Supplementary materials for

Title: Preoperative administration of the 5-HT$_4$ receptor agonist prucalopride reduces intestinal inflammation and shortens postoperative ileus via cholinergic enteric neurons

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Immunohistochemistry.

Eight to ten week old female mice were sacrificed with CO_2 overdose and the jejunum was fixed with 4% paraformaldehyde (PFA) for 30 minutes at room temperature after which the *muscularis* was peeled from the mucosa. Intestinal tissue from patients was immediately transferred to Krebs' solution in the operation room. The mucosa and submucosa were removed and the *muscularis externa* was fixed overnight with 4% PFA at 4ºC.

Both murine and human tissues were blocked with 1% bovine serum albumin (BSA), subsequently incubated overnight with primary antibodies followed by incubation with the appropriate secondary antibody dissolved in 1% BSA + 0.3% Triton X-100. Images were collected under Zeiss LSM880 multiphoton microscope. The following primary antibodies were used: Goat anti-choline acetyltransferase (ChAT; 1:500; Millipore), rat anti-F4/80 (1:500; Biolegend), mouse anti-CD68 (1:500; Biolegend), rabbit anti-α7 nicotinic acetylcholine receptor (human α7nAChR (905 OD1014) and mouse α7nAChR (768 OD1883); 1:1000, kindly provided by C. Gotti of CNR, Institute of Neuroscience, Milano, Italy). The following secondary antibodies were used (all from Jackson ImmunoResearch; 1:800): Alexa Fluor 488-conjugated donkey anti-goat antibody, Cy5-conjugated donkey anti-rat antibody, Cy3-conjugated donkey anti-rabbit antibody and Cy5 conjugated donkey anti-mouse antibody.

Cell culture experiments.

Isolation of lamina propria and muscularis macrophages.

After enzymatic digestion of the jejunal *muscularis*[1] and lamina propria[2] as previously described, the cell suspension was stained using the following surface markers: CD45 (104; eBioscience), CD11c (HL3; BD Pharmingen), IA/IE (M5/114.15.2; eBioscience), CD64 (X54-517.1.1; BD Pharmingen), CD11b (M1/70; BD Pharmingen) to sort lamina propria macrophages (LPMφ) and *muscularis* macrophages (mMφ) ($CD45^+CD11c^-$ $IA/IE^+CD64^+CD11b^+$).

For the Western blotting analysis, mMφ ($CD45^+CD11c^{low}IA/IE^+CD64^+CD11b^+$) and ($CD45^+CD11c^-IA/IE\text{-}CD64^-CD11b^-$) *muscularis* $CD45^+$ immune cells were sorted from the *muscularis* using a FACS Aria (BD Biosciences) up to >95% purity.

Isolation of bone-marrow derived macrophages.

Bone marrow cell suspensions were isolated by flushing femurs 8- to 10-week old female wild type mice with RPMI1640 (Lonza) containing 10% FBS (Gibco) and 1% Pen/Strep (Sigma-Aldrich). The cell suspension was passed through a 70-µm nylon filter and washed twice with RPMI medium. To obtain the bone-marrow-derived macrophages (BMDM), the bone marrow cells were seeded at 10^6 cells/ml and cultured for 9 days in DMEM medium (Lonza) containing 10% FBS (Biowest), 100 µg/ml penicillin (Lonza), 100 µg/ml streptomycin (Lonza), 1% L-glutamine (Lonza), 50 µM β-mercaptoethanol (Gibco) and 20% L929 medium.

Intracellular Ca^{2+} imaging of $Wnt.1^{GCaMP3}$ positive enteric neurons.

The enteric nervous system of $Wnt.1^{GCaMP3}$ mice (8-10 week old; female) was imaged *ex vivo* in mucosa-free murine small intestine preparations as previously described[3]. During imaging, tissue was subjected 3 consecutive times to electrical field stimulation (1, 5 and 20 Hz, pulse width: 1 ms, current: 20 mA) for 10 seconds and 2 minutes apart, using a custom designed electrode (Cibertec). Image analysis was performed using custom-written macros in IGOR PRO (Wavemetrics) as previously described[3]. Fluorescence intensity was normalized to the basal fluorescence at the onset of the recording for each region of interest (F/F0), and fluorescence peaks were analyzed. The peak amplitudes (ΔF/F0) reflect the maximum intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).

Intracellular Ca^{2+} imaging of *muscularis externa* resident macrophages.

GFP-labeled intestinal resident mMφ from $CX_3CR_1^{GFP/+}$ mice (8-10 week old; female) were imaged *ex vivo* in mucosa-free murine small intestine preparations as previously described[1]. In brief, to visualize $[Ca^{2+}]_i$ changes in these resident mMφ, jejunal *muscularis*

preparations were loaded with the Ca^{2+} indicator Fluo-4 AM. Due to the high dynamic range of the camera used, Fluo-4 changes could be recorded in the CX3CR1 positive mMφ as previously proven[1]. Next, jejunal *muscularis* preparations were exposed to 2 consecutive applications of 100 µM ATP (Sigma-Aldrich) for 15 seconds with a 15 minutes interval. Electrical field stimulation (20 Hz, current: 20 mA and pulse width: 1 ms during 5 minutes) was performed using a custom designed electrode (Cibertec) directly after the first ATP administration.

Tetrodotoxin (TTX; 10 µM, Tocris) or ω-conotoxin GVIA (0.1 µM, Tocris) were administered 15 minutes before the first ATP stimulation and for 15 minutes between the 2 ATP applications. Prucalopride (150 µM, Selleckchem) was administered for 15 minutes in between the 2 ATP stimuli. Image analysis and fluorescence quantification was performed as described in the previous section.

Fluorescence intensity was normalized to the basal fluorescence at the onset of the recording for each region of interest (F/F0), and fluorescence peaks were analyzed. The peak amplitudes (ΔF/F0) reflect the maximum intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Delta between peaks (ΔΔF/F0) represents the difference in response from first to the second ATP application. Moreover, the percentage mMφ maximally inhibited was calculated using twice the standard deviation of the control mean as a cut-off value.

Protein expression of α7nAChR in *muscularis* macrophages.

Protein extraction was obtained using 300,000 mMφ and 150,000 *muscularis* $CD45^+$ immune cells from wild type animals (8-10 week old; female) and 200,000 mMφ and 100,000 *muscularis* $CD45^+$ immune cells from α7nAChR KO (8-10 week old; female) animals diluted in 200 µl 2% Triton X-100, solution with 50 mM Tris HCl (pH 7), 120 mM NaCl, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 2 mM PMSF and 10 µg/ml of each of the following protease inhibitors: leupeptin, bestatin, pepstatin A and aprotinin. Mouse brains without cerebellum were included as a positive control. Brain lysates were obtained in 10 ml of 2% Triton X-100, solution with

50 mM Tris HCl (pH 7), 120 mM NaCl, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 2 mM PMSF and 10 µg/ml of proteases inhibitors. α7nAChR subunits were purified using α-bungarotoxin as previously described.[4] The presence of α7nAChR subunits in α-Bgtx–purified nAChR were analyzed via Western blotting. The rabbit α7nAChR (768OD1883) antibody (kindly provided by C. Gotti of CNR, Institute of Neuroscience, Milano, Italy) was used.

Preclinical model of POI

Measurement of gastrointestinal transit.

As previously described[5], mice were gavaged with a liquid non-absorbable FITC-labeled dextran (70,000 Da; Invitrogen) 22.5 hours postoperatively to determine gastrointestinal transit. After 1.5 hours, animals were sacrificed and the contents of stomach, small bowel (divided into 10 segments of equal length), caecum and colon (3 segments of equal length) were collected. The amount of FITC was quantified in each bowel segment using a spectrofluorimeter. The distribution of the fluorescent dextran along the gastrointestinal tract was calculated using the geometric center (GC) formula [5].

Quantification of leukocyte accumulation at the intestinal muscularis.

Influx of MPO positive cells to the jejunal *muscularis* tissue was assessed as previously described[1, 6]. Whole mounts of ethanol-fixed jejunal *muscularis* were prepared and stained for MPO activity as previously described[1, 6].

RNA extraction and inflammatory gene expression.

Total RNA was extracted from the jejunal *muscularis externa* using RNeasy Mini Kit (Qiagen). The cDNA amplification and quantification were performed identical as for cell culture experiments. The primers used are listed in supplementary materials (Supplementary Table 3).

Randomized-controlled clinical pilot study

Abdominal vagus nerve stimulation

The anterior and posterior vagus nerve was dissected free from the esophagus just distally from diaphragmatic hiatus and two cardiac pacing wires (Streamline, model 6494, Medtronic) were placed on each branch at a distance of 1 cm from each other. The other end of the wires was connected to the external stimulator (Inomed). The anterior and posterior vagus nerve were stimulated at the beginning and the end of the surgery with 20 Hz, 1 ms and 2.5 mA during 2 min. In our previous study, these stimulation parameters were shown to induce anti-inflammatory effects in LPS-stimulated whole blood.[7]

Evaluation of the local anti-inflammatory effect.

Two consecutive full thickness biopsies of the distal duodenum were collected to assess the degree of inflammation via gene expression. The first specimen was collected at the beginning of the procedure when the intestine was still unhandled. The second tissue specimen, exposed to the usual handling during surgery, was collected 2 hours later. Following removal of the mucosa, both specimens were partitioned, put in RNA later (Qiagen) and stored at -80˚C until further analysis. The RNA extraction, cDNA amplification and quantification were performed identical as in the animal experiments described above. The primers used are listed in supplementary materials (Supplementary Table 4). The expression levels of the genes of interest were normalized to the expression levels of the reference gene *c1orf43*.

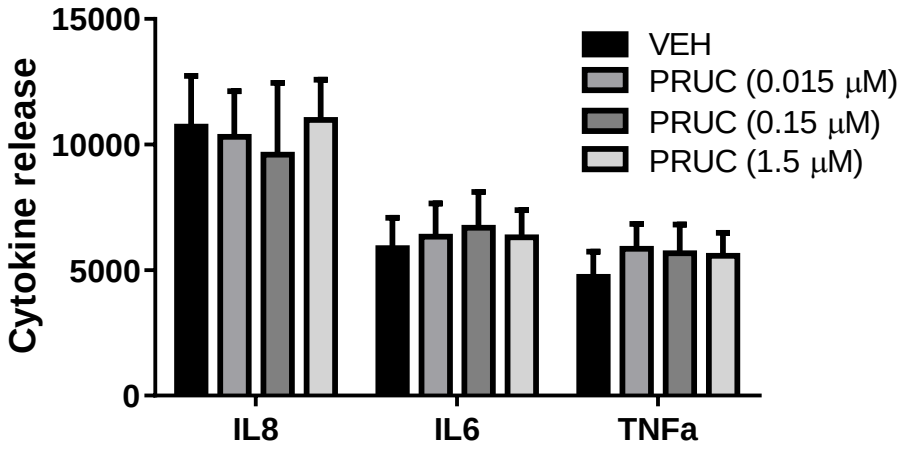
Evaluation of the systemic anti-inflammatory effect.

Blood samples were collected in serum-separating (SST) tubes (both from BD Vacutainer) prior to the induction of general anesthesia; and exactly 2 hours and 1 and 2 days after the end of the surgical procedure. Cytokine levels (IL6, IL8 and TNFα) were determined using the V-Plex human Pro-inflammatory panel (Meso Scale Discovery; MSD). The data were analyzed with the Discovery Workbench 4.0 software (MSD).

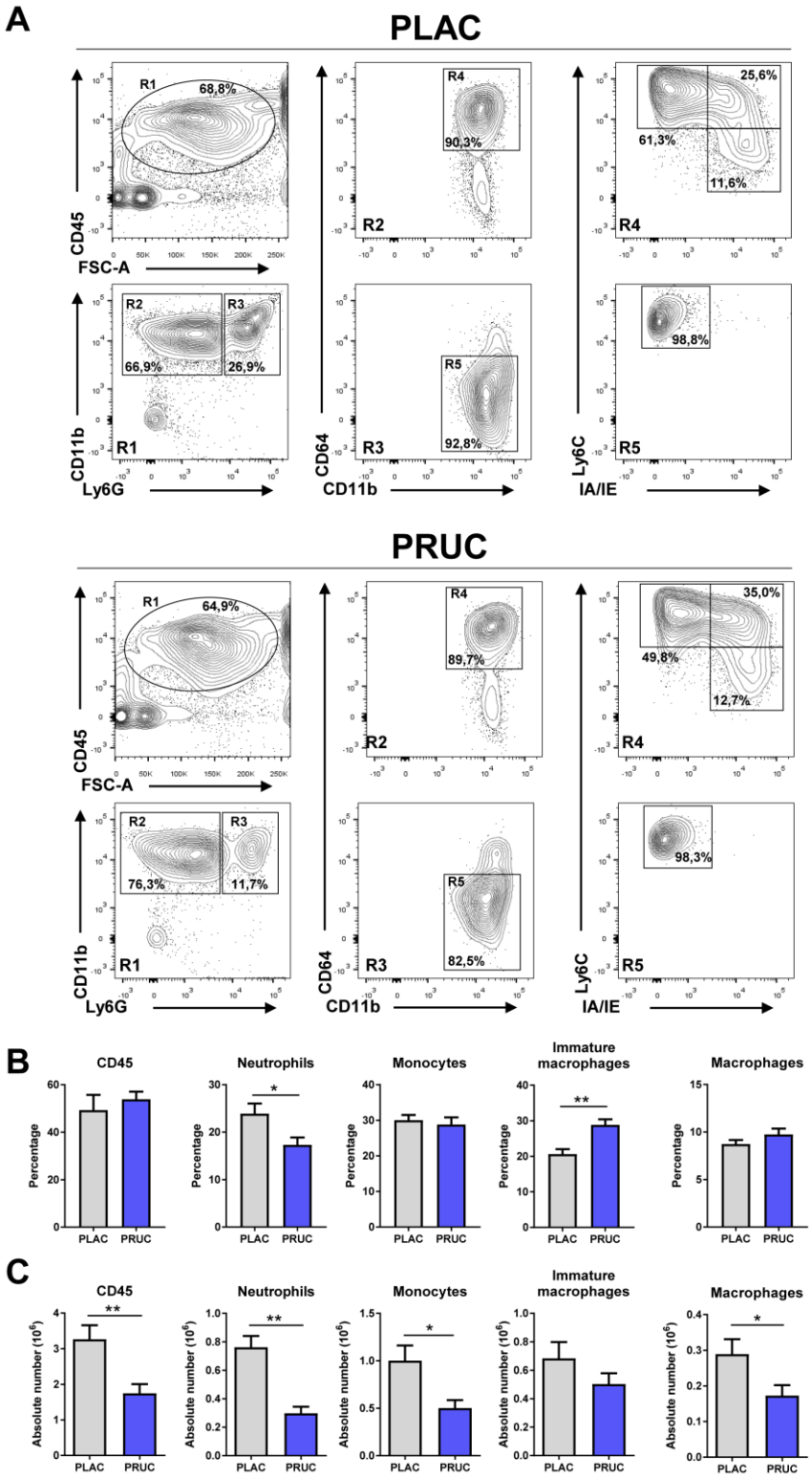
In order to exclude a direct effect of prucalopride on whole blood, heparinized blood (BD Vacutainer) was collected from 8 healthy volunteers with a mean age of 30 ± 4 yrs. Prior to the 4 hour LPS stimulation, whole blood was pretreated with prucalopride (0.015, 0.15 and 1.5 μM in 0.1% DMSO) for 1 hour at 37˚C on a rocking platform after which LPS (1 ng/ml) was added. Cytokine levels (IL6, IL8 and TNFα) were determined using the V-Plex human Pro-inflammatory panel (Meso Scale Discovery; MSD). The data was analyzed with the Discovery Workbench 4.0 software (MSD). The study was approved by the Ethics Committee of University Hospital of Leuven (s61062). All volunteers gave their written informed consent prior to their participation to the study.

Evaluation of clinical recovery.

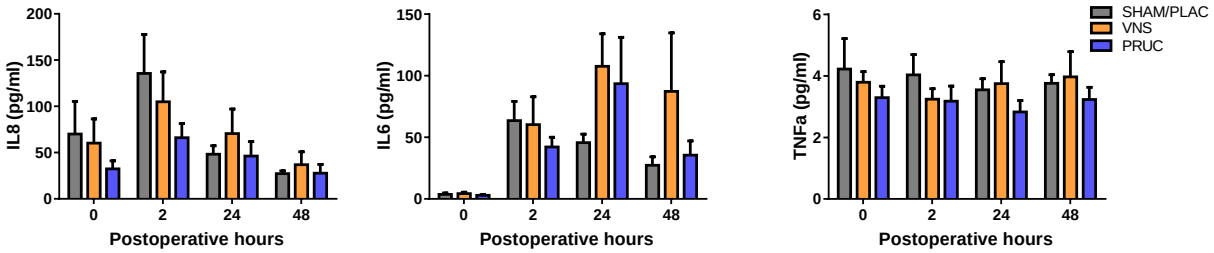
Clinical recovery of gastrointestinal tract was assessed via a daily symptom questionnaire until discharge. The time to removal of NGT, first tolerance of solid food, time to first defecation and length of hospital stay (LOS) were recorded. Since site-specific complications (SSC) are known to be positively correlated with the occurrence of gastroparesis and ileus, signs of clinical recovery were only determined in patients without SSC.[8, 9] Patient follow-up was closed 1 month after hospital discharge.



Supplementary Figure 1. Prucalopride has no direct effect on cytokine production by LPS-stimulated whole blood. The level of IL8, IL6 and TNFα was measured in LPS-stimulated whole blood pretreated with vehicle or prucalopride (0.015-1.5 μM). Data are shown as the mean ± SEM. Repeated one-way ANOVA with Bonferroni correction for multiple testing. n= 8 samples/group



Supplementary Figure 2. Pharmacological activation of enteric neurons by prucalopride reduces intestinal inflammation. Immune cells isolated from the jejunal *muscularis externa* of mice treated with placebo (PLAC) or 5 mg/kg prucalopride (PRUC) 1.5 hour prior to surgery. Mice were sacrificed 24 hours after intestinal manipulation (IM). **(A)** Representative dot plots showing neutrophils ($CD45^+Ly6G^+CD11b^+CD64^-Ly6C^+IA/IE^-$), monocytes ($CD45^+Ly6G^-CD11b^+CD64^+Ly6C^+IA/IE^-$), immature macrophages ($CD45^+Ly6G^-CD11b^+CD64^+Ly6C^+$ IA/IE^+) and macrophages ($CD45^+Ly6G^-CD11b^+CD64^+Ly6C^-IA/IE^+$). Percentage **(B)** and absolute numbers **(C)** of neutrophils, monocytes, immature macrophages and macrophages. Results are expressed as mean ± SEM. *p<0.05, ** p<0.01. Unpaired t-test. n= 7 mice.



Supplementary Figure 3. Effect of abdominal VNS and prucalopride on serum levels of IL8, IL6 and TNFα. Blood samples were collected from Whipple patients treated with abdominal VNS, prucalopride (PRUC) or sham/placebo (SHAM/PLAC) 2 hours prior to and 2, 24 and 48 hours after surgery. Data are expressed as median ± interquartile range. Kruskal-Wallis test with Dunn's correction for multiple testing. n = 10 patients/group.

Standard care

Preoperative phase	
Outpatient department of surgery	- Scheduling of operation
Outpatient department of anesthesiology	- Preassessment for risk adjustment - Open discussion about different possibilities for management of perioperative analgesia
Preadmission counseling and guided tour on surgical ward	- No
Day of admission	
Intake	- Routine & informed consent
Bowel preparation	- No
Administration study medication (1/2)	- Yes (placebo or prucalopride 2 mg), with last meal at 17.00
Diet	- Last meal until midnight
Day of surgery	
Preoperative fasting	- Yes
Intake study medication (2/2)	- intake study medication 2 hours prior to surgery
Preanesthetic medication	- Yes, Xanax 0.5 mg
Anesthetic management	- Placement thoracic epidural (T6-T10); test dose (bupivacaine 0.25% with adrenaline 1:200,000), top-up dose (bupivacaine 0.25% [± 10 mL] with fentanyl 25 μg, followed by continuous infusion (bupivacaine 0.125% with fentanyl 12.5 μg × mL−1) started after last stimulation until postoperative day 3 - Combined with balanced general anesthesia - Standard preoperative fluid infusion regimen - Use of extra fluid challenge as first choice for management of mean blood pressure drop >20% below baseline - Use of noradrenaline drip as second choice for management of persistent mean pressure drop > 20% of baseline - Forced body heating (Bair hugger system and warmed IV fluids) - Insertion of NGT tube - No use of NSAIDs - For PONV management, alizapride hydrochloride was allowed as first line agent, but ondansetron only as a second line agent according to attending anesthesiologist
Surgical management	- Median laparotomy approach - Urine catheter - No infiltration of surgical wounds with local anesthetic drugs

	- Use of 1-2 abdominal drains
	- In case the patient is allocated to VNS, stimulation of the vagus occurred at beginning and end of surgery
Early postoperative management	- Epidural consisting of bupivacaine and fentanyl for 3 days postoperative and paracetamol 4 × 1 g × day^{-1}
	IV fluid administration (1-2 L × day^{-1}) is started until adequate oral fluid intake
Day 1 after surgery	
Postoperative management	- Liquid intake increased on daily basis based on output of NGT tube
	- IV fluid administration is continued until adequate oral fluid intake
	- Removal of NGT tube according to attending surgeon
	- TPN administration is continued until adequate solid intake
	- Solid intake after removal of NGT tube
	- Epidural consisting of bupivacaine and fentanyl for 3-4 days postoperative and paracetamol 4 × 1 g × day^{-1}
	- Mobilization according to attending surgeon
Day 2 after surgery	
Postoperative management	- Continue as on day 1 until discharge criteria fulfilled (removal NGT tube, tolerance of liquids, solids, defecation, adequate pain management and mobilization)
Day 3 after surgery	
Postoperative management	- Continue as on day 1 until discharge criteria fulfilled
	- Measurement of amylase for detection of possible pancreatic fistula
Day 4 after surgery	
Postoperative management	- Continue as on day 1 until discharge criteria fulfilled
Day 5 after surgery	
Postoperative management	- Continue as on day 1 until discharge criteria fulfilled
	- Measurement of amylase for detection of possible pancreatic fistula

Supplementary Table 1. Elaborate treatment protocol

		SHAM/PLAC (n=10)	VNS (n=10)	PRUC (n=10)	p
Age	years	61±5	64±4	71±3	0.2[A]
Gender (%)	M	30	30	80	0.03[B]
	F	70	70	20	
BMI	(kg/m^2)	26 (25-27)	24 (21-26)	25 (23-31)	0.51[C]
ASA (%)	1	10	0	0	0.63[B]
	2	40	60	50	
	3	50	40	50	
Comorbidity (%)	YES	60	60	90	0.24[B]
Smoker (%)	YES	80	40	30	0.22[B]
	PAST	10	20	40	
	NO	10	40	30	
Tumor location (%)	Pancreas	80	70	60	0.54[B]
	Periampuloma	0	10	20	
	Ampuloma	0	10	10	
	Pancreatitis	20	10	0	
	Duodenum	0	0	10	
Surgery time (min)		195 (160-228)	240 (207-252)	240 (195-240)	0.14[C]
Blood loss (ml)		125 (57-525)	450 (137-812)	125(60-525)	0.27[C]

Supplementary Table 2. Patient demographics and clinical characteristics. ASA, American Society of Anesthesiologists. [A] One-way ANOVA test with Bonferroni correcting for multiple testing. [B] two-tailed χ^2-test [C] Kruskal–Wallis test with Dunn's test for multiple correcting.

il6	CAGCCCTGAGAAAGGAGAC	AGGTTCAGGTTGTTTTCTGC
il1α	GAGAGCCGGGTGACAGTATC	ACTTCTGCCTGACGAGCTTC
chrna4	CTAGCAGCCACATAGAGACCC	GACAAGCCAAAGCGGACAAG
chrna7	CACATTCCACACCAACGTCTT	AAAAGGGAACCAGCGTACATC
chrna9	GGAACCAGGTGGACATATTCAAT	GCAGCCGTAGGAGATGACG
chrna10	ATGGATGAACGGAACCAAGTG	GTCCCAATGTAGGTAGGCGT
chrnb2	AGGGGTTTTGGGTACTGACC	AGCTTGTTATAGCGGGAAGGA
5-htr4	ACCTGTGCTGTATTTCCCTG	GGAGAAACGGGATGTAGAAGG

Supplementary Table 3. Primer sequences in mouse.

il6	CAGCCCTGAGAAAGGAGAC	AGGTTCAGGTTGTTTTCTGC
c1orf43	GGATGAAAGCTCTGGATGCC	GCTTTGCGTACACCCTTGAA
il8	ACTGAGAGTGATTGAGAGTGGAC	AACCCTCTGCACCCAGTTTTC
ccl2	CAGCCAGATGCAATCAATGCC	TGGAATCCTGAACCCACTTCT

Supplementary Table 4. Primer sequences in human.

References

1. Matteoli G, Gomez-Pinilla PJ, Nemethova A, et al. A distinct vagal anti-inflammatory pathway modulates intestinal muscularis resident macrophages independent of the spleen. Gut 2013:gutjnl-2013-304676.
2. Sun CM, Hall JA, Blank RB, et al. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. J Exp Med 2007;204:1775-85.
3. Boesmans W, Martens MA, Weltens N, et al. Imaging neuron-glia interactions in the enteric nervous system. Front Cell Neurosci 2013;7:183.
4. Moretti M, Zoli M, George AA, et al. The novel alpha7beta2-nicotinic acetylcholine receptor subtype is expressed in mouse and human basal forebrain: biochemical and pharmacological characterization. Mol Pharmacol 2014;86:306-17.
5. van Bree SH, Nemethova A, Cailotto C, et al. New therapeutic strategies for postoperative ileus. Nat Rev Gastroenterol Hepatol 2012;9:675-83.
6. de Jonge WJ, van der Zanden EP, The FO, et al. Stimulation of the vagus nerve attenuates macrophage activation by activating the Jak2-STAT3 signaling pathway. Nat Immunol 2005;6:844-51.
7. Stakenborg N, Wolthuis AM, Gomez-Pinilla PJ, et al. Abdominal vagus nerve stimulation as a new therapeutic approach to prevent postoperative ileus. Neurogastroenterol Motil 2017.
8. Qu H, Sun GR, Zhou SQ, et al. Clinical risk factors of delayed gastric emptying in patients after pancreaticoduodenectomy: a systematic review and meta-analysis. Eur J Surg Oncol 2013;39:213-23.
9. Topal B, Fieuws S, Aerts R, et al. Pancreaticojejunostomy versus pancreaticogastrostomy reconstruction after pancreaticoduodenectomy for pancreatic or periampullary tumours: a multicentre randomised trial. Lancet Oncol 2013;14:655-62.