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Mechanisms contributing to visceral hypersensitivity: focus on splanchnic afferent nerve signaling

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Key Words:	mechanosensitivity, afferent nerve signaling, visceral hypersensitivity, colitis, splanchnic nerves

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3 **1. Title page**
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7 **MECHANISMS CONTRIBUTING TO VISCERAL HYPERSENSITIVITY: FOCUS ON SPLANCHNIC AFFERENT**
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9 **NERVE SIGNALING**
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14 **Short running page heading: Splanchnic afferent nerve signaling in colitis**
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2. Abstract

Background: Visceral hypersensitivity is a main characteristic of functional bowel disorders and is mediated by both peripheral and central factors. We investigated whether enhanced splanchnic afferent signaling *in vitro* is associated with visceral hypersensitivity *in vivo* in an acute and postinflammatory rat model of colitis.

Methods: TNBS-colitis was monitored individually by colonoscopy to confirm colitis and follow convalescence and endoscopic healing in each rat. Experiments were performed in controls, rats with acute colitis and in post-colitis rats. Colonic afferent mechanosensitivity was assessed *in vivo* by quantifying visceromotor responses (VMRs), and by making extracellular afferent recordings from splanchnic nerve bundles *in vitro*. Multi-unit afferent activity was classified into single units identified as low threshold (LT), wide dynamic range (WDR), high threshold (HT) and mechanically insensitive afferents (MIA).

Key results: During acute TNBS-colitis, VMRs were significantly increased and splanchnic nerve recordings showed proportionally less MIA and increased WDR and HT afferents. Acute colitis gave rise to an enhanced spontaneous activity of both LT and MIA and augmented afferent mechanosensitivity in LT, WDR and HT afferents. Post-colitis, VMRs remained significantly increased while splanchnic nerve recordings showed that the proportion of LT, WDR, HT and MIA had normalized to control values. However LT and MIA continued to show increased spontaneous activity and WDR and HT remained sensitized to colorectal distension.

Conclusions & Inferences: Visceral hypersensitivity *in vivo* is associated with sensitized splanchnic afferent responses both during acute colitis and in the post-inflammatory phase. However splanchnic afferent subpopulations are affected differentially at both time points.

Keywords: mechanosensitivity, afferent nerve signaling, visceral hypersensitivity, TNBS, colitis, splanchnic nerves

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3 **Key messages:**
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- 5 • The role of splanchnic afferent nerves in visceral hypersensitivity remains unclear.
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- 7 • Visceral sensitivity during acute TNBS-colitis and in the post-inflammatory phase was
8 assessed *in vitro* by recording splanchnic afferent nerve discharge and *in vivo* by recording
9 visceromotor responses to colorectal distension.
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- 11 • Sensitization of splanchnic afferents *in vitro* is associated with visceral hypersensitivity *in vivo*
12 both during acute colitis and in the post-inflammatory phase but the different
13 subpopulations of afferent nerves are differentially affected in the acute versus the post-
14 inflammatory phase of colitis.
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3. Introduction

Visceral hypersensitivity is a main characteristic of bowel disorders such as inflammatory bowel disease (IBD) or irritable bowel syndrome (IBS) (1-4). It is involved in pain perception and is mediated by both peripheral and central factors. Growing evidence suggests that sensory nerve pathways play a key role in the peripheral pain transmission.

Sensory information from colon and rectum is conveyed via thoracolumbar splanchnic nerves and lumbosacral pelvic nerves to the spinal cord that synapse onto second-order neurons in the spinal cord, which in turn transmit the sensory signal to the higher brain regions (5). Various studies have shown that lumbosacral pelvic nerves are the main signaling pathway for visceral sensory processing under physiological conditions including peristaltic movement of fecal contents into the rectum; in contrast, thoracolumbar splanchnic nerves convey primarily noxious signals derived from rapid gut distension and mesentery torsion (6-11). This pathway is less active under physiological conditions.

Using different animal models, several studies corroborate the involvement of both pelvic and splanchnic afferents in mediating visceral hypersensitivity, however splanchnic afferents seem specifically equipped for noxious nerve signaling during inflammation (6, 9, 12-16). For instance, splanchnic afferents are more likely to respond to inflammatory mediators such as adenosine 5'-triphosphate (ATP) and bradykinin and more frequently increase their firing rate when exposed to capsaicin (8, 17). Moreover, silent nociceptors recruited by these mediators were exclusive to the splanchnic signaling pathway (8, 17). In addition, electrophysiological recordings in mice corroborated the predominant involvement of splanchnic afferents in acute trinitrobenzene sulphonic acid (TNBS)-colitis (6). In contrast, splanchnic afferent discharge in rats remained unaffected by dextran sulphate sodium (DSS)-colitis (18, 19). In addition, Chen *et al.* (20) recently demonstrated that sensitized responses of spinal afferents do not always result in visceral hypersensitivity *in vivo* as assessed using behavioral assays such as the visceromotor response (VMR) to colorectal distension. Besides, acute colitis can induce long-lasting visceral hypersensitivity which persists after the initial inflammatory insult itself has already fully subsided (21, 22). Whether and to

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3 what extent altered splanchnic nerve signaling correlates with post-inflammatory hypersensitivity
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5 remains unclear. Therefore we studied rat splanchnic afferent nerve discharge in response to
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7 colorectal distension either in the acute phase of TNBS-colitis or in the post-inflammatory phase. In
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9 addition, we assessed whether sensitized responses were associated with visceral hypersensitivity *in*
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11 *vivo* by quantifying the VMRs to colorectal distension.

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14 Of note, part of this work was presented as an abstract at nEUROgastro 2015 (23).
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18 4. Material and methods

19 *Animals*

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21 Adult male Sprague-Dawley rats (200-225g, Charles River, Lecco, Italy) were allowed to acclimatize to
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23 housing conditions for one week prior to experimentation and had free access to water and food. All
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25 experiments were approved by the Committee for Medical Ethics and the use of Experimental
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27 Animals at the University of Antwerp (file number 2010-18).
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33 *Colitis*

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35 Distal colitis was induced by the intrarectal instillation of 0.5 mL of 15 mg TNBS in 50% ethanol under
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37 pentobarbital anesthesia (60 mg.kg⁻¹ ip). Control animals received a 0.5 mL saline enema. The
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39 relatively high concentration of ethanol relates to strain differences in susceptibility to TNBS-colitis as
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41 previously described (24).
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46 *Colonoscopy*

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48 Under pentobarbital anesthesia (45 mg.kg⁻¹ ip), the lubricated tip of a baby gastroscope (Olympus
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50 Europa GmbH, Hamburg, Germany) was gently introduced into the distal colon and advanced under
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52 direct endoscopic vision up to 10 cm proximal to the anal verge. During withdrawal, mucosal damage
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54 was assessed using our previously published scoring system (25, 26). The colonoscopic score (total 0-
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56 19) takes into account the degree of ulceration (0-6), the extent of damage (0-10) and presence of
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3 edema (0-1), active bleeding (0-1) and/or stenosis (0-1). We previously demonstrated that
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5 colonoscopic damage scores correlate highly with the traditional methods to assess TNBS-colitis,
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7 namely macroscopic and microscopic scoring systems and myeloperoxidase (MPO) activity (25).
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10 11 *Post-mortem markers of inflammation*

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13 At the end of the *in vivo* experiments, the distal colon was rapidly excised to evaluate macroscopic
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15 mucosal damage using a validated scoring system (score 0-10) (25, 27). A representative section was
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17 fixed in 4% formaldehyde for 24 h and embedded in paraffin for hematoxylin-eosin staining.
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19 Histological specimens (5 μm slices) were scored microscopically for the presence of an inflammatory
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21 infiltrate, the number of infiltrated layers, mucosal architectural distortion and edema (score 0-10)
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23 (25, 28). A second representative 1 cm segment was used to assay MPO activity according to our
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25 previously published methods (25). In brief, specimens were blotted dry and placed in a potassium
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27 phosphate buffer pH 6.0 containing 0.5% hexadecyltrimethylammonium bromide a ratio of 5 g per
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29 100 mL buffer. The samples were arranged on ice, homogenized for 30 s and subjected to two
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31 sonication and freeze-thawing cycles. The suspension was centrifuged at 15 000 g for 15 min at 4 °C.
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33 Aliquots (100 μL) of the supernatant were added to 2.9 mL of *o*-dianisidine solution (16.7 mg of *o*-
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35 dianisidine in 1 mL saline, 98 mL of 50 mmol potassium phosphate buffer, pH 6.0 and 1 mL of a 0.05
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37 % H_2O_2 solution as a substrate for MPO enzyme). The change in absorbance was read at 460 nm over
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39 60 s using a Spectronic Genesys 5 spectrophotometer (Milton Roy, Rochester, NY, USA). One unit of
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41 MPO activity was defined as the quantity able to convert 1 μmol H_2O_2 to H_2O per min at 25 °C and
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43 was expressed as units per gram of tissue ($\text{U}\cdot\text{g}^{-1}$).
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50 51 *Visceromotor responses to colorectal distension*

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53 VMRs to colorectal distension were used as an objective measure of visceral sensitivity. This
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55 validated nociceptive reflex consists of the contraction of the abdominal musculature in response to
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57 noxious colorectal distension and integrates both peripheral and central processing (26, 28, 29).
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3 Other reliable outcome parameters to assess visceral sensitivity *in vivo* include variations in heart
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5 rate and blood pressure or changes in colonic balloon pressure (29, 30).
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7 Three days prior to VMR assessment, two EMG electrodes were sutured into the oblique abdominal
8 muscle and were exteriorized at the base of the neck for future access. On the day of VMR
9 assessment, the electrodes were connected to a data acquisition system. A lubricated balloon (4 cm
10 length) was gently introduced through the anus and inserted in the colorectum up to 0.5 cm passed
11 the anal verge. The balloon catheter was secured to the base of the tail and connected to a barostat
12 (Distender Series II™ Barostat, G&J Electronics, Willowdale, Ontario, Canada) for graded and
13 pressure-controlled balloon distension (10-60 mmHg, 20 s duration, 4 min interval) as previously
14 published (28). The abdominal EMG signal was recorded (NL100AK headstage), amplified (NL104),
15 filtered (NL 125/126, Neurolog, Digitimer Ltd, bandpass 50–5000 Hz) and digitized (CED 1401,
16 Cambridge Electronic Design, Cambridge, UK) to a PC for off-line analysis using Spike2 version 5.16
17 for Windows (Cambridge Electronic Design). The analog EMG signal was rectified and integrated. To
18 quantify the magnitude of the VMR at each distension pressure, the area under the curve (AUC)
19 during distension (20 s) was corrected for the baseline activity (AUC pre-distension, 20 s).
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37 *Colonic compliance*

38 Rats were anesthetized (pentobarbital 45 mg.kg⁻¹) and graded volumes (0-2.5 mL) were applied to
39 the balloon inserted in the colorectum while recording the corresponding intracolonic pressure (31).
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46 *Splanchnic afferent nerve activity*

47 Under deep pentobarbital anesthesia (60 mg.kg⁻¹), the distal colon with the mesenteric bundle in
48 place was excised and transferred to a Sylgard-lined organ chamber. This chamber was maintained at
49 34°C and continuously perfused with oxygenated Krebs solution (118 mM NaCl, 4.75 mM KCl, 1 mM
50 NaH₂PO₄, 22 mM NaHCO₃, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 11 mM D-glucose, 3 μM indomethacin; 10
51 mL.min⁻¹). The proximal and distal end of the colon were secured to an in- and outflow port
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3 respectively for intraluminal perfusion with Krebs solution which was maintained at a constant rate
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5 of 1 mL.min⁻¹ via a perfusion syringe pump. Colorectal distension was induced by closure of the
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7 outflow while maintaining the inflow. The mesenteric bundle was pinned out at the base of the
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9 chamber and the splanchnic nerve was identified at the origin of the inferior mesenteric artery and
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11 dissected free. After transection, the myelin sheet was carefully peeled off and the bare nerve
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13 bundle was teased into fine strands. One of these was drawn into a suction electrode that was
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15 relayed to a data acquisition system to record action potential discharge as previously described (32,
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20 Multi-unit recordings contained action potentials of different amplitude, width and shape. Individual
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22 wavemarks were matched to spike templates by a computerized analysis, allowing single-unit
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24 discrimination. A signal-noise ratio of at least 2:1 was enforced before spike allocation. All action
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26 potential responses were expressed as impulses per second (imp.s⁻¹, 10 s bin size). Afferent unit
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28 discharge to colorectal distension was quantified by deducting the spontaneous activity from the
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30 response to distension. This allowed us to differentiate the spontaneous activity from the actual
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32 response to colorectal distension. Based on the firing discharge profile during colorectal distension,
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34 units were categorized as low threshold (LT), wide dynamic range (WDR), high threshold (HT) and
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36 mechanically insensitive afferents (MIA) as previously published (34-36). First, the firing rate at 20
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38 mmHg distension is expressed as the percentage of the response at 60 mmHg. This parameter,
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40 referred to as LT%, reflects the relative firing at low compared to high distension pressures and was
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42 used to further characterize afferent responses based on their functional features as previously
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44 published (35). An LT% of 40% represents a linear increase in afferent discharge. An LT% of >55%,
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46 indicates the main increase in afferent firing occurs during limited colorectal distension which is
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48 characteristic for LT. An LT% of <15% is characteristic for HT as this implies a disproportionate
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50 increase in afferent firing during noxious distension. WDR have an LT% between 15 and 55. Finally,
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52 MIA are defined as afferent fibers displaying spontaneous activity that does not increase during
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54 colonic distension.
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Experimental design

Two experimental set-ups were performed (Figure 1).

In set-up 1 (acute colitis), rats were randomized to receive saline (control) or TNBS enema. VMRs or splanchnic afferent nerve signaling were evaluated 3 days later during the acute inflammatory phase.

In addition, colonoscopy was performed in both TNBS and saline treated rats to confirm the presence of colitis in addition to macroscopic and microscopic evaluation of the tissue and an MPO activity assay. Set-up 2 (post-colitis) focused on visceral sensitivity in the post-inflammatory phase. Rats were administered saline (control) or TNBS (colitis) and the extent of inflammation was verified colonoscopically on day 3 in all rats, similar to set-up 1. However, from day 10 onwards, convalescence was monitored individually by repeated colonoscopy that was performed every 4 days as described previously (28). If at any time point colonoscopy still showed signs of mucosal inflammation, the animal was allowed to recover further and colonoscopy was repeated every 4 days. If colonoscopy showed complete mucosal healing, the EMG electrodes were implanted and VMR or splanchnic afferent nerve firing were recorded 3 days later. In addition, the post-inflammatory status was confirmed using colonoscopic, macroscopic and microscopic evaluation of the colonic tissue in addition to the MPO activity assay.

Data presentation and statistical analysis

Data are presented as mean \pm sem for N, the number of animals and n, the number of units. Preliminary analysis indicated that afferent signaling was similar in control rats of the acute set-up and control rats of the post-inflammatory set-up (data not shown). Therefore data from splanchnic nerve firing in controls from the acute and post-inflammatory set-ups were pooled to reduce the number of animals needed. Variables were analyzed using unpaired Student's *t*-test and generalized estimating equations (GEE) followed by least significant difference (LSD) post-hoc test when appropriate. Frequency distributions to compare the proportion of the different afferent fibers was performed using a Chi-square test. Statistical significance was set at $p < 0.05$ for all tests.

5. Results

VMRs and splanchnic afferent nerve signaling during acute TNBS-colitis

Intrarectal instillation of TNBS resulted in acute colitis on day 3 post-induction characterized by the presence of multiple serpiginous ulcerations in the distal colon and an increased colonoscopic damage score (7.3 ± 0.3 for TNBS vs 0.0 ± 0.0 for control; $N=17-22$; $p<0.001$). In addition, acute colitis was further confirmed by the macroscopic damage scores (7.0 ± 1.3 vs 0.0 ± 0.0 for control; $p<0.001$), the microscopic damage scores (6.0 ± 0.6 vs 0.0 ± 0.0 for control; $p<0.001$) and the MPO activity ($16.3 \pm 4.3 \text{ U.g}^{-1}$ vs 0.0 ± 0.0 for control; $p<0.001$).

VMRs to colorectal distension increased in a pressure-dependent manner (Figure 2A). During acute TNBS-colitis, VMRs were significantly increased compared to controls for the full range of distension pressures, indicating the presence of marked visceral hypersensitivity.

Distending the balloon in the colorectum with fixed volumes of water heightened the intracolonic pressure in a volume-dependent manner, reflecting colonic compliance. Intracolonic pressures increased more rapidly during acute TNBS-colitis, indicating reduced colonic compliance compared to controls (Figure 2B).

Splanchnic afferent nerves of both control and acute TNBS-colitis rats showed irregular spontaneous activity in the absence of colorectal distension (Figure 3A-C). Mechanosensitivity was assessed by performing ramp distension up to 60 mmHg, increasing afferent nerve discharge in a pressure-dependent fashion (Figure 3A-C). Each nerve bundle contained in the suction electrode consisted of several different units that could be discriminated based on their spike waveform. In total, 57 single units were discriminated in the multi-unit recordings of 12 controls and 60 units were identified in 17 rats with acute TNBS-colitis. On average, 5 ± 1 units were discriminated in each multi-unit recording in controls compared to 4 ± 1 units in those of rats with acute TNBS-colitis ($N=12-17$; ns). When afferent firing was normalized for the number of units in each recording, spontaneous activity was not significantly different between controls and acute colitis rats ($0.8 \pm 0.1 \text{ imp.s}^{-1}$ for acute colitis vs 0.5 ± 0.1 for controls; $N=12-17$; $p=0.07$). In contrast, afferent nerve discharge to colorectal distension

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3 normalized for the number of units in each recording, was significantly increased during acute TNBS-
4 colitis compared to control from a distension pressure of 10 mmHg onwards, indicating the presence
5 of sensitized afferent responses (Figure 3D).
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9 Based on their response to colorectal distension, the 57 units identified in control specimens were
10 identified as 11 LT, 16 WDR, 13 HT and 17 MIA; the 60 units of TNBS-colitis rats were categorized as
11 11 LT, 19 WDR, 22 HT and 8 MIA (Figure 4A). The proportion of these four fiber subtypes was
12 significantly different for acute TNBS-colitis compared to control conditions (Chi-square test; $p < 0.05$).
13 Spontaneous activity of LT and MIA was significantly more vigorous in rats with acute TNBS-colitis
14 compared to controls, whereas WDR and HT units displayed similar spontaneous firing rates in both
15 groups (Table 1). Each of the four different unit subtypes elicited a characteristic response to ramp
16 distension as described previously (Figure 4B-E) (36). LT disproportionally enhanced their firing rate
17 to limited increases in intraluminal pressure and their discharge rapidly saturated at relatively low
18 distension levels (approximately 20 mmHg) reaching a plateau. In contrast, WDR responded linearly
19 to increasing distension pressures: these fibers were activated by low levels of distension and
20 afferent discharge continued to rise as the intraluminal pressure increased further. HT typically
21 displayed little activity to low distension levels but responded vigorously to noxious distension.
22 Finally, MIA were unresponsive to ramp distension. LT afferent discharge to colorectal distension in
23 acute colitis rats was significantly more vigorous at the plateau phase (from 20 mmHg onwards;
24 Figures 4A). LT, WDR and HT units from rats with acute TNBS-colitis displayed markedly enhanced
25 afferent firing in response to colorectal distension compared to their control counterparts (Figure 4B-
26 D). Of note, all afferent responses to colorectal distension were corrected for spontaneous activity.
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52 *Splanchnic afferent nerve signaling and VMRs after the resolution of TNBS-colitis*

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54 Three days after the induction of TNBS-colitis, ulcerations were present in all TNBS-instilled rats
55 (colonoscopic score 5.6 ± 0.4 for TNBS vs 0.0 ± 0.0 for control; $N=20-23$; $p < 0.001$). During
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3 colonoscopic follow-up, TNBS-colitis resolved spontaneously and colonoscopic resolution was
4 present after a median of 14 days (range 10-22 days). Resolution of TNBS-colitis was confirmed by
5 macroscopic (0.0 ± 0.0 for TNBS vs 0.0 ± 0.0 for control) and microscopic evaluation of the colonic
6 tissue (0.0 ± 0.0 vs 0.0 ± 0.0 for control) in addition to similar MPO activity in both groups at the end
7 of the experiment (0.8 ± 0.3 vs 1.1 ± 0.3 for control).
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13 After colonoscopically-proven resolution of colitis, VMRs to colorectal distension remained markedly
14 increased in post-colitis rats compared to controls from 20 to 60 mmHg of distension, indicating the
15 presence of post-inflammatory visceral hypersensitivity (Figure 5A). Colonic compliance was similar
16 in both groups (Figure 5B).
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22 In total, 57 single units were discriminated in the recordings from 12 controls (mean 5 ± 1 unit per
23 recording) and 56 units were identified in the recordings from 13 rats with colonoscopically-proven
24 resolution of TNBS-colitis (mean 4 ± 1 unit per recording; ns). Spontaneous activity, normalized for
25 the number of units in each recording, tended to be higher in the post-colitis group (0.8 ± 0.1 imp. s^{-1}
26 for post-colitis vs 0.4 ± 0.1 for controls; N=12-13; $p=0.06$) (Figure 6B-C). Afferent nerve discharge to
27 colorectal distension, normalized for the number of units in each recording, was significantly
28 increased in rats that had recovered from colitis from a distension pressure of 15 mmHg onwards,
29 indicating sensitized afferent nerve responses (Figure 6D).
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39 The 57 single units from control specimens were identified as 11 LT, 16 WDR, 13 HT and 17 MIA,
40 whereas the 56 units from post-colitis rats were categorized as 10 LT, 18 WDR, 17 HT and 11 MIA
41 (Figure 7A). Although numerically there were less MIA in the preparations of post-colitis rats, in favor
42 of an increased presence of WDR and HT, the proportions were not significantly different between
43 control and post-colitis groups ($p=0.2$). Spontaneous afferent nerve discharge of LT and MIA was
44 more vigorous in post-colitis rats, whereas spontaneous activity of WDR and HT was similar in both
45 groups (table 2). The afferent response to colorectal distension was significantly increased in WDR
46 and HT fibers of rats that had recovered from TNBS-colitis compared to controls (Figure 7B-E).
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6. Discussion

In this study we studied whether enhanced splanchnic afferent signaling *in vitro* is associated with visceral hypersensitivity *in vivo* during acute TNBS-colitis and in the postinflammatory phase. Based on their discharge profile during colorectal distension, afferent units were categorized as LT, WDR, HT and MIA. During acute TNBS-colitis, spontaneous afferent discharge was significantly increased in LT and MIA, sensitization of LT, WDR and HT was evident as a leftward shift of the afferent response to colorectal distension and VMRs to colorectal distension were increased. In post-colitis rats, hypersensitivity to mechanical gut distension was mainly mediated by WDR and HT with little effect on LT. In addition, LT and MIA continued to display enhanced spontaneous activity after the resolution of TNBS-colitis. Also VMRs remained significantly increased in the post-inflammatory phase.

The characteristic features of visceral nociceptor sensitization include i) increased spontaneous afferent discharge, ii) enhanced response to suprathreshold stimulation, iii) activation by subthreshold stimuli and/or iv) recruitment of silent nociceptors (37). Several of these features were present in our experiments. Firstly, both LT and MIA demonstrated increased afferent discharge in the absence of colorectal distension. These LT most likely correspond to mucosal and muscular-mucosal afferents that signal sensory information in the physiological range, for instance on the presence and/or propulsion of fecal matter. Therefore sensitization of LT as evidenced by their enhanced spontaneous discharge (in addition to greater responses to limited levels of distension) could contribute to symptom generation and spontaneous abdominal pain in IBS patients. Also MIA displayed enhanced spontaneous activity. This class of afferent fibers is insensitive to colorectal distension per definition, and therefore does not contribute to the increased mechanosensitivity observed *in vivo*. Although we cannot fully exclude that some of these fibers may originate from other structures (such as the bladder for example), the enhanced spontaneous activity of these MIA most likely represent thermo- and chemosensitive signaling afferents. Considering the reports of increased spontaneous release of inflammatory and pro-nociceptive mediators in the colonic mucosa

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3 of patients with IBD and IBS, increased activity of such MIA could contribute to symptoms of
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5 abdominal pain.
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7 Secondly, enhanced responses to suprathreshold stimulation were evident for LT, WDR and HT fibers
8 during acute TNBS-colitis and sensitization of WDR and HT persisted in the post-inflammatory phase.
9 These findings are in agreement with a previous report of Hughes *et al.* (6). Using a different set-up
10 in which colonic segments were opened longitudinally and pinned out flat in a recording chamber,
11 Hughes *et al.* demonstrated predominant involvement of serosal and mesenteric fibers both during
12 acute TNBS-colitis and in the post-inflammatory phase of colitis in C57BL/6 mice (6). Serosal and
13 mesenteric afferents constitute the main fiber types of mouse splanchnic nerves and their peripheral
14 endings are located near the mesenteric attachment (7). They respond to distortion of the outer
15 layers of the colonic wall and levels of stretch into the noxious range (6, 38) and therefore most likely
16 correspond to the WDR and HT population in our experiments. In addition, we found evidence of
17 sensitization towards mechanical distension of LT. The data regarding the sensitization of LT fibers
18 are difficult to interpret as during acute TNBS-colitis LT responses were not significantly increased at
19 the lower levels of distension, reaching significance only for the plateau phase (20 mmHg onwards).
20 In the post-inflammatory phase of colitis, LT responses were only more vigorous at 40 to 45 mmHg of
21 distension, indicating that this subtype of splanchnic afferents most likely does not contribute
22 significantly to visceral hypersensitivity in the post-colitis rats. Previously, LT slowly-adapting fibers
23 were reported to display enhanced responses to circumferential stretch during acute TNBS-colitis in
24 the guinea pig (39). Similarly to our findings, LT responses recovered to normal once the
25 inflammation resolved. Finally, sensitization of afferent neurons can manifest as a lowering of the
26 activation threshold or through recruitment of silent nociceptors (37). Both would result in a shift of
27 the afferent functional phenotype. During acute TNBS-colitis, we found less MIA in splanchnic
28 afferent recordings in favor of mainly HT and also WDR whereas the proportion of LT remained
29 constant. In the post-inflammatory phase of colitis however, the fiber proportions were no longer
30 significantly different compared to control. Therefore our findings suggest that silent nociceptors,
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3 most likely MIA, are recruited during acute TNBS-colitis and may revert back into their silent
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5 phenotype after the inflammation has subsided. This hypothesis coincides with previous reports that
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7 MIA can be sensitized by local exposure to inflammatory mediators and can acquire a
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9 mechanosensitive phenotype (40), however this was not supported by all studies (8, 17).

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13 Both during acute TNBS-colitis as in the post-colitis phase, sensitized afferent responses were
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15 associated with enhanced VMRs to colorectal distension *in vivo*. Changes were more pronounced
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17 during acute colitis than after the resolution of colitis in both the *in vitro* and *in vivo* set-ups. Several
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19 reasons may underlie this difference. First, colonic compliance was reduced during acute TNBS-colitis
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21 but returned to baseline values after resolution of colitis. Colonic compliance has been shown to
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23 account for approximately 25% of visceral sensations in healthy subjects (41) and therefore may have
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25 altered the response profile during acute colitis. However by opting for a pressure-controlled
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27 distension of the colon, lower volumes will have been administered to the colon in the acute colitis
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29 group versus the control and post-colitis animals to achieve the same distension pressure.
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31 Nevertheless, reduced colonic compliance and altered connective tissue matrix and muscle tone will
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33 have influenced how colon is distended, which in turn can affect visceral sensation (42). Secondly,
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35 during acute colitis excessive amounts of cytokines and mediators among which ATP, protons and
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37 bradykinin are released that can act on sensory receptors located at the afferent nerve endings in the
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39 wall thus contributing to visceral hypersensitivity (5, 43, 44). While sensitizing effects can persist long
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41 after this 'inflammatory soup' has resolved, it is not surprising that this post-inflammatory visceral
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43 hypersensitivity might be of a lesser degree compared to the acute inflammation-induced
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45 hypersensitivity.

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52 Of note, in our study, we assessed the splanchnic mechanosensory signaling in relation of visceral
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54 hypersensitivity during acute colitis and in the post-inflammatory phase. However, sensory
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56 information from the distal colon is conveyed by both the splanchnic and pelvic nerve pathways.
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3 Although traditionally the pelvic nerve is thought to be tuned more towards the transmission of
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5 colonic sensations within the physiological range, our group as well as others have demonstrated
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7 altered pelvic mechanosensory function in different models of visceral hypersensitivity (6, 9, 12-16,
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9 45). With our study we do not wish to refute or contest the pelvic contribution to visceral
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11 hypersensitivity but mainly aimed to highlight the additional importance of splanchnic afferent nerve
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13 signaling (6, 8, 17, 46, 47).

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15 We have standardized the stimulus in both the *in vitro* and *in vivo* set-ups, namely mechanical
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17 distension which is the most commonly used stimulus to assess visceral sensory function in human
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19 experimental designs (48). In contrast to the flat-sheet preparation, our intact colonic segments do
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21 not allow us to identify the location of the afferent ending in the colonic wall (mucosal, muscular,
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23 serosal or mesenteric). Nevertheless, segmental distension is a physiological relevant way of
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25 demonstrating that these afferents are activated using an integrated stimulus and that sensitized
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27 responses contribute toward increased afferent discharge when the gut is distended. In addition we
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29 used the same methods and equipment in order to relate our *in vitro* to *in vivo* outcomes. Doing so,
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31 we demonstrate a good correlation between *in vitro* electrophysiological recordings and *in vivo*
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33 behavioral assays.
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40 In conclusion, we demonstrated that sensitization of splanchnic primary afferent neurons contributes
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42 significantly to visceral hypersensitivity during acute TNBS-colitis and in the post-inflammatory phase
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44 of colitis. Spontaneous afferent nerve discharge was increased in LT and MIA and could be associated
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46 with increased chemosensitivity. Sensitization to mechanical distension was a prominent feature of
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48 LT, WDR and HT fibers during acute colitis and the enhanced afferent discharge of WDR and HT
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50 persisted in the post-inflammatory phase. Importantly, we demonstrated that this enhanced afferent
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52 discharge to colorectal distension *in vitro* was correlated with visceral hypersensitivity *in vivo* as
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54 assessed by the VMRs. Although not indicative of a causative relationship, these findings may suggest
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that sensitization of splanchnic afferent nerves is an important factor in the pathogenesis of mechanical visceral hypersensitivity in the TNBS-colitis model.

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Author contribution: AD performed the experiments and wrote the article. AD, JDM, CK and WJ optimized the experimental methods. AD, JDM, CK, WJ, HUDS, PAP, SMF and BYDW provided intellectual contents, extensively revised the article and approved the final version.

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9. Abbreviations

AUC	area under the curve
DSS	dextran sulphate sodium
GEE	generalized estimating equations
HT	high threshold
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
LSD	least significant difference
LT	low threshold
MIA	mechanically insensitive afferent
MPO	myeloperoxidase
TNBS	trinitrobenzene sulphonic acid
VMR	visceromotor response
WDR	wide dynamic range

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10. Tables

Table 1. Spontaneous firing rate of single units in controls and rats with acute TNBS-colitis.

	Control (imp.s ⁻¹)	Colitis (imp.s ⁻¹)
LT	0.3 ± 0.1	1.2 ± 0.3 *
WDR	0.6 ± 0.2	0.9 ± 0.1
HT	0.6 ± 0.2	0.6 ± 0.2
MIA	0.4 ± 0.1	1.1 ± 0.1 **

Results expressed as impulses per second and presented as mean ± sem
n=8-22. LT, low threshold; WDR, wide dynamic range; HT, high
threshold; MIA, mechanically insensitive afferents. Unpaired Student's *t*-
test; * p<0.05, ** p<0.01, significantly different from control.

Table 2. Spontaneous firing rate of single units in controls and rats that had recovered from TNBS-colitis.

	Control	Post-colitis
	(imp.s⁻¹)	(imp.s⁻¹)
LT	0.3 ± 0.1	0.8 ± 0.3 *
WDR	0.6 ± 0.2	0.8 ± 0.2
HT	0.6 ± 0.2	0.4 ± 0.1
MIA	0.4 ± 0.1	1.0 ± 0.2 *

Results expressed as impulses per second and presented as mean ± sem for N=12-13 and n=10-18. LT, low threshold; WDR, wide dynamic range; HT, high threshold; MIA, mechanically insensitive afferents. Unpaired Student's *t*-test; * *p*<0.05, significantly different from control.

Pre-Review

Figures legends

Figure 1. Schematic diagram of the experimental design. In set-up 1 (acute colitis), rats were treated with TNBS (colitis) or saline (control) and visceral sensitivity was assessed 3 days later by quantifying VMRs and splanchnic afferent nerve discharge to colorectal distension. In set-up 2, rats were treated with TNBS or saline and the extent and healing process of colitis was monitored individually by repeated colonoscopy: first on day 3 to confirm the presence of colitis and thereafter, starting from day 10, every 4 days until complete mucosal healing (score = 0) had occurred. Visceral sensitivity was evaluated 3 days after colonoscopic mucosal healing by quantifying VMRs and splanchnic afferent nerve discharge to colorectal distension. In both set-up 1 and set-up 2, the inflammatory status was assessed by colonoscopic, macroscopic and microscopic evaluation of the colonic tissue in addition to a myeloperoxidase activity assay. TNBS, trinitrobenzene sulphonic acid; VMR, visceromotor response.

Figure 2. The effect of acute TNBS-colitis (filled squares) compared to controls (open triangles) on visceromotor responses (VMRs) to colorectal distension (**A**) and colonic compliance (**B**). Generalized estimating equations, LSD post-hoc test; N=5/group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significantly different compared to controls (open triangles).

Figure 3. The effect of acute TNBS-colitis on spontaneous and distension-induced splanchnic afferent nerve firing. Closure of the segmental outflow while maintaining the inflow resulted in colonic ramp distension (**A**). Representative tracings of spontaneous and distension-induced afferent discharge are shown in **B** and **C** respectively. Afferent firing during colorectal distension (**D**) was corrected for spontaneous activity, normalized to the number of units in each recording and expressed as impulses per second (imp/s). Generalized estimating equations, LSD post-hoc test; N=12-17/group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significantly different compared to controls.

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3 **Figure 4.** The proportion of LT, WDR, HT and MIA in recordings from 12 control rats and 17 rats with
4 acute TNBS-colitis. Chi-square test for n=57-60; * p<0.05, proportions significantly different
5 compared to controls (**A**). Afferent firing to colorectal distension for LT, WDR, HT and MIA are shown
6 in **B, C, D** and **E** respectively. Afferent firing was corrected for spontaneous activity. Generalized
7 estimating equations, LSD post-hoc test; n=10-22/group; * p<0.05, ** p<0.01, *** p<0.001,
8 significantly different compared to controls. LT, low threshold; WDR, wide dynamic range; HT, high
9 threshold; MIA, mechanically insensitive afferents.
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20 **Figure 5.** Visceromotor responses (VMRs) to colorectal distension (**A**) and colonic compliance (**B**) in
21 control rats (open triangles) and rats with colonoscopically-proven resolution of TNBS-colitis (filled
22 circles). Generalized estimating equations, LSD post-hoc test; N=5-10/group; * p<0.05, ** p<0.01, ***
23 p<0.001, significantly different compared to controls.
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31 **Figure 6.** Spontaneous and distension-induced splanchnic afferent nerve firing in control rats and rats
32 with colonoscopically-proven resolution of TNBS-colitis. Closure of the segmental outflow while
33 maintaining the inflow resulted in colonic ramp distension (**A**). Representative tracings of
34 spontaneous and distension-induced afferent discharge are shown in **B** and **C** respectively. Afferent
35 firing during colorectal distension (**D**) was corrected for spontaneous activity, normalized to the
36 number of units in each recording and expressed as impulses per second (imp/s). Generalized
37 estimating equations, LSD post-hoc test; N=12-13/group; * p<0.05, ** p<0.01, *** p<0.001,
38 significantly different compared to controls.
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50 **Figure 7.** The proportion of LT, WDR, HT and MIA in recordings from 12 control rats and 13 rats with
51 colonoscopically-proven resolution of TNBS-colitis. Chi-square test for n=56-57; no significant
52 difference (**A**). Afferent firing to colorectal distension for LT, WDR, HT and MIA are shown in **B, C, D**
53 and **E** respectively. Afferent firing was corrected for spontaneous activity. Generalized estimating
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3 equations, LSD post-hoc test; n=10-18; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significantly different
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5 compared to controls. LT, low threshold; WDR, wide dynamic range; HT, high threshold; MIA,
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7 mechanically insensitive afferents.
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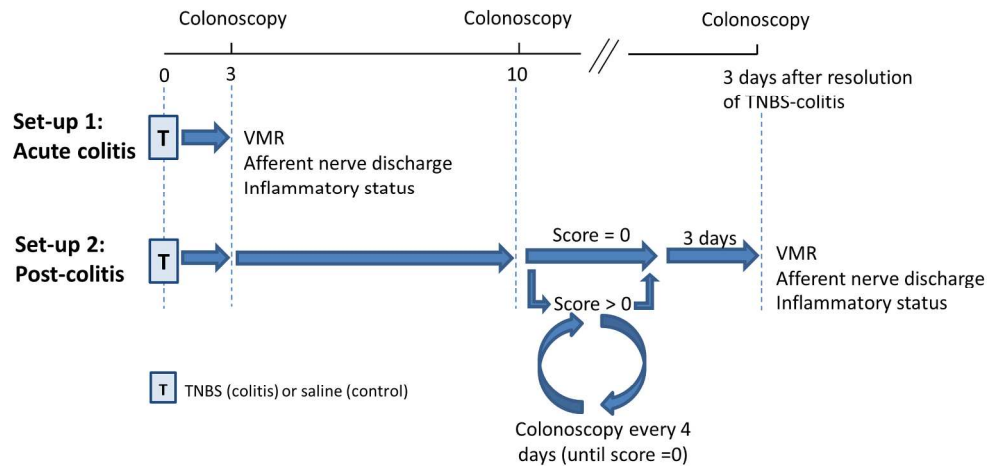


Figure 1. Schematic diagram of the experimental design. In set-up 1 (acute colitis), rats were treated with TNBS (colitis) or saline (control) and visceral sensitivity was assessed 3 days later by quantifying VMRs and splanchnic afferent nerve discharge to colorectal distension. In set-up 2, rats were treated with TNBS or saline and the extent and healing process of colitis was monitored individually by repeated colonoscopy: first on day 3 to confirm the presence of colitis and thereafter, starting from day 10, every 4 days until complete mucosal healing (score = 0) had occurred. Visceral sensitivity was evaluated 3 days after colonoscopic mucosal healing by quantifying VMRs and splanchnic afferent nerve discharge to colorectal distension. In both set-up 1 and set-up 2, the inflammatory status was assessed by colonoscopic, macroscopic and microscopic evaluation of the colonic tissue in addition to a myeloperoxidase activity assay. TNBS, trinitrobenzene sulphonic acid; VMR, visceromotor response.

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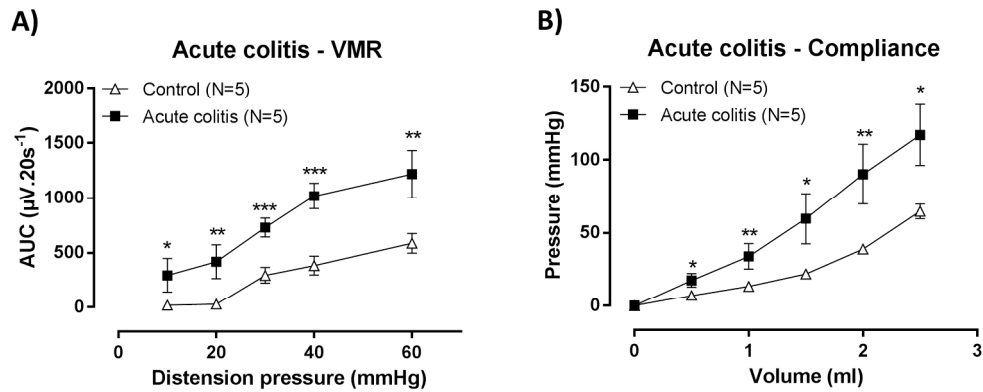


Figure 2. The effect of acute TNBS-colitis (filled squares) compared to controls (open triangles) on visceromotor responses (VMRs) to colorectal distension (A) and colonic compliance (B). Generalized estimating equations, LSD post-hoc test; N=5/group; * p<0.05, ** p<0.01, *** p<0.001, significantly different compared to controls (open triangles).

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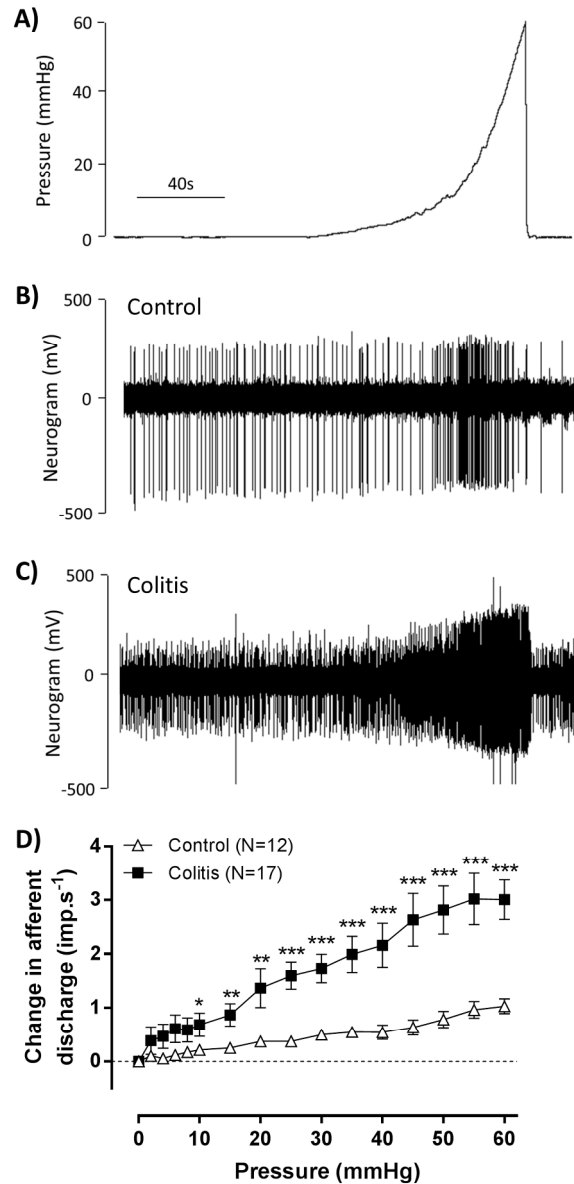


Figure 3. The effect of acute TNBS-colitis on spontaneous and distension-induced splanchnic afferent nerve firing. Closure of the segmental outflow while maintaining the inflow resulted in colonic ramp distension (A). Representative tracings of spontaneous and distension-induced afferent discharge are shown in B and C respectively. Afferent firing during colorectal distension (D) was corrected for spontaneous activity, normalized to the number of units in each recording and expressed as impulses per second (imp/s). Generalized estimating equations, LSD post-hoc test; N=12-17/group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significantly different compared to controls.

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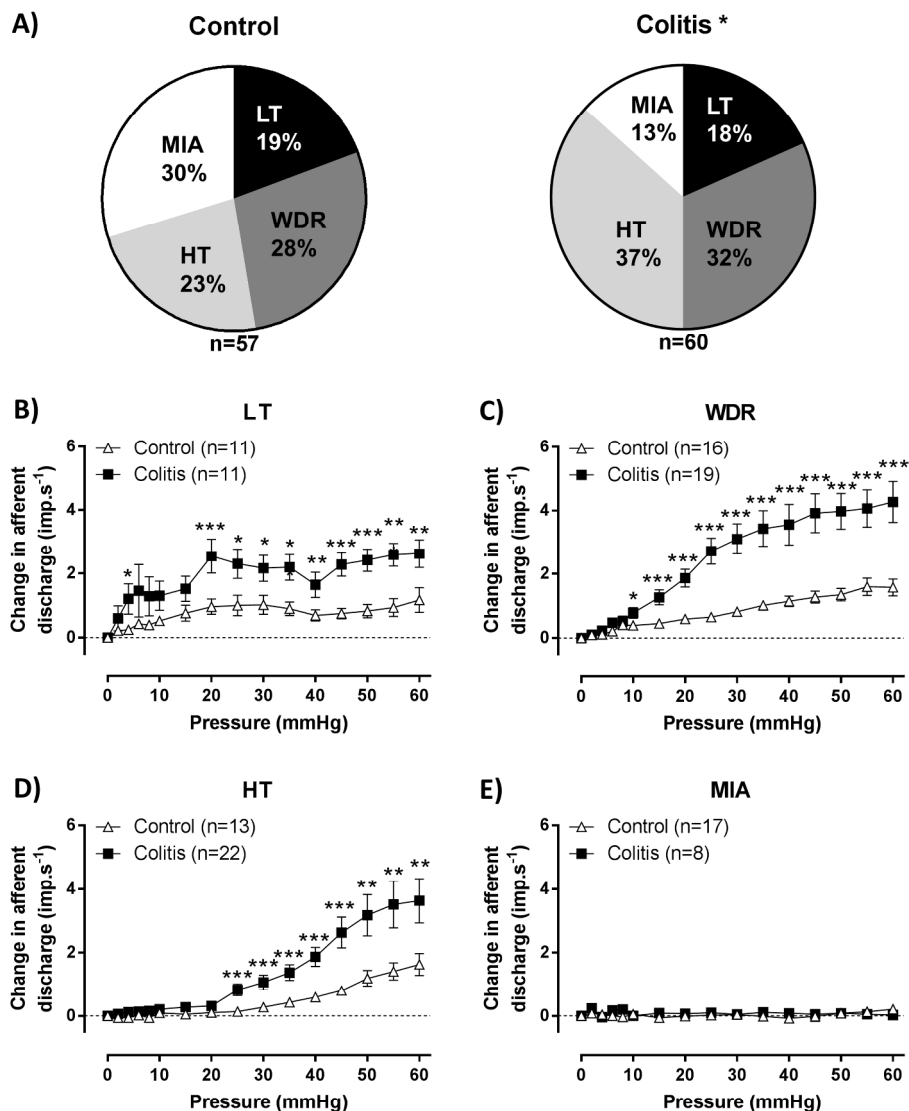


Figure 4. The proportion of LT, WDR, HT and MIA in recordings from 12 control rats and 17 rats with acute TNBS-colitis. Chi-square test for $n=57-60$; * $p<0.05$, proportions significantly different compared to controls (A). Afferent firing to colorectal distension for LT, WDR, HT and MIA are shown in B, C, D and E respectively. Afferent firing was corrected for spontaneous activity. Generalized estimating equations, LSD post-hoc test; $n=10-22$ /group; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, significantly different compared to controls. LT, low threshold; WDR, wide dynamic range; HT, high threshold; MIA, mechanically insensitive afferents.
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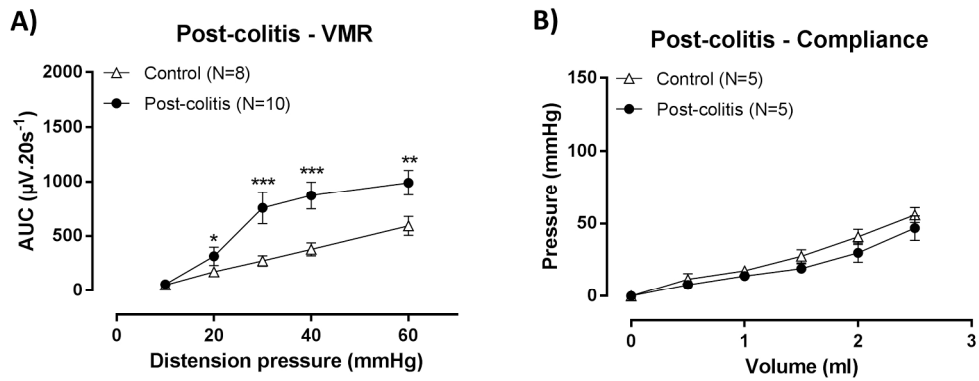


Figure 5. Visceromotor responses (VMRs) to colorectal distension (A) and colonic compliance (B) in control rats (open triangles) and rats with colonoscopically-proven resolution of TNBS-colitis (filled circles). Generalized estimating equations, LSD post-hoc test; N=5-10/group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significantly different compared to controls.
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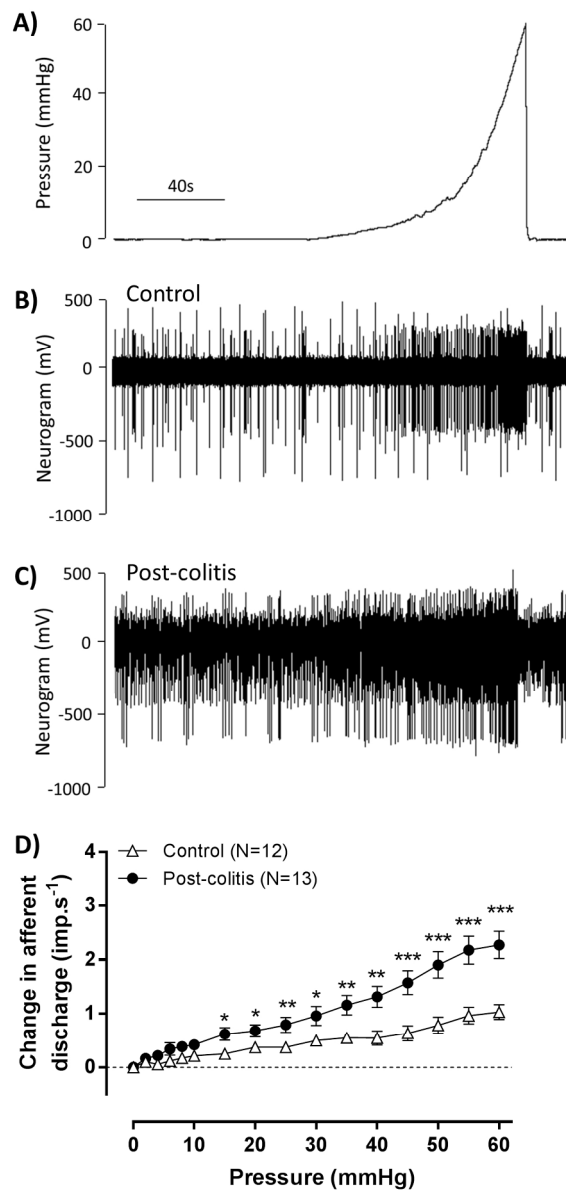


Figure 6. Spontaneous and distension-induced splanchnic afferent nerve firing in control rats and rats with colonoscopically-proven resolution of TNBS-colitis. Closure of the segmental outflow while maintaining the inflow resulted in colonic ramp distension (A). Representative tracings of spontaneous and distension-induced afferent discharge are shown in B and C respectively. Afferent firing during colorectal distension (D) was corrected for spontaneous activity, normalized to the number of units in each recording and expressed as impulses per second (imp/s). Generalized estimating equations, LSD post-hoc test; N=12-13/group; * p<0.05, ** p<0.01, *** p<0.001, significantly different compared to controls.

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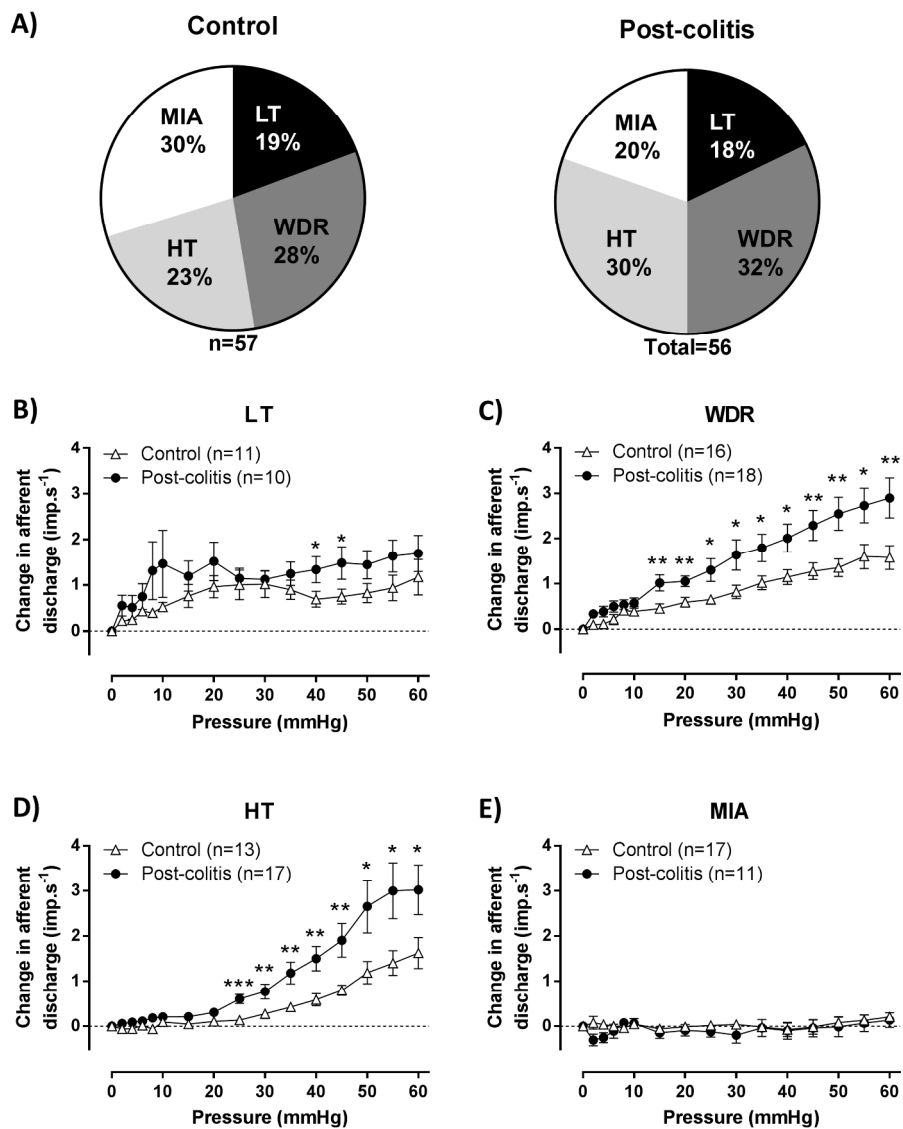


Figure 7. The proportion of LT, WDR, HT and MIA in recordings from 12 control rats and 13 rats with colonoscopically-proven resolution of TNBS-colitis. Chi-square test for n=56-57; no significant difference (A). Afferent firing to colorectal distension for LT, WDR, HT and MIA are shown in B, C, D and E respectively. Afferent firing was corrected for spontaneous activity. Generalized estimating equations, LSD post-hoc test; n=10-18; * p<0.05, ** p<0.01, *** p<0.001, significantly different compared to controls. LT, low threshold; WDR, wide dynamic range; HT, high threshold; MIA, mechanically insensitive afferents. 190x254mm (300 x 300 DPI)

1 We are very pleased that the editorial board has shown interest in our manuscript entitled
2 “Mechanisms contributing to visceral hypersensitivity: focus on splanchnic afferent nerve signaling”.
3 We thank you and the two expert referees for the valuable and positive comments. In accord with
4 their suggestions we have revised the manuscript to address their concerns. Please find below a
5 detailed point-to-point reply to the comments that were raised. We hope we addressed the questions
6 satisfactorily.
7

8 9 Response to the editor

10 Part of this work was presented at nEUROgastro 2015 [1], sponsored by the European
11 Neurogastroenterology and Motility Society, as now stated at the end of the introduction, page 5 of the
12 revised manuscript.
13

14 15 Response to reviewer 1

16 1. The authors need to explicitly describe in this submission how low threshold, WDR and HT
17 afferents are characterized from one another. This is crucial as the authors are reporting changes in
18 the proportion of these afferent subtypes in the colitis model.
19 Based on the firing discharge profile during colorectal distension, units were categorized as low
20 threshold (LT), wide dynamic range (WDR), high threshold (HT) and mechanically insensitive
21 afferents (MIA) as previously published [2-4]. First, the firing rate at 20 mmHg distension is expressed
22 as the percentage of the response at 60 mmHg. This parameter, referred to as LT%, reflects the
23 relative firing at low compared to high distension pressures and was used to further characterize
24 afferent responses based on their functional features as previously published [3]. An LT% of 40%
25 represents a linear increase in afferent discharge. An LT% of >55%, indicates the main increase in
26 afferent firing occurs during limited colorectal distension which is characteristic for LT. An LT% of
27 <15% is characteristic for HT as this implies a disproportionate increase in afferent firing during
28 noxious distension. WDR have an LT% between 15 and 55. Finally, MIA are defined as afferent fibers
29 displaying spontaneous activity that does not increase during colonic distension.
30 This is described in the revised manuscript on page 8.
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32 2. In addition, MIA afferents have been defined as ‘afferent fibers displaying spontaneous activity
33 but do not increase during colonic distension’. However, depending on exactly where the recordings
34 are performed from this firing is not necessarily indicative of an afferent innervating the colon. If the
35 recording is the spinal side of the inferior mesenteric ganglion it could in fact be an afferent
36 innervating another organ (bladder for example). This needs to be clarified by the authors.
37 We agree with the reviewer that although we dissected the tissue in such a way that only the colon
38 and a minimum of mesenteric tissue was present, we cannot exclude that some non-mechanosensitive
39 afferent firing to relate to innervation of other tissue beside the colon.
40 This is now included in the discussion of the revised manuscript on page 13-14.
41

42 3. The authors should discuss why they used a relatively high concentration of ethanol (50%) in
43 their TNBS studies when other studies have used lower ethanol concentrations. This may be a simply
44 issue of comparing rats vs. mice but it warrants a comment for comparative purposes.
45 Indeed, the concentration of ethanol is relatively high. However, we found this dose of TNBS (15 mg)
46 in combination with 50% of ethanol optimal to induce a reproducible degree of colitis in our Sprague-
47 Dawley rats. This is higher compared to the dose and concentration we previously used in Wistar rats
48 (7.5 mg of TNBS in 40% of ethanol) [5] and Swiss mice (10 mg of TNBS in 30% ethanol) [6] and most
49 likely relates to species and strain differences in the susceptibility to TNBS-induced colitis as was
50 previously discussed by others [7].
51

52 A comment on this was inserted in the methods section of the revised manuscript on page 5.
53

54 4. The authors have measured colonic compliance in the control, acute colitis and post-colitis
55 animals. Notably, they show that compliance is only altered in acute colitis and not in the post-colitis
56 animals. However, they make no point of comment in the discussion on how this may alter their
57 findings. This should be addressed, as the altered compliance is likely to alter the response profiles in
58 the acute colitis animals. Essentially there is a leftward shift in the pressure curve vs. volume and the
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1 authors will actually be administering higher pressures at any given volume in the acute colitis vs the
2 control and post-colitis animals. This will have a direct effect on afferent sensitivity and may explain
3 some of their alterations in afferent and VMR responses. This should be clarified and commented on
4 in the discussion.

5 Indeed, colonic compliance was reduced in the acute colitis setting compared to control and post-
6 colitis rats as evidenced by a leftward shift of the pressure-volume curve. In a volume-controlled
7 setting this would indeed mean that higher pressures are administered at any given volume in the
8 acute colitis vs the control and post-colitis animals. However, as we have opted for a pressure-
9 controlled system, this means that to achieve the same distension pressures lower volumes will be
10 administered in the acute colitis versus the control and post-colitis animals. Nevertheless, reduced
11 colonic compliance and altered connective tissue matrix and muscle tone will have influenced how
12 colon is distended, which in turn can affect visceral sensation [8]. This paragraph is now included in
13 the discussion of the revised manuscript (page 15-16).

14 5. Discussion. Page 14 Line 18. The authors statement starting with "However it remains
15 uncertain whether distending a segment to 60mmHg is a large enough stimulus to activated these
16 mesenteric/serosal afferents" and continues until line 34. The authors should read "Selective Role
17 for TRPV4 Ion Channels in Visceral Sensory Pathways" by Brierley et al., 2008 Gastroenterology and
18 adjust their conclusion accordingly. This paper compares distension in intact preparations, vs. flat
19 sheet von frey hair and stretch responses. This would suggest that that these afferents are indeed
20 activated by the various stimuli at quite specific mechanical stimulation intensities in the noxious
21 range. If the authors accordingly altered their comments based on these finding, it would actually help
22 to explain their own results.

23 We thank the reviewer for the suggestion. In the paper of Brierley et al, it was shown that colonic
24 mechanoreceptors with receptive fields on serosa and mesentery responded to high intensities of
25 mechanical stimulation with von Frey hairs (>50% above 400 mg). This was correlated with a high
26 threshold to distention of the intact colon by balloon (45 ± 3.2 mmHg) or circumferential stretch of
27 the preparation as a flat sheet (10.6 ± 0.3 g) indicating that indeed 60 mmHg is a large enough
28 stimulus to activate mesenteric/serosal afferents.

29 The paragraph was adjusted accordingly (page 14 of the revised manuscript).

30 Response to reviewer 2

31 1. This paper represents a solid piece of work from a solid group with a good reputation in their
32 field. The methods are well-established and the interpretation is spot on up to a point. The point at
33 which interpretation becomes too speculative is unfortunately the whole point of the study, which is
34 to determine the importance of the splanchnic pathway in visceral pain. What the authors do is to
35 correlate changes in splanchnic afferent fiber mechanosensitivity with changes in behavioral
36 responses to mechanical stimuli. It is commendable that they have standardized the stimulus in both
37 cases, which has not been done before. The problem is they have done nothing to prove that the
38 splanchnic pathway actually mediates the behavioral response. To do this they would have to lesion
39 the nerves somehow and measure VMR before and after. If they can do this and add it to the paper it
40 would make a massive difference to its impact.

41 It is clear in several species that the splanchnic afferent innervation extends as far as the distal colon,
42 whereupon the pelvic pathway takes over, with a small area of overlap of the two. The group of NJ
43 Spencer has shown that the VMR to rectal distension is abolished by rectal nerve section (which are
44 branches of the pelvic nerve). This is not at all surprising. Similarly I would expect that responses to
45 distension of the distal colon would be abolished by section of the lumbar colonic or splanchnic
46 nerves. It is all a simple matter of anatomy. What is the important question is the pathway of pain in
47 the clinic and/or the pathway of pain in response to a particular type of stimulus. To this end it is
48 critical to know where is the balloon relative to the two sources of innervation.

49 I think the authors need to either do the extra experiments to determine the contribution of the
50 splanchnic nerve, or change their conclusions substantially to make it clear that they are seeing a
51 correlation, not a causative relationship, between splanchnic afferent activation and VMR.
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1 The paper is largely in agreement with others on effects of inflammation on sensory pathways. Thus
2 its uniqueness in its current form is performing VMR and afferent studies using the same methods and
3 equipment, which makes it easier to correlate the two. This needs to be better advertised instead of
4 the current conclusions which are too bold.

5 We agree with the reviewer that we might have been too bold in drawing conclusions from our data as
6 it is indeed a correlation or association that we see between the afferent recordings and the
7 visceromotor responses, which by itself does not suggest a causative relationship.

8 The idea of leaisioning the splanchnic nerve in vivo followed by VMR assessment is intriguing. Indeed,
9 in the paper of Nick Spencer's group, referred to by the reviewer, transection of the rectal nerves
10 abolished VMRs to rectal distension [9]. In the same paper, the authors described that similarly
11 leaisioning the lumbar nerves did not affect VMRs to rectal distension in healthy control mice under
12 general anesthesia. It would be interesting to evaluate the effect of splanchnic afferent nerve
13 transection on the VMRs in our model to determine the exact contribution of this sensory pathway to
14 the visceral hypersensitivity.

15 However, there are some difficulties in performing these experiments. First, the surgery is quite
16 challenging as the splanchnic afferents are located deep in the abdomen close to the aorta, however
17 this is only a matter of practice. Nevertheless, to perform these experiments in sufficient number of
18 rats in the three experimental conditions (control, acute colitis and post-colitis) is almost a completely
19 new project by itself. Secondly, a major difference between the methodology of the Spencer group and
20 our approach is that we assess VMRs in non-sedated, fully awake rats, as anesthetics are known to
21 significantly alter or even abolish VMRs [10]. This makes it nearly impossible to transect the
22 splanchnic nerve in vivo and afterwards perform VMR assessments as effects due to the surgery itself
23 such as post-operative ileus and other motility dysfunctions due to severing the efferent pathways
24 will undoubtedly impede correct interpretation of the results [11]. Also, transecting the splanchnic
25 nerve will impact bladder function, which due to the crosstalk between bladder and colon might also
26 affect colonic sensitivity as previously published [12]. Finally, there is the aspect of postoperative pain
27 as you open the abdominal muscles and go deep into the abdominal cavity which could affect afferent
28 function.

29 Therefore, although we agree that these experiments would be highly interesting, we decided to
30 follow the reviewer's advice of revising our conclusions as currently we have insufficient evidence to
31 demonstrate a causative relationship and instead focus on the correlation between splanchnic
32 afferent activation and VMR.

33 This is now altered in the abstract and throughout the discussion.

34 Regarding the comment of the reviewer on where the balloon is relative to the splanchnic and pelvic
35 innervation we believe that with our balloon distension in vivo we stimulate both nervous pathways
36 as the balloon covers the segment of the colon that is dissected for the in vitro electrophysiological
37 recordings as can be seen in the picture below. However, we fully acknowledge that this is purely
38 speculative.

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46 2. It is important to note that the VMR has some major weaknesses when it comes to correlation
47 with pain. How much is an effort to expel the balloon and how much is a wincing response to pain is
48 very difficult to say. The fact that the response can be evoked by low intensity stimuli suggests it is not
49 all pain-related. The group led by Larsson investigated this a few years ago, showing that blood
50 pressure and heart rate are much better indices of pain perception. Just because VMR has been used
51 extensively in this field does not mean it is the gold standard. It would be good to see discussion of
52 these aspects.

53
54 It is true that although the VMR remains the most commonly used method to assess visceral
55 hypersensitivity to colorectal distension in vivo other techniques are available such as using telemetry
56 to monitor heart rate and blood pressure during distensions. Both the cardiovascular and
57 visceromotor responses act via brainstem loops and are reduced or abolished by classis analgesics
58 among which morphine [10]. There are few papers that compared VMRs and cardiovascular
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1 responses head-to-head. As the reviewer already mentioned, the group of Larsson published several
2 papers evaluating the antinoceptive potential of among others baclofen and pregabalin on visceral
3 pain looking at VMR, blood pressure and heart rate simultaneously [13,14]. However, in their
4 discussion they state that 'the VMR is most sensitive [to inhibition] when compared to cardiovascular
5 responses'. They hypothesize that this 'might point toward the VMR being more tightly controlled by
6 nociceptive input, with cardiovascular responses to colorectal distension being affected by other
7 factors such as emotional state' [13]. Therefore, we are not sure which paper of the group of Larsson
8 the reviewer refers to. Nevertheless, we fully acknowledge that the VMR is not the only technique to
9 assess visceral sensitivity, nor did we intend to claim that it is the gold standard. However, as a
10 discussion on the different methods to assess visceral hypersensitivity is beyond the scope of our
11 manuscript, we hope we comply to the concern of the referee by referring to alternative techniques to
12 assess visceral hypersensitivity in vivo in the methods section on page 7.

13 3. The discussion of the paper is too long, and strays often from the subject onto matters
14 unrelated to this study.

15 The discussion has been extensively reworked to address the reviewer's remark.

16 REFERENCE LIST

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18 mechanisms contributing to visceral hypersensitivity in a rat model of acute and postinflammatory
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