Analgesic effects of a novel pH-dependent \( \mu \)-opioid receptor agonist in models of neuropathic and abdominal pain

Antonio Rodriguez-Gazelumendi, Viola Spahn, Dominika Labuz, Halina Machelska, Christoph Stein*  

Abstract  
Recently, (±)-N-(3-fluoro-1-phenethylpiperidine-4-yl)-N-phenyl propionamide (NFEPP), a newly designed \( \mu \)-opioid receptor (MOR) agonist with a low pKa, has been shown to produce injury-restricted analgesia in models of inflammatory and postoperative pain, without exhibiting typical opioid side effects. Here, we investigated MOR binding of NFEPP in brain and dorsal root ganglia, pH in injured tissues, and the analgesic efficacy of NFEPP compared with fentanyl in a chronic constriction injury model of neuropathic pain, and in the acetic acid–induced abdominal writhing assay in rats. Binding experiments revealed significantly lower affinity of NFEPP compared with fentanyl at pH 7.4. In vivo, pH significantly dropped both at injured nerves after chronic constriction injury and in the abdominal cavity after acetic acid administration. Intravenous NFEPP as well as fentanyl dose-dependently diminished neuropathy-induced mechanical and heat hypersensitivity, and acetic acid–induced abdominal constrictions. In both models, NFEPP-induced analgesia was fully reversed by naloxone methiodide, a peripherally restricted opioid receptor antagonist, injected at the nerve injury site or into the abdominal cavity. Our results indicate that NFEPP exerts peripheral opioid receptor–mediated analgesia exclusively in damaged tissue in models of neuropathic and abdominal pain.  
Keywords: NFEPP, Peripheral opioid receptors, Acidic pH, Neuropathic pain, Abdominal pain

1. Introduction

Both conventional opioids and nonsteroidal analgesics (nonsteroidal anti-inflammatory drugs) produce detrimental side effects. Opioids exert sedation, apnoea, nausea, addiction, and constipation mediated in brain or gut, whereas cyclooxygenase inhibitors can elicit ulcers, bleeding, myocardial infarction, or stroke.8,12,13 Previous strategies in drug development have focused on central opioid receptors in noninjured environments.8,9,14 However, a large number of painful syndromes (eg, arthritis, neuropathy, and surgery) are driven by peripheral opioid receptors and are typically accompanied by inflammation with tissue acidosis.1,14,19,34 Under such circumstances, opioid receptors and their signaling pathways in dorsal root ganglion (DRG) neurons are upregulated.5 Targeting peripheral opioid receptors in damaged tissue avoids adverse opioid effects in the brain or gut, as well as detrimental side effects of NSAIDs, as demonstrated in animal models and humans with acute and chronic pain.11,29,32,37,41 Moreover, pharmacological, genetic, and clinical studies have shown that a large proportion of opioid analgesia is mediated by peripheral opioid receptors10,15,39 and that such receptors can confer significant anti-inflammatory effects.4,8

By computer simulations at low pH, a hallmark of injured tissue, we recently designed the novel opioid (±)-N-(3-fluoro-1-phenethylpiperidine-4-yl)-N-phenyl propionamide (NFEPP) which, due to its low acid dissociation constant (pKa = 6.8), selectively activates peripheral \( \mu \)-opioid receptors (MORs) in inflamed tissue.32 NFEPP showed pH-sensitive binding, G-protein subunit dissociation, and 3', 5'-cyclic adenosine monophosphate inhibition in MOR-transfected human embryonic kidney 293 cells. It did not produce typical side effects mediated by central or intestinal MOR exposed to normal pH (about 7.4), such as respiratory depression, sedation, addiction, and constipation. Both NFEPP and conventional fentanyl inhibited pain with similar efficacy in rats with complete Freund adjuvant–induced inflammation or incision of one hind paw.32 In this study, we investigated MOR binding of NFEPP in native tissues using membranes of brain and DRG, in vivo pH of damaged tissue, and the analgesic efficacy of NFEPP compared with fentanyl in the chronic constriction injury (CCI) model of neuropathic pain and in the acetic acid–induced abdominal writhing assay in rats.

2. Methods

2.1. Chemicals/drugs

Fentanyl citrate (F3886), naloxone hydrochloride (NLX; N7758), naloxone methiodide (NLXM; N129), and acetic acid were purchased...
from Sigma-Aldrich (Taufkirchen, Germany), and [\(^{3}H\)]-D-Ala\(^2\)N-Me-Phe\(^4\), Gly\(^5\)-ol]-enkephalin ([\(^{3}H\)]-DAMGO; NET902250UC) from Perkin Elmer (Waltham, MA). NFEPF was synthesized according to our design by a contractor (ASCA GmbH, Berlin, Germany).\(^{32}\) NFEPF was dissolved in dimethyl-sulfoxide (DMSO; Sigma-Aldrich) and diluted with 0.9% NaCl or in binding assay buffer. The maximum DMSO concentration for intravenous (i.v.) injections was 0.5%. Fentanyl and NLXM were dissolved in 0.9% NaCl. Control groups were treated with the respective vehicles.

### 2.2. Animals

All experiments were approved by the state animal care committee (Landesamt für Gesundheit und Soziales, Berlin) and performed according to the ARRIVE guidelines.\(^{16}\) Male Wistar rats (200-300 g; Janvier Laboratories, Le Genest-Saint-Isle, France) were kept on a 12-hour light/dark schedule, in groups of 3 in cages lined with ground corncob bedding, with free access to standard laboratory food and tap water. Room temperature was 22 ± 0.5°C and humidity 60% to 65%. Statistical power calculations were performed a priori to determine minimum sample sizes. Rats were handled once per day for 1 to 2 minutes or habituated to the test cages (1-2 times for 15 minutes), starting 4 days before experiments. After completion of experiments, animals were killed with an overdose of isoflurane (AbbVie, Wiesbaden, Germany).

### 2.3. Isolation of brain, dorsal root ganglion, and membrane preparation

Naive rats were killed by an overdose of isoflurane. Brains without cerebella were removed, collected in ice-cold binding assay buffer (Trizma, 50 mM, pH 7.4), homogenized, and centrifuged at 42,000 g for 20 minutes at 4°C. The pellet was resuspended in assay buffer, followed by centrifugation at 42,000 g and 4°C for 20 minutes. Protein concentration was determined using the Bradford method.\(^{5}\) Membrane preparation of lumbar and thoracic DRG was performed in the same way except for an additional incubation step at 37°C for 10 minutes after the first centrifugation, and the addition of 1 mM EGTA to the assay buffer, as described by us before.\(^{31,42}\)

The corrected half maximal concentration (IC\(_{50}\)) of fentanyl or NFEPP necessary to displace 4 nM of the standard MOR ligand ([\(^{3}H\)]-DAMGO) was determined at different pH values (5.5, 6.5, and 7.4), as described previously.\(^{32}\) A protein amount of 100 μg was incubated for 90 minutes at room temperature with the radioligand (53.7 Ci/mmol) and the competing ligands (fentanyl or NFEPP) dissolved in binding assay buffer at the respective pH values. Nonspecific binding was determined by the addition of 10 μM of NLX. Filters were soaked in 0.1% polyethyleneimine solution before use. Bound and free ligands were separated by rapid filtration under vacuum through Whatman GF/B glass fiber filters. Bound radioactivity was assessed by liquid scintillation spectrophotometry with a counting efficiency of 69% for [\(^{3}H\)] after overnight extraction of the filters in the scintillation fluid.

### 2.4. Chronic constriction injury model

Chronic constriction injury of the sciatic nerve was induced under isoflurane anesthesia as described elsewhere.\(^{3}\) Briefly, the sciatic nerve was exposed at the level of the left midthigh, 4 loose 4/0 silk ligatures were placed around the nerve with approximately 1 to 2 mm spacing, and the wound was closed with silk sutures.

### 2.4.1. Mechanical hyperalgesia (Randall–Selitto test)

Rats were gently restrained under paper wadding and incremental pressure was applied using a wedge-shaped, blunt piston onto the dorsal surface of the hind paws by means of an automated gauge (Ugo Basile, Comerio, Italy). The paw pressure threshold (PPT; cutoff at 250 g) required to elicit paw withdrawal was determined by averaging 3 consecutive trials separated by 15-second intervals. The sequence of paws was alternated between animals to avoid “order” effects, as described previously.\(^{32}\)

### 2.4.2. Mechanical allodynia (von Frey test)

Animals were individually placed in clear Plexiglas cubicles located on a stand with anodized mesh (Model 410; IITC Life Science, Woodland Hills, CA). The plantar surface of each hind paw was stimulated using von Frey filaments (Stoetling, Wood Dale, IL) of increasing force until the filament that produced withdrawal responses to 3 stimuli (paw withdrawal threshold [PWT]) was reached, as described previously.\(^{22}\) The strengths of calibrated von Frey filaments were 0.6, 1, 1.4, 2, 4, 6, 8, 10, 15, and 26 g.

### 2.4.3. Heat hyperalgesia (Hargreaves test)

Rats were individually placed in clear Plexiglas cubicles positioned on a stand with glass surface. Radiant heat generated by a high-intensity light bulb was applied to the plantar surface of the hind paws from underneath the glass surface, and paw withdrawal latency (PWL) was measured using an electronic timer (Model 930; IITC Life Science), as described earlier.\(^{13}\) Three measurements separated by at least 10 seconds were averaged. The heat intensity was adjusted to obtain a baseline withdrawal latency of about 10 to 12 seconds in uninjured paws, and the cutoff was 20 seconds.

### 2.5. Writhing model

Animals received 1% acetic acid intraperitoneally (i.p.; 10 mL/kg) under brief isoflurane anesthesia, and were placed individually in transparent cages for observation of abdominal constrictions (“writhing”).\(^{38}\) The total number of writhes between 5 and 35 minutes after acetic acid injection was counted.

### 2.6. pH measurements

For in vivo measurements, a pH-sensitive glass microelectrode (model IC-401 combination pH electrode; Warner Instruments, Hamden, CT) was calibrated using reference solutions of pH 4.0, 7.0, and 9.2. Measurements were performed at 3, 7, and 14 days after CCI, or before, 5, 15, and 30 minutes after i.p. acetic acid injections, under isoflurane anesthesia. In the CCI model, the microelectrode mounted in the lumen of the 20-gauge needle was inserted close to the sciatic nerve, in an area of 2 to 6 mm around the ligations, or in a similar location on the contralateral uninjured limb. In the writhing model, the needle was introduced until it perforated the skin and moved freely into the abdominal cavity. Stable readings were obtained 2 to 3 minutes after the electrode insertion in both cases. In vitro, we measured the pH of saline (0.9% NaCl; vehicle) and NLXM dissolved in saline (50 μg/100 μL) using a pH-sensitive glass electrode (Mettler Toledo; InLab Routine Pro, Giessen, Germany) calibrated using reference solutions of pH 4.01, 7.0, and 9.21.
2.7. Injections and experimental protocols

Injections of opioid agonists and antagonist were performed i.v. (200 μL), at the nerve injury site (100 μL), or i.p. (100 μL) under brief isoflurane anesthesia. In the CCI model, effects on nociceptive thresholds (PPT, PWT, and PWL) of fentanyl (4-16 μg/kg i.v.) and NFEPP (4-16 μg/kg i.v.) were evaluated before and 15 to 60 minutes after injections, on day 14 after CCI. To examine the contribution of peripheral opioid receptors, we used NLXM, an opioid receptor antagonist that does not readily cross the blood–brain barrier.6 NLXM (50 μg/rat) was injected at the nerve injury site immediately before i.v. injection of agonists, and nociceptive thresholds were measured 15 minutes later.

In the writhing model, fentanyl (4-16 μg/kg i.v.) and NFEPP (4-16 μg/kg i.v.) were injected immediately after acetic acid, and the total number of writhes was counted thereafter within 5 to 35 minutes. NLXM (50 μg/rat i.p.) was injected immediately after injection of acetic acid and right before i.v. injection of agonists, and the number of writhes was counted thereafter within 5 to 35 minutes. All dosages were determined in pilot experiments. The experimenter was blinded to the treatments and dosages.

2.8. Data handling and statistical analyses

All data were assessed for normal distribution and equal variances by Kolmogorov–Smirnov normality tests. In binding experiments, means of values at each agonist concentration and each pH were determined to calculate IC\textsubscript{50} by nonlinear regression, which were then subjected to Friedman 1-way analysis of variance (ANOVA) and Dunn tests. All behavioral data were expressed as raw values. Two-sample comparisons were made using paired or unpaired t tests for normally distributed data, or Wilcoxon or Mann–Whitney tests for non-normally distributed data. Two-way repeated-measures ANOVA and Bonferroni test were used to compare 2 parameters over time. To analyze one parameter over time, 1-way repeated-measures ANOVA and Bonferroni test were used. Multiple comparisons at one time point were performed using 1-way ANOVA followed by Bonferroni test for normally distributed data, or Kruskal–Wallis 1-way ANOVA followed by Dunn test for non-normally distributed data. Differences were considered significant if P < 0.05. Prism 5 (GraphPad, San Diego, CA) was used for all tests and graphs, and all data are expressed as mean ± SEM.

3. Results

3.1. NFEPP binding affinity is decreased at pH 7.4

In membranes of brain, the IC\textsubscript{50} values to displace DAMGO binding did not significantly differ between fentanyl and NFEPP at pH 5.5 (P > 0.05). However, at pH 6.5 and 7.4, the IC\textsubscript{50} values of NFEPP significantly increased (ie, its affinity decreased) compared with fentanyl (P < 0.05) (Fig. 1; Fig. S1, available online as supplemental digital content at http://links.lww.com/PAIN/A612—legend, http://links.lww.com/PAIN/A618; and Table S1, available online as supplemental digital content at http://links.lww.com/PAIN/A615). We also detected impaired binding of NFEPP to MOR at pH 7.4 in membranes of DRG. Due to high variability of the DRG binding values (possibly resulting from the additional incubation step at 37°C), we did not perform statistical analysis, and present descriptive data only (Fig. S2, available online as supplemental digital content at http://links.lww.com/PAIN/A613—legend, http://links.lww.com/PAIN/A618; Fig. S3, available online as supplemental digital content at http://links.lww.com/PAIN/A614—legend, http://links.lww.com/PAIN/A618; and Table S1, available online as supplemental digital content at http://links.lww.com/PAIN/A615).

3.2. pH values

In vivo, compared with contralateral uninjured nerves, the pH values at the site of nerve injury (CCI) were significantly decreased on 3, 7, and 14 days after CCI (7.19 ± 0.007 vs 7.01 ± 0.019; 7.14 ± 0.018 vs 6.91 ± 0.022; and 7.22 ± 0.016 vs 6.98 ± 0.024, respectively; P < 0.001; Fig. 2A). After i.p. injection of 1% acetic acid, intra-abdominal pH values were significantly decreased at 5 minutes (4.52 ± 0.058) and 15 minutes (6.97 ± 0.032) compared with baseline (7.3 ± 0.031) (P < 0.001), and returned to baseline by 30 minutes (P > 0.05; Fig. 2B). The in vitro pH of 0.9% NaCl was 5.36 ± 0.01 and that of NLXM was 5.18 ± 0.05 (n = 3; P = 0.25; Wilcoxon test), consistent with the literature.25

3.3. NFEPP produces analgesia selectively through peripheral opioid receptors in the neuropathic pain model

Fourteen days after CCI, rats developed mechanical hyperalgesia (reduced PPT; P < 0.05; Figs. 3A and D), mechanical allodynia (reduced PWT to von Frey filaments; P < 0.05; Figs. 3B and E), and heat hyperalgesia (reduced PWL; P < 0.05;
Figs. 3C and F) in ipsilateral compared with contralateral paws. After injection into the tail vein, both fentanyl and NFEPP (4-16 μg/kg) elevated PPT, PWT, and PWL at 15 to 45 minutes in the ipsilateral paw (P < 0.05, P < 0.01, and P < 0.001 depending on the time point; Figs. 3A–F) in a dose-dependent manner (Figs. 4A–C). In contralateral paws, fentanyl significantly increased PWL (P < 0.05; Fig. 3C) but not PPT and PWT (P > 0.05; Figs. 3A and B), whereas NFEPP was not effective (P > 0.05; Fig. 3). In all 3 tests, the analgesic effects induced by fentanyl (12 μg/kg i.v.) were only partially reversed by injection of...
NLXM (50 μg) at the nerve injury site because the effects were significantly different from baseline levels before injections \( (P < 0.05; \# P < 0.01) \) (Fig. 4D, F, and H). The local injection of 0.9% NaCl (vehicle) (Figs. 4E, G, and I) did not significantly change the analgesic effects of i.v. NFEPP (12 μg/kg i.v.) compared to control animals treated with acetic acid and vehicle \( (P > 0.05; \# P < 0.01) \) (Fig. 5C). This indicates that NFEPP produces analgesia through abdominal peripheral opioid receptors.

**3.4. NFEPP produces analgesia selectively through peripheral opioid receptors in the abdominal pain model**

Within 5 to 35 minutes after i.p. injection of 1% acetic acid, rats demonstrated abdominal constrictions (writhes) indicative of abdominal pain. Intravenous fentanyl (4-16 μg/kg) dose-dependently inhibited the writhes (Fig. 5A). NFEPP (4-16 μg/kg i.v.) also dose-dependently but only partially (by about 51%) decreased the number of writhes (Fig. 5A). Analgesic effects induced by i.v. fentanyl (12 μg/kg) were partially attenuated by i.p. NLXM (50 μg) \( (P < 0.001) \) because the effects were significantly different from control animals treated with acetic acid and vehicle \( (P < 0.001; \# P < 0.01) \) (Fig. 5B), implying the activation of both peripheral and central opioid receptors. By contrast, analgesic effects of i.v. NFEPP (12 μg/kg) were fully reversed by i.p. NLXM (50 μg) \( (P > 0.05; \# P < 0.01) \) because there was no significant difference compared with control animals treated with acetic acid and vehicle \( (P > 0.05; \# P < 0.01) \) (Fig. 5C). This indicates that NFEPP produces analgesia through abdominal peripheral opioid receptors.

**4. Discussion**

Previous attempts to develop new analgesics without side effects have met various obstacles. In contrast to other strategies (eg,
blockade of individual excitatory ion channels or receptors on sensory neurons,27 the activation of peripheral opioid receptors on DRG neurons presents advantages such as reduced tolerance development and simultaneous modulation of multiple ion currents.33 We took advantage of injury-specific MOR–ligand tolerance development and simultaneous modulation of multiple sensory neurons,27 the activation of peripheral opioid receptors (both at normal and low pH), whereas NFEPP produced analgesia of similar efficacy to fentanyl by selective activation of peripheral MOR in inflamed tissue, but did not act in healthy central or peripheral compartments. Therefore, the actions of NFEPP were devoid of typical opioid side effects such as reward, sedation, motor impairment, respiratory depression, and constipation.32

We now extended our studies to examine binding of NFEPP to MOR in native membranes of brain and DRG, and its analgesic efficacy in 2 additional models involving inflammation and pain transmission through DRG neurons.1,19,20,26,38 In line with our previous findings in human embryonic kidney 293 cells (Table S1, available online as supplemental digital content at http://links.lww.com/PAIN/A615, and Ref. 32), NFEPP binding to MOR at pH 7.4 was about one order of magnitude lower than that of conventional fentanyl, whereas the 2 ligands had similar affinities at pH 5.5, both in the brain and DRG. In the CCI model, pH values at the site of nerve injury were decreased in comparison with noninjured nerves for at least 2 weeks, in line with previously demonstrated inflammatory response 26 as well as a lack of upregulation of opioid receptors on peripheral terminals of sensory neurons in the peritoneum18 may explain the moderate analgesic efficacy of NFEPP in this short-lasting (30 minutes) pain assay. Nevertheless, also in this model, NFEPP exerted its actions solely through peripheral opioid receptors, supported by the complete antagonism by i.p. NLXM.

By contrast, the analgesic effects of fentanyl were only partially reversed by NLXM in both models. In line with our previous studies, these data suggest that fentanyl acts at both central and peripheral opioid receptors (both at normal and low pH), whereas NFEPP selectively activates peripheral opioid receptors in injured tissues at low pH.32 This is consistent with the fact that the acid dissociation constant (pKa) of fentanyl (and other conventional opioid ligands) is above 7.4, whereas that of NFEPP is 6.8.32 Consequently, fentanyl is protonated and activates MOR at both normal (eg, in brain) and low pH, whereas NFEPP is only protonated at low pH.32 It is noteworthy that fentanyl and NFEPP exert similar analgesic effects in the CCI model; however (at least at 14 days), central sensitization should be present and opioid effects in the spinal cord or brain might contribute to analgesia. However, because the effects of NFEPP were abolished (and those of fentanyl were significantly reduced) by locally administered NLXM, it seems that peripheral opioid receptors mediate a large part of the overall analgesic effects, similar to clinical reports.15 This is consistent with the notion that continuing input from primary sensory neurons is essential for the maintenance of central sensitization, and that blocking those neurons (eg, by peripheral opioid receptor activation, local anesthetics, or capsaicin) can effectively reduce even chronic neuropathic pain (reviewed in Ref. 2,17,27).
In summary, compared with fentanyl, NFEPP showed markedly diminished MOR binding in native tissues at pH 7.4 and produced analgesia exclusively through peripheral opioid receptors in models of neuropathic and abdominal pain.

Conflict of interest statement

C. Stein is listed as inventor on patents US 14/239,461 and EP 2,801,046. The remaining authors have no conflicts of interest to declare.

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Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/A612, http://links.lww.com/PAIN/A613, http://links.lww.com/PAIN/A614, http://links.lww.com/PAIN/A615, and http://links.lww.com/PAIN/A618.

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