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

Jiménez-Vargas NN, *et al. Gut* 2022; 71:695–704. doi: 10.1136/gutjnl-2021-324070

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.).

Jiménez-Vargas NN, *et al. Gut* 2022; 71:695–704. doi: 10.1136/gutjnl-2021-324070

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.

REFERENCES

1 Kest B, Sarton E, Dahan A*, et al.* Gender Differences in Opioid-mediated Analgesia: Animal and Human Studies. Anesthesiology 2000;**93**:539-47.

2 Spahn V, Del Vecchio G, Labuz D*, et al.* A nontoxic pain killer designed by modeling of pathological receptor conformations. Science 2017;**355**:966-9.

3 Kim JJ, Shajib MS, Manocha MM*, et al.* Investigating intestinal inflammation in DSS-induced model of IBD. J Vis Exp 2012.

4 Wallace JL, Keenan CM. An orally active inhibitor of leukotriene synthesis accelerates healing in a rat model of colitis. Am J Physiol 1990;**258**:G527-34.

5 Jimenez-Vargas NN, Pattison LA, Zhao P*, et al.* Protease-activated receptor-2 in endosomes signals persistent pain of irritable bowel syndrome. Proc Natl Acad Sci U S A 2018;**115**:E7438-E47.

6 Robertson MJ, Deane FM, Stahlschmidt W*, et al.* Synthesis of the Pitstop family of clathrin inhibitors. Nature protocols 2014;**9**:1592-606.

7 Israelyan N, Del Colle A, Li Z*, et al.* Effects of Serotonin and Slow-Release 5-Hydroxytryptophan on Gastrointestinal Motility in a Mouse Model of Depression. Gastroenterology 2019;**157**:507-21 e4.

8 Jensen DD, Lieu T, Halls ML*, et al.* Neurokinin 1 receptor signaling in endosomes mediates sustained nociception and is a viable therapeutic target for prolonged pain relief. Sci Transl Med 2017;**9**.

9 Robertson MJ, Deane FM, Robinson PJ*, et al.* Synthesis of Dynole 34-2, Dynole 2-24 and Dyngo 4a for investigating dynamin GTPase. Nature protocols 2014;**9**:851-70.