Original research

Agonist that activates the μ-opioid receptor in acidified microenvironments inhibits colitis pain without side effects

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ABSTRACT

Objective The effectiveness of μ-opioid receptor (MOPr) agonists for treatment of visceral pain is compromised by constipation, respiratory depression, sedation and addiction. We investigated whether a fentanyl analogue, (±)-N-(3-fluoro-1-phenethylpiperidine-4-yl)-N-phenyl propionamide (NFEPP), which preferentially activates MOPr in acidified diseased tissues, would inhibit pain in a preclinical model of inflammatory bowel disease (IBD) without side effects in healthy tissues.

Design Antinociceptive actions of NFEPP and fentanyl were compared in control mice and mice with dextran sodium sulfate colitis by measuring visceromotor responses to colorectal distension. Patch clamp and extracellular recordings were used to assess nociceptor activation. Defecation, respiration and locomotion were assessed. Colonic migrating motor complexes were assessed by spatiotemporal mapping of isolated tissue. NFEPP-induced MOPr signalling and trafficking were studied in human embryonic kidney 293 cells.

Results NFEPP inhibited visceromotor responses to colorectal distension in mice with colitis but not in control mice, consistent with acidification of the inflamed colon. Fentanyl inhibited responses in both groups. NFEPP inhibited the excitability of dorsal root ganglion neurons and suppressed mechanical sensitivity of colonic afferent fibres in acidified but not physiological conditions. Whereas fentanyl decreased defecation and caused respiratory depression and hyperactivity in mice with colitis, NFEPP was devoid of these effects. NFEPP did not affect colonic migrating motor complexes at physiological pH. NFEPP preferentially activated MOPr in acidified extracellular conditions to inhibit cAMP formation, recruit β-arrestins and evoke MOPr endocytosis.

Conclusion In a preclinical IBD model, NFEPP preferentially activates MOPr in acidified microenvironments of inflamed tissues to induce antinociception without causing respiratory depression, constipation and hyperactivity.

Significance of this study

What is already known on this subject?

► The use of opioids to manage inflammatory bowel disease pain is restricted by side effects of respiratory depression, constipation, sedation and addiction. The μ-opioid receptor (MOPr) mediates the analgesic and detrimental actions of opioids such as fentanyl. We investigated whether a fentanyl analogue, N-(3-fluoro-1-phenethylpiperidine-4-yl)-N-phenyl propionamide (NFEPP), engineered to preferentially bind to the MOPr under acidic conditions found in diseased tissues could selectively inhibit colitis pain without side effects.

What are the new findings?

► In sharp contrast to fentanyl, which inhibited colonic nociception in healthy mice and mice with acute colitis, NFEPP selectively inhibited nociception in mice with colitis but not healthy controls. NFEPP inhibited activity of colonic nociceptors, but only under acidified conditions. Unlike fentanyl, NFEPP did not induce constipation, respiratory depression or altered locomotion. The acidified extracellular fluid of the inflamed colon allowed NFEPP to engage the MOPr and activate antinociceptive signalling pathways.

How might it impact on clinical practice in the foreseeable future?

► Opioids designed to selectively engage receptors in diseased tissues offer the prospect of treating pain without life threatening side effects mediated by receptors in heathy tissues. Since G protein-coupled receptors such as MOPr are the target of over thirty percent of approved drugs, agonists and antagonists designed to selectively engage receptors in diseased tissues might provide enhanced efficacy and specificity for treatment of widespread disorders.

INTRODUCTION

Opioid receptors (OPrs), members of the large family of G protein-coupled receptors, provide an endogenous mechanism for pain control and are thus targets for the treatment of pain.[1] Opioids

that activate the μ-, ∂- and κ-opioid receptors (MOPr, DOPr) on primary sensory, spinal and supraspinal neurons depress activity and inhibit pain. In the inflamed intestine, opioids from infiltrating immune cells activate MOPr and DOPr on primary sensory neurons and suppress inflammatory bowel disease (IBD) pain.[2 3] Drugs that activate MOPr on neurons of the pain pathway are powerful analgesics.[1] However, the usefulness of opioids for the treatment of pain, including IBD pain, is limited by on-target side effects mediated by OPrs in other neurons. MOPr hyperpolarises neurons of the central nervous system, resulting in diminished respiratory drive and sedation.[1] Opioids can also inhibit peristalsis and electrolyte and fluid secretion in the digestive tract by activating MOPr on enteric and central neurons.[4 5] Whereas the analgesic properties of MOPr agonists diminish with continued use (ie, induce tolerance), on-target side effects such as respiratory depression are more sustained, with life-threatening consequences as escalating doses are required to control pain. The addictive properties of opioids exacerbate these problems. The heavy use of opioids is an independent predictor of mortality in IBD patients.[6] Given these problems, non-opioid analgesics have been identified as targets for visceral pain, including antagonists of pronociceptive G protein-coupled receptors and transient receptor potential ion channels.[7]

Insights into the structure and function of MOPrs have facilitated the development of pharmacological approaches to mitigate the detrimental side effects of opioids.[8] Biased agonists, which stabilise MOPr conformations that favour activation of G protein pathways that might underlie analgesia at the expense of β-arrestin (βARR) pathways that may mediate side effects, could provide analgesia with fewer side effects.[9–13] Some G protein biased MOPr agonists show promise in preclinical and human studies,[11 12 14] which has not always been replicated.[15] The premise that different signalling pathways underlie the beneficial and detrimental actions of MOPr agonists and the promise of biased agonists has been questioned.[16] An alternative approach has been to exploit the acidified extracellular environment of diseased tissues (eg, cancer, inflammation) to minimise the side effects of MOPr agonists. Through molecular modelling of MOPr in an acidified environment, a fluorinated analogue of fentanyl, (±)-N-(3-fluoro-1-phenethylpiperidine-4-yl)-N-phenyl propionamide (NFEPP), with a low pKa was developed and found to preferentially activate MOPr in acidified tissues.[17–20] NFEPP inhibits nociception emanating from acidified, diseased tissues without on-target side effects in healthy tissues. It is not known whether NFEPP suppresses inflammatory pain of the colon without affecting defecation, ventilation or locomotion.

Herein, we compared the actions of NFEPP and fentanyl in a preclinical mouse model of IBD. Our results show that NFEPP preferentially activates MOPr in acidified inflamed colon to inhibit nociception without side effects in healthy tissues. Ligands designed to preferentially activate MOPrs in diseased tissues offer the potential for treatment of visceral pain without life-threatening side effects. The approach presented here may be advantageous compared with G protein-biased ligands because G protein activation is an underlying mechanism of opioid-induced respiratory depression and addiction.[21–23]

MATERIALS AND METHODS

Additional materials and methods are included in online supplemental information.

MOPr agonists

NFEPP has been described.[19] Fentanyl was from Sandoz.

Dextran sodium sulfate-induced colitis

Colitis was induced by the administration of 2.5% dextran sodium sulfate (DSS) in drinking water for 5 days.

Visceromotor response to colorectal distention

A telemetric transmitter was implanted to measure electromyographic activity of external oblique muscles. At 10 days after surgery, mice received DSS in the drinking water or water alone for 5 days and were then switched to water alone for 2 days. Visceromotor responses were assessed day 8 after commencing DSS for NFEPP treatment and day 9 for fentanyl treatment. A catheter was inserted 0.5 cm into the colorectum for distension (20, 40, 60, 80 μL). Visceromotor responses were measured 30 min after vehicle, NFEPP or fentanyl (0.2 mg/kg, s.c.). Compliance of the excised colorectum was measured using a pressure transducer.

Colonic pH measurement

pH of colon segments was measured using the pH indicator SNARF 4 F-5 (and 6) carboxylic acid.

Colonic inflammation

Inflammation was assessed by measurement of myeloperoxidase (MPO) activity and by histological scoring.[24]

Tail flick test

Tail flick latency to noxious heat was recorded.

Defecation

Faecal pellets were counted for 1 hour.

Heart rate, oxygen saturation

A pulse oximeter sensor was used to measure heart rate and oxygen saturation.

Locomotion

Distance travelled, speed and resting time were recorded in an open field test.

Patch clamp recording

The excitability of nociceptors was assessed by measuring rheobase.[25]

Extracellular recording

Extracellular recordings were made from the splanchnic nerve innervating isolated segments of mouse distal colon.[25]

Colonic migrating motor complexes

Spatiotemporal maps along of segments of isolated mouse colon were constructed to assess colonic migrating motor complexes (CMMCs).[26]

cDNAs, transfection

Human embryonic kidney 293 (HEK293) cells were transiently transfected using polyethylenimine.[27]

Bioluminescence resonance energy transfer

cAMP formation, βARR2 recruitment and MOPr trafficking to Rab5a-positive endosomes were measured in HEK293 cells using bioluminescence resonance energy transfer (BRET).[27]

Förster resonance energy transfer

Nuclear extracellular signal regulated kinase (ERK) activity was measured in HEK293 cells expressing the Nuc-EKAR Förster resonance energy transfer (FRET) sensor.[27]

Statistics

Results are expressed as mean±SEM Statistical significance was assessed using Student unpaired t-test with Welch's correction or Mann-Whitney test or one-way or two-way analysis of variance (ANOVA) with Tukey's, Bonferroni's or Dunnett's post hoc test.

RESULTS

NFEPP inhibits visceral nociception only in mice with colitis

An agonist that selectively activates MOPr in diseased tissues might obviate on-target side effects mediated by MOPr in healthy tissues. The fentanyl analogue NFEPP was designed to preferentially activate MOPr in the acidified microenvironment of diseased tissues.[19] To assess whether NFEPP would activate MOPr in the inflamed colon and thereby inhibit colitis-induced pain, we compared the efficacy with which NFEPP and fentanyl inhibit visceral nociception in mice with acute colitis and healthy control mice. Mice were treated with DSS (2.5% drinking water, 5 days, 2 days recovery) to induce acute colitis. Time-matched control mice received normal drinking water. Mice were treated with doses of NFEPP, fentanyl (both 0.2 mg/kg, s.c.) or vehicle (control) based on published work.[19] After 30 min, visceral nociception was examined by measuring visceromotor responses to graded colorectal distension (20, 40, 60, 80 µL). Although NFEPP did not affect the visceromotor responses to graded colorectal distension in healthy control mice (% vehicle at 80 µL: NFEPP 108.6%±32.5%, vehicle 100%, N=7, p=0.999, two-way ANOVA, Bonferroni correction), NFEPP inhibited the visceromotor responses in mice with DSS colitis by 65.2%±2.0% (80 µL) (NFEPP 34.8%±2.0%, vehicle 100%, N=5, p<0.001) (figure 1A–D). In contrast, fentanyl inhibited visceromotor responses to colorectal distension both in control mice by 67.8%±11.1% (80 µL: fentanyl 32.3%±11.1%, vehicle 100%, N=6, p<0.01) (figure 1E) and in mice with DSS colitis by 79.7%±15.5% (80 µL: fentanyl 20.3%±15.5%, vehicle 100%, N=4, p<0.001) (figure 1F). The visceromotor responses to colorectal distension at baseline did not differ significantly between control and colitis mice but trended towards higher values in mice with colitis (area under curve; DSS-colitis 7.19±2.65, control 5.27±2.47, N=5–7, p=0.215, two-way ANOVA) (figure 1G). The production of endogenous opioids in the inflamed colon likely explains the absence of significant hyperalgesia in mice with colitis.[28] Compliance of the colorectum from control and colitis mice was not different (increased volume (µL) DSS-colitis 66.1±8.65, control 61.64±9.73, N=4–5, p=0.736, two-way ANOVA) (figure 1H). The results suggest that NFEPP preferentially activates MOPr on nociceptors innervating the inflamed but not healthy colon to inhibit nociception, whereas fentanyl activates MOPr in healthy and diseased mice.

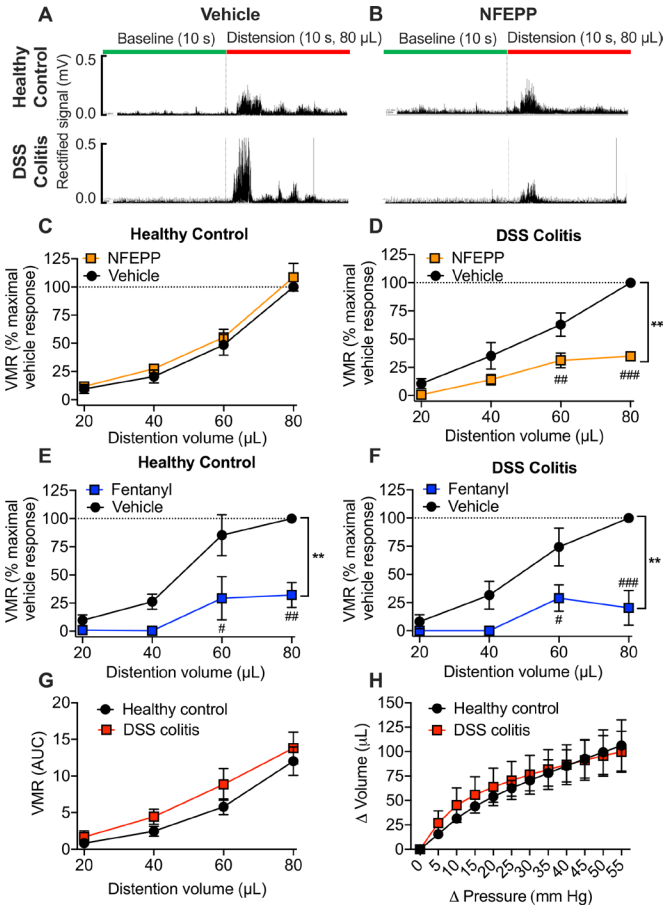

Figure 1 Effects of NFEPP and fentanyl on visceromotor responses to colorectal distension. (A, B) Representative traces showing the effects of vehicle (A) and NFEPP (B) on visceromotor responses to colorectal distension (80 µL) in healthy control and acute DSS colitis mice. (C, D) Effects of vehicle and NFEPP on visceromotor responses to colorectal distension in healthy control mice (C) (N=7, p=0.305) and DSS colitis mice (D) (N=5, p=0.0036). (E, F) Effects of vehicle and fentanyl on visceromotor responses to colorectal distension in healthy control mice (E) (N=6, p=0.0053) and DSS colitis mice (F) (n=4, p=0.0031). Two-way ANOVA, Bonferroni test. (G) Comparison of visceromotor responses to colorectal distension in healthy control (N=7) and DSS colitis (N=5) mice. The area under curve (AUC) of visceromotor responses of control and colitis mice were not significantly different (p=0.215, two-way ANOVA, Bonferroni test. (H) Colorectal compliance in healthy control (N=4) and DSS colitis (N=5) mice. There was no significant difference between control and colitis mice (p=0.736, two-way ANOVA, Bonferroni test). #P<0.05, **##p<0.01, ###p<0.001. In (D–F) # volume distention compared with vehicle, **curve compared with vehicle. ANOVA, analysis of variance; DSS, dextran sodium sulfate; NFEPP, N-(3-fluoro-1-phenethylpiperidine-4-yl)-N-phenyl propionamide; VMR, visceromotor response.

Inflammation acidifies extracellular fluid of the colon

The selectivity of NFEPP depends on its enhanced activity at MOPr in acidified extracellular fluid.[19] To assess whether the inflamed colon is acidified, colon segments from healthy control mice and mice with acute DSS colitis were incubated with the fluorescent pH indicator probe SNARF 4 F-5 (and 6) carboxylic acid, which fluoresces dependent on the protonation state. The inflamed colon (pH 6.71±0.09, N=17) was more acidic (ΔpH 0.33±0.1, p<0.01) than the non-inflamed colon (figure 2A). Colonic MPO activity, an indicator of neutrophil infiltration, was higher in DSS-treated mice (4.08±0.75 U/mg tissue, N=11) than in control mice (0.39±0.13 U/mg tissue, N=11, p<0.001) (figure 2B). Histological examination and measurement of the histological damage score confirmed transmucosal inflammation in the colon of DSS-treated mice (figure 2C,D). Inflammation was more pronounced in the mucosa and submucosa than the muscle layer (figure 2E).

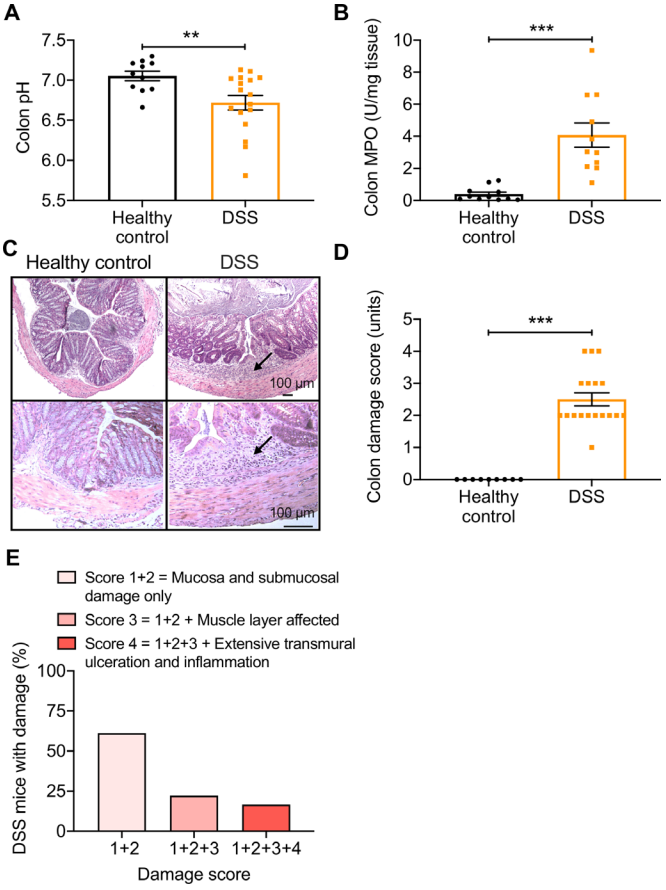

Figure 2 Acidification and inflammation of the colon. (A) pH of extracts of colon from healthy control mice and DSS colitis mice. (B) Myeloperoxidase activity in extracts of colon from healthy control mice and DSS colitis mice. (C–E) Histology (C) and histological damage score (D, E) of the colon from healthy control mice and DSS colitis mice. Arrows in C denote the inflammatory infiltrate in the mucosa of DSS-treated mice. (E) Proportion of specimens with damage score in different layers of colon. N=18 mice. Data points are responses of individual mice. **P<0.01, ***p<0.001. Unpaired Welch's t-test or Mann Whitney test. DSS, dextran sodium sulfate; MPO, myeloperoxidase.

NFEPP does not inhibit somatic thermal nociception, defecation, heart rate, ventilation or locomotion

Conventional agonists activate MOPr in the peripheral and central nervous systems, causing constipation, respiratory depression and sedation.[1 4 5] These on-target side effects limit their usefulness to treatment of IBD pain. We investigated whether NFEPP caused these side effects in healthy control and DSS colitis mice, with the expectation that even defecation might be unaffected in mice with colitis if the pH of the uninflamed musculature is normal. In mice with colitis, NFEPP (0.2 mg/kg, s.c.) did not affect withdrawal responses to noxious heat, assessed by a tail flick assay, whereas fentanyl strongly inhibited the response (p<0.001, figure 3A). These results are in line with the lack of efficacy of NFEPP on visceral nociception in control mice. NFEPP (0.2 or 0.4 mg/kg, s.c.) did not inhibit faecal pellet output in control or colitis mice, whereas fentanyl abolished pellet output in colitis mice (figure 3B,C). NFEPP (0.2 mg/kg, s.c.) did not affect heart rate or blood oxygen saturation in mice with colitis, whereas fentanyl reduced heart rate (ΔBPM 73.33±4.94, N=6, p<0.05 compared with basal) and oxygen saturation (decreased to 85.41%,±1.26, N=6, p<0.05) within 5 min, which returned to basal after 60 min (figure 3D,E). In an open field test of locomotion behaviour, NFEPP did not affect distance travelled, mean speed of travel or resting time (figure 3F–L). In contrast, fentanyl increased distance travelled and speed of travel and decreased resting time as previously shown.[29] These findings suggest that NFEPP does not activate MOPr on peripheral or central neurons of healthy tissues and thus does not cause the typical on-target side effects of fentanyl.

NFEPP preferentially inhibits colonic nociceptors in an acidified extracellular environment

MOPr activates K^+ channels of nociceptors and thereby reduces excitability.[1] Given its pH-dependence, NFEPP might inhibit nociceptors innervating the acidified inflamed colon, without affecting nociceptors in healthy tissues with normal extracellular pH.[17–20] To examine this possibility, we exposed mouse DRG neurons equilibrated at pH 6.5, 6.8 or 7.4 to NFEPP (300 nM), the MOPr agonist DAMGO (100 nM) or vehicle (control) for 15 min (figure 4A). Concentrations were based on published work.[19] Neurons were washed and excitability was assessed by measuring the rheobase (minimum input current required to fire an action potential) of small diameter neurons by patch clamp. NFEPP increased rheobase by 21.32%±8.62% at pH 6.5 and by 29%±8.62% at pH 6.8 when compared with vehicle (p<0.05) but had no effect at pH 7.4 (figure 4B–E). DAMGO increased rheobase by 25.21%±8.07% compared with vehicle (p<0.05) at pH 7.4 (figure 4E) as reported.[25 30] DAMGO had no effect at pH 6.5 (figure 4C). We then examined the duration of NFEPP-mediated inhibition of nociceptor excitability. After measurement of baseline rheobase at pH 7.4, neurons were incubated with NFEPP (300 nM) or vehicle at pH 6.5 for 10 min, and then rapidly switched to agonist-free buffer at pH 7.4. Rheobase was measured at 0, 15 or 30 min after NFEPP or vehicle (figure 4F). At T=0 min, NFEPP increased the rheobase compared with baseline (53.84%±10.5%, p<0.01) and vehicle (36.36%±9.31%, p<0.05) (figure 4F). However, the effect of NFEPP was not sustained at T=15 or T=30 min. When neurons were incubated with NFEPP (300 nM, 15 min) at pH 6.5 and then washed, there was an immediate increase in rheobase (figure 4G). After washing and recovery for 30 min at pH 6.5, the effect of NFEPP was also not sustained.

Although G protein-coupled receptors were once thought to signal exclusively from the plasma membrane, accumulating evidence suggests certain receptors signal from endosomes to control pain transmission.[3 25 27 31–33] Inhibitors of endocytosis blunt the inhibitory actions of DOPr agonists on excitability of DRG neurons, suggesting that endosomal signalling of DOPr underlies the sustained inhibitory effects of certain opioids.[3] However, the clathrin inhibitor PitStop2 did not block the immediate effect (T=0 min) of NFEPP on rheobase at pH 6.5 (figure 4G). Thus, endosomal signalling does not mediate the inhibitory actions of NFEPP-activated MOPr in primary afferent neurons.

To determine whether NFEPP could similarly depress the activation of the peripheral processes of nociceptors that would innervate diseased, acidified tissues, we made extracellular recordings from lumbar splanchnic nerves innervating isolated segments of mouse colon. Nociceptors were characterised by their responsiveness to stimulation of the colon or mesentery with von Frey filaments (VFF) (1 g). Tissues were equilibrated at pH 6.5 or 7.4 (10 min) and basal VFF responses were measured and found to be the same (figure 4H,I). NFEPP (300 nM) or vehicle (control) was superfused into the organ bath for 5 min

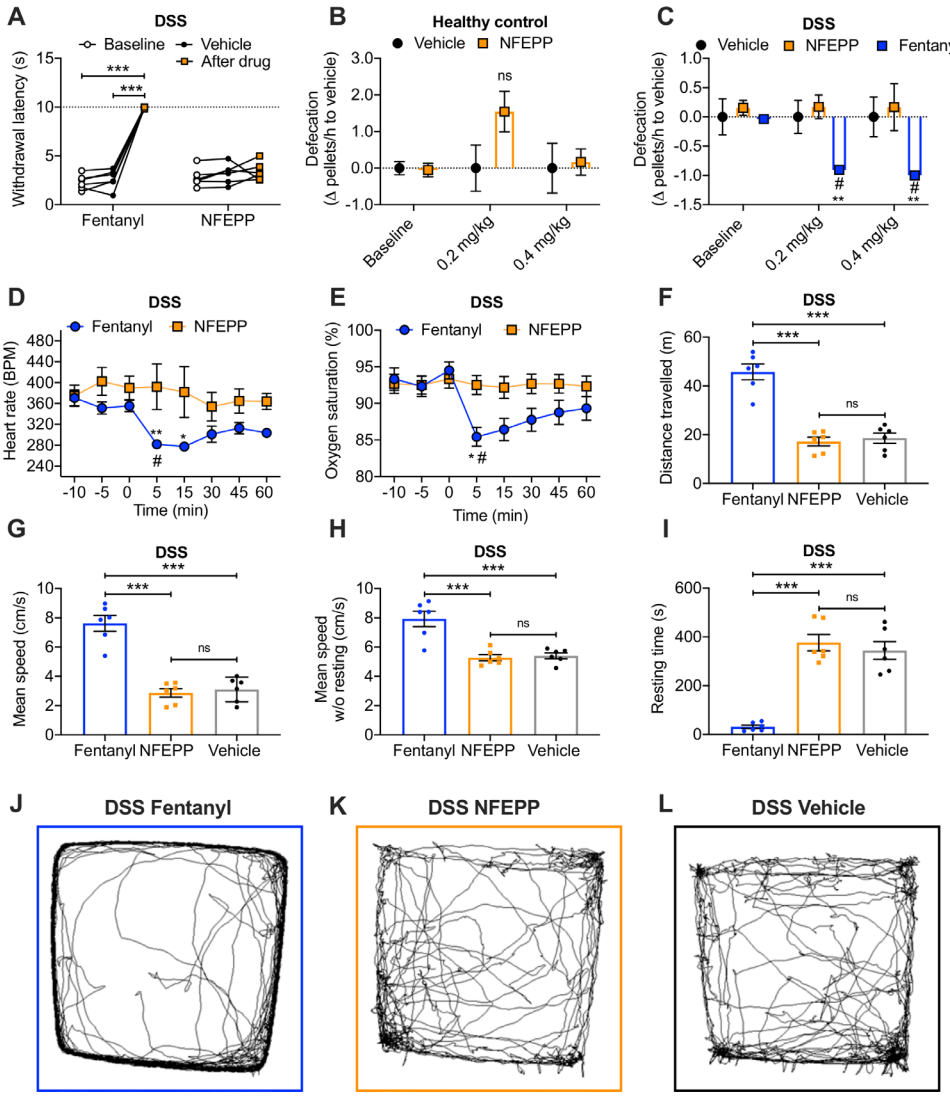

Figure 3 Evaluation of on-target side effects of NFEPP and fentanyl. The effects of NFEPP and fentanyl on withdrawal responses to noxious heat in a tail flick assay (A), N=6 mice per treatment, defecation (B, C), N=6–11 mice per treatment, heart rate (D), N=6 mice per treatment, oxygen saturation (E), N=6 mice per treatment and locomotion (open field test) (F–L), N=6 mice per treatment. (J–L) Are representative travel maps of mice treated with fentanyl, NFEPP or vehicle. Data are from DSS mice with the exception of (B), which is from control mice. Data points are responses of individual mice. #*P<0.05, **p<0.01, ***p<0.001. One-way or two-way ANOVA, Tukey's or Bonferroni's or Dunnett's tests. In C, #compared with vehicle, **fentanyl compared with NFEPP. in D, E, #compared with baseline, *fentanyl compared with NFEPP. ANOVA, analysis of variance; DSS, dextran sodium sulfate; NFEPP, N-(3-fluoro-1-phenethylpiperidine-4-yl)-N-phenyl propionamide; ns, not significant.

and then VFF responses were measured. At pH 6.5, NFEPP attenuated afferent responses to VFF probing compared with basal responses (27.3%±3.5% inhibition, p<0.01) (figure 4H). This effect was reversed after a 15 min washout at pH 7.4 (figure 4H). In contrast, the exposure to NFEPP at pH 7.4 had no effect on afferent mechanical sensitivity (figure 4I). Thus, NFEPP preferentially activates MOPr at the soma and in peripheral processes of nociceptors in acidic environments to transiently depress excitability and mechanical sensitivity.

NFEPP does not alter CMMCs at physiological extracellular pH

Fentanyl and morphine activate MOPr on interneurons of the myenteric plexus, which inhibits release of acetylcholine and nitric oxide and thereby depresses peristaltic contractions of the colon.[4] We made spatiotemporal maps of isolated segments of mouse colon to analyse whether NFEPP would affect the frequency, velocity and length of CMCCs in a pH-dependent manner. Segments of colon were equilibrated in organ baths at extracellular pH 7.4 or 6.8. Spatiotemporal maps were made under basal conditions, in tissues exposed to NFEPP (300 nM), and after NFEPP washout. Under basal conditions, the frequency, velocity and length of CMMCs were not significantly different at pH 7.4 or 6.8 (figure 5A,D,G–I). pH-dependent differences were detected in NFEPP-treated tissues. At pH 7.4, NFEPP had no significant effect on frequency, velocity or length of migrating motor complexes compared with baseline (figure 5B,G–I). These parameters were also unchanged after drug washout (figure 5C,G–I). At pH 6.8 NFEPP abolished migrating motor complexes (figure 5E,G–I). The frequency, velocity and length of migrating motor complexes recovered after drug wash-out (figure 5F,G–I). These findings suggest that NFEPP does not alter colonic peristalsis at physiological pH but may inhibit peristalsis in acidified tissues.

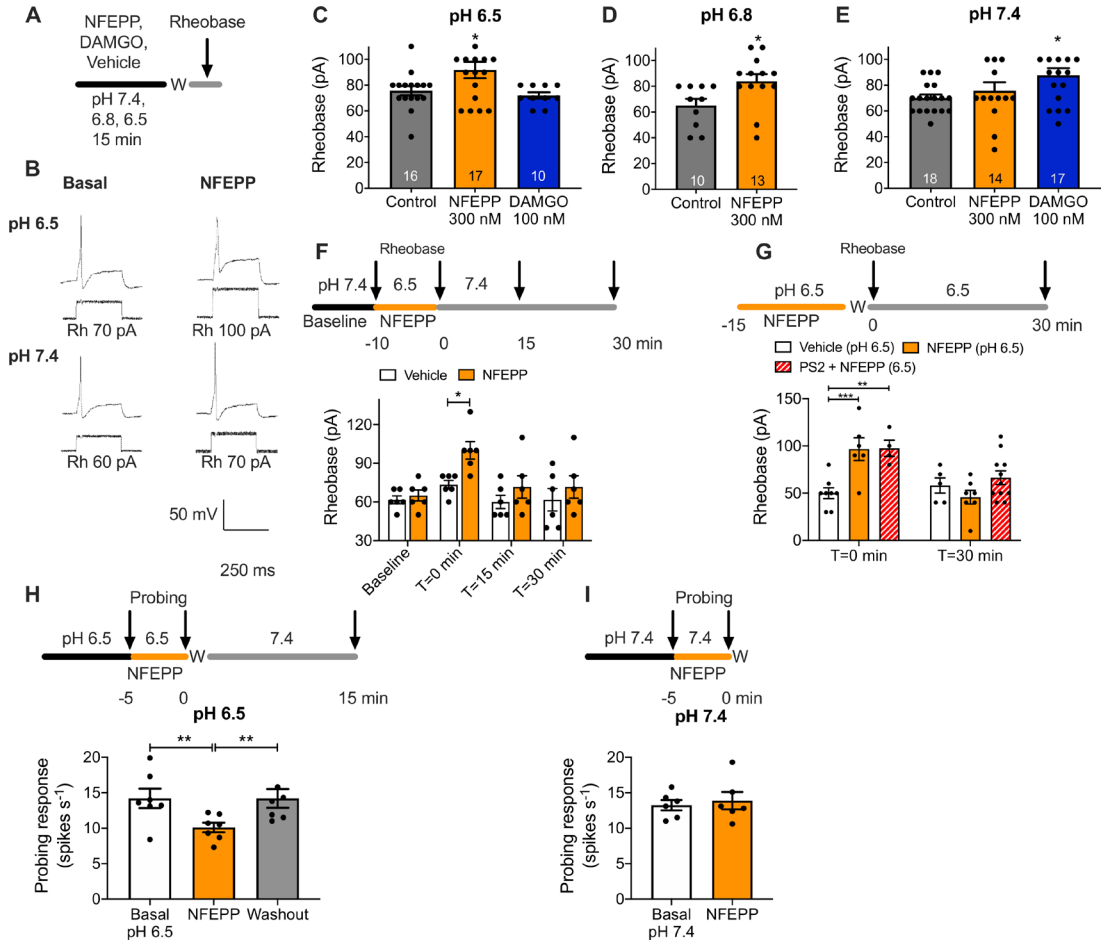

Figure 4 pH-dependent effects of NFEPP on nociceptor excitability. (A–G) Rheobase measurements of excitability of DRG neurons, showing effects of NFEPP and DAMGO at extracellular pH 7.4, 6.8 or 6.5. DRG neurons were preincubated with NFEPP and rheobase (Rh) was measured after washing. (A) protocol. (B) Representative traces under control conditions and after NFEPP at pH 6.5 or pH 7.4. (C–E) effects of NFEPP and DAMGO on rheobase at pH 6.5 (C), 6.8 (D) and 7.4 (E). Data points are responses of individual neurons from n=5–6 mice for each treatment. *P<0.05, 1-way ANOVA, Dunnett's test. (F, G) Duration of NFEPP effects. Neurons were incubated with NFEPP at pH 6.5 and then recovered at pH 7.4 (F) or pH 6.5 (G). Some neurons were treated with PitStop2 (pS2) in G). Data points are responses of individual neurons from N=6–7 mice for each treatment. *P<0.05, **p<0.01, ***p<0.001, two-way ANOVA, Tukey's test. (H, I) Excitability of colonic afferent nociceptors to mechanical stimulation of the mesentery with VFF. Basal responses, effects of NFEPP and recovery of responsiveness on mechano-sensitivity at pH 6.5 (H) and 7.4 (I). Data points are responses of individual receptive fields from n=4 mice. (H) **P<0.01, one-way ANOVA, Bonferroni test. (I) p=0.371, paired t-test. ANOVA, analysis of variance; NFEPP, N-(3-fluoro-1-phenethylpiperidine-4-yl)-N-phenyl propionamide; VFF, von Frey filaments.

NFEPP activates MOPr in an acidified extracellular environment

MOPr couples to $G_{\alpha i}$, which inhibits cAMP formation, and recruits βARRs.[34] We assessed the pH-dependence with which NFEPP might activate these signalling and trafficking pathways. The effects of NFEPP on forskolin-stimulated cAMP generation at extracellular pH 7.4, 6.8 or 6.5 was assessed in HEK293 cells expressing MOPr. NFEPP (300 nM) inhibited cAMP formation at pH 6.5 and 6.8 but not at pH 7.4 for at least 20 min (figure 6A,B). The inhibitory effects of NFEPP at pH 6.5 or 6.8 were comparable to that of the MOPr agonist DAMGO (100 nM) at pH 7.4. Although higher concentrations of NFEPP inhibited cAMP formation at pH 7.4, the effects were less potent and efficacious than at pH 6.5 or 6.8 (figure 6C).

βARRs associate with G protein-coupled receptors to mediate desensitisation, endocytosis and endosomal signalling.[35] To examine the pH-dependence of βARR recruitment to MOPr, we coexpressed in HEK293 cells MOPr tagged with *Rinella* luciferase (MOPr-Rluc8) and βARR2 tagged with yellow fluorescent protein (βARR-YFP). BRET was measured to monitor the proximity between MOPr-Rluc8 and βARR2-YFP. NFEPP (300 nM) increased MOPr-Rluc8/βARR2-YFP BRET at pH 6.5 and 6.8 but not at pH 7.4 (figure 6D,E). To assess pH-dependent MOPr endocytosis, we used BRET to examine the proximity between MOPr-Rluc8 and Rab5a (a resident protein of early endosomes) tagged with Venus (Rab5a-Venus). NFEPP (300 nM) increased MOPr-Rluc8/Rab5a-Venus BRET at pH 6.5 and 6.8, but not at pH 7.4 (figure 6F,G). Thus, NFEPP preferentially activates MOPr under acidic extracellular conditions, leading to inhibition of cAMP formation, recruitment of βARR2 and endocytosis.

Some G protein-coupled receptors, including OPrs, signal in endosomes by G protein-mediated and βARR-mediated mechanisms.[3 25 27 31–33] Receptors in endosomes generate compartmentalised signals that regulate neuronal excitability and pain. For neuropeptide receptors, the acidic microenvironment of the endosome (pH 5.5–6) promotes peptide/receptor dissociation and peptide degradation, which terminate endosomal signalling.[36–40] NFEPP (a non-peptide) might robustly activate MOPr in acidic early endosomes given its pH-dependent

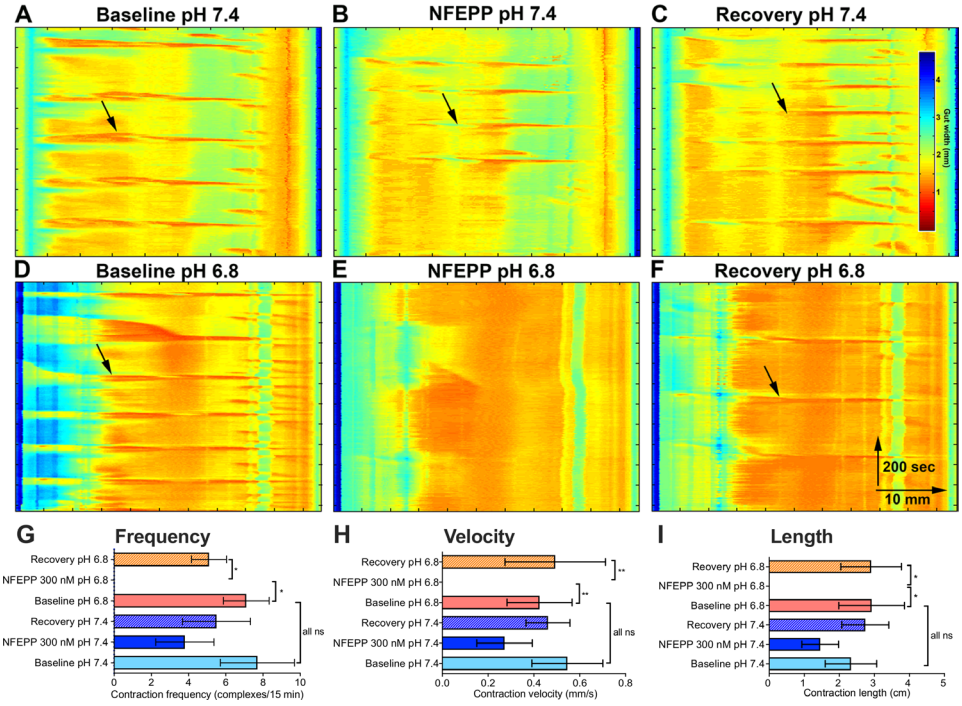

Figure 5 pH-dependent effects of NFEPP on colonic migrating motor complexes. (A–F) Representative spatiotemporal maps of mouse colon at pH 7.4 (A–C) and pH 6.8 (D–F) under baseline conditions (A, D), in the presence of NFEPP 300 nM, (B, D) and after drug wash-out and recovery (C, F). Black arrows indicate sample contractions. (G) Frequency. (H) Velocity. (I) Contraction length. N=6 mice per group. *P<0.05, **p<0.01, Student's unpaired t-tests with Welch's correction were used to compare means. NFEPP, N-(3-fluoro-1-phenethylpiperidine-4-yl)-N-phenyl propionamide; ns, not significant.

properties and presumed resistance to peptidases. To assess endosomal signalling, we expressed in HEK293 cells MOPr and a genetically encoded FRET biosensor for ERK activity in the nucleus (Nuc-EKAR), which is regulated by endosomal receptor signalling.[25 27 33] We measured ERK activity by FRET, which allowed analysis of signalling with high spatial and temporal fidelity. When incubated with cells at pH 6.5 or 6.8, but not at pH 7.4, NFEPP (300 nM) stimulated a sustained activation of nuclear ERK (figure 6H,I). The clathrin inhibitor PitStop2 (30 µM) and the dynamin inhibitor Dyngo4a (50 µM) blocked NFEPP-stimulated nuclear ERK activity (figure 6J,K). The inactive analogues, PitØ and DynØ, had no effect (figure 6J,K). The results suggest that NFEPP-induced MOPr signalling in endosomes activates nuclear ERK.

DISCUSSION

The main finding of our study is that NFEPP, which preferentially activates MOPr in the acidic extracellular environment of diseased tissues, selectively inhibits distension-evoked nociception in the inflamed mouse colon but not in the healthy colon. This finding is consistent with the acidification of the inflamed colon. Under acidic but not physiological conditions, NFEPP also inhibited excitability of nociceptors and suppressed mechanosensitivity of colonic afferent fibres. NFEPP did not affect defecation, ventilation or locomotion in mice with colitis, and did not affect peristaltic contractions of the isolated colon at physiological pH. In line with these findings, NFEPP evoked MOPr $G_{\alpha i}$ signalling, βARR recruitment and endocytosis only under acidic conditions. In contrast, fentanyl inhibited distention-evoked nociception in both the inflamed and healthy colon and induced the expected side effects of a conventional MOPr agonist.

Ischaemia, inflammation and cancer are associated with acidification of the extracellular fluid. During acute and chronic inflammation, the influx of activated immune cells, increased energy expenditure and oxygen use, and accelerated rate of glycolysis result in accumulation of lactic acid, which can acidify extracellular fluid by several pH units.[41] The hypoxic and proliferative zones of solid tumours are also markedly acidified.[42] Extracellular acidification can itself alter the inflammatory response and influence proliferation and metastasis of tumours.[42 43] In the present study, we exploited the acidification of the inflamed colon to increase the selectivity of a MOPr agonist for the treatment of pain, without side effects in healthy tissues. We observed that acute colitis caused a mild acidification of the colon (pH 6.7). pH was measured by incubating the pH indicator probe SNARF 4F-5 (and 6) carboxylic acid with segments of colon in vitro, which might explain the lower-than-expected pH of the uninflamed colon (pH 7.05). Our results support reports of colonic acidosis in patients with ulcerative colitis and Crohn's disease.[44] NFEPP was designed by modelling the docking of fentanyl with MOPr.[19] Since its pKa exceeds 8, fentanyl is protonated and binds to and activates MOPr in healthy (pH 7.4) and inflamed (pH 5–7) tissues. Replacement of hydrogen of fentanyl by fluorine in NFEPP decreased the pKa of NFEPP to 6.8, which resulted in preferential NFEPP activation of MOPr in acidified extracellular fluid (pH 5.5–6.5). We observed that NFEPP more potently activated MOPr at pH 6.5 and 6.8 than at pH 7.4, leading to inhibition of cAMP formation, recruitment of βARR2, and stimulation of MOPr endocytosis.

The ability of fentanyl to interact with MOPr at the pH of both normal and inflamed tissues accounts for its capacity to inhibit mechanically evoked nociception in the non-inflamed and inflamed colon, and explains the side effects of constipation, respiratory depression and altered locomotion that are mediated by MOPr in the peripheral and central nervous systems. The preference of NFEPP for activating MOPr in the acidified

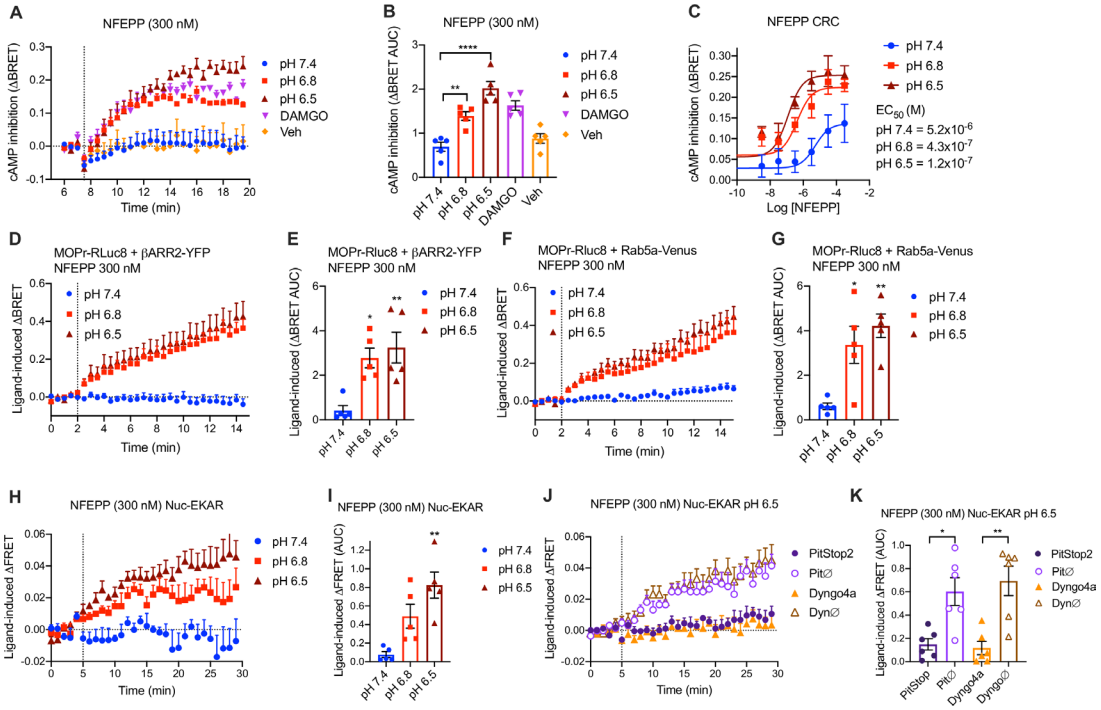

Figure 6 pH-dependent effects of NFEPP on MOPr signalling and endocytosis in HEK293 cells. (A–C) pH-dependency of NFEPP-induced inhibition of forskolin-stimulated formation of cAMP compared with effects of DAMGO at pH 7.4. (A) Time course. (B) Areas under curves (AUC). (C) Concentration-response curve (CRC). (D, E) pH-dependency of NFEPP-induced recruitment of βARR2 to MOPr. (D) Time course. (E) Areas under curves. (F, G) pH-dependency of NFEPP-induced recruitment of MOPr to Rab5a early endosomes. (F) Time course. (G) Areas under curves. (H-K) (H, I) pH-dependency of NFEPP-induced activation of nuclear ERK (Nuc-EKAR). (H) Time course. (I) Areas under curves. (J, K) Effects of PitStop2, Dyngo4a and inactive analogues (PitØ, DynØ) on NFEPP-induced activation of nuclear ERK at pH 6.5. (J) Time course. (K) Areas under curves. Data points are responses of independent experiments. N=5 independent experiments. *P<0.05, **p<0.01, ****p<0.0001, one-way ANOVA, Tukey's test. ANOVA, analysis of variance; ERK, extracellular signal regulated kinase; MOPr, μ-opioid receptor; NFEPP, N-(3-fluoro-1-phenethylpiperidine-4-yl)-N-phenyl propionamide.

extracellular fluid accounts for its selectivity for inhibiting nociception in the inflamed but not non-inflamed colon, and accounts for the lack of observed on-target side effects. We observed that NFEPP decreased the excitability of dorsal root ganglion nociceptors and suppressed mechanically evoked activation of colonic nociceptors in acidic but not physiological conditions. These findings are in line with the capacity of NFEPP to inhibit colonic nociception in mice with colitis. Together, our results suggest that NFEPP selectively activates MOPr at the terminals of nociceptors innervating the inflamed and acidified colonic mucosa. Most experiments in mice compared a single dose of fentanyl and NFEPP. Higher doses of NFEPP may activate MOPr in normal tissues since we observed that NFEPP could activate MOPr expressed in HEK293 cells at normal extracellular pH, although with a reduced potency and efficacy. Further studies are required to examine this possibility.

The effects of NFEPP on colonic motility and defecation deserves further study. Whereas NFEPP did not inhibit defecation in mice with colitis, NFEPP did suppress propulsive contractions of isolated segments of colon at acidic pH. One explanation of the lack of effect of NFEPP on defecation in mice with colitis is that inflammation and thus acidosis is restricted to the mucosa whereas the muscularis externa and myenteric plexus remain at physiological pH. In contrast, in studies of the isolated colon the entire bath and thus all layers were acidified. We observed that the mucosa was the predominant site of DSS-evoked inflammation, which supports the hypothesis that the muscularis externa is not acidified and thus NFEPP does not activate MOPr in myenteric neurons to suppress motility. In addition, MOPr can also regulate defecation by a central mechanism, which would not be activated by NFEPP.[5] A limitation of our experiments is that we were only able to measure pH of the entire colon wall rather than different layers (mucosa, submucosa, muscularis externa), and thus cannot be certain that the pH of the muscularis mucosa is unaffected in mice with DSS colitis. pH-dependent peptide probes, which have been used to localise acidified tumour microenvironments in intact tissues,[42] may enable precise mapping of acidified regions of the inflamed colon. It will also be important to study the effects of NFEPP on defecation in preclinical models of transmural colitis, such as the trinitrobenzene sulphonic acid model, to explore further the potential effects of NFEPP on motility in colitis. Such studies are clinically relevant since whereas ulcerative colitis is largely confined to the mucosa and submucosa, Crohn's disease is transmural. It will be of interest to study the efficacy of NFEPP in preclinical models of chronic colitis and in postinflammatory states that are relevant to irritable bowel syndrome. It will be important to determine if NFEPP, like some other opioids, can predispose the development of toxic megacolon.[45] Whether repeated administration of NFEPP attenuates responsiveness (ie, induces tolerance) remains to be studied. Our results showing that NFEPP selectively inhibits inflammatory pain in the colon are in agreement with other reports of NFEPP selectivity and efficacy for treatment of somatic inflammatory and neuropathic pain, acid-induced abdominal pain, and cancer pain in mice and rats.[18–20 46]

We examined the effects of NFEPP on MOPr signalling and trafficking in HEK293 cells. Our results show that NFEPP activates MOPr at pH 6.5 and 6.8 to inhibit formation of cAMP, recruit βARR2 and stimulate MOPr trafficking to early endosomes

expressing Rab5a. Once viewed as a conduit for receptor trafficking, endosomes are now considered an important site of signal transduction of G protein-coupled receptors that control pain transmission.[3 25 27 31–33] Both MOPr and DOPr can signal from endosomes,[32] and endosomal signalling of DOPr mediates the sustained antinociceptive actions of opioids, including those released from the inflamed colon.[3] The capacity of NFEPP to promote MOPr endocytosis and to engage MOPr at the pH of early endosomes may predispose NFEPP to activate MOPr in endosomes. In support of this possibility, we observed that NFEPP activated ERK in the nucleus, and that inhibitors of clathrin- and dynamin-mediated endocytosis abolished this signal, which thus requires endosomal signalling. DOPr similarly signals from endosomes to activate nuclear ERK.[3] DOPr agonists cause a sustained inhibition of nociceptor excitability, which is suppressed by endocytic inhibitors and thus requires endosomal signalling.[3] In contrast, the inhibitory actions of NFEPP on nociceptor excitability were not sustained despite its ability to activate MOPr in endosomes of HEK293 cells. Other MOPr agonists similarly exert transient inhibitory effects. Together, these findings suggest that NFEPP inhibits nociception primarily be activating MOPr at the plasma membrane of acidified tissues.

Agonists designed to preferentially engage MOPr in diseased tissues offer the potential for effective pain relief without the side effects that are mediated by MOPr in healthy tissues. These drugs would represent a major advance in the treatment of painful digestive diseases, including IBD, irritable bowel syndrome, pancreatitis and pancreatic cancer. Further work is required before NFEPP can be advanced to clinical trials, including toxicology, pharmacokinetic analysis, and studies of efficacy in preclinical models of painful inflammatory and functional diseases of the digestive system. Since G protein-coupled receptors are the single largest target of approved drugs, similar principles might allow the enhanced targeting of other ligands for the treatment of diverse diseases with fewer on-target side effects.

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Competing interests NWB is a founding scientist of Endosome Therapeutics Inc.

Patient consent for publication Not required.

Ethics approval Queen's University and Columbia University ethics committees approved procedures on C57BL/6 mice (male, 6–8 weeks).

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MATERIALS AND METHODS

Mice. C57BL/6 mice (male, 6-8 weeks) were from Charles River Laboratory. Since there are gender differences in some responses to opioids, male mice were used to limit potential gender-specific effects.[1] Mice were maintained in a light (12 h cycle) and temperature (25°C) controlled environment with free access to food and water. Queen's University and Columbia University ethics committees approved procedures. Mice were randomly assigned to treatments and investigators were blinded to treatments for studies of inflammation.

MOPr agonists. NFEPP has been described.[2] Fentanyl citrate was from Sandoz. [D-Ala^2, N-Me-Phe^4, Gly-ol^5]-enkephalin (DAMGO) was from Sigma Aldrich (St Louis, MO).

Dextran sodium sulphate (DSS) -induced colitis. Acute colitis was induced by the administration of 2.5% (w/v) DSS (Cat J14489-22, LOT 178017, Thermo Fisher Scientific) to mice in drinking water for 5 days. Mice were studied after 2-5 days recovery.

Visceromotor response to colorectal distention. Mice were anesthetized with isoflurane (1.5-2.5%), placed on a heating pad, and given bupivacaine (2 mg/kg, s.c.) and tramadol (20 mg/kg, s.c.). A PhysioTel ETA-F10 telemetric transmitter (Data Science International) was inserted in the abdominal cavity. Electrode tips were sutured onto the external oblique muscle (~5-10 mm apart) to measure electromyographic activity. Mice received tramadol (20 mg/kg, s.c.) for three post-operative days. At 10 days after surgery, mice received DSS in the drinking water or water alone (control) for 5 days and were then switched to water alone for 2 days. Visceromotor responses were assessed day 8 after commencing DSS for NFEPP treatment and day 9 for fentanyl treatment. Mice were acclimatized in a restrainer (Kent Scientific Corporation) for 30 min daily for two days before visceromotor response recording. On the day of recording, mice were sedated with isoflurane, placed in a restrainer, and a 4F arterial embolectomy catheter (Fogerty 120804FF, Edwards Lifesciences) was inserted 0.5 cm into the colorectum and taped to the tail. After 15 min recovery from anaesthesia, the catheter was distended in a stepwise manner (20, 40, 60, 80 µl, duplicate 10 s distensions, 3-min interval between tests). Visceromotor responses were measured 30 min after treatment with vehicle (0.5% DMSO, s.c.) followed by NFEPP (0.2 mg/kg, s.c.) on the same

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day within a 3-4 h interval. On the following day, visceromotor responses were measured 30 min after treatment with vehicle (0.9% NaCl, s.c.) followed by fentanyl (0.2 mg/kg, s.c., Sandoz) within a 3-4 h interval. Visceromotor responses were analysed with Ponemah v6.5 software (Data Science International). Mean basal electromyographic activity recorded 10 s before colorectal distension was subtracted from the mean electromyographic activity recorded during the 10 s distension. Results are presented as % visceromotor response relative to the maximal response after vehicle injection for a given mouse. Compliance of the excised colorectum was measured using a pressure transducer (NL108, Digitimer) and Spike 2 software (Version 7, Cambridge Electronic Design).

Colonic pH measurement. At 5 d post DSS or vehicle, the colon was excised, flushed with Krebs solution, and cut into 2-3 mm segments. Tissues were incubated in 20 μM SNARF 4F-5 (and 6) carboxylic acid (Molecular Probes) in PBS (10 mM $NaHPO_4$, 2.7 mM KCl, 137 mM NaCl, 25 mM Glucose, pH 6.4) (1 h, 37°C), and was then washed in PBS (pH 7.4). Tissue SNARF fluorescence was measured (488 nm excitation, 580, 640 nm emission) (Spectra MaxM3) and data were processed using SoftMax Pro 6.5 software (Molecular Devices). Tissue fluorescence was converted to pH by comparison to a standard curve of SNARF fluorescence with NIH-3T3 cells ($1.2x10^6$ cells per 0.1 ml) in PBS (pH 5.2 - 8.0, 0.4 increments).

Colonic inflammation. Inflammation of the colon was assessed by measurement of myeloperoxidase activity and haematoxylin and eosin staining of tissue sections.[3] Histological damage of the entire colon wall was evaluated using a modified scoring procedure: 0 = normal, 1 = damage limited to mucosa, 2 = ulceration limited to submucosa, 3 = focal transmural inflammation and ulceration, 4 = extensive transmural ulceration and inflammation, and 5 = extensive transmural ulceration and inflammation involving the whole section.[4]

Tail flick test. At 4 d post DSS or vehicle, mice were habituated to the room for 1 h and were placed in a restrainer. The distal 3 cm of the tail was immersed in a water bath at 52°C. The latency until withdrawal (rapid flick) was video recorded. To prevent tissue damage, the cut off time was set to 10 s. Latency was measured before and 10 min after vehicle, NFEPP or fentanyl (0.2 mg/kg, s.c.).

Defecation. At 5 d post DSS or vehicle, mice received vehicle or NFEPP or fentanyl (0.2 or 0.4 mg/kg, s.c.). Faecal pellets were counted for 1 h at the same time of day (9 am-12 pm). The number of pellets was normalized to the vehicle response, and the difference of the means of each treatment to vehicle were analysed.

Heart rate, oxygen saturation. At 3 d post DSS or vehicle, mice were anesthetized (1.5% isoflurane) and maintained at 37°C on a heating pad. A paw pulse oximeter sensor (MoseSTAT Jr., Kent Scientific) was used to measure heart rate and oxygen saturation. After 10 min baseline recording, mice received NFEPP or fentanyl (0.2 mg/kg, s.c.). Data were collected every 15 min for 1 h.

Locomotion. At 4 d post DSS or vehicle, mice were placed in an open field (45x45 cm, Harvard Apparatus). Locomotion was recorded by video and the data were processed using the Smart Video Tracking System V3.0 (Panlab) software. Mice were habituated to the room for 1 h prior to the experiment. Mice received vehicle or NFEPP or fentanyl (0.2 mg/kg, s.c.). Distance travelled, speed and resting time were recorded for 10 min.

Patch clamp recording. DRG (T9 - T13) were digested and cultured overnight.[5] Changes in excitability of small-diameter (<30 pF capacitance) neurons with properties of nociceptors were quantified by measuring rheobase (minimum input current to elicit an action potential) by whole-cell perforated patch-clamp recordings.[5] The pH of the external solution was adjusted to 6.5, 6.8 or 7.4 with 3 M NaOH. Neurons were incubated with NFEPP (300 nM) or DAMGO (100 nM) for 15 min and rheobase was measured with external solution at the corresponding pH for each condition. To assess response duration, neurons were perfused (5 min, pH 7.4) and baseline rheobase was measured. Neurons were rapidly switched to pH 6.5 with NFEPP (300 nM) or vehicle (10 min) using a hydraulic micromanipulator (MMO-203, Narishige). Neurons were then rapidly switched back to pH 7.4. Rheobase was measured at time T = 0, T = 15 and T = 30 min after NFEPP or vehicle. To investigate the role of endosomal signalling, neurons were preincubated with the clathrin inhibitor PitStop2 (15 μM, 30 min) or vehicle.[6]

Extracellular recording. Extracellular recordings were made from the splanchnic nerve innervating isolated segments of mouse distal colon.[5] Receptive fields were identified by stroking the mucosa and

mesentery with a brush and were classified on the basis of responses to distinct stimuli: probing (1 g von Frey filament, VFF), mucosal stroking (0.4 g VFF) and stretch. Afferents that responded only to probing were considered nociceptors. For studies at pH 7.4, tissues were maintained in Krebs solution pH 7.4 (5% CO_2, 95% O_2). For studies at pH 6.5, normal Krebs solution was replaced by pH 6.5 Krebs (adjusted with hydrochloride acid without gassing) 10 min prior to application of NFEPP. Baseline responses were recorded (3 times, 3 s, 1 g VFF). Tissues were superfused with NFEPP (300 nM) or vehicle for 5 min and probing responses were re-examined. Tissues were washed, recovered in Krebs pH 7.4 for 15 min, and probing responses were measured. Single unit activity was analysed.[5]

Colonic migrating motor complexes. Spatiotemporal maps along the proximo-distal length of segments of isolated mouse colon were constructed to quantify the frequency, velocity and length of colonic migrating motor responses, which were defined as diameter constrictions that propagated for ≥ 50% length of the preparation.[7] An intraluminal pressure of +2 cm H_2O was used to evoke colonic migrating motor responses. Two baseline recordings were made in Krebs buffer at pH 7.4 or 6.8. Two recordings were made after the bath was refilled with Krebs buffer containing NFEPP (300 nM). Two final recordings were made after NFEPP washout.

cDNAs, transfection. cDNA for human MOPr with three N-terminal HA epitopes (HA-MOPr) was from cDNA.org (#OPRM10TN00, Bloomsberg, PA). cDNAs for CAMYEL, MOPr-RLuc8, βARR2-Venus, Rab5a-Venus and Nuc-EKAR have been described.[8] Human embryonic kidney 293 (HEK293) cells were transiently transfected using polyethylenimine with the following cDNAs: 1 µg HA-MOPr + 4 µg CAMYEL, 1 µg MOPr-RLuc8 + 4 µg βARR2-YFP, 1 µg MOPr-RLuc8 + 4 µg Rab5a-Venus or 1 µg MOPr + 4 µg Nuc-EKAR.[8] Cells were plated and maintained as described.[8]

Bioluminescence resonance energy transfer (BRET). HEK293 cells were equilibrated in Hank's balanced salt solution (HBSS) + HEPES (10 mM) at pH 7.4, 6.8 or 6.5. Cells were incubated with the RLuc8 substrate coelenterazine (5 µM, 10 min). To evaluate MOPr activation, cells expressing HA-MOPr + CAMYEL (cAMP sensor) were challenged with forskolin (10 µM, 5 min) to stimulate cAMP formation, and exposed to NFEPP (3 nM - 3 µM), DAMGO (100 nM) or vehicle. To evaluate βARR2 recruitment and

MOPr trafficking to Rab5a-positive endosomes, cells expressing MOPr-RLuc8 + βARR2-YFP or Rab5a-Venus were challenged with NFEPP (300 nM). BRET was recorded and normalized to baseline and controls.[8]

Förster resonance energy transfer (FRET). To analyse nuclear ERK activity, HEK293 cells expressing MOPr and Nuc-EKAR FRET sensor were serum-starved overnight and equilibrated in HBSS + HEPES (10 mM) at pH 7.4, 6.8 or 6.5 (15 min). Cells were challenged with NFEPP (300 nM), phorbol 12,13-dibutyrate (PDBu, 10 μM) or vehicle. To assess role of endosomal signalling, cells at pH 6.5 were treated with clathrin inhibitor PitStop2 (50 μM),[6] dynamin inhibitor Dyngo4a (50 μM),[9] or inactive analogues (controls) for 30 min. FRET was measured and normalized to baseline and controls.[8]

Statistics. Results were analysed and graphs prepared using Prism 9. Results are expressed as mean±SEM. Statistical significance was assessed using Student unpaired *t* test with Welch's correction or Mann-Whitney test or 1-way or 2-way ANOVA with Tukey's, Bonferroni's or Dunnett's *post hoc* test.

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