

Evolving acidic microenvironments during colitis provide selective analgesic targets for a pH-sensitive opioid

Claudius E. Degro^{a,b}, Nestor Nivardo Jiménez-Vargas^a, Quentin Tsang^a, Yang Yu^a, Mabel Guzman-Rodriguez^a, Elahe Alizadeh^c, David Hurlbut^{a,d}, David E. Reed^a, Alan E. Lomax^a, Alan E. Lomax^a, Christoph Stein^e, Nigel W. Bunnett^{f,g}, Stephen J. Vanner^{a,*}

Abstract

Targeting the acidified inflammatory microenvironment with pH-sensitive opioids is a novel approach for managing visceral pain while mitigating side effects. The analgesic efficacy of pH-dependent opioids has not been studied during the evolution of inflammation, where fluctuating tissue pH and repeated therapeutic dosing could influence analgesia and side effects. Whether pH-dependent opioids can inhibit human nociceptors during extracellular acidification is unexplored. We studied the analgesic efficacy and side-effect profile of a pH-sensitive fentanyl analog, (\pm)-*N*-(3-fluoro-1-phenethylpiperidine-4-yl)-*N*-phenyl propionamide (NFEPP), during the evolution of colitis induced in mice with dextran sulphate sodium. Colitis was characterized by granulocyte infiltration, histological damage, and acidification of the mucosa and submucosa at sites of immune cell infiltration. Changes in nociception were determined by measuring visceromotor responses to noxious colorectal distension in conscious mice. Repeated doses of NFEPP inhibited nociception throughout the course of disease, with maximal efficacy at the peak of inflammation. Fentanyl was antinociceptive regardless of the stage of inflammation. Fentanyl inhibited gastrointestinal transit, blocked defaecation, and induced hypoxemia, whereas NFEPP had no such side effects. In proof-of-principle experiments, NFEPP inhibited mechanically provoked activation of human colonic nociceptors under acidic conditions mimicking the inflamed state. Thus, NFEPP provides analgesia throughout the evolution of colitis with maximal activity at peak inflammation. The actions of NFEPP are restricted to acidified layers of the colon, without common side effects in normal tissues. *N*-(3-fluoro-1-phenethylpiperidine-4-yl)-*N*-phenyl propionamide could provide safe and effective analgesia during acute colitis, such as flares of ulcerative colitis.

Keywords: IBD, Ulcerative colitis, Opioids, pH, Visceromotor reflexes, Analgesia, Motility, Constipation

1. Introduction

Abdominal pain is a major cause of morbidity for patients suffering from inflammatory bowel disease (IBD), with nearly 50% of patients reporting pain.³⁸ Adequate acute pain control in patients with IBD relies largely on the administration of opioids, with up to 62% of patients receiving opioids while hospitalized and 21% as outpatients.²⁹

The actions of opioids are mediated by a subfamily of transmembrane G-protein-coupled receptors: μ -, δ -, and κ -opioid receptors (MORs, DORs, and KORs, respectively).

Activation of these receptors reduces the excitability of nociceptive, spinal and supraspinal neurons and leads to effective analgesia.^{9,43} Although opioids are the most potent analgesics for acute pain, the ubiquitous expression of opioid receptors at both central and peripheral sites invariably leads to serious on-target side effects (ie, activation of opioid receptors in all tissues in the body). In the gastrointestinal tract, inhibition of motility and secretion causes constipation, nausea, and bloating, resulting in significant morbidity.^{13,44} In the central nervous system, sedation

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

C. E. Degro and N. N. Jiménez-Vargas contributed equally to this work.

N. W. Bunnett, S. J. Vanner contributed equally to this work.

^a Gastrointestinal Diseases Research Unit, Kingston General Hospital, Queen's University, Kingston, ON, Canada, ^b Department of General and Visceral Surgery, Charité—Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Campus Benjamin Franklin, Berlin, Germany, ^c Queen's Cardiopulmonary Unit (QCPU), Translational Institute of Medicine (TIME), Department of Medicine, Queen's University, Kingston, ON, Canada, ^d Department of Pathology and Molecular Medicine, Queen's University, Kingston, ON, Canada, ^e Department of Experimental Anaesthesiology, Charité—Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Campus Benjamin Franklin, Berlin, Germany, ^f Department of Molecular Pathobiology, New York University College of Dentistry, New York, NY, United States, ^g Department of Neuroscience and Physiology, Neuroscience Institute, Grossman School of Medicine, New York University, New York, NY, United States

*Corresponding author. Address: GI Diseases Research Unit, Kingston General Hospital, 78 Stuart St, K7L 2V7 Kingston, ON, Canada. Tel.: 613.549.6666 x6518. E-mail address: stephen.vanner@kingstonhsc.ca (S. J. Vanner).

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).

© 2023 International Association for the Study of Pain

<http://dx.doi.org/10.1097/j.pain.0000000000002956>

and respiratory depression can be life-threatening in hospitalized patients and chronic opioid users.^{2,23}

An approach to mitigating the on-target side effects has been to modify the pKa of opioids such that they preferentially activate opioid receptors in acidic tissues, such as at sites of inflammation and cancer.⁴² We recently studied the pH-sensitive opioid analogue (\pm)-*N*-(3-fluoro-1-phenethylpiperidine-4-yl)-*N*-phenyl propionamide (NFEP) as a strategy to inhibit nociception in the inflamed intestine without affecting healthy tissues with normal extracellular pH. This fluorinated fentanyl analogue has a lower pKa than the parent compound, facilitating a preferential activation of MORs in the acidic environment of inflamed tissues.⁴⁰ We found that a single dose of NFEP inhibited visceral hyperalgesia in the inflamed colon with similar efficacy to fentanyl, without the side effects of respiratory depression, sedation, or constipation.²⁰ Similar findings have been reported for other models of inflammatory pain.^{34,40} However, the analgesic properties of NFEP have not been studied during the evolution of inflammation, where alterations in tissue pH during the initiation and resolution of disease and repeated dosing could affect analgesic efficacy and occurrence of on-target side effects. The effects of colitis on acidification at the cellular level are unexplored. Whether NFEP has pH-dependent inhibitory actions on human colonic nociceptors has not been examined. Herein, we investigated the antinociceptive actions and on-target side effects of repeated dosing of NFEP during the course of inflammation in a preclinical mouse model that mimics acute episodes of ulcerative colitis. We related the antinociceptive efficacy of NFEP to acidification at the cellular level. Observations of the effects of extracellular acidification on antinociceptive actions in human colonic nociceptors advance NFEP as a therapy for IBD pain.

2. Methods

2.1. Animals

Male C57BL/6 mice (6–8 weeks) were obtained from Charles River Laboratory and kept in a light- (12-hour cycle) and temperature-controlled (22°C) environment with free access to food and water. Mice were randomly assigned to treatment groups, and investigators were blinded to experimental regimens. Experiments on animals and animal handling were in accordance with the Canadian Council of Animal Care and approved by the Queen's University Animal Care Committee.

2.2. Chemicals and pharmacological drugs

N-(3-fluoro-1-phenethylpiperidine-4-yl)-*N*-phenyl propionamide is a fluorinated analogue of fentanyl, as previously described.^{20,40} Fentanyl citrate was obtained from Sandoz and naloxone hydrochloride from Teligent. Acetylcholine (ACh) chloride (TLC) and naloxone hydrochloride dihydrate (TLC) were purchased from Sigma Aldrich (St Louis, MO).

2.3. Dextran sulphate sodium-induced colitis

Acute colitis in mice was induced by administration of 2.5% (wt/vol) dextran sulphate sodium (DSS) (MW: 165,192 g/mol, Cat J14489-22; Thermo Fisher Scientific) diluted in drinking water for 5 days (2.5% DSS protocol) followed by normal water.^{20,32,51}

2.4. Telemetric transmitter implantation and visceromotor responses to colorectal distension

Surgical procedures and measurements of visceromotor responses (VMRs) to colorectal distension (CRD) were performed as previously described.²⁰ Briefly, for telemetric transmitter implantation, mice were anesthetized with isoflurane in 1 L/minute oxygen (2–2.5% isoflurane) and treated with tramadol (20 mg/kg s.c.) and bupivacaine (2 mg/kg, i.d.) to counteract pain. A PhysioTel ETA-F10 telemetric transmitter (Data Science International, St. Paul, MN) was implanted into the abdominal cavity with electrode tips sutured onto the external oblique muscle (~5–10 mm apart) to measure electromyographic (EMG) activity. After surgery, mice received tramadol (20 mg/kg s.c.) and 1 mL NaCl 0.9% s.c. for 3 postoperative days to support recovery and rehydration. After 8 to 10 days, mice underwent the 2.5% DSS protocol. Before performing VMR recordings, mice were acclimatized in a restrainer for 30 minutes daily for 2 days. At the day of recording, mice were anesthetized with isoflurane, placed in a restrainer, and a 4F arterial embolectomy catheter (Fogarty 120804FF; Edwards Lifesciences, Mississauga, ON, Canada) was inserted 0.5 cm into the colorectum. After the mice regained consciousness (10–15 minutes later), the catheter was distended in a stepwise manner (20, 40, 60, and 80 μ L, duplicate 10-second distensions, 3-minute interval between distensions). Visceromotor responses were measured 30 minutes after vehicle (0.5% dimethyl sulfoxide, DMSO), NFEP, or fentanyl s.c. administrations, unless stated otherwise. Visceromotor responses were analysed with Ponemah v6.5 software (Data Science International, St. Paul, MN). Mean basal EMG activity recorded 10 seconds before CRD was subtracted from the mean EMG activity recorded during the 10-second CRD. Results are expressed as % VMR in proportion to the maximal VMR recorded after vehicle administration (baseline) in the same mouse (baseline recording 4 hours before vehicle duplicate, NFEP, or fentanyl administration).

2.5. Study design of opioid treatments

After application of 2.5% DSS for 5 days, mice were switched to normal water (day 1) and experiments were then performed as follows:

2.5.1. Cohort 1

Mice were randomly assigned to NFEP, fentanyl (each opioid at 0.4 mg/kg s.c.), or vehicle (0.5% DMSO s.c.) treatment groups. Experiments commenced at day 3, based on previous DSS colitis studies showing a robust colonic inflammation after 2 days of normal water,^{20,25,26} and consisted of s.c. injections administered for 5 days b.i.d. (bis in die, twice daily: 11 AM and 5 PM) until day 7 followed by a final injection in the morning of day 8. Previous studies have shown that fentanyl analgesia persists >4 hours after s.c. injection.²¹

2.5.1.1. Computed tomography group

Oral contrast-enhanced computed tomography (CT) scans were performed at day 3 (pretreatment, baseline) and at day 8 (1 hour after the last drug injection). At day 3 and day 6, defaecation was assessed over 1 hour immediately after each drug administration. Pulse oximeter experiments were performed at day 4 and day 7 after each drug administration.

2.5.1.2. Visceromotor response group

Visceromotor response recordings were performed at day 3 (after the first drug injection) and at day 8 (after the last drug injection) in response to stepwise CRDs (20, 40, 60, and 80 μ L). A baseline

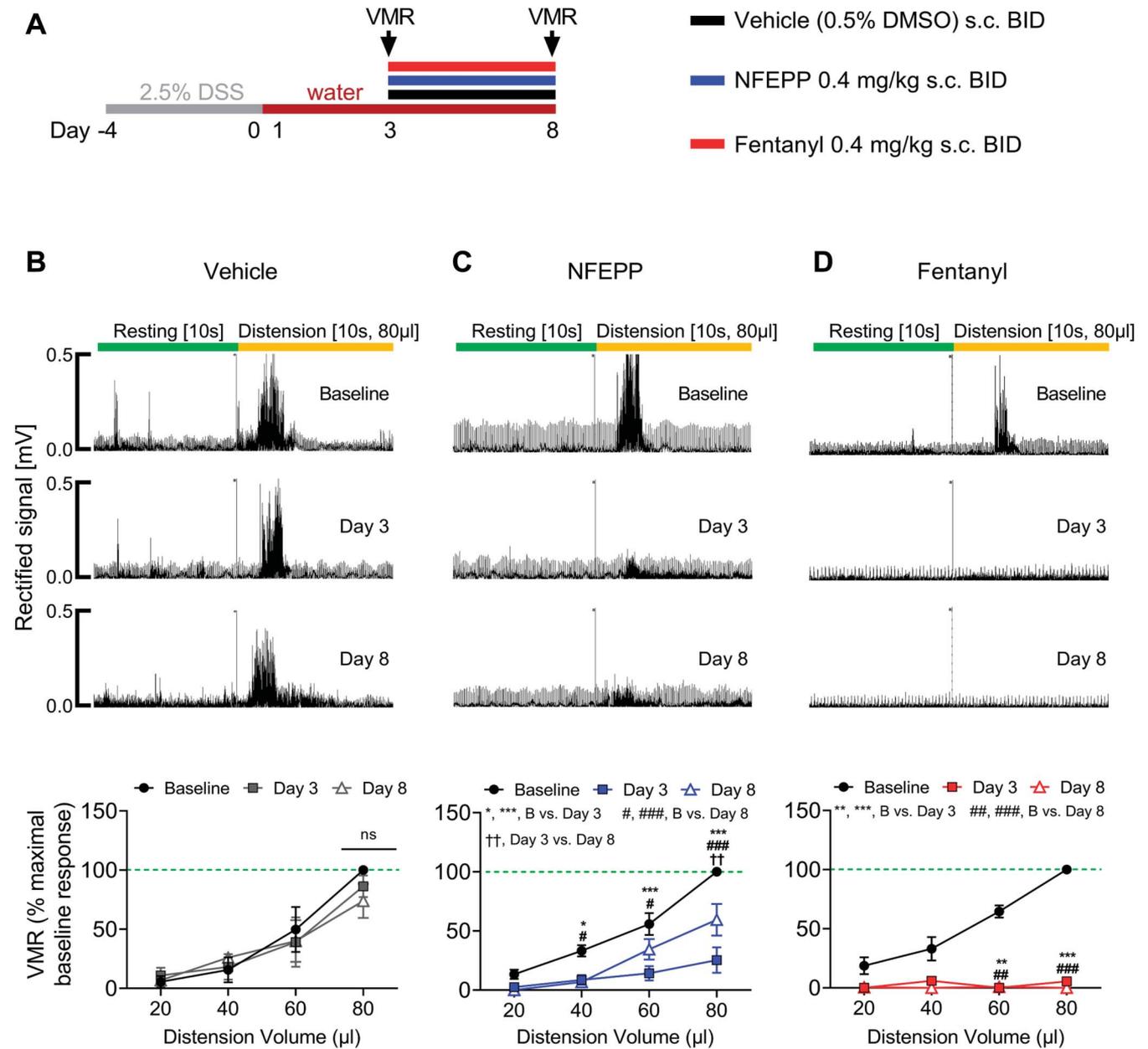


Figure 1. Effects of repeated NFEPP and fentanyl application on visceromotor responses (VMRs) to colorectal distension during acute DSS colitis. (A) Study design with injection regimen (*color coded*). VMRs were measured at day 3 and day 8. (B–D) Effects of vehicle (B, 0.5% DMSO s.c., $P = 0.48$, $N = 4$), NFEPP (C, 0.4 mg/kg s.c., $P < 0.001$, $N = 5$), and fentanyl (D, 0.4 mg/kg s.c., $P < 0.001$, $N = 4$) on VMRs to colorectal distension at day 3 and day 8 with representative traces. *, # $P < 0.05$. **, ##, †† $P < 0.01$. ***, ### $P < 0.001$. Two-way ANOVA, Tukey test. ANOVA, analysis of variance; B, baseline; b.i.d., bis in die (twice daily); DMSO, dimethyl sulfoxide; DSS, dextran sulphate sodium; NFEPP, *N*-(3-fluoro-1-phenethylpiperidine-4-yl)-*N*-phenyl propionamide.

VMR recording (0.5% DMSO s.c.) was first performed in each group at day 3 and was used as a reference to characterize the VMRs at day 3 and at day 8 after drug administration, individually within each treatment group. Visceromotor responses during acute DSS colitis did not change significantly over time (**Fig. 1**).

Mice of the CT and VMR groups were used for isometric tension recordings of colonic longitudinal muscle contractile activity or additional pulse oximeter experiments (each opioid at 0.8 mg/kg s.c.) at day 8 after the above-described experiments.

2.5.2. Cohort 2

N-(3-fluoro-1-phenethylpiperidine-4-yl)-*N*-phenyl propionamide (0.4 mg/kg) was administered s.c. b.i.d. (11 AM and 5 PM) starting

at day 0 until day 4 followed by a last injection at day 5. Visceromotor response recordings in response to 80 µL CRD were performed daily after NFEPP administration starting at day 1 until day 5.

2.5.3. Cohort 3

Locomotor activity, tail immersion assay, and pulse oximeter experiments were performed in healthy control mice after vehicle (0.5% DMSO s.c.) and NFEPP (0.8 mg/kg s.c.) administration. The same group of mice was then exposed to the 2.5% DSS protocol to induce acute colonic inflammation. Experiments were then repeated at day 4 and day 5 after NFEPP administration (0.8 mg/kg s.c.).

Downloaded from http://journals.lww.com/pain by BMDM5epHkav1ZEoum1QIN4e+kLHEZ9bsHh04XMI0hCwCk1AAW nYQp/llqH-D3i3D00dRy7T/SF14C3V4OA/pDa8KKGKv0Ymy+78= on 10/14/2023

2.6. Disease activity index assessment

Mice were monitored daily for colitis disease activity starting at day 1 after the 2.5% DSS protocol until the end of each experiment. The disease activity index (DAI), used in this study, has been previously described¹⁸ and included stool consistency, presence of faecal blood, and change in body weight (compared with the body weight one day before starting the 2.5% DSS protocol) to assess the following score: stool consistency: 0 = normal, 1 = moist and sticky, 2 = soft, and 3 = diarrhoea; stool blood: 0 = normal, 1 = brown reddish colour, 2 = visible blood, and 3 = rectal bleeding; body weight loss: 0 = 0 to 1%, 1 = 1 to 5%, 2 = 5 to 10%, and 3 = >10%. Each category was then added up to calculate the final DAI.

2.7. Colonic inflammation

Colonic inflammation was assessed by quantification of myeloperoxidase (MPO) activity²⁴ and by evaluation of haematoxylin and eosin (H&E)-stained tissue sections of healthy control and DSS colitis mice. Histological damage of the entire colon wall was assessed, as previously described,²⁰ using a modified scoring: 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.⁴⁷

2.8. Whole colon tissue pH measurement

Colonic pH was measured as previously described.²⁰ Healthy control and DSS colitis mice were euthanized and the colon was excised. After flushing with Krebs solution to remove the intraluminal content, the colon was cut into 2- to 3-mm flat segments. Tissues were consequently incubated in 20 μ M SNARF 5F-5 (and 6) carboxylic acid (Molecular Probes) in phosphate-buffered saline (PBS, in mM: 10 NaHPO₄, 2.7 KCl, 137 NaCl, and 25 glucose, pH 7.2) for 1 hour at 37°C and then washed in PBS (pH 7.4). Tissue SNARF fluorescence was measured (488 nm excitation, 580 and 640 nm emission; Spectra MaxM3, Molecular Devices, San Jose, CA), and acquired data were processed using SoftMax Pro 6.5 software (Molecular Devices). Tissue fluorescence (ratio 580/640 nm) conversion to pH was achieved by comparison with a standard curve of SNARF fluorescence in NIH-3T3 cells (1.2×10^6 cells per 0.1 mL, in PBS; pH 5.2–8.0 with 0.4 increments).

2.9. pH low insertion peptide imaging

At day 4 after the 2.5% DSS protocol, healthy control and DSS colitis mice received a tail vein injection of a Cyanin (Cy)-7-labelled pH low insertion peptide (pHLIP) (40 μ M, 100 μ L: [N > C: AC-(Cy7)-DDQNFWRAYLDLLFPTDLLLLDLLW-DLys-DLys]; CPC Scientific Inc, San Jose, CA). Then, 6 to 10 hours after injection, mice were euthanized and the colon, small intestine, heart, brain, liver, and lung were immediately excised and fixed in 10% formaldehyde overnight. Tissues were then treated with 30% sucrose in PBS (pH 7.4) for cryopreservation (24 hours), before embedding in an optimal cutting temperature (OCT) compound (Tissue-Tek, Torrance, CA) combined with liquid nitrogen freezing to prepare 9- μ m-thick slices using a Cryostat (CryoStar NX50; Thermo Fisher Scientific). Tissue slices were then blocked for 1 hour in a solution containing 2% bovine serum albumin (BSA), 5% normal goat serum, and 0.5% Triton X-100 in PBS followed by incubation with a sheep anti-Cy-fluorescein

isothiocyanate (FITC)-conjugated antibody (1:100, Abcam/ab7628, diluted in PBS containing 1% BSA) overnight. Finally, tissue slices were rinsed in PBS and mounted in a liquid mounting medium formulated with 4',6-diamidino-2-phenylindole (DAPI; ProLong Gold antifade reagent with DAPI; Thermo Fisher Scientific) before cover-slipped. The presence of DAPI (emission: 455 nm) and FITC (emission: 518 nm) immunofluorescence was then tested by confocal microscopy (Leica TCS SP8; Leica Microsystems, Concord, ON, Canada). pHLIP-FITC particle analysis of colon sections was performed based on $\times 10$ magnification images (HC PL APO CS2 10x, NA 0.40) by normalizing the number of pHLIP-FITC particles within the mucosa/submucosa and muscle layer against the DAPI density (no. of DAPI positive cells per square millimeter) within the same area. The average value of 2 to 3 different colon sections per mouse was used for further analysis.

2.10. In vivo micro-computed tomography

Micro-CT images were acquired exploiting VECTOr⁴CT preclinical imaging system (MILabs B.V., Utrecht, the Netherlands) equipped with a cone beam X-ray CT scanner. The X-ray source rotated around a fixed bed, allowing the mouse to be kept in a horizontal position during the scan. Mice were anesthetized with isoflurane in 1 L/min oxygen (2–2.5% isoflurane during the scan), and image acquisition was immediately started after tail vein injection of 200 μ L iohexol (300 mg/mL Omnipaque; GE Healthcare, Princeton, NJ) to increase the abdominal contrast. Measurements were performed with an acceleration voltage of 50 kVp and an X-ray tube current of 430 μ A in an accurate total-body mode to take 720 projections over a 360° scan with 40 milliseconds of exposure time. Computed tomography images were consequently processed using MILabs reconstruction software implementing a Hann filter of 50 mm voxel grid to generate 3-dimensional (3D) CT images with a slice thickness of 30 μ m. A post hoc Gaussian filter of 100 μ m was additionally applied to a subset of images using PMOD 3.9 software (PMOD Technologies Ltd., Zurich, Switzerland). Image quantification including gastrointestinal transit (GIT) analysis and diameter calculation was then performed based on axial, coronal, and sagittal projections using the Fiji software package (<https://imagej.net>), and 3D image rendering was finally fulfilled with the OsiriX Lite DICOM Viewer.³⁶

2.11. Computed tomography-based gastrointestinal transit scoring and colonic diameter calculation

To assess the CT-based GIT, mice were orally gavaged with 0.2 mL barium sulphate (0.7 g/mL, E-Z-HD; E-Z-EM Canada Inc, QC, Canada) 3 hours before each CT scan. After image acquisition and processing, a scoring system was then applied to describe the location of the leading front of the barium sulphate bolus within the gastrointestinal tract: 0 = stomach and duodenum, 1 = mid small intestine, 2 = terminal ileum, 3 = cecum, 4 = colon, and 5 = rectum and expelled. The extent of the large intestine was assessed by calculating the maximal diameter of the cecum and colon based on axial, coronal, and sagittal projections using the Fiji software package (<https://imagej.net>). The largest diameter measured was used for further analysis.

2.12. Defaecation

After s.c. administration of NFEPP, fentanyl (each opioid at 0.4 mg/kg), or vehicle (0.5% DMSO), faecal pellets were counted over 1 hour. The total number of pellets was used to compare between groups and time points.

2.13. Isometric tension recordings and electrical field stimulation

Mice were anesthetized with isoflurane and euthanized by cervical dislocation. One-centimeter-long segments of the mid-colon were subsequently excised, dissected from the mesentery, and flushed from the luminal content. Tissue samples were then transferred into organ bath recording chambers (Radnoti, Covina, CA) containing carbogenated (95% O₂/5% CO₂) Krebs solution (in mM: 118 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 25 NaHCO₃, 1.2 NaH₂PO₄, and 11 glucose, pH 7.4), warmed to 37°C, and changed every 20 minutes. Colon segments were placed in a vertical position, aligned with the longitudinal muscle, and stretched between a glass hook and an isometric force transducer (MLT 0201/RAD; AD Instruments Inc, Colorado Springs, CO) using silk suture 5-0. Specimens were allowed to equilibrate for 45 minutes under a pretension of 1 g before starting experiments. Experimental protocols included spontaneous activity recordings over a 30-minute period to assess frequency and amplitude (baseline-to-peak difference) of spontaneous contractions and to calculate the basal tone defined as the average resting tension between spontaneous contractions. Subsequently, neurogenic-induced contractions were provoked via electrical field stimulation (EFS) through 2 horizontal platinum ring electrodes (Radnoti) using a multichannel stimulator with automation control system (RADSTIM, Radnoti). Pulse trains of 1, 3, 5, and 10 Hz were applied in 3-minute intervals to elicit neurogenic-induced contractions (EFS parameters: 32 V, 0.5 ms pulse width, 10 seconds duration) measured as the difference between baseline and peak amplitude. The average of the contractions elicited by 2 subsequent EFS trains was used for further analysis. In a subset of experiments, naloxone hydrochloride (10 μM) was additionally applied to the organ bath, and EFS was repeated after a 5-minute wash-in period. Stimulation parameters were set based on preceding experiments showing an abolishment of contractile responses to EFS in the presence of 1 μM tetrodotoxin, indicating no or little effect on direct smooth muscle depolarization (data not shown). Finally, contractility of the smooth muscle was assessed through bath application of ACh chloride in cumulative logarithmic increasing concentrations (10⁻¹⁰-10⁻³ M, 2-minute intervals). All signals were filtered online at 50 Hz and recorded at 1 kHz using LabChart 8.1.10 software (AD Instruments Inc). Tension was normalized against the weight of the tissue used, expressed as g/mg tissue. Data were analysed offline using Clampfit 10.7 software.

2.14. Pulse oximeter measurements

Heart rate and blood oxygen saturation were measured in anesthetized mice (1.5% isoflurane) on a heating pad (37°C) using a paw pulse oximeter (Mouse STAT Jr.; Kent Scientific, Torrington, CT). After a 15-minute baseline recording, mice were s.c. administered NFEPP, fentanyl, or vehicle (0.5% DMSO). Data were subsequently collected every 15 minutes over 1 hour.

2.15. Tail immersion assay

Mice were acclimatized to the experimental room for 1 hour and placed in a restrainer. The distal 3 cm of the tail was immersed in a water bath maintained at 52°C. The latency to tail withdrawal (rapid flick) was subsequently video recorded. The cut-off time was set to 10 seconds to prevent any damage to the tail. Withdrawal latency was measured before and 10 minutes after NFEPP (0.8 mg/kg) or vehicle (0.5% DMSO) s.c. administration.

2.16. Locomotor activity

Mice were acclimatized to the experimental room for 1 hour before each experiment. Locomotor activity was then tested by placing the mice in an open-field apparatus (45 × 45 cm; Harvard Apparatus). Locomotion was assessed by video footages, and data were processed using the Smart Video Tracking System V3.0 (Panlab) software. Distance travelled, speed, and resting time were recorded over 10 minutes after NFEPP (0.8 mg/kg) or vehicle (0.5% DMSO) s.c. administration. Repeated locomotor activity experiments in the same mice were performed 7 days apart to prevent habituation to the open-field apparatus.

2.17. Human extracellular afferent nerve recordings

Colon specimens (descending and sigmoid colon) were obtained from 3 patients (1 male and 2 female patients) who underwent left hemicolectomy at the Kingston General Hospital, Queen's University, Kingston, Ontario, CA. All participants signed a written informed consent, and experiments were approved by the Queen's University Human Research Ethics Committees (#6015649). Afferent nerve recordings in human colon preparations were performed as previously described.⁴⁹ Colon sections were opened and pinned flat, with the mucosal layer face down in a tissue bath perfused with carbogenated (95% O₂/5% CO₂) pH 7.4 Krebs solution (in mM: 120 NaCl, 25 NaHCO₃, 1.2 MgSO₄, 1.2 KH₂PO₄, 11.7 glucose, and 2.0 CaCl₂). Mesenteric nerve bundles in the mesentery were identified and aspirated into a glass pipette containing a recording electrode. The receptive fields of the identified nerves were localized by systematically stroking the mesentery and serosa with a brush. Afferents that were responsive to von Frey filament (VFF) probing (10 g), but were not responsive to stretch, were used for further experiments and considered nociceptors. After a 30-minute stabilization period, pH 6.5 Krebs solution (adjusted with hydrochloride acid and without carbogen) was applied to the tissue bath for 10 minutes followed by VFF probing (3 times, 3 seconds each, 10 g). Next, colon preparations were superfused with NFEPP (300 nM) for 10 minutes in pH 6.5 Krebs solution and VFF probing was repeated. Thereafter, washout was performed for 20 minutes with carbogenated pH 7.4 Krebs solution, followed by a final VFF probing. Single units were analysed offline using the Spike 2 software (Version 7; Cambridge Electronic Design, Milton, Cambridge, United Kingdom).

2.18. Statistics

Group differences were analysed using 1-way or 2-way analysis of variance (ANOVA) with Tukey test or Bonferroni post hoc test, unless stated otherwise. Nonparametric data were compared with either the Kruskal–Wallis or the Friedman test combined with Dunn post hoc test. A *P*-value <0.05 was assigned to be statistically significant. Data are shown as mean ± SEM.

3. Results

3.1. Repeated N-(3-fluoro-1-phenethylpiperidine-4-yl)-N-phenyl propionamide administration has antinociceptive activity that is maintained throughout the evolution of colitis

To assess the antinociceptive effects of repeated administration of NFEPP during colitis, equivalent doses of NFEPP or fentanyl (0.4 mg/kg s.c.) or vehicle (0.5% dimethyl sulfoxide, DMSO s.c.) were administered twice daily (bis in die, b.i.d.) between day 3 and day 8 (Fig. 1A, cohort 1). Visceromotor responses to stepwise CRDs (20, 40, 60, and 80 μL) were measured at day 3 and day 8 and did not change during colitis after vehicle injection (*P* = 0.48,

$N = 4$, 2-way ANOVA; **Fig. 1B**). However, NFEPP significantly inhibited VMRs over the entire course of colitis (day 3: $74.7 \pm 10.8\%$ reduction, $P < 0.001$; day 8: $40.5 \pm 13.4\%$ reduction, $P < 0.001$, $N = 5$, all compared with baseline at $80 \mu\text{L}$, 2-way ANOVA, Tukey test; **Fig. 1C**). Nevertheless, the analgesic effect of NFEPP was less at day 8 compared with day 3 ($80 \mu\text{L}$ CRD: $P < 0.01$, 2-way ANOVA, Tukey test). Fentanyl inhibited VMRs at day 3 and day 8 to a similar extent (day 3: $94.6 \pm 3.2\%$ reduction, $P < 0.001$; day 8: $100\% \pm 0.0\%$ reduction, $P < 0.001$, $N = 4$, all compared with baseline at $80 \mu\text{L}$, 2-way ANOVA, Tukey test; **Fig. 1D**). Thus, repeated administrations of NFEPP provide effective antinociceptive actions over the course of acute colitis, and this inhibition is blocked by naloxone hydrochloride (Supplementary digital content Fig. 1, available at <http://links.lww.com/PAIN/B857>), as shown in our previous studies.⁴⁰

3.2. Antinociceptive actions of N-(3-fluoro-1-phenethylpiperidine-4-yl)-N-phenyl propionamide are closely related to the degree of inflammation during colitis

Because NFEPP preferentially activates MORs in the acidified microenvironment of actively inflamed tissues, the decline in efficacy at 8 days might be related to a resolution of inflammation. We therefore investigated whether the antinociceptive effects of NFEPP reflect the degree of inflammation during the evolution of colitis. Daily assessment of DAI revealed that inflammation increased from 1 to 4 days after initial DSS and then declined from days 5 to 8 ($P < 0.001$, $N = 15$ –43, Kruskal–Wallis test; **Fig. 2A**). Consequently, for histological as well as molecular studies of inflammation and tissue pH measurements, colon samples were collected at 3 different time points reflecting the initiation, peak, and resolution of the inflammatory course (days 2, 5, and 8, respectively). Histological damage was predominantly localized in the mucosal and submucosal layer and also demonstrated a change in the degree of inflammation over time (**Fig. 2B**). Colon damage score was greatest at day 5 ($P < 0.001$, $N = 9$) and lower at day 2 ($P < 0.05$, $N = 10$) and day 8 ($P < 0.01$, $N = 7$, all compared with healthy control, Kruskal–Wallis test, Dunn test; **Fig. 2C**). Colonic MPO activity, an indicator of granulocyte infiltration, was significantly increased at all 3 times, but also peaked at day 5 (MPO activity: $4.6 \pm 0.9 \text{ U/mg tissue}$, $P < 0.001$, $N = 9$, compared with healthy control, Kruskal–Wallis test, Dunn test; **Fig. 2D**). Consistent with these findings, colonic pH was lowest at day 5 ($\text{pH } 6.3 \pm 0.05$, $P < 0.01$, $N = 5$, compared with healthy control, Welch ANOVA, Dunnett T3 test; **Fig. 2E**). To relate these measures of evolving inflammation during colitis to the antinociceptive effects of NFEPP, mice with DSS colitis were treated daily with NFEPP ($0.4 \text{ mg/kg s.c. b.i.d.}$) during the initiation of disease activity for 6 days and VMRs to CRD ($80 \mu\text{L}$ only) were measured each day (**Fig. 2F**, cohort 2). *N*-(3-fluoro-1-phenethylpiperidine-4-yl)-*N*-phenyl propionamide did not affect VMRs at day 1, reduced VMRs at day 2, 3, and 4, and maximally inhibited VMRs at day 5 ($P < 0.05$, $N = 5$, compared with baseline, Friedman test, Dunn test; **Fig. 2F**, bar chart). Thus, the maximal antinociceptive action coincides with the peak of inflammation and tissue acidification, in line with the preferential ability of NFEPP to activate MORs in acidified extracellular environments.

3.3. Localization of sites of acidification in the inflamed colon using pH low insertion peptide reveals tissue-specific pH gradients

pH low insertion peptides allow identification of sites of acidification at the cellular level.^{39,48} Although soluble at physiological pH, protonation of these specialized peptides at low pH leads to a

subsequent insertion into biological membranes, thus allowing target specificity for cells located in acidic regions.³ To reveal acidification at the cellular level of the inflamed colon, we administered a Cy-labelled pHLIP via tail vein injection. At the peak of inflammation in DSS colitis (days 4–5), the pHLIP signal was predominantly identified in the mucosal and submucosal layer, whereas much less signal was detected in the adjacent muscle layer (**Fig. 3A**). Analysis of pHLIP density reflected a spatially restricted acidification within the inflamed colon wall (pHLIP-FITC/(DAPI/ mm^2): mucosal/submucosal layer: 34.1 ± 6.6 vs muscle layer: 7.1 ± 2.3 , $P < 0.01$, $N = 8$, paired *t* test; **Fig. 3B**). Analysis of the subcellular localization of pHLIP particles revealed a perinuclear signal suggesting insertion into biological membranes in line with their proposed mechanism (Supplementary digital content Fig. 2, available at <http://links.lww.com/PAIN/B857>). In control experiments, fluorescent signals could neither be detected in DSS colitis mice without prior pHLIP injection (**Fig. 3C**) nor in the colon of healthy control mice after pHLIP administration (**Fig. 3D**). To further validate the specificity of pHLIP for acidic environments, we examined its signal in additional extraintestinal organs of DSS colitis mice and could not detect pHLIP particles in these areas except for sporadic appearances along blood vessels (eg, heart and liver; Supplementary digital content Fig. 3, available at <http://links.lww.com/PAIN/B857>). H&E-stained sections of the same area revealed a distinctive overlap of the identified pHLIP signal with infiltrating inflammatory cells (**Fig. 3E**). Thus, colitis is associated with acidification of mucosal and submucosal tissues at sites of infiltration of inflammatory cells. Nociceptors expressing MORs innervate these regions of the intestine,^{6,16} facilitating a highly specific and restricted NFEPP-mediated inhibition.

3.4. Repeated N-(3-fluoro-1-phenethylpiperidine-4-yl)-N-phenyl propionamide application does not inhibit gastrointestinal transit and defaecation during colitis

To examine the effect of repeated dosing of NFEPP and fentanyl on intestinal motility during colitis, oral contrast-enhanced CT scans were recorded to evaluate GIT and defaecation was assessed. Between day 3 and day 8, equivalent doses of NFEPP or fentanyl (0.4 mg/kg s.c.) or vehicle ($0.5\% \text{ DMSO s.c.}$) were administered b.i.d. (**Fig. 4A**, cohort 1, CT-group). Oral contrast-enhanced CT scans were recorded at day 3 (before the first drug injection) and at day 8 (after the last drug injection) 3 hours after barium sulphate gavage (**Fig. 4B**). During acute colitis, GIT was not affected by vehicle injections (day 3 vs day 8: $P > 0.99$, $N = 5$, 2-way ANOVA, Bonferroni test; **Figs. 4B and C**). Likewise, NFEPP did not alter GIT during the course of acute colitis (day 3 vs day 8: $P = 0.60$, $N = 5$, 2-way ANOVA, Bonferroni test), whereas fentanyl reduced GIT by 74% (day 3 vs day 8: $P < 0.05$, $N = 6$, 2-way ANOVA, Bonferroni test; **Figs. 4B and C**). Similarly, no change in defaecation (number of faecal pellets) was observed after NFEPP administration at day 3 and day 6 ($N = 6$) compared with the vehicle ($N = 5$) group (day 3: $P > 0.99$; day 6: $P = 0.70$, 2-way ANOVA, Bonferroni test) in sharp contrast to fentanyl, which completely inhibited defaecation at day 3 and day 6 (day 6: $P < 0.05$, $N = 6$, compared with vehicle, 2-way ANOVA, Bonferroni test; **Fig. 4D**).

Because treatment with MOR agonists in patients with IBD is a risk factor for developing toxic megacolon,²² we calculated the maximal diameter of the colon and cecum based on the acquired CT images. In all 3 treatment groups, no change of the colon and cecum maximal diameter was observed over the treatment period (**Figs. 4E and F**). *N*-(3-fluoro-1-phenethylpiperidine-4-yl)-*N*-phenyl propionamide or fentanyl did not affect the colonic MPO activity or histological damage score of cohort 1 (CT and VMR

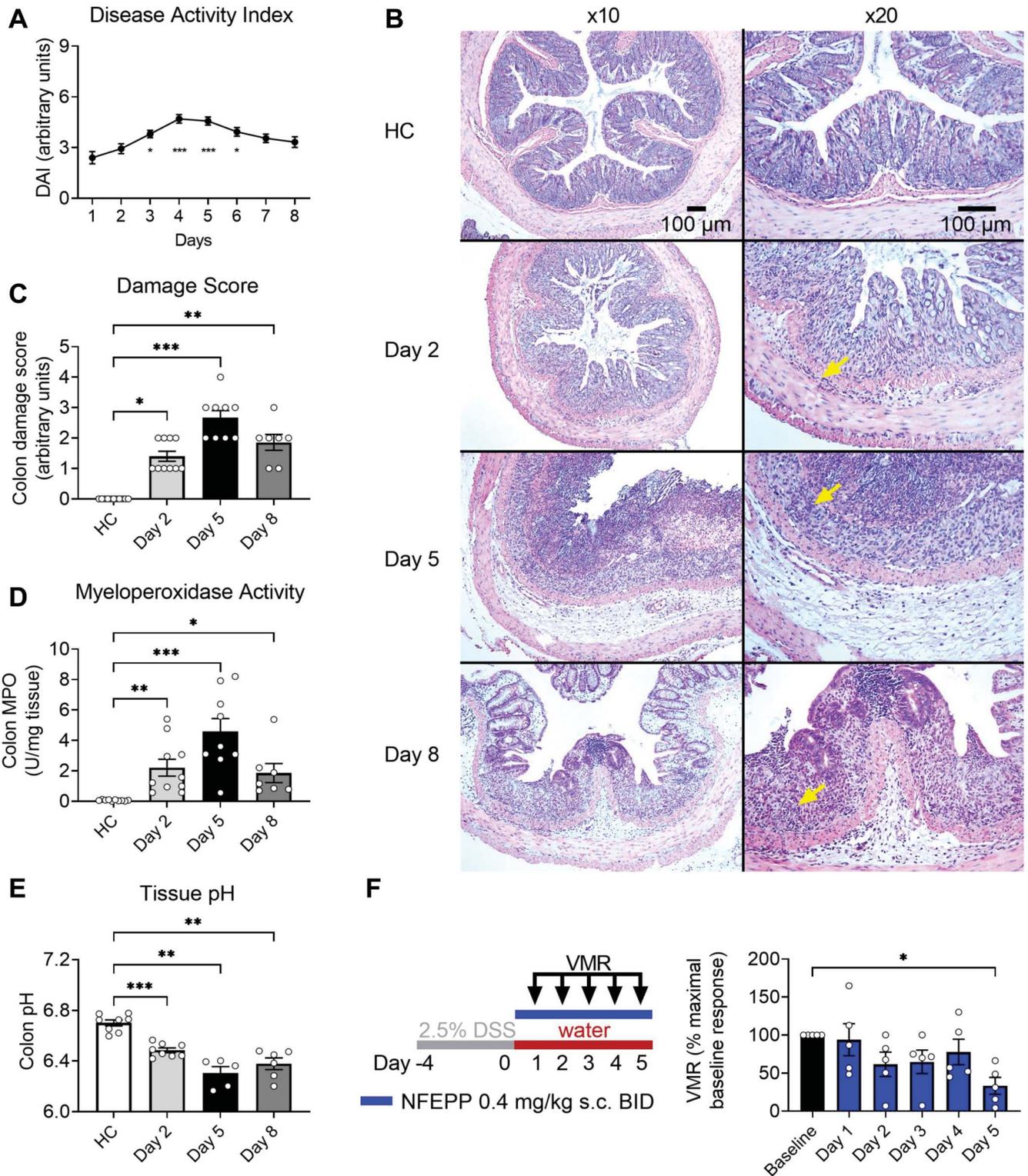


Figure 2. Changes of colonic inflammation and NFEPP effects on visceromotor responses (VMRs) during acute DSS colitis. (A) DAI of DSS colitis mice over time (N = 15-43, combined data of all cohorts, compared with day 1). (B–E) Histology (B, H&E staining), histological damage score (C), MPO activity (D), and tissue pH (E) of the colon from healthy control (HC, N = 8-11) and DSS colitis mice (N = 5-10) at different time points. Arrows in (B) denote infiltrating inflammatory cells. (F) Study design with injection regimen (color coded) and daily VMR measurements (80 μL colorectal distension, day 1-day 5) after NFEPP administration (0.4 mg/kg s.c.) in DSS colitis mice (N = 5). *P < 0.05. **P < 0.01. ***P < 0.001. (A, C, and D): Kruskal–Wallis test, Dunn test. (E) Welch ANOVA, Dunnett T3 test. (F) Friedman test, Dunn test. ANOVA, analysis of variance; b.i.d., bis in die (twice daily); DAI, disease activity index; DSS, dextran sulphate sodium; H&E, haematoxylin and eosin; MPO, myeloperoxidase; NFEPP, N-(3-fluoro-1-phenethylpiperidine-4-yl)-N-phenyl propionamide.

Downloaded from http://journals.lww.com/pain by BHDMM5EP#HKav1ZEoum1tQIN4a+kLHEZ9bsHh04XMM0hCwCXC1A1AV nYQp/llqH-D3i3D00dRy/TVSF14C3V/C4/OA/VpDDa8KKGKAV0Ymy+78= on 10/14/2023

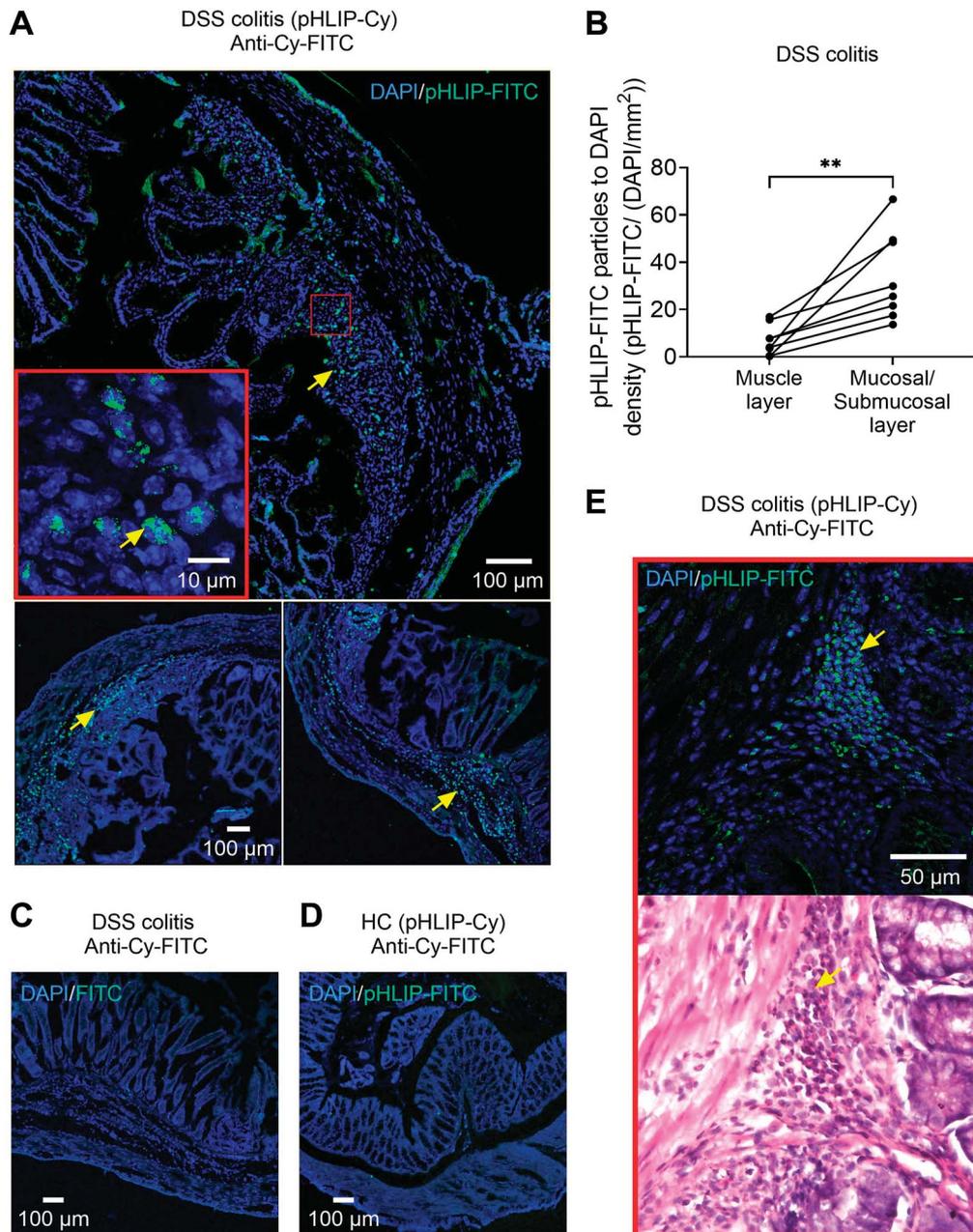


Figure 3. pHILIP sublocalization in the colon of healthy control and acute DSS colitis mice. (A) DAPI/anti-Cy-FITC labelling in DSS colitis mice treated with pHILIP (representative images of $N = 2$ mice). Staining (arrows) is predominantly localized in the mucosal and submucosal layer. Inset shows the subcellular localization of pHILIP. See also Supplementary digital content Fig. 2 (available at <http://links.lww.com/PAIN/B857>) for expanded confocal images illustrating the insertion of pHILIP into cell membranes within the inflamed layers of the colon. (B) pHILIP-FITC particle distribution within the colon wall of DSS colitis mice ($N = 8$). (C) DAPI/anti-Cy-FITC labelling in a DSS colitis mouse that was not treated with pHILIP. (D) DAPI/anti-Cy-FITC labelling in a healthy control (HC) mouse treated with pHILIP. (E) DAPI/anti-Cy-FITC labelling and H&E staining in a DSS colitis mouse treated with pHILIP. Arrows denote the co-localization of pHILIP particles and infiltrating inflammatory cells. $**P < 0.01$. Paired t test. Cy, cyanin; DAPI, 4',6-diamidino-2-phenylindole; DSS, dextran sulphate sodium; FITC, fluorescein isothiocyanate; H&E, haematoxylin and eosin; pHILIP, pH low insertion peptide.

groups; Supplementary digital content Fig. 4, available at <http://links.lww.com/PAIN/B857>).

To further test whether repeated NFEPP administrations induce an impaired colon contractility and myenteric excitability of the inflamed colon, we additionally measured the colonic longitudinal muscle contractile activity by recording **isometric tension** during phasic activity, in response to EFS and after bath application of ACh of mid-colon segments from mice with DSS colitis treated with equivalent doses of NFEPP or fentanyl (0.4 mg/kg s.c) or vehicle (0.5% DMSO s.c.) b.i.d. over 6 days

(Supplementary digital content Fig. 5, available at <http://links.lww.com/PAIN/B857>).

3.5. Repeated N-(3-fluoro-1-phenethylpiperidine-4-yl)-N-phenyl propionamide application during colitis has no effect on blood oxygen saturation and less effect on heart rate than fentanyl

On-target side effects compromising the cardiorespiratory system seriously limit the acute and chronic use of

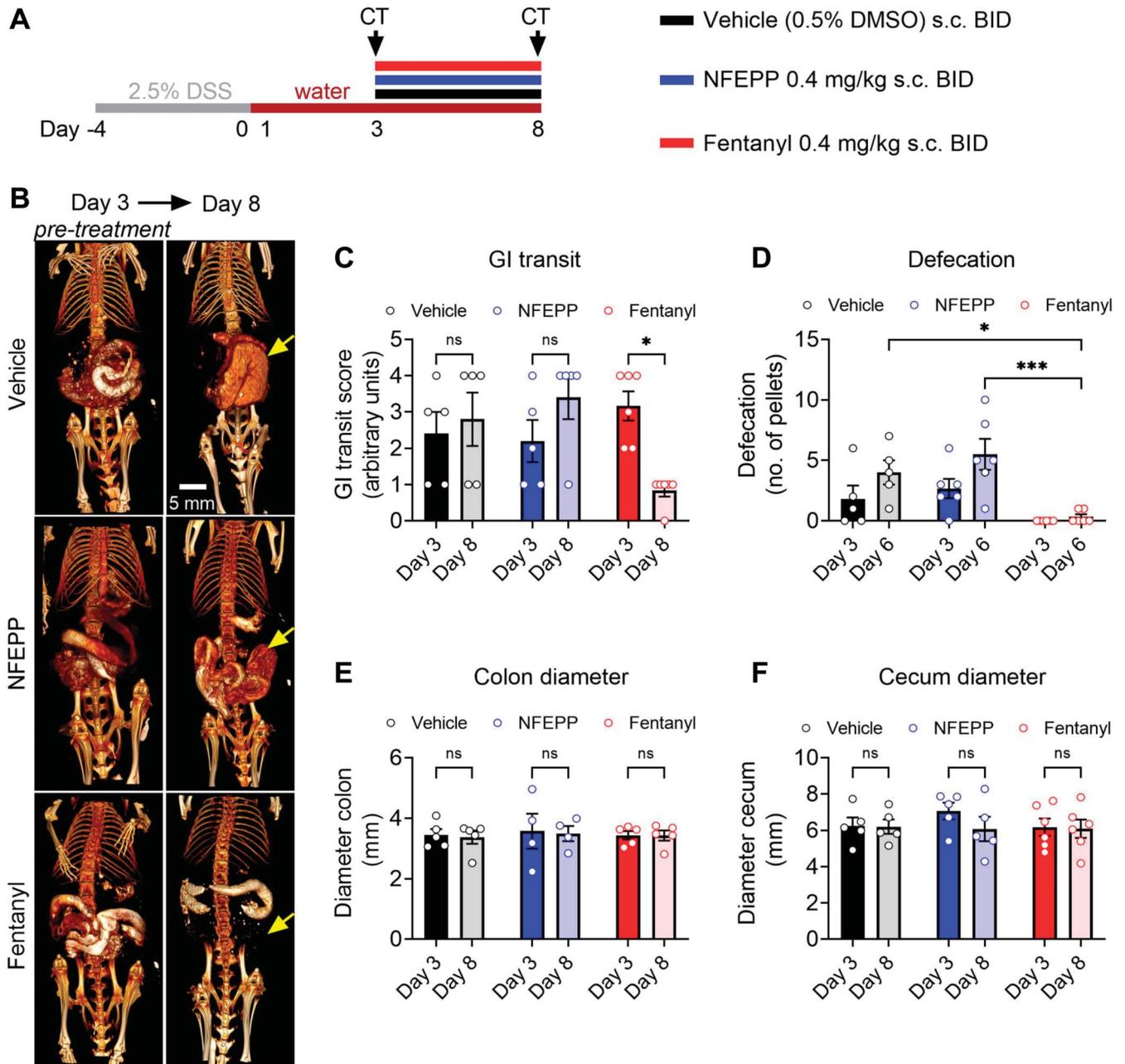


Figure 4. Effects of repeated NFEPP and fentanyl application on gastrointestinal (GI) transit and defaecation during acute DSS colitis. (A) Study design with injection regimen (color coded). (B) Oral contrast-enhanced CT scans before (day 3, drug naive) and after (day 8) repeated dosing of vehicle (0.5% DMSO s.c., N = 5), NFEPP (0.4 mg/kg s.c., N = 5), or fentanyl (0.4 mg/kg s.c., N = 6) b.i.d.. Arrows denote the cecum. (C) Effects of NFEPP and fentanyl on CT-based GI transit. (D) Effects of NFEPP and fentanyl administration (each opioid at 0.4 mg/kg s.c.) on defaecation. (E and F) Effects of NFEPP and fentanyl on colon diameter (E) and cecum diameter (F). * $P < 0.05$. *** $P < 0.001$. Two-way ANOVA, Bonferroni test. ANOVA, analysis of variance; b.i.d., bis in die (twice daily); CT, computed tomography; DMSO, dimethyl sulfoxide; DSS, dextran sulphate sodium; NFEPP, *N*-(3-fluoro-1-phenethylpiperidine-4-yl)-*N*-phenyl propionamide.

conventional MOR agonists.^{2,46} To test whether daily repeated applications of NFEPP cause these side effects in our IBD mouse model, we administered equivalent doses of NFEPP or fentanyl (0.4 mg/kg s.c.) or vehicle (0.5% DMSO s.c.) b.i.d. for 6 days and monitored blood oxygen saturation and heart rate (Fig. 5A, cohort 1). *N*-(3-fluoro-1-phenethylpiperidine-4-yl)-*N*-phenyl propionamide did neither affect blood oxygen saturation at day 4 and day 7 nor at day 8 after doubling the initial dose to 0.8 mg/kg, whereas fentanyl significantly reduced blood oxygen saturation at each of these time points compared with baseline ([15 minutes]: day 4: $85.0 \pm 1.5\%$

SpO₂, $P < 0.01$, N = 6; day 7: $87.5 \pm 1.4\%$ SpO₂, $P < 0.05$, N = 6; day 8: $80.0 \pm 2.9\%$ SpO₂, $P < 0.001$, N = 3, 2-way ANOVA, Bonferroni test; Figs. 5B–D). Both fentanyl and NFEPP reduced the heart rate at day 4, day 7, and at day 8 (Figs. 5E–G). However, compared with fentanyl, the NFEPP effect was smaller and more transient, with a partial or full recovery after 60 minutes compared with baseline (day 8: Δ BPM $+11.9 \pm 14.6$ from baseline, $P > 0.99$, N = 5, 2-way ANOVA, Bonferroni test; Fig. 5G). At 0.8 mg/kg s.c., the maximal decline in heart rate from baseline averaged 31.7% for fentanyl and 16.5% for NFEPP. Within all treatment groups, no

Downloaded from http://journals.lww.com/pain by BHDMM5EPHKav1ZEumr1tQIN4e+kLHEZgbsHh04XIM0hCwWCX1AW nYQp/llqH-D3i3D00D0Ry7T/SF14C3V4/OAVpDda8KKGKv0Ymy+78= on 10/14/2023

differences of heart rate or blood oxygen saturation were observed comparing day 4 with day 7 experiments. Thus, after repeated administration, NFEPP has no effect on blood oxygenation and thus respiration and minimal effect on heart rate compared with fentanyl, which markedly reduces both parameters.

3.6. High doses of *N*-(3-fluoro-1-phenethylpiperidine-4-yl)-*N*-phenyl propionamide do not affect blood oxygen saturation, somatic thermal nociception, and locomotion in mice with and without colitis

On-target effects in the central nervous system (eg, sedation) are frequent limitations of conventional MOR agonists.^{7,14} To test whether high doses of NFEPP have central on-target side effects, we administered NFEPP in a concentration of 0.8 mg/kg s.c., a dose approximately 25-fold higher than the ED₅₀ of fentanyl for thermal nociception.²⁸ Experiments were first performed in healthy mice followed by application of the 2.5% DSS protocol in the same set of mice and repetition of central side effects experiments (Fig. 6A, cohort 3). In healthy mice,

NFEPP did not affect somatic thermal nociception, assessed by a tail immersion assay ($P = 0.11$, $N = 8$, 2-way ANOVA), and had no effect on locomotor behavior, measured by total distance travelled ($P = 0.19$, $N = 7$, 1-way ANOVA), mean speed ($P = 0.19$, $N = 7$, 1-way ANOVA), and resting time ($P = 0.06$, $N = 7$, 1-way ANOVA) in a locomotion open-field test (Figs. 6B–F). After induction of DSS colitis, tail immersion assay and open-field experiments were repeated at peak inflammation (days 4–5). As found in healthy mice, NFEPP (0.8 mg/kg) had no effect on thermal nociception or on locomotor behavior in DSS colitis mice (Figs. 6B–F). This higher concentration of NFEPP also had no effect on blood oxygen saturation in healthy and DSS colitis mice ($N = 6$; Fig. 6G). However, as found with repeated dosing of NFEPP at a lower dose (Fig. 5), a single dose of a higher concentration of NFEPP (0.8 mg/kg s.c.) caused a small reduction in heart rate that recovered after 60 minutes in both mice with and without colitis (DSS NFEPP [60 minutes]: Δ BPM $+8.9 \pm 21.3$ from baseline, $P > 0.99$, $N = 6$, compared with baseline, 2-way ANOVA, Bonferroni test; Fig. 6H).

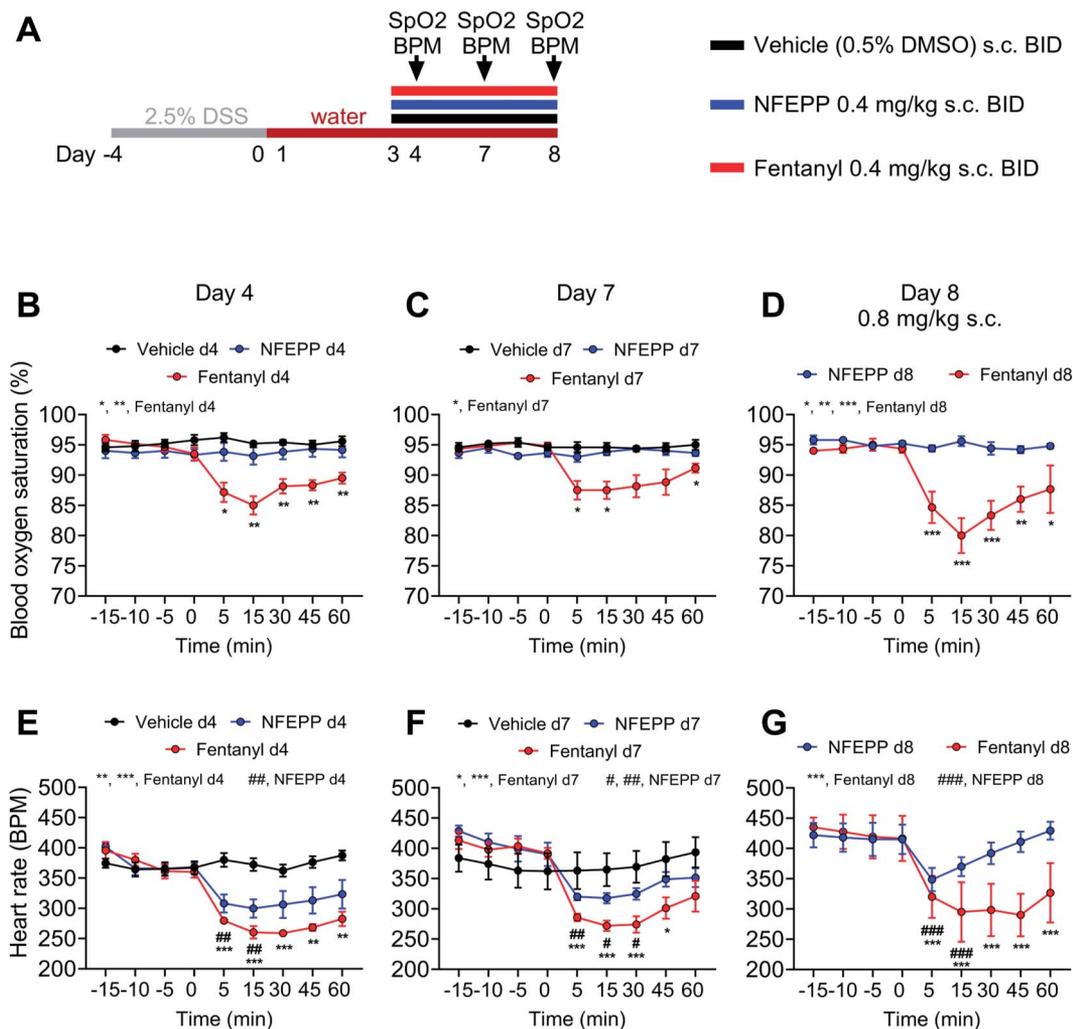


Figure 5. On-target side effects of repeated NFEPP and fentanyl application during acute DSS colitis at different time points. (A) Study design with injection regimen (color coded). Pulse oximeter measurements were performed at day 4, day 7, and day 8 in vehicle-treated (0.5% DMSO s.c. b.i.d., $N = 5$), NFEPP-treated (0.4 mg/kg s.c. b.i.d., $N = 6$), and fentanyl-treated (0.4 mg/kg s.c. b.i.d., $N = 6$) DSS colitis mice. (B–D) Effects of NFEPP and fentanyl on blood oxygen saturation (B–D) and heart rate (E–G) at different time points and concentrations. *, $\#P < 0.05$. **, $\#\#P < 0.01$. ***, $\#\#\#P < 0.001$. Two-way ANOVA, Bonferroni test. (D and G) Fentanyl: $N = 3$; NFEPP: $N = 5$. ANOVA, analysis of variance; b.i.d., bis in die (twice daily); BPM, beats per minute; DMSO, dimethyl sulfoxide; DSS, dextran sulphate sodium; NFEPP, *N*-(3-fluoro-1-phenethylpiperidine-4-yl)-*N*-phenyl propionamide; SpO₂, blood oxygen saturation.

3.7. N-(3-fluoro-1-phenethylpiperidine-4-yl)-N-phenyl propionamide inhibits colonic nociception in isolated human colon preparations in acidic conditions

To test whether NFEPP also inhibits nociception in human tissues, afferent nerve recordings from mesenteric nerves were made in isolated preparations of the left colon (descending and sigmoid colon) from female and male patients. Colonic nociceptors were identified based on their responsiveness to VFF probing (10 g, serosal or mesenteric probing). Control responses to VFF probing were measured after tissue equilibration (10 minutes) at pH 6.5. At pH 6.5, NFEPP application (300 nM) to the tissue bath (10 minutes wash-in) caused a 49% reduction in afferent nerve activity in response to VFF probing compared with pH 6.5 control responses ($P < 0.05$, $N = 3$, Friedman test, Dunn test; **Figs. 7A–C**). A subsequent 20-minute

washout period at pH 7.4 did not provide complete washout of the observed effect. These results show that NFEPP is able to inhibit the activity of human colonic nociceptors in the setting of extracellular acidification that replicates inflammation. These findings, that mirror our previous findings in the mouse colon,²⁰ support the concept of using NFEPP to treat pain associated with IBD, with fewer side effects than conventional opioids such as fentanyl.

4. Discussion

Opioids are the most effective analgesics for the management of acute pain and are often required for pain management of patients with IBD, especially those requiring hospital admission.²⁹ Patients typically require repeated dosing of analgesics

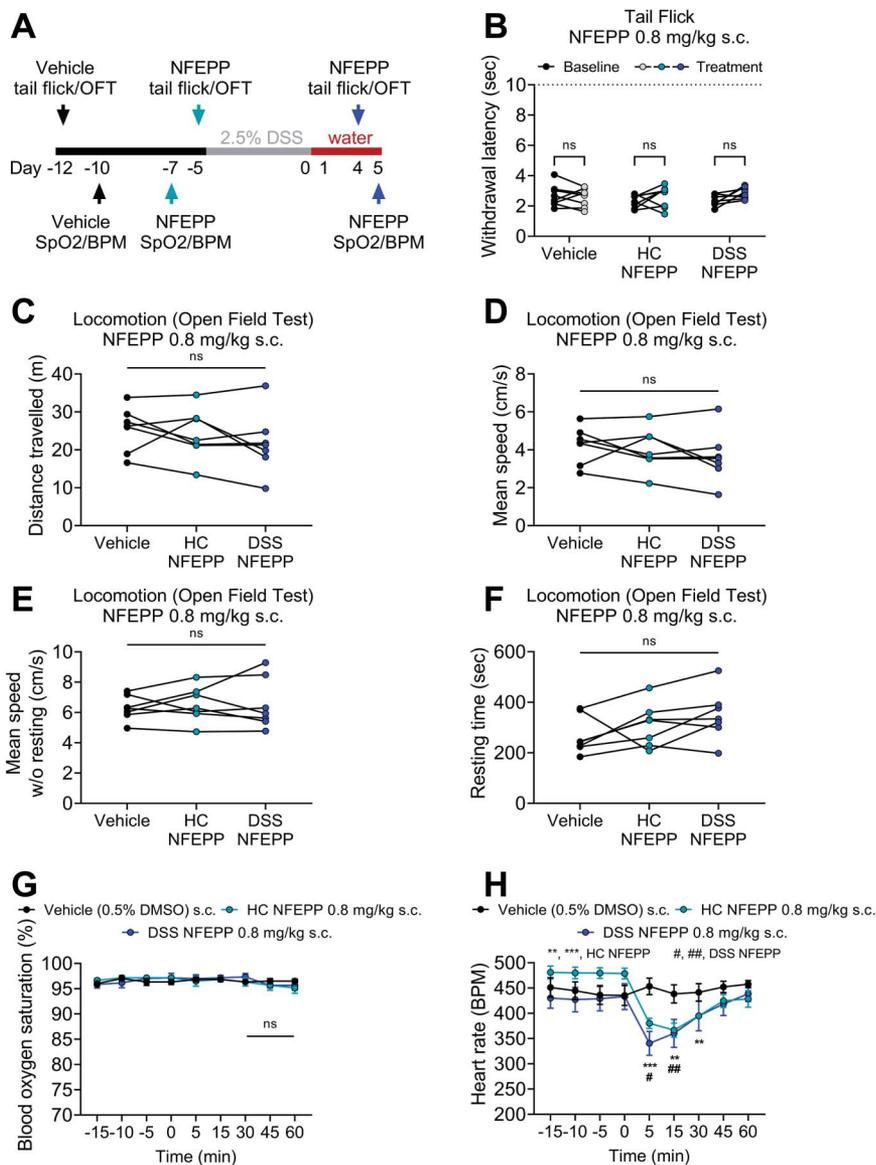


Figure 6. On-target side effects of increased NFEPP doses in healthy control and acute DSS colitis mice. (A) Study design. Tail immersion assay ($N = 8$), locomotor activity ($N = 7$), and pulse oximeter measurements ($N = 6$) were performed in the same mice at different time points. (B–H) Effects of increased doses of NFEPP (0.8 mg/kg s.c.) on withdrawal responses to noxious heat in a tail immersion assay (B), locomotor activity (C–F), blood oxygen saturation (G), and heart rate (H) in healthy control (HC) and DSS colitis mice. # $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$. (B, G, and H) Two-way ANOVA, Bonferroni test. (C–F) One-way ANOVA. ANOVA, analysis of variance; BPM, beats per minute; DMSO, dimethyl sulfoxide; DSS, dextran sulphate sodium; NFEPP, N-(3-fluoro-1-phenethylpiperidine-4-yl)-N-phenyl propionamide; OFT, open field test; SpO₂, blood oxygen saturation.

Downloaded from http://journals.lww.com/pain by BHDMD5ePHKav1ZEoum1QIN4a+kLlHEZ9b5Ih04XIM0hCwWCX1AW nYOp/llQH3D000dRy/TVSFI4C3VCA4OAVpDDa8KKGAKV0Ymy+78= on 10/14/2023

to manage their visceral pain, and they often experience unpleasant on-target side effects and occasionally life-threatening events.^{2,7,44} In the current study, we have shown that repeated application of the pH-sensitive opioid NFEPP inhibits visceral nociceptive responses during the evolution of acute colitis in a preclinical model of ulcerative colitis. This antinociceptive action of NFEPP was targeted to sites of inflammation and acidification within the colon and was sustained during the period of inflammation. The common on-target side effects of opioids, such as constipation, delayed GIT, respiratory depression, and adverse central effects, were not observed, even with increased dosing. We strengthened the translational relevance of these findings by demonstrating that NFEPP inhibits human colonic nociceptors in acidified conditions, mimicking the acidic intramural and intraluminal microenvironments found in many human inflammatory disorders such as ulcerative colitis, Crohn disease, ischemic colitis, or colorectal cancer.^{27,30,37}

N-(3-fluoro-1-phenethylpiperidine-4-yl)-*N*-phenyl propionamide was designed by modifying fentanyl (hydrogen replacement with fluorine on its piperidine moiety) to decrease its

pKa and thereby enable MOR activation preferentially in acidified tissues.^{20,34,40} We have extensively investigated the activity of fentanyl and NFEPP at different types of opioid receptors in injured/inflamed vs noninjured/noninflamed tissues by using μ -, δ -, and κ -selective antagonists in rats⁴⁰ and in mice.⁴ NFEPP was selective for MORs. Fentanyl was active in both normal and inflamed tissues, whereas NFEPP was not active in noninflamed tissues.^{4,40} Based on the chemical structures and on our previous in vivo data, there is no evidence for significant differences in drug distribution between fentanyl, NFEPP, and related compounds.^{4,10,40,41} Both ligands are lipophilic and will rapidly penetrate the blood–brain (and other) barriers. We have also thoroughly investigated related compounds with varying pKa values (6.82–8.44) in vitro and in vivo in 2 further articles.^{10,41} Finally, our previous studies have shown that the analgesic actions of NFEPP are blocked by the peripherally restricted opioid receptor antagonist naloxone methiodide, demonstrating that these analgesic effects are mediated by activation of peripheral MORs.^{34,40} Together, these data indicated that ligands with pKa values close to the pH of inflamed/injured tissues

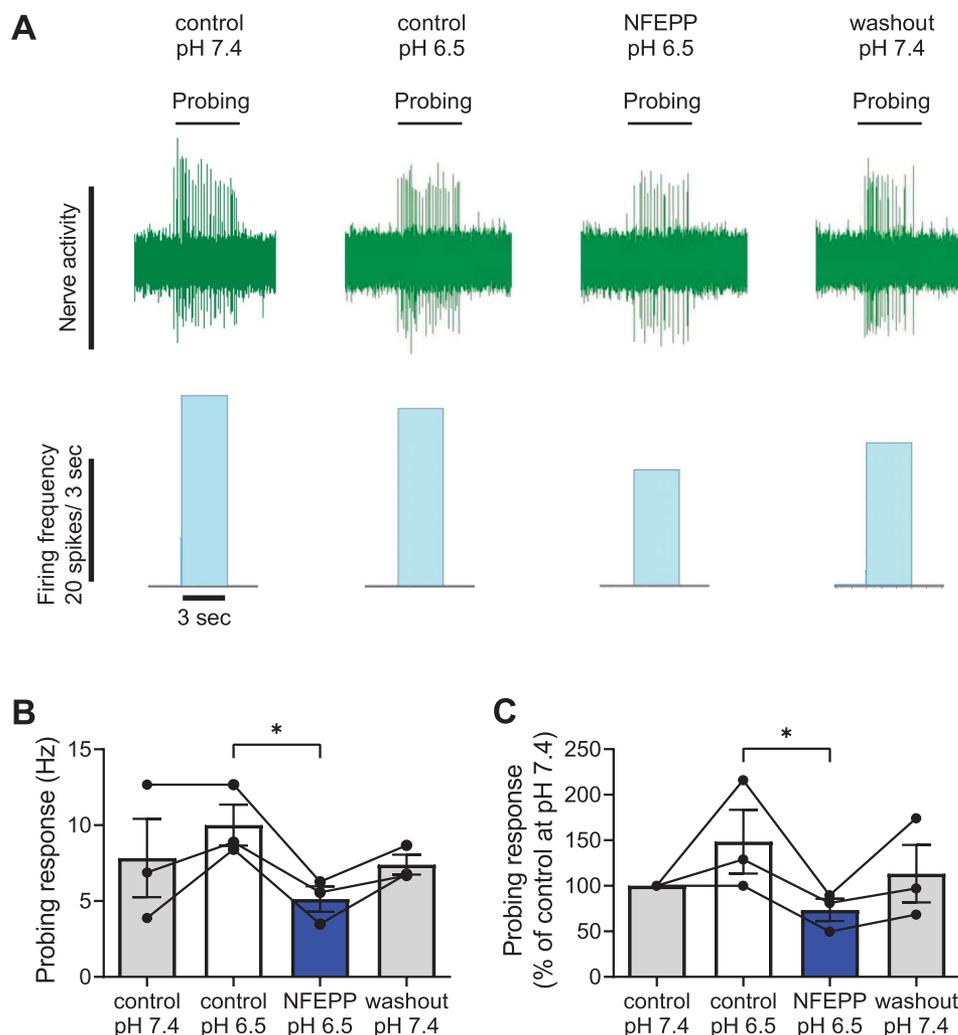


Figure 7. Effects of NFEPP on human colonic nociceptors. (A) Representative traces and histograms of colonic afferent nerve firing in response to von Frey filament probing (10 g) at pH 7.4 (control), pH 6.5 (control), in the presence of NFEPP (300 nM) at pH 6.5 and after washout at pH 7.4. (B and C) Summary bar charts of afferent nerve probing responses (N = 3) presented as Hz (B) and as % of control at pH 7.4 (C). **P* < 0.05, Friedman test, Dunn test. Hz, hertz; NFEPP, *N*-(3-fluoro-1-phenethylpiperidine-4-yl)-*N*-phenyl propionamide.

selectively activate peripheral opioid receptors, and that progressively decreasing pKa values correlate with diminishing central adverse effects.

Tissue pH will vary with the degree of inflammation, and thus, the magnitude of analgesia for a given dose of a pH-sensitive compound would be anticipated to reflect the severity of inflammation. Indeed, we found that NFEPP's antinociceptive effects were greatest at the peak of colonic inflammation, based on DAI, MPO activity, histological scoring, and measurements of colonic pH. Together, these findings provide further support for the selective mechanism of action of NFEPP and its clinical utility. Peripheral nociceptive signaling and resulting pain would be greatest at peak inflammation in the clinical course, and these results indicate that NFEPP's analgesic activity for a given dose would be maximal at this time point. Although the analgesic actions of NFEPP and fentanyl seem similar at peak inflammation, it is possible that the overall magnitude of analgesia compared with fentanyl, which would also inhibit pain central pathways including the dorsal horn, may be less.

The distribution of inflammation in the tissue layers of the colon resulting from acute DSS colitis also offers insights into the mechanism of action of NFEPP. The inflammation in this self-limiting colitis model is predominantly found in the mucosa and submucosa, as shown by our histological analysis and previous studies.⁹ It is generally considered a model of ulcerative colitis,³¹ as opposed to the transmural inflammation that characterizes Crohn disease.³³ We localized sites of colonic acidification by using specialized peptides that insert into cell membranes at a low pH, a strategy that was recently validated in cancer models¹ where acidic pH is among the lowest observed in tissues.³⁵ Using Cy-labelled pHLIP, we found its highest accumulation in the colonic mucosal and submucosal layers, whereas much less pHLIP was detected in the adjacent muscle layer. These findings are consistent with our histological scores, suggesting that the greatest inflammation occurred within these regions. The nerve terminals of visceral nociceptors travel into the colon along the arterioles that terminate within the submucosa. Acidosis within the submucosal microenvironment that activates NFEPP with ensuing inhibition of these nerves is highly consistent with the proposed mechanism of action. The lack of acidification of the musculature explains the inability of NFEPP to inhibit GIT and defaecation.

Our study examined male mice because of previous reports demonstrating that female mice show a reduced inflammatory response to DSS consumption compared with male mice.^{5,15} Furthermore, our previous studies have shown that opioid responses in visceral DRG neurons did not differ between female and male mice.^{19,50} Such findings are consistent with the current evidence on sex differences in opioid analgesia, suggesting sex-specific therapeutic interventions are not warranted.^{11,12}

The cumulative actions of opioids and their metabolites with repeated dosing could amplify potential on-target side effects or could cause tolerance. Fentanyl caused an inhibition of transit and defaecation, whereas NFEPP had neither an effect on defaecation nor on GIT, even after repeated dosing. These results are consistent with the finding that inflammation and acidification were largely confined to the mucosa and submucosa. Thus, NFEPP activated MORs at these sites as opposed to the muscle layer. Future studies of models of transmural inflammation, which more closely approximates the distribution of inflammation in Crohn disease, are required to further elucidate NFEPP activity within the gut wall.

We did not detect common on-target side effects in organs outside the gastrointestinal tract after repeated dosing of NFEPP, in contrast to fentanyl. Chronic treatment with NFEPP did not affect blood oxygen saturation at any point during acute colitis. We also showed that acute application of increased doses of NFEPP (multiples of the fentanyl ED₅₀ for thermal nociception) had no effect. In contrast, fentanyl caused significant hypoxemia and a reduction of heart rate even at lower doses. Our previous study with a single lower dose of NFEPP (0.2 mg/kg s.c.) revealed no effect on central regulation of locomotion or peripherally on somatic sensory testing, whereas fentanyl at the same dose had profound effects.²⁰ Herein, we also showed that even with doses 4 times higher than those initially studied, NFEPP had no effect on locomotion and somatic nociception compared with vehicle. We did observe a distinctive inhibition of heart rate with fentanyl and a smaller, transient effect with NFEPP when used at higher doses. Although opioid receptor expression is present in the heart,^{17,45} the explanation for why NFEPP has a transient effect is unclear, but potentially could reflect a small and local acidic microenvironment. Our studies with pHLIP detected a small signal in the heart, whereas nothing was found in the brain or small intestine, but these signals were found along blood vessels and their significance is unclear.

In summary, repeated dosing of NFEPP provides sustained antinociception during the evolution of an acute colonic inflammation, and this cumulative dosing does not lead to typical on-target side effects. Because of its pKa that favors acidic environments, the activity of NFEPP is maximal at peak inflammation when nociceptive stimulation from tissue mediators is greatest and its actions are highly restricted to sites of acidification within the inflamed colon wall. Thus, this novel opioid shows considerable promise to provide targeted, effective, and safe analgesia by actions in peripheral nociceptive nerves throughout the course of an acute inflammatory disorder such as that occurs in IBD and other chronic remitting inflammatory diseases.

Conflict of interest statement

The authors have no conflicts of interest to declare. The Charité Universitätsmedizin Berlin has filed US patent 14/239,461 on pH-dependent opioid ligands.

Acknowledgments

S. J. Vanner, D. E. Reed, and A. E. Lomax were funded by an operating grant from Crohn's and Colitis Canada. N. W. Bunnett was funded by grants from the National Institutes of Health (NS102722, DE026806, DK118971, and DE029951) and Department of Defense (W81XWH1810431, W81XWH-22-1-0239, Expansion Award). C. Stein was funded by grants from Deutsche Forschungsgemeinschaft (STE 477/19, EXC 2046 AA1-15, FOR 5177, STE 477/21) and Bundesministerium für Bildung und Forschung (01GQ2109A).

Patient consent for publication: Not required.

Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Supplemental digital content

Supplemental digital content associated with this article can be found online at <http://links.lww.com/PAIN/B857>.

Article history:

Received 25 July 2022

Received in revised form 7 March 2023

Accepted 25 April 2023

Available online 15 June 2023

References

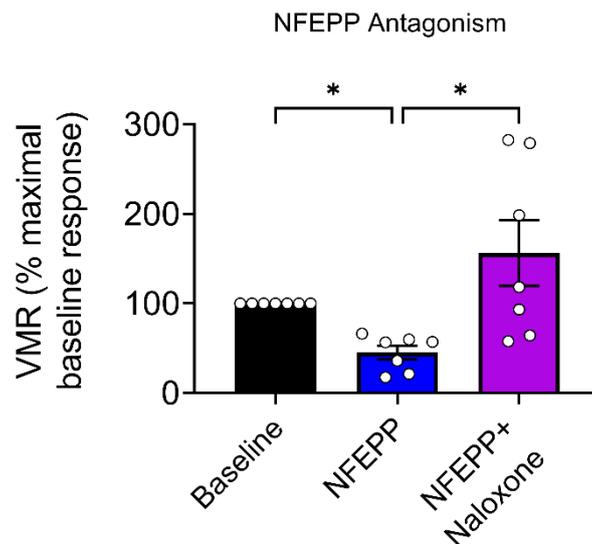
- [1] Adochite RC, Moshnikova A, Carlin SD, Guerrieri RA, Andreev OA, Lewis JS, Reshetnyak YK. Targeting breast tumors with pH (Low) insertion peptides. *Mol Pharmaceutics* 2014;11:2896–905.
- [2] Algera MH, Olofsen E, Moss L, Dobbins RL, Niesters M, van Velzen M, Groeneveld GJ, Heuberger J, Laffont CM, Dahan A. Tolerance to opioid-induced respiratory depression in chronic high-dose opioid users: a model-based comparison with opioid-naïve individuals. *Clin Pharmacol Ther* 2021;109:637–45.
- [3] Andreev OA, Dupuy AD, Segala M, Sandugu S, Serra DA, Chichester CO, Engelman DM, Reshetnyak YK. Mechanism and uses of a membrane peptide that targets tumors and other acidic tissues in vivo. *Proc Natl Acad Sci U S A* 2007;104:7893–8.
- [4] Baamonde A, Menéndez L, González-Rodríguez S, Lastra A, Seitz V, Stein C, Machelska H. A low pKa ligand inhibits cancer-associated pain in mice by activating peripheral mu-opioid receptors. *Sci Rep* 2020;10:18599.
- [5] Bábíčková J, Tóthová Ľ, Lengyelová E, Bartoňová A, Hodosy J, Gardlík R, Celec P. Sex differences in experimentally induced colitis in mice: a role for estrogens. *Inflammation* 2015;38:1996–2006.
- [6] Bagnol D, Mansour A, Akil H, Watson SJ. Cellular localization and distribution of the cloned mu and kappa opioid receptors in rat gastrointestinal tract. *Neuroscience* 1997;81:579–91.
- [7] Bateman JT, Saunders SE, Levitt ES. Understanding and countering opioid-induced respiratory depression. *Br J Pharmacol* 2021;180:813–28.
- [8] Chassaing B, Aitken JD, Malleshappa M, Vijay-Kumar M. Dextran sulfate sodium (DSS)-induced colitis in mice. *Curr Protoc Immunol* 2014;104:15.25.1–14.
- [9] Corder G, Castro DC, Bruchas MR, Scherrer G. Endogenous and exogenous opioids in pain. *Annu Rev Neurosci* 2018;41:453–73.
- [10] Del Vecchio G, Labuz D, Temp J, Seitz V, Klöner M, Negrete R, Rodríguez-Gaztelumendi A, Weber M, Machelska H, Stein C. pKa of opioid ligands as a discriminating factor for side effects. *Sci Rep* 2019;9:19344.
- [11] Fillingim RB, King CD, Ribeiro-Dasilva MC, Rahim-Williams B, Riley JL. Sex, gender, and pain: a review of recent clinical and experimental findings. *J Pain* 2009;10:447–85.
- [12] Fullerton EF, Doyle HH, Murphy AZ. Impact of sex on pain and opioid analgesia: a review. *Curr Opin Behav Sci* 2018;23:183–90.
- [13] Galligan JJ, Sternini C. Insights into the role of opioid receptors in the GI tract: experimental evidence and therapeutic relevance. *Handb Exp Pharmacol* 2017;239:363–78.
- [14] Gaudreau JD, Gagnon P, Harel F, Roy MA, Tremblay A. Psychoactive medications and risk of delirium in hospitalized cancer patients. *J Clin Oncol* 2005;23:6712–8.
- [15] Goodman WA, Havran HL, Quereshy HA, Kuang S, De Salvo C, Pizarro TT. Estrogen receptor α loss-of-function protects female mice from DSS-induced experimental colitis. *Cell Mol Gastroenterol Hepatol* 2018;5:630–33.e1.
- [16] Guerrero-Alba R, Valdez-Morales EE, Jiménez-Vargas NN, Bron R, Poole D, Reed D, Castro J, Campaniello M, Hughes PA, Brierty SM, Bunnett N, Lomax AE, Vanner S. Co-expression of μ and δ opioid receptors by mouse colonic nociceptors. *Br J Pharmacol* 2018;175:2622–34.
- [17] He SF, Jin SY, Yang W, Pan YL, Huang J, Zhang SJ, Zhang L, Zhang Y. Cardiac μ -opioid receptor contributes to opioid-induced cardioprotection in chronic heart failure. *Br J Anaesth* 2018;121:26–37.
- [18] Huynh E, Penney J, Caswell J, Li J. Protective effects of protegrin in dextran sodium sulfate-induced murine colitis. *Front Pharmacol* 2019;10:156.
- [19] Jaramillo-Polanco J, Lopez-Lopez C, Yu Y, Neary E, Hegron A, Canals M, Bunnett NW, Reed DE, Lomax AE, Vanner SJ. Opioid-induced pronociceptive signaling in the gastrointestinal tract is mediated by delta-opioid receptor signaling. *J Neurosci* 2022;42:3316–28.
- [20] Jiménez-Vargas NN, Yu Y, Jensen DD, Bok DD, Wisdom M, Latorre R, Lopez C, Jaramillo-Polanco JO, Degro C, Guzman-Rodríguez M, Tsang Q, Snow Z, Schmidt BL, Reed DE, Lomax AE, Margolis KG, Stein C, Bunnett NW, Vanner SJ. Agonist that activates the μ -opioid receptor in

- acidified microenvironments inhibits colitis pain without side effects. *Gut* 2021;71:695–704.
- [21] Kalvass JC, Olson ER, Cassidy MP, Selley DE, Pollack GM. Pharmacokinetics and pharmacodynamics of seven opioids in P-glycoprotein-competent mice: assessment of unbound brain $EC_{50,u}$ and correlation of in vitro, preclinical, and clinical data. *J Pharmacol Exp Ther* 2007;323:346–55.
 - [22] Katagiri N, Sakai R, Izutsu T, Kawana H, Sugino S, Kido K. Postoperative pain management in patients with ulcerative colitis. *Anesth Prog* 2020;67:158–63.
 - [23] Khanna AK, Bergese SD, Jungquist CR, Morimatsu H, Uezono S, Lee S, Ti LK, Urman RD, McIntyre R, Tornero C, Dahan A, Saager L, Weingarten TN, Wittmann M, Auckley D, Brazzi L, Le Guen M, Soto R, Schramm F, Ayad S, Kaw R, Di Stefano P, Sessler DI, Uribe A, Moll V, Dempsey SJ, Buhre W, Overdyk FJ, Tanios M, Rivas E, Mejia M, Elliott K, Ali A, Fiorda-Diaz J, Carrasco-Moyano R, Mavarez-Martinez A, Gonzalez-Zacarias A, Roeth C, Kim J, Esparza-Gutierrez A, Weiss C, Chen C, Taniguchi A, Mihara Y, Ariyoshi M, Kondo I, Yamakawa K, Suga Y, Ikeda K, Takano K, Kuwabara Y, Carignan N, Rankin J, Egan K, Waters L, Sim MA, Liew LL, Liew QEL, Siu-Chun Law L, Gosnell J, Shrestha S, Okponyia C, Al-Musawi MH, Gonzalez MJP, Neumann C, Guttenthaler V, Männer O, Delis A, Winkler A, Marchand B, Schmal F, Aleskerov F, Nagori M, Shafi M, McPhee G, Newman C, Lopez E, Har SM, Asbahi M, Nordstrom McCaw K, Theunissen M, Smit-Fun V. Prediction of opioid-induced respiratory depression on inpatient wards using continuous capnography and oximetry: an international prospective, observational trial. *Anesth Analg* 2020;131:1012–24.
 - [24] Kim JJ, Shajib MS, Manocha MM, Khan WI. Investigating intestinal inflammation in DSS-induced model of IBD. *J Visual Exp* 2012;:3678.
 - [25] Lama A, Provensi G, Amoriello R, Pirozzi C, Rani B, Mollica MP, Raso GM, Ballerini C, Meli R, Passani MB. The anti-inflammatory and immunomodulatory effects of OEA limit DSS-induced colitis in mice. *Biomed Pharmacother* 2020;129:110368.
 - [26] López-Estévez S, Gris G, de la Puente B, Carceller A, Martínez V. Intestinal inflammation-associated hypersensitivity is attenuated in a DSS model of colitis in Sigma-1 knockout C57BL/6 mice. *Biomed Pharmacother* 2021;143:112126.
 - [27] Meyer KA, Kammerling EM, Amtman L, Koller M, Hoffman SJ. pH studies of malignant tissues in human beings. *Cancer Res* 1948;8:513–8.
 - [28] Minami K, Hasegawa M, Ito H, Nakamura A, Tomii T, Matsumoto M, Orita S, Matsushima S, Miyoshi T, Masuno K, Torii M, Koike K, Shimada S, Kanemasa T, Kihara T, Narita M, Suzuki T, Kato A. Morphine, oxycodone, and fentanyl exhibit different analgesic profiles in mouse pain models. *J Pharmacol Sci* 2009;111:60–72.
 - [29] Niccum B, Moninuola O, Miller K, Khalili H. Opioid use among patients with inflammatory bowel disease: a systematic review and meta-analysis. *Clin Gastroenterol Hepatol* 2021;19:895–907.e4.
 - [30] Nugent SG, Kumar D, Rampton DS, Evans DF. Intestinal luminal pH in inflammatory bowel disease: possible determinants and implications for therapy with aminosaliclates and other drugs. *Gut* 2001;48:571–7.
 - [31] Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R. A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. *Gastroenterology* 1990;98:694–702.
 - [32] Park YH, Kim N, Shim YK, Choi YJ, Nam RH, Choi YJ, Ham MH, Suh JH, Lee SM, Lee CM, Yoon H, Lee HS, Lee DH. Adequate dextran sodium sulfate-induced colitis model in mice and effective outcome measurement method. *J Cancer Prev* 2015;20:260–7.
 - [33] Podolsky DK. Inflammatory bowel disease. *N Engl J Med* 2002;347:417–29.
 - [34] Rodríguez-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.
 - [35] Rohani N, Hao L, Alexis MS, Joughin BA, Krismer K, Moufarrej MN, Soltis AR, Lauffenburger DA, Yaffe MB, Burge CB, Bhatia SN, Gertler FB. Acidification of tumor at stromal boundaries drives transcriptome alterations associated with aggressive phenotypes. *Cancer Res* 2019;79:1952–66.
 - [36] Rosset A, Spadola L, Ratib O. OsiriX: an open-source software for navigating in multidimensional DICOM images. *J Digit Imaging* 2004;17:205–16.
 - [37] Schiedler MG. Sigmoid intramural pH for prediction of ischemic colitis during aortic surgery. *Arch Surg* 1987;122:881.
 - [38] Schirbel A, Reichert A, Roll S, Baumgart DC, Büning C, Wittig B, Wiedenmann B, Dignass A, Sturm A. Impact of pain on health-related quality of life in patients with inflammatory bowel disease. *World J Gastroenterol* 2010;16:3168–77.
 - [39] Sosunov EA, Anyukhovskiy EP, Sosunov AA, Moshnikova A, Wijesinghe D, Engelman DM, Reshetnyak YK, Andreev OA. pH (low) insertion peptide (pHLIP) targets ischemic myocardium. *Proc Natl Acad Sci U S A* 2013;110:82–6.

- [40] 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.
- [41] Spahn V, Del Vecchio G, Rodriguez-Gaztelumendi A, Temp J, Labuz D, Kloner M, Reidelbach M, Machelska H, Weber M, Stein C. Opioid receptor signaling, analgesic and side effects induced by a computationally designed pH-dependent agonist. *Sci Rep* 2018;8:8965.
- [42] Stein C. New concepts in opioid analgesia. *Expert Opin Investig Drugs* 2018;27:765–75.
- [43] Sternini C, Patierno S, Selmer IS, Kirchgessner A. The opioid system in the gastrointestinal tract. *Neurogastroenterol Motil* 2004;16:3–16.
- [44] Swegle JM, Logemann C. Management of common opioid-induced adverse effects. *Am Fam Physician* 2006;74:1347–54.
- [45] Theisen MM, Schlottmann S, August C, Herzog C, Theilmeier G, Maas M, Blumenstiel JM, Weber TP, Van Aken HK, Kaerlein KT. Detection and distribution of opioid peptide receptors in porcine myocardial tissue. *Pharmacol Res* 2014;84:45–9.
- [46] Urman RD, Khanna AK, Bergese SD, Buhre W, Wittmann M, Le Guen M, Overdyk FJ, Di Piazza F, Saager L. Postoperative opioid administration characteristics associated with opioid-induced respiratory depression: results from the PRODIGY trial. *J Clin Anesth* 2021;70:110167.
- [47] Wallace JL, Keenan CM. An orally active inhibitor of leukotriene synthesis accelerates healing in a rat model of colitis. *Am J Physiol Gastrointest Liver Physiol* 1990;258:G527–34.
- [48] Yao L, Daniels J, Moshnikova A, Kuznetsov S, Ahmed A, Engelman DM, Reshetnyak YK, Andreev OA. pHLIP peptide targets nanogold particles to tumors. *Proc Natl Acad Sci U S A* 2013;110:465–70.
- [49] Yu Y, Daly DM, Adam IJ, Kitsanta P, Hill CJ, Wild J, Shorthouse A, Grundy D, Jiang W. Interplay between mast cells, enterochromaffin cells, and sensory signaling in the aging human bowel. *Neurogastroenterol Motil* 2016;28:1465–79.
- [50] Yu Y, Tsang QK, Jaramillo-Polanco J, Lomax AE, Vanner SJ, Reed DE. Cannabinoid 1 and mu-opioid receptor agonists synergistically inhibit abdominal pain and lack side effects in mice. *J Neurosci* 2022;42:6313–24.
- [51] Zhai Z, Zhang F, Cao R, Ni X, Xin Z, Deng J, Wu G, Ren W, Yin Y, Deng B. Cecropin A alleviates inflammation through modulating the gut microbiota of C57BL/6 mice with DSS-induced IBD. *Front Microbiol* 2019;10:1595.

Supplementary digital content: Degro et al., Evolving acidic microenvironments during acute colitis provide selective analgesic targets for a pH-sensitive opioid

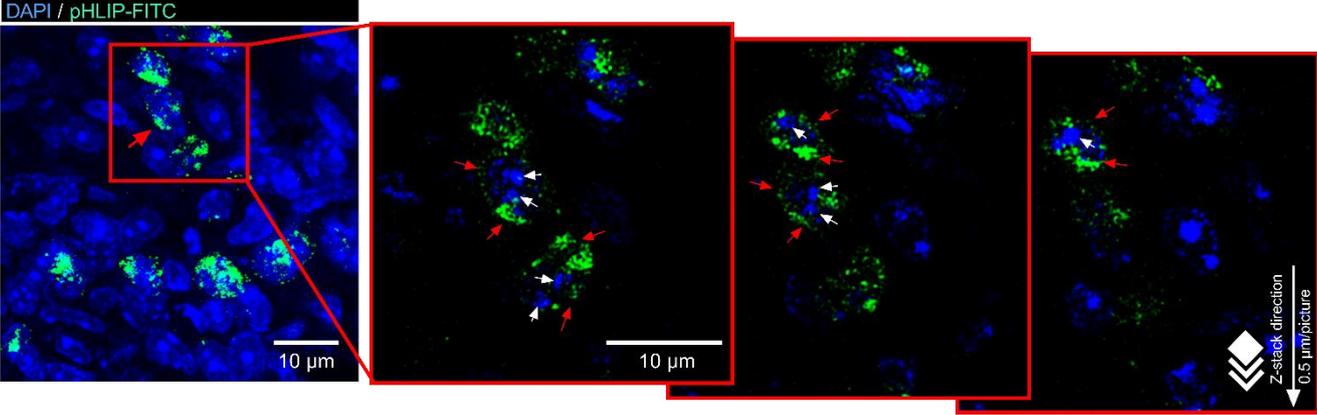
Supplementary digital content Figure 1:



Supplementary digital content Fig. 1: Reversal of NFEPP induced inhibition of VMRs (80 μ l colorectal distension) by the opioid antagonist naloxone in DSS colitis mice. Mice received NFEPP 0.4 mg/kg s.c. and after 4 hours a re-administration of NFEPP (0.4 mg/kg s.c.) together with the opioid antagonist naloxone hydrochloride (1 mg/kg s.c., 15 min prior to VMR recording). *Abbreviations: DSS, dextran sulphate sodium. VMR, visceromotor response.* * $p < 0.05$, $N = 7$, Friedman test, Dunn's test.

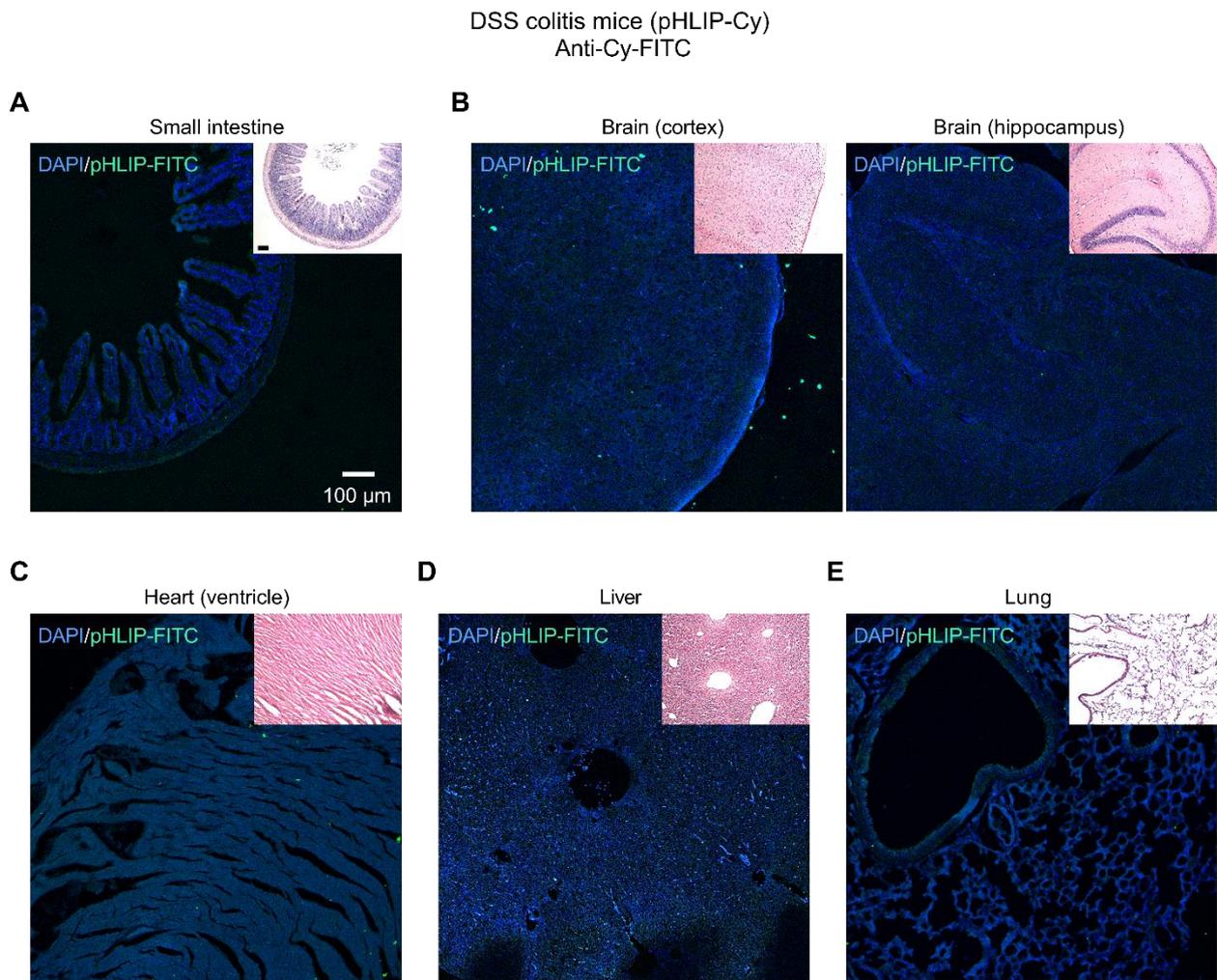
Supplementary digital content Figure 2:

DSS colitis mice (pHLIP-Cy)
Anti-Cy-FITC



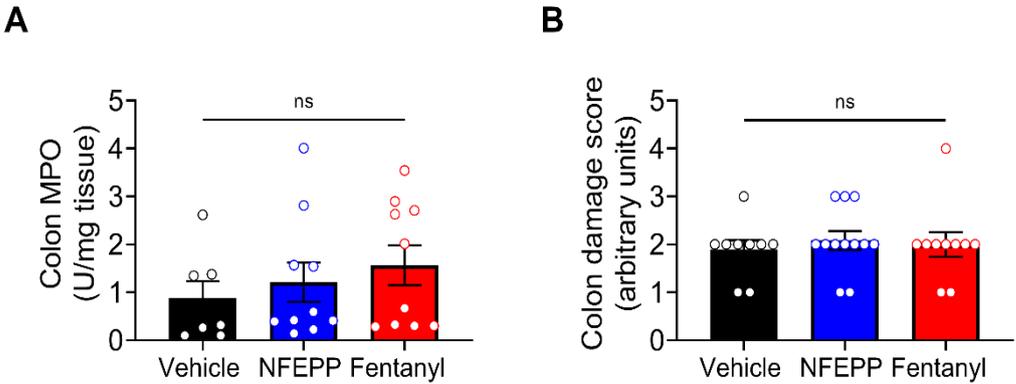
Supplementary digital content Fig. 2: Subcellular localization of pHLIP. Z-axis sections (0.5 µm) of the inflamed colon from a DSS colitis mouse, obtained by confocal microscopy. White arrows denote the nucleus labelled with DAPI. Red arrows indicate the pHLIP-FITC signal in the plasma membrane surrounding the nucleus. *Abbreviations: Cy, cyanin. DSS, dextran sulphate sodium. DAPI, 4',6-diamidino-2-phenylindole. FITC, fluorescein isothiocyanate. pHLIP, pH low insertion peptide.*

Supplementary digital content Figure 3:



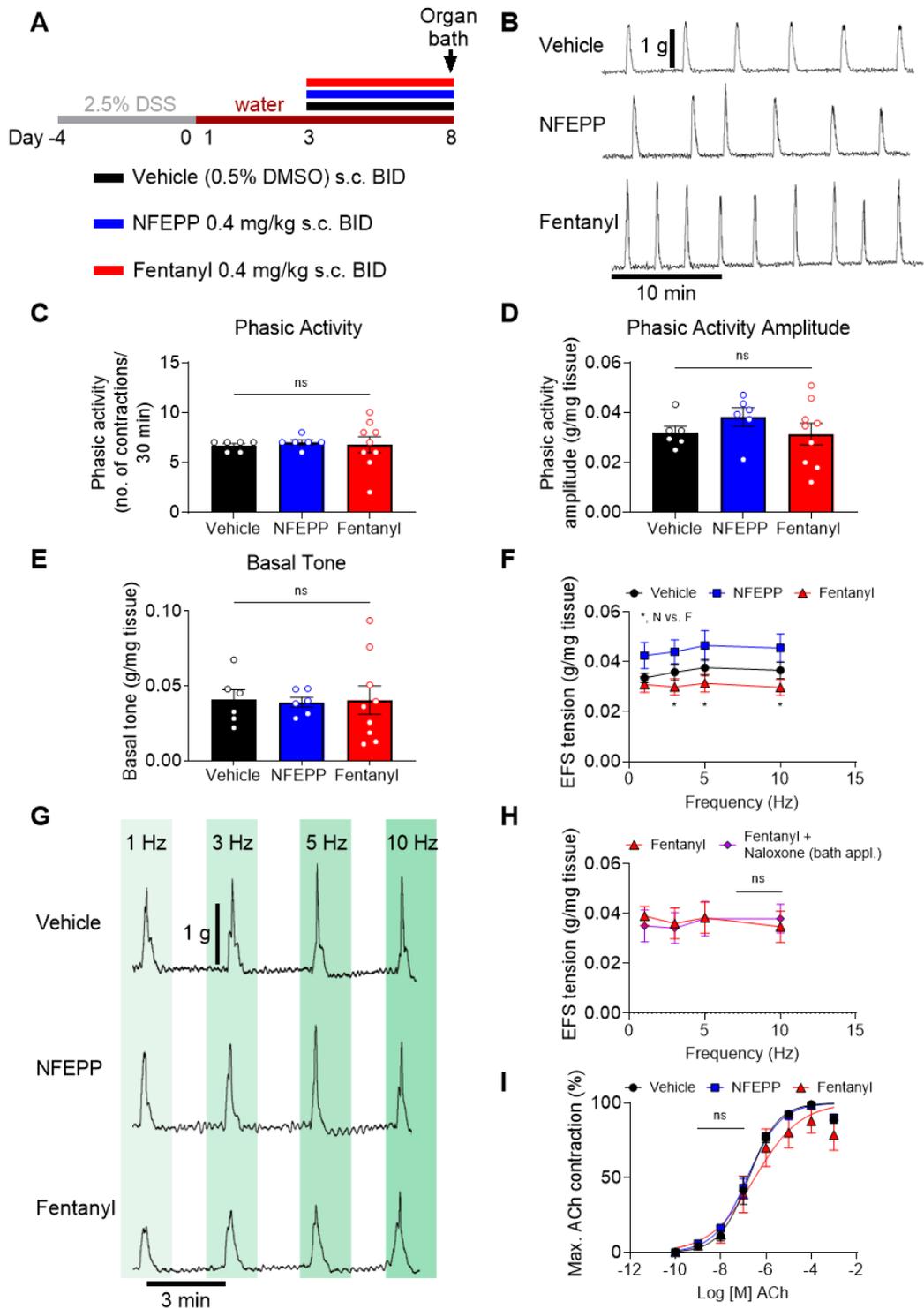
Supplementary digital content Fig. 3: pHLIP detection in extraintestinal organs from DSS colitis mice. DAPI/ anti-Cy-FITC labelling of the small intestine (**A**), the brain (**B**, cortex and hippocampus), the heart (**C**, ventricle), the liver (**D**) and the lung (**E**) from DSS colitis mice treated with pHLIP. Insets illustrate H&E stained sections of the corresponding organ (black scale bar: 100 µm). *Abbreviations: Cy, cyanin. DSS, dextran sulphate sodium. DAPI, 4',6'-diamidino-2-phenylindole. FITC, fluorescein isothiocyanate. H&E, haematoxylin and eosin. pHLIP, pH low insertion peptide.*

Supplementary digital content Figure 4:



Supplementary digital content Fig. 4: MPO activity and colon damage score. Combined MPO activity (**A**) and histological damage score (**B**) of the inflamed colon from the CT- and VMR- group (cohort 1). *Abbreviations: MPO, myeloperoxidase. VMR, visceromotor response. A: N=7-10. B: N=9-12. Kruskal-Wallis test, Dunn’s test.*

Supplementary digital content Figure 5:



Supplementary digital content Fig. 5: Effects of repeated NFEPP and fentanyl application on colonic contractility during acute DSS colitis assessed by in-vitro isometric tension recordings and electrical field stimulation (EFS). **A.** Study design with injection regimen (color coded). Isometric tension recordings were performed at day 8 after chronic treatment with vehicle (0.5% DMSO s.c., N=6), NFEPP (0.4 mg/kg s.c., N=6) or fentanyl (0.4 mg/kg s.c., N=9) BID. **B.** Representative traces of phasic contractions of colon segments from vehicle, NFEPP and fentanyl treated DSS colitis mice. **C-E.** Phasic activity frequency (**C**), phasic activity amplitude (**D**) and basal tone (**E**) of colon segments from vehicle, NFEPP and fentanyl treated DSS colitis mice. **F.** EFS-induced contractility of colon segments from vehicle, NFEPP and fentanyl treated DSS colitis mice. **G.** Representative traces of colon segment contractions induced by EFS with different stimuli (1, 3, 5 and 10 Hertz, Hz) **H.** EFS-induced contractility of colon segments before and after bath application of naloxone hydrochloride (10 μ M) in a subset of fentanyl treated DSS colitis mice (N=4). **I.** Smooth muscle contractility in response to bath application of cumulative increasing concentrations of ACh chloride of colon segments from vehicle (N=6), NFEPP (N=6) and fentanyl (N=5) treated DSS colitis mice. *Abbreviations: BID, bis in die (twice daily). DMSO, dimethyl sulfoxide. DSS, dextran sulphate sodium. ACh, acetylcholine. * p<0.05. C: Kruskal-Wallis test. D, E: Welch ANOVA. F, H, I: Two-way ANOVA, Bonferroni test.*