Research Paper

PAIN®

Uncovering the analgesic effects of a pH-dependent mu-opioid receptor agonist using a model of nonevoked ongoing pain

Nicolas Massaly, Julia Temp, Halina Machelska, Christoph Stein*

Abstract

Currently, opioids targeting mu-opioid receptors are the most potent drugs for acute and cancer pain. However, opioids produce adverse side effects such as constipation, respiratory depression, or addiction potential. We recently developed (±)-*N*-(3-fluoro-1-phenethylpiperidine-4-yl)-*N*-phenyl propionamide (NFEPP), a compound that does not evoke central or intestinal side effects due to its selective activation of mu-opioid receptors at low pH in peripheral injured tissues. Although we demonstrated that NFEPP effectively abolishes injury-induced pain, hyperalgesia, and allodynia in rodents, the efficacy of NFEPP in nonevoked ongoing pain remains to be established. Here, we examined reward, locomotor activity, and defecation in rats with complete Freund's adjuvant-induced paw inflammation to compare fentanyl's and NFEPP's potentials to induce side effects and to inhibit spontaneous pain. We demonstrate that low, but not higher, doses of NFEPP produce conditioned place preference but not constipation or motor disturbance, in contrast to fentanyl. Using a peripherally restricted antagonist, we provide evidence that NFEPP-induced place preference is mediated by peripheral opioid receptors. Our results indicate that a low dose of NFEPP produces reward by abolishing spontaneous inflammatory pain.

Keywords: Ongoing pain, Inflammatory pain, Mu-opioid receptor, Conditioned place preference, Analgesia

1. Introduction

Pain is a multifaceted experience composed of both sensory and emotional components. Current strategies for the development of novel pain medication mostly aim to dampen evoked nociceptive sensory information and to reverse hyperalgesia and allodynia. Although these investigations provide evidence for pain relief and prevention of further noxious injury, the affective (aversive) component of the pain experience remains understudied.[5,15,18,26,30,34] Recently, growing interest in studying nonevoked ongoing pain and associated negative affective states has evolved.[5,15,18,26,30,34] This represents a step forward in translational value of preclinical research. Using negative reinforcement in the conditioned place preference (CPP) paradigm represents a way to assess nonevoked ongoing pain.[15,28]

Currently, opioids targeting mu-opioid receptors (MOR) are still the most potent drugs for acute and cancer-associated pain. However, MOR agonists also produce peripherally and centrally mediated deleterious side effects such as constipation, locomotor impairment, abuse liability, and respiratory depression that can lead to lethal outcomes.[8,41] Several approaches have been pursued to design safer analgesics. Biased agonism is on the forefront of such strategies.[33] Indeed, MOR agonists selectively activating G proteins over β-arrestins were thought to promote analgesia without inducing abuse liability or respiratory depression.[11,16,33] However, recent studies have uncovered that biased MOR agonists still retain nausea, vomiting, and respiratory depression.[1,8,36] Another auspicious approach consists of restricting the activity of opioids to peripheral sensory neurons in injured tissues.[37,41,43,45] Indeed, peripheral MOR significantly participate in the analgesic effects produced by systemically applied opioids.[9,38–40] In that sense, our team recently developed a milieu-selective MOR agonist, taking advantage of increased proton concentrations in inflamed tissues.[37] All conventional opioid analgesics are protonated and activate MOR in both normal (pH 7.4) and inflamed milieus (pH 5-7). We designed an opioid derivate with reduced pK_a (6.8) allowing protonation only under acidic conditions to selectively target MOR in inflamed tissue. This compound, (±)-N-(3-fluoro-1-phenethylpiperidine-4-yl)-N-phenyl propionamide (NFEPP), exhibited enhanced potency at low pH in vitro, and abolished evoked hyperalgesia and allodynia observed in the presence of inflammatory pain in vivo.[32,37] However, the efficacy of NFEPP to reduce spontaneous pain remains to be investigated. Here, we uncover, using a combination of CPP and classic pharmacology, that NFEPP is able to selectively relieve ongoing nonevoked pain through the activation of peripheral opioid receptors.

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

Department of Anesthesiology, Charité—Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany. Dr. Massaly is now with the Department of Anesthesiology, Washington University Pain Center, School of Medicine, Washington University in St. Louis, St. Louis, MO, United States.

**Corresponding author. Address: Department of Experimental Anesthesiology, Charité—Universitätsmedizin Berlin, Campus Benjamin Franklin, Hindenburgdamm 30, 12203 Berlin, Germany. Tel.: +49-30-450551586; fax: +49-30-450551939. E-mail address: Christoph.Stein@charite.de (C. Stein).*

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.painjournalonline.com).

PAIN 161 (2020) 2798–2804

http://dx.doi.org/10.1097/j.pain.0000000000001968

2. Materials and methods

2.1. Animals

All protocols were approved by the state animal care committee (Landesamt für Gesundheit und Soziales, Berlin) and were performed according to the ARRIVE guidelines.[12] Male Wistar rats (200-300 g) were kept in groups of 2 to 3 per cage, on a 12-hour light/dark schedule, with food and water ad libitum. Room temperature was 22 ± 0.5°C and humidity was 60% to 65%. Animals were randomly placed in cages by an animal caretaker who was not involved in the study. Based on statistical power calculations performed a priori using the G*Power 3.1.2 program, 8 to 16 rats were used per group. Rats were handled once per day for 1 to 2 minutes and habituated to the test cages (1-2 times for 15 minutes), starting at least 4 days before experiments. After completion of experiments, animals were euthanized with isoflurane overdose (AbbVie, Wiesbaden, Germany).

2.2. Paw inflammation induced by complete Freund's adjuvant

Rats received an intraplantar (i.pl.) injection of complete Freund's adjuvant (CFA) (150 μL; 0.1% *Mycobacterium butyricum*; Calbiochem, Merck, Germany) into the right hind paw under brief isoflurane anesthesia.[29,41]

2.3. Drugs and injections

Fentanyl citrate (F3886) and naloxone methiodide (NLXM) were purchased from Sigma-Aldrich (Taufkirchen, Germany). NFEPP was synthesized according to our design by a contractor (ASCA GmbH, Berlin, Germany), as described in detail in Spahn et al.[37] Fentanyl and NLXM were dissolved in 0.9% NaCl. NFEPP was dissolved in dimethyl sulfoxide (DMSO) and diluted with 0.9% NaCl to obtain the final concentrations (maximum DMSO: 4.2%).

Fentanyl (20-60 $\mu g \cdot kg^{-1}$) and NFEPP (20-150 $\mu g \cdot kg^{-1}$) were injected subcutaneously (s.c.; volume 1 $mL.kg^{-1}$) into a skinfold on the neck without anesthesia. Naloxone methiodide (100 μg) was injected i.pl. (100 μL) into the inflamed paw under brief isoflurane anesthesia immediately before s.c. injection of agonists. The dosages are based on our earlier studies[37] and pilot experiments. Control groups were treated with the respective vehicles. The experimenters were blinded to the treatments (drugs and dosages).

2.4. Conditioned place preference

We used an unbiased counterbalanced CPP protocol as described previously.[37] Briefly, light- and sound-attenuating chambers (60 × 30 × 30 cm) consisted of 2 compartments separated by a removable door. The 2 compartments differed in wall color (gray or white with black stripes) and floor texture ("grid" or "hole"). All rats received i.pl. CFA on day 1 (**Table 1**). For habituation, each rat was placed into the CPP apparatus (without separator) for 2 days after CFA injection, and was allowed to freely explore it for 30 minutes (days 3 and 4) (**Table 1**). For the preconditioning test (day 5), each rat was placed into the CPP apparatus (without separator) for 15 minutes and the time spent in each compartment was recorded (**Table 1**). If rats showed high unconditioned preference (more than 67% spent in one compartment) for 1 of the 2 compartments in this phase, they were excluded from further analysis (ca. 10% of animals). During conditioning (days 6-11, **Table 1**), the treatment and treatment-associated compartment were assigned randomly. Each rat underwent three 60-minute conditioning sessions receiving the agonist (s.c. fentanyl or NFEPP) in one compartment (one session every other day), and three 60-minute sessions receiving vehicle in the other compartment on the alternate days (**Table 1**). The agonist was given to one half of the animals on odd days and to the other half on even days after CFA injection. On the test day (day 12, **Table 1**), no agonist or vehicle was administered and each rat was allowed to freely explore the entire CPP apparatus (without separator) for 15 minutes. Time spent in each compartment was recorded using the AnyMaze software. Preference was calculated as time spent in drug compartment minus time spent in the vehicle compartment. To examine the contribution of peripheral opioid receptors to fentanyl- and NFEPP-induced CPP, the antagonist NLXM was injected i.pl. immediately before each s.c. injection of fentanyl, NFEPP, or vehicle (**Table 1**). The procedure was then performed as described above, except that each conditioning session lasted 30 minutes (instead of 60 minutes), i.e., the duration of NLXM's action.

Table 1

Timeline of treatments and tests.

Day #	1	2	3	4	5	6	7	8	9	10	11	12
i.pl. CFA	X											
Habituation (CPP)			x	x								
Preconditioning test					x							
s.c. fentanyl (x)/vehicle (o)						x	o	x	o	x	o	
s.c. NFEPP (x)/vehicle (o)						o	x	o	x	o	x	
i.pl. NLXM/vehicle						x	x	x	x	x	x	
CPP test												x
Locomotor test (fentanyl)						x						
Locomotor test (NFEPP)							x					
Defecation (fentanyl)						x						
Defecation (NFEPP)							x					
Habituation (rotarod)		x	x	x								
Rotarod test (fentanyl)					x							
Rotarod test (NFEPP)					x							

CFA, complete Freund's adjuvant; CPP, conditioned place preference; NLXM, naloxone methiodide.

2.5. Locomotor activity

On the first 2 days of conditioning (days 6 and 7, **Table 1**), the animals' horizontal locomotor activity and location in CPP boxes were monitored by an infrared camera using AnyMaze software, as previously described.[37] Locomotion was measured as the distance (in meters) traveled during the first 30 minutes of the conditioning session after administration of fentanyl, NFEPP, or vehicle. Data were analyzed using AnyMaze software. After unblinding, the data recorded on day 6 and day 7 were used to document fentanyl's and NFEPP's effects, respectively.

2.6. Defecation

On the first 2 days of conditioning (days 6 and 7, **Table 1**), the number of fecal boli per animal was counted after completion of the 60-minute conditioning session.[37] Data were analyzed using AnyMaze software.

After unblinding, the data recorded on day 6 and day 7 were used to document fentanyl's and NFEPP's effects, respectively.

2.7. Motor coordination (rotarod test)

On day 2, rats were habituated to the rotarod at 5 and 10 rotations per min (rpm) until they were able to stay on the rotarod for 300 seconds (maximum 5 trials) (**Table 1**). On the test day (day 5, **Table 1**), data were recorded for 3 baseline trials before injection of fentanyl, NFEPP, or vehicle, followed by 3 trials at 2, 30, and 60 minutes after agonist/vehicle injection at an accelerating speed (10-35 rpm over 300 seconds). The latency to fall was recorded for 3 successive attempts and averaged for each trial, similar to our earlier studies.[37] Data were analyzed after unblinding of the experimenter to group assignment.

2.8. Statistics

All data were assessed for normal distribution and equal variances by D'Agostino–Pearson omnibus normality test. Multiple comparisons at one time point were performed using one-way analysis of variance (ANOVA) followed by Dunnett test for normally distributed data, or Kruskal–Wallis one-way ANOVA followed by Dunn test for nonnormally distributed data. Two-way repeated-measures ANOVA followed by Bonferroni test were used to compare 2 groups over time (more than 2 time points). Differences were considered significant if $P < 0.05$. The detailed statistical evaluation is presented in Table S1, http://links.lww.com/PAIN/B86. Prism 8 (GraphPad, San Diego, CA) was used for all tests and graphs, and all data are expressed as individual animal data points and as mean ± SEM.

3. Results

3.1. NFEPP produces place preference

All animals received i.pl. CFA before starting the CPP procedure (**Fig. 1A** and **Table 1**). After 6 days of conditioning sessions with either NFEPP or fentanyl, animals were given the ability to freely explore both compartments on day 12 (**Fig. 1A** and **Table 1**). Rats showed a preference for the compartment associated with 20 $\mu g \cdot kg^{-1}$ s.c. NFEPP treatment (**Fig. 1B**). When conditioned with higher doses of NFEPP (30-150 $\mu g \cdot kg^{-1}$ s.c.), animals did not demonstrate a preference for the agonist compartment. Fentanyl induced a preference at the dose of 30 $\mu g \cdot kg^{-1}$, but not at doses 20, 50, and 60 $\mu g \cdot kg^{-1}$ s.c.

3.2. Effects of naloxone methiodide

Using separate cohorts of animals, we confirmed that s.c. administration of 20 $\mu g \cdot kg^{-1}$ NFEPP and 30 $\mu g \cdot kg^{-1}$ fentanyl produced CPP (**Fig. 2B**). Naloxone methiodide (100 μg)

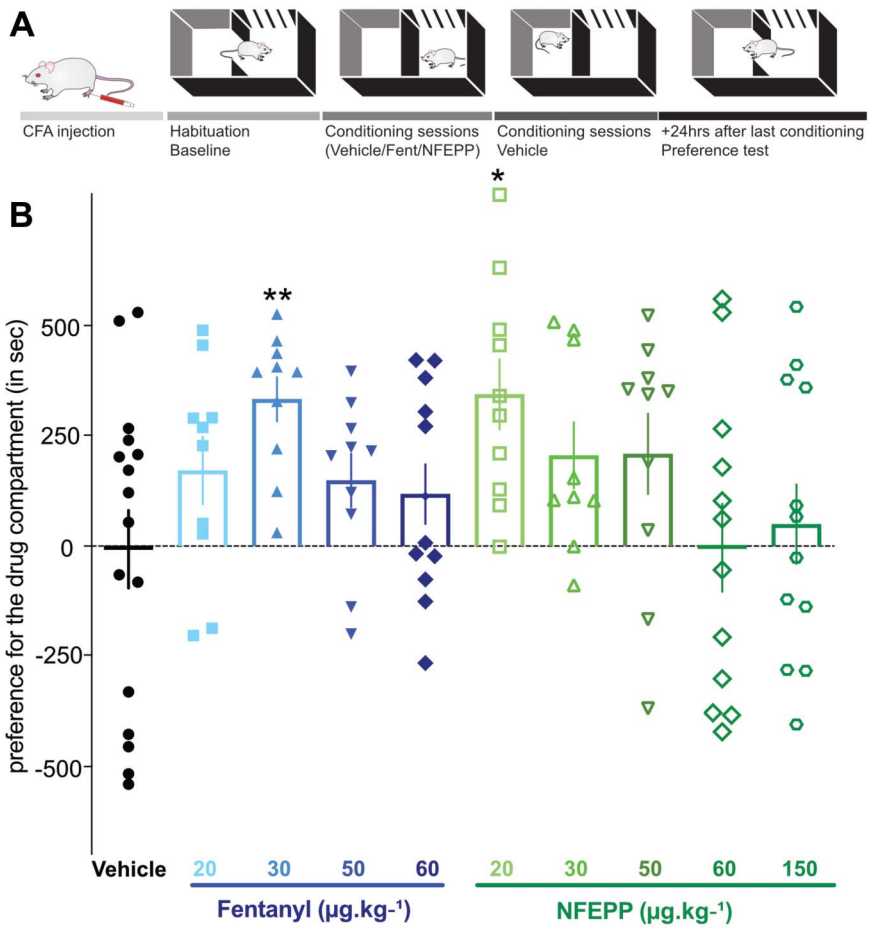

Figure 1. Fentanyl and NFEPP produce CPP. (A) Schematic representation of the CPP protocol (see Table 1 for more details). (B) Fentanyl (30 $\mu g \cdot kg^{-1}$ s.c.) and NFEPP (20 $\mu g \cdot kg^{-1}$ s.c.) induced a preference for the agonist-associated compartment during the postconditioning test. *$P = 0.0283$, **$P = 0.0075$ (one-way ANOVA and Dunnett test; see also supplementary statistical evaluation, available at http://links.lww.com/PAIN/B86). Data are expressed as individual animal data points and as mean ± SEM. N = 9 to 16 rats per group. ANOVA, analysis of variance; CPP, conditioned place preference.

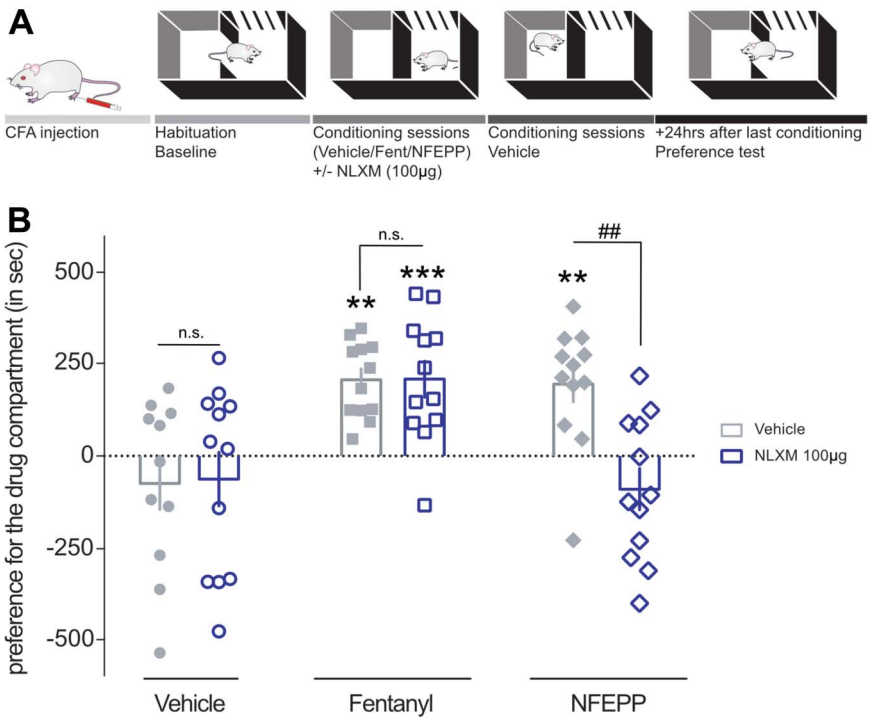

Figure 2. NFEPP-induced CPP, but not fentanyl-induced CPP, is mediated through peripheral opioid receptors. (A) Schematic representation of the CPP protocol (see Table 1 for more details). (B) Both fentanyl (30 $\mu g \cdot kg^{-1}$) and NFEPP (20 $\mu g \cdot kg^{-1}$) (s.c.) induced a preference for the agonist compartment as compared to vehicle-treated animals. NFEPP-induced CPP was reversed by NLXM (##$P < 0.01$, Kruskal–Wallis and Dunn test; see also supplementary statistical evaluation, available at http://links.lww.com/PAIN/B86). Fentanyl-induced CPP was not affected by NLXM ($P > 0.05$, one-way ANOVA; see also supplementary statistical evaluation, available at http://links.lww.com/PAIN/B86). **$P < 0.01$ and ***$P < 0.001$ compared to Vehicle-Vehicle group (Kruskal–Wallis and Dunn test). Data are expressed as individual animal data points and as mean ± SEM. N = 11 to 12 rats per group. ANOVA, analysis of variance; CPP, conditioned place preference.

injected into the animal's inflamed paw abolished the preference for the agonist-associated compartment induced by NFEPP, but not by fentanyl treatment. Naloxone methiodide administration alone did not produce aversion or preference for the associated compartment (**Fig. 2B**).

3.3. NFEPP does not impair locomotion, bowel movement, or motor coordination

NFEPP (s.c.) increased locomotor behavior compared to vehicle-injected control animals when a 60 $\mu g \cdot kg^{-1}$ dose was administered, but no significant effects were observed at doses of 20 to 50 $\mu g \cdot kg^{-1}$ and 150 $\mu g \cdot kg^{-1}$. Fentanyl (30-60 $\mu g \cdot kg^{-1}$ s.c.) induced a strong decrease in locomotion (**Fig. 3**). Fentanyl (20-60 $\mu g \cdot kg^{-1}$) also induced a strong decrease in the number of fecal boli 1 hour after treatment as compared to vehicle. By contrast, NFEPP did not cause constipation at any tested dose (20-150 $\mu g \cdot kg^{-1}$) (**Fig. 4**). At all time points tested, fentanyl (60 $\mu g \cdot kg^{-1}$), but not NFEPP (60-150 $\mu g \cdot kg^{-1}$), impaired motor coordination assessed by the rotarod test (**Fig. 5**).

4. Discussion

In rats with ongoing inflammatory pain, we demonstrate that 20 $\mu g \cdot kg^{-1}$ (but not higher doses) of NFEPP induces a preference for the drug-associated compartment, which is mediated by peripheral opioid receptors. Using spontaneous locomotor activity, motor coordination, and constipation tests, we also show that NFEPP does not induce typical MOR-mediated side effects in this model.

A vast variety of strategies, including chemistry, genetics, and alteration of molecular and cellular pathways, is currently used by the pain research community to develop safer analgesics.[11,16,33,37] However, the translation from preclinical studies to the development of treatments applicable in patients remains

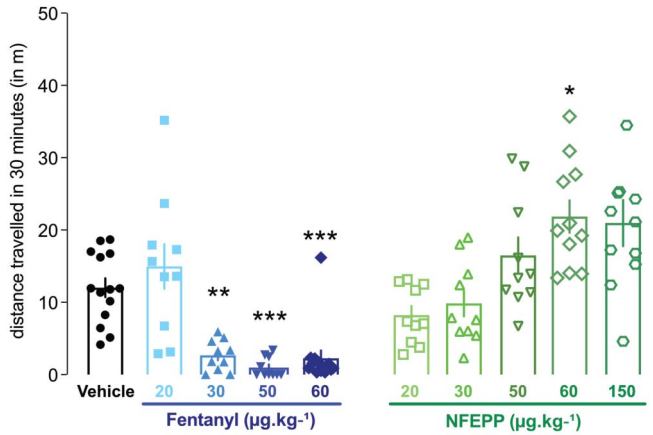

Figure 3. Fentanyl, but not NFEPP, impairs locomotion. Fentanyl (20-60 $\mu g \cdot kg^{-1}$ s.c.) dose-dependently impaired locomotor activity, whereas NFEPP at high doses (60 $\mu g \cdot kg^{-1}$ s.c.) increased locomotor activity. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to vehicle (Kruskal–Wallis and Dunn test; see also supplementary statistical evaluation, available at http://links.lww.com/PAIN/B86). Data show the distance travelled in meters during 30 minutes, and are expressed as individual animal data points and as mean ± SEM. N = 10 to 14 rats per group.

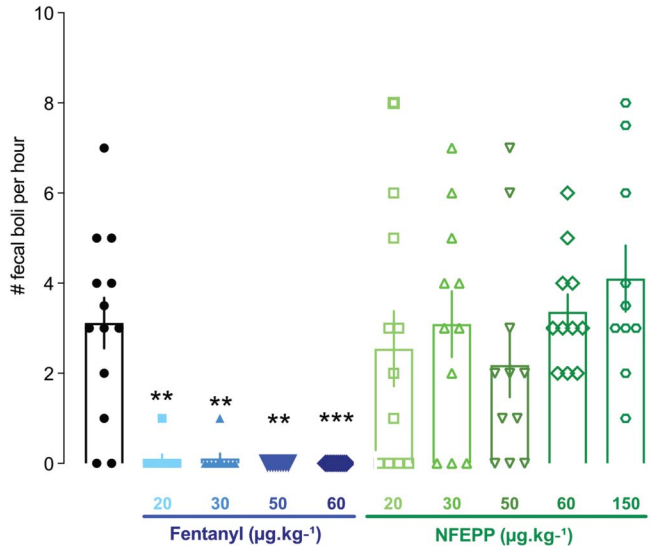

Figure 4. Fentanyl, but not NFEPP, induces constipation. Fentanyl (20-60 $\mu g \cdot kg^{-1}$ s.c.) induced constipation manifested by decreased number of excreted fecal boli during 1 hour. NFEPP (20-150 $\mu g \cdot kg^{-1}$ s.c.) did not induce constipation. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to vehicle (Kruskal–Wallis and Dunn test; see also supplementary statistical evaluation, available at http://links.lww.com/PAIN/B86). Data are expressed as number of fecal boli per individual animal and as mean ± SEM. N = 9 to 13 rats per group.

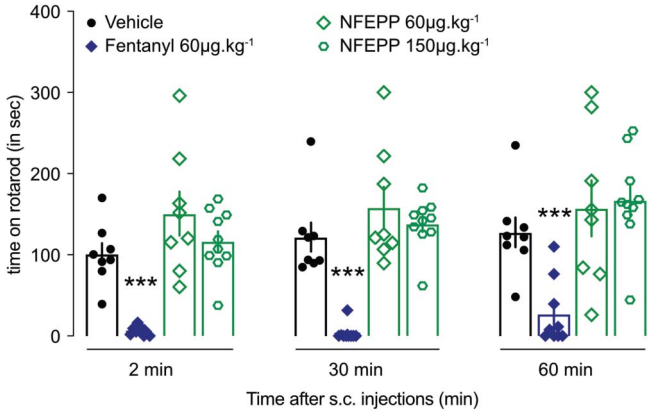

Figure 5. Fentanyl, but not NFEPP, impairs motor coordination. Fentanyl (60 $\mu g \cdot kg^{-1}$ s.c.) impaired motor coordination as manifested by decreased time spent on the accelerating rotarod. NFEPP (60-150 $\mu g \cdot kg^{-1}$ s.c.) did not alter motor coordination. $^{***}P < 0.0001$ compared to vehicle (two-way RM ANOVA and Bonferroni test; see also supplementary statistical evaluation, available at http://links.lww.com/PAIN/B86). Data are expressed as individual animal data points and as mean ± SEM. N = 8 to 10 rats per group. ANOVA, analysis of variance.

rare due to the complexity of pain and the many limitations of the current cellular and behavioral models.

Using the known low pH conditions observed at injury sites,[40] we recently developed NFEPP, a low pKa opioid derivate targeting peripheral MOR in acidic milieu. We demonstrated that NFEPP reversed hyperalgesia and allodynia in animals experiencing inflammatory and neuropathic pain.[31,37] In addition, this compound was devoid of reinforcing properties and did not produce respiratory depression, locomotor impairment, or constipation in animals without tissue damage.[37]

Despite these promising findings, the value of NFEPP to relieve ongoing pain remained to be established. In the current article, we used CPP as an assessment of drug effects on nonevoked pain and its negative consequences. The CPP paradigm has been extensively used to reveal aversive and reinforcing properties of drugs.[2,6,17,24,35,37,44,47] More recently, laboratories have used this procedure as a measure for nonevoked pain.[24] Indeed, when an animal experiences pain, the rewarding effects of an analgesic treatment coupled to a selective context can lead to reinforcing memories, allowing us to study nonevoked ongoing pain.

In the current study, we uncover that the association between NFEPP and a selective environment in an animal experiencing inflammatory pain is sufficient to establish a contextual preference. This indicates that NFEPP is also capable to relieve ongoing nonevoked pain. Interestingly, higher doses of NFEPP produced progressively decreasing preference for the agonist compartment. This may be due to either the development of tolerance to the analgesic effects of NFEPP upon several administrations, or it may represent a bell-shaped dose–response curve, which is not unusual for ligands of G-protein-coupled receptors (see also fentanyl's effects in **Fig. 1B**). Further investigations are needed to clarify these points and to rule out potential actions of high doses of NFEPP outside of the acidic milieu or on other opioid receptors. Nonetheless, it should be noted that neither in inflamed (this article) nor in naive conditions,[37] NFEPP exhibited rewarding properties at higher doses (up to 150 $\mu g \cdot kg^{-1}$). Future experiments testing self-administration should be implemented to confirm a possible lack of abuse potential of this compound.

In addition, we observed a bell-shaped response in the reinforcing properties of fentanyl using CPP in this model of inflammatory pain (**Fig. 1B**). This is reminiscent of previous reports that uncovered a decreased ability of MOR agonists to induce CPP in pain conditions.[21]

Numerous supraspinal sites including the mesolimbic pathway,[13,15,18,24,30,34] the anterior cingulate cortex,[3,19,25,29] and the amygdala[5,22,23,26,46] are involved in mediating the unpleasantness and negative affective states associated with pain. To further investigate the sites of action of NFEPP and fentanyl in producing CPP, we administered NLXM, a peripherally restricted antagonist, into the inflamed paw before the conditioning sessions. This treatment reversed NFEPP-induced CPP but not fentanyl-induced CPP, indicating that NFEPP-induced relief of ongoing pain is mediated by peripheral opioid receptors at the inflammation site. This result is in line with former studies from Dr. Porreca's laboratory, which demonstrated a role of peripheral circuits in mediating the aversiveness of ongoing pain.[7,14,27] By contrast, peripheral opioid receptor activation is apparently not necessary for the reinforcing properties of fentanyl in this model. This can be explained by the actions of fentanyl at supraspinal structures such as the mesolimbic pathway to either relieve ongoing inflammatory pain or trigger positive reinforcement. Overall, these findings are consistent with the well-known central effects of fentanyl and expand our previous results demonstrating that NFEPP-induced reversal of hyperalgesic and allodynic states was restricted to its actions on MOR in low pH milieus.[20,31,37]

Although we confirmed the known deleterious effects of fentanyl on locomotor activity and motor coordination, we observed that increasing doses of NFEPP progressively increased locomotor activity in CFA animals. Previous reports have used spontaneous locomotor activity and voluntary wheel running to assess nonevoked inflammatory pain.[4,10,42] Together, these results indicate that, in addition to the CPP paradigm, the observed NFEPP-induced increase in locomotor activity may represent a useful measure to uncover relief of ongoing inflammatory pain.

Finally, we found that NFEPP, unlike fentanyl, did not induce constipation or motor coordination impairment. In addition to the

lack of reward and the analgesic properties of NFEPP, these findings further support the concept of developing safer non-addictive analgesics based on peripherally restricted and milieu-specific opioid receptor activation.

Together, we provide evidence that the selective activation of opioid receptors in peripheral injured tissue can mediate efficient relief of both hyperalgesic/allodynic states and of ongoing pain, while lacking centrally mediated side effects.

Conflict of interest statement

The Charité-Universitätsmedizin Berlin and the Zuse Institute Berlin have filed a patent on pH-dependent opioid receptor agonists (US 9133120 B2). The authors have no other conflicts of interest to declare.

Acknowledgements

This project was supported by Bundesministerium für Bildung und Forschung (VIP 0272/03V0364) and by Deutsche Forschungsgemeinschaft (STE 477/19-1). The experiments were conducted by N. Massaly and J. Temp at the Department of Anesthesiology and Intensive Care Medicine, Charité Campus Benjamin Franklin, Berlin, Germany. All authors contributed to writing of the manuscript. N. Massaly's current address is Department of Anesthesiology and Pain Center, Washington University, St. Louis, MO, USA. The Charité-Universitätsmedizin Berlin and the Zuse Institute Berlin have filed patents on pH-dependent opioid receptor agonists (US 9133120 B2) and computational methods (PCT/EP2013/102681). C. Stein was also supported by Charité 3^R| Replace-Reduce-Refine.

Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/B86.

Article history:
Received 20 March 2020
Received in revised form 13 May 2020
Accepted 15 June 2020
Available online 24 June 2020

References

[1] Altarifi AA, David B, Muchhala KH, Blough BE, Akbarali H, Negus SS. Effects of acute and repeated treatment with the biased mu opioid receptor agonist TRV130 (oliceridine) on measures of antinociception, gastrointestinal function, and abuse liability in rodents. J Psychopharmacol (Oxford) 2017;31:730–9.
[2] Bardo MT, Bevins RA. Conditioned place preference: what does it add to our preclinical understanding of drug reward? Psychopharmacol (Berl) 2000;153:31–43.
[3] Becerra L, Navratilova E, Porreca F, Borsook D. Analogous responses in the nucleus accumbens and cingulate cortex to pain onset (aversion) and offset (relief) in rats and humans. J Neurophysiol 2013;110:1221–6.
[4] Cobos EJ, Ghasemlou N, Araldi D, Segal D, Duong K, Woolf CJ. Inflammation-induced decrease in voluntary wheel running in mice: a nonreflexive test for evaluating inflammatory pain and analgesia. PAIN 2012;153:876–84.
[5] Corder G, Ahanonu B, Grewe BF, Wang D, Schnitzer MJ, Scherrer G. An amygdalar neural ensemble that encodes the unpleasantness of pain. Science 2019;363:276–81.
[6] Fakira AK, Massaly N, Cohensedgh O, Berman A, Morón JA. Morphine-associated contextual cues induce structural plasticity in hippocampal CA1 pyramidal neurons. Neuropsychopharmacol 2016;41:2668–78.
[7] Havelin J, Imbert I, Cormier J, Allen J, Porreca F, King T. Central sensitization and neuropathic features of ongoing pain in a rat model of advanced osteoarthritis. J Pain 2016;17:374–82.
[8] Hill R, Disney A, Conibear A, Sutcliffe K, Dewey W, Husbands S, Bailey C, Kelly E, Henderson G. The novel μ-opioid receptor agonist PZM21 depresses respiration and induces tolerance to antinociception. Br J Pharmacol 2018;175:2653–61.
[9] Jagla C, Martus P, Stein C. Peripheral opioid receptor blockade increases postoperative morphine demands–a randomized, double-blind, placebo-controlled trial. PAIN 2014;155:2056–62.
[10] Kandasamy R, Calsbeek JJ, Morgan MM. Home cage wheel running is an objective and clinically relevant method to assess inflammatory pain in male and female rats. J Neurosci Methods 2016;263:115–22.
[11] Kennedy NM, Schmid CL, Ross NC, Lovell KM, Yue Z, Chen YT, Cameron MD, Bohn LM, Bannister TD. Optimization of a series of mu opioid receptor (MOR) agonists with high G protein signaling bias. J Med Chem 2018;61:8895–907.
[12] Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol 2010;8:e1000412.
[13] King T, Vera-Portocarrero L, Gutierrez T, Vanderah TW, Dussor G, Lai J, Fields HL, Porreca F. Unmasking the tonic-aversive state in neuropathic pain. Nat Neurosci 2009;12:1364–6.
[14] King T, Qu C, Okun A, Mercado R, Ren J, Brion T, Lai J, Porreca F. Contribution of afferent pathways to nerve-injury induced spontaneous pain and evoked hypersensitivity. PAIN 2011;152:1997–2005.
[15] Liu SS, Pickens S, Burma NE, Ibarra-Lecue I, Yang H, Xue L, Cook C, Hakimian JK, Severino AL, Lueptow L, Komarek K, Taylor AMW, Olmstead MC, Carroll FI, Bass CE, Andrews AM, Walwyn W, Trang T, Evans CJ, Leslie F, Cahill CM. Kappa opioid receptors drive a tonic aversive component of chronic pain. J Neurosci 2019;39:4162–78.
[16] Manglik A, Lin H, Aryal DK, McCorvy JD, Dengler D, Corder G, Levit A, Kling RC, Bernat V, Hübner H, Huang XP, Sassano MF, Giguère PM, Löber S, Da Duan, Scherrer G, Kobilka BK, Gmeiner P, Roth BL, Shoichet BK. Structure-based discovery of opioid analgesics with reduced side effects. Nature 2016;537:185–90.
[17] Massaly N, Dahan L, Baudonnat M, Hovnanian C, Rekik K, Solinas M, David V, Pech S, Zajac JM, Roullet P, Mouledous L, Frances B. Involvement of protein degradation by the ubiquitin proteasome system in opiate addictive behaviors. Neuropsychopharmacol 2013;38:596–604.
[18] Massaly N, Copits BA, Wilson-Poe AR, Hipólito L, Markovic T, Yoon HJ, Liu S, Walicki MC, Bhatti DL, Sirohi S, Klaas A, Walker BM, Neve R, Cahill CM, Shoghi KI, Gereau RW, McCall JG, Al-Hasani R, Bruchas MR, Morón JA. Pain-induced negative affect is mediated via recruitment of the nucleus accumbens kappa opioid system. Neuron 2019;102:564–73.e6.
[19] Meda KS, Patel T, Braz JM, Malik R, Turner ML, Seifikar H, Basbaum AI, Sohal VS. Microcircuit mechanisms through which mediodorsal thalamic input to anterior cingulate cortex exacerbates pain-related aversion. Neuron 2019;102:944–59.e3.
[20] Meyer J, Del Vecchio G, Seitz V, Massaly N, Stein C. Modulation of mu-opioid receptor activation by acidic pH is dependent on ligand structure and an ionizable amino acid residue. Br J Pharmacol 2019;176:4510–20.
[21] Narita M, Kishimoto Y, Ise Y, Yajima Y, Misawa K, Suzuki T. Direct evidence for the involvement of the mesolimbic kappa-opioid system in the morphine-induced rewarding effect under an inflammatory pain-like state. Neuropsychopharmacol 2005;30:111–18.
[22] Narita M, Kaneko C, Miyoshi K, Nagumo Y, Kuzumaki N, Nakajima M, Nanjo K, Matsuzawa K, Yamazaki M, Suzuki T. Chronic pain induces anxiety with concomitant changes in opioidergic function in the amygdala. Neuropsychopharmacol 2006;31:739–50.
[23] Nation KM, De Felice M, Hernandez PI, Dodick DW, Neugebauer V, Navratilova E, Porreca F. Lateralized kappa opioid receptor signaling from the amygdala central nucleus promotes stress-induced functional pain. PAIN 2018;159:919–28.
[24] Navratilova E, Xie JY, Okun A, Qu C, Eyde N, Ci S, Ossipov MH, King T, Fields HL, Porreca F. Pain relief produces negative reinforcement through activation of mesolimbic reward-valuation circuitry. Proc Natl Acad Sci U S A 2012;109:20709–13.
[25] Navratilova E, Xie JY, Meske D, Qu C, Morimura K, Okun A, Arakawa N, Ossipov M, Fields HL, Porreca F. Endogenous opioid activity in the anterior cingulate cortex is required for relief of pain. J Neurosci 2015;35:7264–71.
[26] Navratilova E, Ji G, Phelps C, Qu C, Hein M, Yakhnitsa V, Neugebauer V, Porreca F. Kappa opioid signaling in the central nucleus of the amygdala promotes disinhibition and aversiveness of chronic neuropathic pain. PAIN 2019;160:824–32.
[27] Okun A, Liu P, Davis P, Ren J, Remeniuk B, Brion T, Ossipov MH, Xie J, Dussor GO, King T, Porreca F. Afferent drive elicits ongoing pain in a model of advanced osteoarthritis. PAIN 2012;153:924–33.
[28] Porreca F, Navratilova E. Reward, motivation and emotion of pain and its relief. PAIN 2017;158:S43–9.

[29] Rainville P, Duncan GH, Price DD, Carrier B, Bushnell MC. Pain affect encoded in human anterior cingulate but not somatosensory cortex. Science 1997;277:968–71.
[30] Ren W, Centeno MV, Berger S, Wu Y, Na X, Liu X, Kondapalli J, Apkarian AV, Martina M, Surmeier DJ. The indirect pathway of the nucleus accumbens shell amplifies neuropathic pain. Nat Neurosci 2016;19: 220–2.
[31] Rodriguez-Gaztelumendi A, Spahn V, Labuz D, Machelska H, Stein C. Analgesic effects of a novel pH-dependent μ-opioid receptor agonist in models of neuropathic and abdominal pain. PAIN 2018;159:2277–84.
[32] Rosas R, Huang XP, Roth BL, Dockendorff C. β-Fluorofentanyls are pH-sensitive mu opioid receptor agonists. ACS Med Chem Lett 2019;10: 1353–6.
[33] Schmid CL, Kennedy NM, Ross NC, Lovell KM, Yue Z, Morgenweck J, Cameron MD, Bannister TD, Bohn LM. Bias factor and therapeutic window correlate to predict safer opioid analgesics. Cell 2017;171: 1165–75.e13.
[34] Schwartz N, Temkin P, Jurado S, Lim BK, Heifets BD, Polepalli JS, Malenka RC. Chronic pain. Decreased motivation during chronic pain requires long-term depression in the nucleus accumbens. Science 2014; 345:535–42.
[35] Shippenberg TS, Herz A. Differential effects of mu and kappa opioid systems on motivational processes. NIDA Res Monogr 1986;75:563–6.
[36] Soergel DG, Subach RA, Sadler B, Connell J, Marion AS, Cowan CL, Violin JD, Lark MW. First clinical experience with TRV130: pharmacokinetics and pharmacodynamics in healthy volunteers. J Clin Pharmacol 2014;54:351–7.
[37] Spahn V, Del Vecchio G, Labuz D, Rodriguez-Gaztelumendi A, Massaly N, Temp J, Durmaz V, Sabri P, Reidelbach M, Machelska H, Weber M, Stein C. A nontoxic pain killer designed by modeling of pathological receptor conformations. Science 2017;355:966–9.
[38] Stein C, Gramsch C, Hassan AH, Przewlocki R, Parsons CG, Peter K, Herz A. Local opioid receptors mediating antinociception in inflammation: endogenous ligands. Prog Clin Biol Res 1990;328:425–7.
[39] Stein C, Gramsch C, Herz A. Intrinsic mechanisms of antinociception in inflammation: local opioid receptors and beta-endorphin. J Neurosci 1990;10:1292–8.
[40] Stein C, Hassan AH, Przewłocki R, Gramsch C, Peter K, Herz A. Opioids from immunocytes interact with receptors on sensory nerves to inhibit nociception in inflammation. Proc Natl Acad Sci U S A 1990;87:5935–9.
[41] Stein C. Opioids, sensory systems and chronic pain. Eur J Pharmacol 2013;716:179–87.
[42] Tanimoto A, Shinozaki Y, Nozawa K, Kimoto Y, Amano W, Matsuo A, Yamaguchi T, Matsushita M. Improvement of spontaneous locomotor activity with JAK inhibition by JTE-052 in rat adjuvant-induced arthritis. BMC Musculoskelet Disord 2015;16:339.
[43] Tiwari V, Yang F, He SQ, Shechter R, Zhang C, Shu B, Zhang T, Tiwari V, Wang Y, Dong X, Guan Y, Raja SN. Activation of peripheral μ-opioid receptors by dermorphin [D-Arg2, Lys4] (1-4) amide leads to modality-preferred inhibition of neuropathic pain. Anesthesiology 2016;124: 706–20.
[44] Tzschentke TM. Measuring reward with the conditioned place preference paradigm: a comprehensive review of drug effects, recent progress and new issues. Prog Neurobiol 1998;56:613–72.
[45] Vanderah TW, Schteingart CD, Trojnar J, Junien JL, Lai J, Riviere PJM. FE200041 (D-Phe-D-Phe-D-Nle-D-Arg-NH2): a peripheral efficacious kappa opioid agonist with unprecedented selectivity. J Pharmacol Exp Ther 2004;310:326–33.
[46] Veinante P, Yalcin I, Barrot M. The amygdala between sensation and affect: a role in pain. J Mol Psychiatry 2013;1:9.
[47] Wise RA. Opiate reward: sites and substrates. Neurosci Biobehav Rev 1989;13:129–33.

Supplementary Table S1: Detailed statistical evaluation of the data shown in the corresponding figures.

Figure 1

Vehicle vs. Fentanyl

one-way ANOVA	$F_{4,53}$=2.731, p = 0.0385	
Dunnett's post-hoc test		Adjusted p value
	Veh. vs. 20	0.2867
	Veh. vs. 30	0.0075
	Veh. vs. 50	0.4065
	Veh. vs. 60	0.5582

Vehicle vs. NFEPP

one-way ANOVA	$F_{5,63}$=2.414, p = 0.0458	
Dunnett's post-hoc test		Adjusted p value
	Veh. vs. 20	0.0283
	Veh. vs. 30	0.3611
	Veh. vs. 50	0.3140
	Veh. vs. 60	>0.9999
	Veh. vs. 150	0.9885

Figure 2

Vehicle vs. Fentanyl

one-way ANOVA	$F_{3,43}$=9.098, p < 0.0001	
Dunnett's post-hoc test		Adjusted p value
	Veh./Veh. vs Fent./Veh.	0.0011
	Veh./Veh. vs Fent./NLXM	0.0008
	Veh./ NLXM vs Fent./ NLXM	0.0030
	Veh./ NLXM vs Fent./Veh.	0.0044
	Veh./Veh. vs Veh./NLXM	0.8996
	Fent./Veh. vs Fent./NLXM	0.9984

Vehicle vs. NFEPP

Kruskal-Wallis	H = 14.43; p = 0.0024	
Dunn's post-hoc test		Adjusted p value
	Veh./Veh. vs NFEPP/Veh.	0.0071
	Veh./Veh. vs NFEPP /NLXM	>0.9999
	Veh./ NLXM vs NFEPP / NLXM	>0.9999
	Veh./ NLXM vs NFEPP /Veh.	0.0337
	Veh./Veh. vs Veh./NLXM	>0.9999
	NFEPP /Veh. vs NFEPP./NLXM	0.0091

Figure 3

Vehicle vs. Fentanyl

Kruskal-Wallis	H = 37.39; p < 0.0001	
Dunn's post-hoc test		Adjusted p value
	Veh. vs. 20	>0.9999
	Veh. vs. 30	0.0087
	Veh. vs. 50	<0.0001
	Veh. vs. 60	0.00

Vehicle vs. NFEPP

Kruskal-Wallis	H = 25.96; p < 0.0001	
Dunn's post-hoc test		Adjusted p value
	Veh. vs. 20	0.9750
	Veh. vs. 30	>0.9999
	Veh. vs. 50	>0.9999
	Veh. vs. 60	0.0110
	Veh. vs. 150	0.0982

Figure 4

Vehicle vs Fentanyl

Kruskal-Wallis	H = 35.69; p < 0.0001	
Dunn's post-hoc test		Adjusted p value
	Veh. vs. 20	<0.0001
	Veh. vs. 30	0.0002
	Veh. vs. 50	<0.0001
	Veh. vs. 60	<0.0001

Vehicle vs. NFEPP

Kruskal-Wallis	H = 5.872; p = 0.3189	
Dunn's post-hoc test		Adjusted p value
	Veh. vs. 20	>0.9999
	Veh. vs. 30	>0.9999
	Veh. vs. 50	0.8289
	Veh. vs. 60	>0.9999
	Veh. vs. 150	>0.9999

Figure 5

Vehicle vs. Fentanyl

2-way RM ANOVA	Time x Treatment	$F_{2,30} = 1.082$, P = 0.3519
	Time	$F_{2,30} = 4.095$, P = 0.0268
	Treatment	$F_{1,15} = 48.18$, P < 0.0001
Bonferroni's post-hoc test	Veh. vs. Fentanyl	Adjusted p value
	2 minutes	<0.0001
	30 minutes	<0.0001
	60 minutes	<0.0001

Vehicle vs. NFEPP

2-way RM ANOVA	Time x Treatment	$F_{4,46} = 0.8742$, P = 0.4867
	Time	$F_{2,46} = 3.729$, P = 0.0316
	Treatment	$F_{2,23} = 1.142$, P = 0.3365
Bonferroni's post-hoc test	Veh. vs. Fentanyl	Adjusted p value
	2 minutes	<0.0001
	30 minutes	<0.0001
	60 minutes	<0.0001