peptides in morphine tolerance and dependence. Synapse 12: 129–138.

Roumy M, Zajac J (1999). Neuropeptide FF selectively attenuates the effects of nociceptin on acutely dissociated neurons of the rat dorsal raphe nucleus. Brain Res 845: 208–214.

Roumy M, Garnier M, Zajac JM (2003). Neuropeptide FF receptors 1 and 2 exert an anti-opioid activity in acutely dissociated rat dorsal raphe and periventricular hypothalamic neurones. Neurosci Lett 348: 159–162.

Rutten K, De Vry J, Bruckmann W, Tzschentke TM (2011). Pharmacological blockade or genetic knockout of the NOP receptor potentiates the rewarding effect of morphine in rats. Drug Alcohol Depend 114: 253–256.

Scoto GM, Arico G, Iemolo A, Ronsisvalle G, Parenti C (2010). Selective inhibition of the NOP receptor in the ventrolateral periaqueductal gray attenuates the development and the expression of tolerance to morphine-induced antinociception in rats. Peptides 31: 696–700.

Simonin F (2006). Neuropeptide FF receptors as therapeutic targets. Drugs Future 31: 603–609.

Simonin F, Valverde O, Smadja S, Slowe S, Kitchen I, Dierich A *et al*. (1998). Disruption of the κ-opioid receptor gene in mice enhances sensitivity to chemical visceral pain, impairs pharmacological actions of the selective κ-agonist U-50,488H and attenuates morphine withdrawal. EMBO J 17: 886–897.

Simonin F, Schmitt M, Laulin J-P, Laboureyras E, Jhamandas JH, MacTavish D *et al*. (2006). RF9, a potent and selective neuropeptide FF receptor antagonist, prevents opioid-induced tolerance associated with hyperalgesia. Proc Natl Acad Sci U S A 103: 466–471.

Simonnet G, Rivat C (2003). Opioid-induced hyperalgesia: abnormal or normal pain? Neuroreport 14: 1–7.

Tan PP, Chen JC, Li JY, Liang KW, Wong CH, Huang EY (1999). Modulation of naloxone-precipitated morphine withdrawal syndromes in rats by neuropeptide FF analogs. Peptides 20: 1211–1217.

Trigo JM, Zimmer A, Maldonado R (2009). Nicotine anxiogenic and rewarding effects are decreased in mice lacking beta-endorphin. Neuropharmacology 56: 1147–1153.

Ueda H (2004). Locus-specific involvement of anti-opioid systems in morphine tolerance and dependence. Ann N Y Acad Sci 1025: 376–382.

Ueda H, Ueda M (2009). Mechanisms underlying morphine analgesic tolerance and dependence. Front Biosci 14: 5260–5672.

Wang YQ, Guo J, Wang SB, Fang Q, He F, Wang R (2008). Neuropeptide FF receptors antagonist, RF9, attenuates opioid-evoked hypothermia in mice. Peptides 29: 1183–1190.

Wilson GR, Reisfield GM (2003). Morphine hyperalgesia: a case report. Am J Hosp Palliat Care 20: 459–461.

Yang HY, Fratta W, Majane EA, 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.

Zeng Z, McDonald TP, Wang R, Liu Q, Austin CP (2003). Neuropeptide FF receptor 2 (NPFF2) is localized to pain-processing regions in the primate spinal cord and the lower level of the medulla oblongata. J Chem Neuroanat 25: 269–278.

Zhou HY, Chen SR, Chen H, Pan HL (2010). Opioid-induced long-term potentiation in the spinal cord is a presynaptic event. J Neurosci 30: 4460–4466.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Effect of RF9 given i.c.v. (10 nmol) or s.c. (5 mg·kg^{-1}) on the NPFF-induced anti-morphine analgesia. Nociceptive thresholds were evaluated in the tail immersion test at 30 min. after i.c.v. Injection. NPFF (10 nmol) and morphine (1.5 nmol) were injected i.c.v. alone or simultaneously co-injected in a volume of 5 µl. Results are expressed as mean ± S.E.M. from 6–10 mice depending on the group. *$p < 0.05$, **$p < 0.01$ (One-way ANOVA followed by Bonferroni's test). One way ANOVA showed that there is significant differences between tested groups ($F_{(4, 32)} = 6.63$, $p < 0.001$). Post-hoc analyses with Bonferroni's test showed that i.c.v. Injection of morphine (1.5 nM) displayed a strong analgesic effect ($p < 0.01$ versus saline group) which is significantly inhibited when it was co-injected with NPFF (10 nmol) ($p < 0.01$ versus NPFF + morphine group). Pre-treatment with RF9 (5 mg·kg^{-1}, s.c.) 30 min before i.c.v. injection of morphine and NPFF reversed the NPFF effect and restored morphine analgesia ($p < 0.05$ versus NPFF + morphine group). Similarly, when it was co-injected i.c.v. with morphine and NPFF, RF9 reversed the NPFF effect ($p < 0.05$ versus NPFF + morphine group).

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.