Postoperative ileus: Pathophysiology & treatment strategies
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Chapter 6

Neuroanatomical evidence demonstrating the existence of the vagal anti-inflammatory reflex in the intestine


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Abstract

**Background:** The cholinergic anti-inflammatory pathway is proposed to be part of the so-called vago-vagal “inflammatory reflex”. The aim of this study is to provide neuro-anatomical evidence to support the existence of a functional neuronal circuit and its activation in response to intestinal inflammation.

**Methods:** The expression of c-Fos was evaluated at different levels of the neurocircuitry in the course of postoperative ileus (POI) in a mouse model. Specific activation of the motor neurons innervating the inflamed intestine and the spleen was monitored by retrograde tracing using cholera toxin-b. The role of the vagal afferent pathway nerve was evaluated by selective vagal denervation of the intestine.

**Results:** Abdominal surgery resulted in subtle inflammation of the manipulated intestine at 24hrs (late phase), but not after 2 and 6 hrs (early) after surgery. This local inflammation was associated with activation of neurons in the nucleus of the solitary tract and in the dorsal nucleus of the vagus. The vagal output mainly targeted the inflamed zone: 42% of motor neurons innervating the intestine expressed cFos IR in contrast to 7% of those innervating the spleen. Vagal denervation of the intestine abolished cFos expression in the brain nuclei involved in the neuronal network activated by intestinal inflammation.

**Conclusions:** Our data demonstrate that intestinal inflammation triggers a vagally mediated circuit leading mainly to activation of vagal motor neurons connected to the inflamed intestine. These findings for the first time provide neuro-anatomical evidence for the existence of the endogenous “inflammatory reflex” and its activation during inflammation.
Introduction

Vago-vagal reflexes coordinate gastric and intestinal digestive functions and motility\(^1\). These functions are controlled through functionally distinct pathways, called neurocircuits, connecting the intestine with the brain stem nuclei, i.e., the nucleus of the solitary tract (NTS) and the dorsal motor nucleus of the vagus (DMV). Mechanical (i.e., contraction, distention) and chemical (i.e., nutrients) signals are transmitted to the NTS via the vagal ascending fibers. After integration of the incoming information and through neuronal communication between the NTS and the DMV, vagal efferent output is triggered to adjust gastrointestinal (GI) functions such as secretion, absorption and motility.

Recently, it was suggested that also the immune system is modulated by the vagus nerve. Tracey and coworkers demonstrated that electrical stimulation of the vagus nerve prevents the development of endotoxin-induced shock by reduction of pro-inflammatory cytokine production, in particular TNF\(_\alpha\) in the spleen\(^4, 5\). This anti-inflammatory effect is mediated by activation of nicotinic receptors located on immune cells (in particular macrophages) in response to acetylcholine released by vagal efferent nerve terminals\(^6, 7\). The discovery of the cholinergic anti-inflammatory vagal efferent pathway introduced the concept of the “inflammatory reflex” by which the central nervous system is capable of sensing inflammation and subsequently modulating the immune response\(^8, 9\). To date, however no anatomical evidence has been reported supporting the existence of this neurocircuit.

Recently, we extended the anti-inflammatory role of the vagal efferent route in a confined/subtle model of inflammation, i.e. post-operative ileus (POI)\(^10\). POI is characterized by a generalized hypomotility of the GI tract, and occurs after almost every abdominal surgical procedure. The prolonged paralytic phase of POI is mediated by an inflammatory response in the muscular layer of the intestine triggered by activation of resident macrophages following intestinal handling\(^11, 12\). Previously, we demonstrated that electrical stimulation of the vagus nerve inhibits the production of pro-inflammatory cytokines, reduces intestinal inflammation and shortens POI\(^10\). This effect was blocked by incubation of the intestine with the nicotinic receptor antagonist hexamethonium, indicating vagal modulation of the intestinal immune response within the intestinal wall. To what extent this anti-inflammatory mechanism is also endogenously activated during inflammatory conditions, such as POI, and is part of a vago-vagal neurocircuitry remains however unclear.

The expression of the nucleoprotein Fos, a product of the c-fos immediate-early gene, is widely considered a marker of neuronal activity and has been used repeatedly to map functional brainstem pathways in response to various stimuli\(^13-15\). The
distribution of cFos expression induced by abdominal surgery enlightened specific neural circuits including brainstem nuclei (NTS, locus coeruleus, caudal ventral medulla and cuneate, lateral parabrachial nuclei) but also hypothalamic ones such as the paraventricular nucleus PVN. In the current study, we focused on the expression of cFos at different levels of the neuronal pathway proposed to mediate the vagal anti-inflammatory mechanism. Finally, selective vagal denervation of the small intestine was performed to further evaluate the role of the vagus nerve in the modulation of peripheral inflammation.

**Materials and Methods**

**Animals**

Mice (female BALB/c; Harlan Nederland, Horst, The Netherlands) were kept in 12h light/12h dark cycle (lights on at 8:00 AM to 8:00 PM) under constant conditions of temperature (20± 2°C) and humidity (55% humidity) with water and food *ad libitum*. Mice were studied at 10-12 weeks of age. All experiments were conducted in accordance with the guidelines of the Ethical Animal Research Committee of the Academic Medical Center.

**CTB injection in peripheral organs: small intestine and spleen**

The cholera toxin-B conjugated with Alexa Fluor 555 (1%; CTB-Alexa Fluor 555) (Molecular Probes, USA) was used to label the neuronal innervation of the small intestine and the spleen. 0.5µl of the retrograde tracer was injected (flow of 0.5µl/min) at different spots (5) along the ileum. 3µl of the tracer was injected in both ends of the spleen. The injection was performed with a fused silica tubing (40µm i.d., 105µm o.d.) (Aurora Borealis control, Schoonebeek, The Netherlands) protected at its end by a 30-gauge 1/2 needle. The probe was connected to an injection pump via a guide PEEK tube (PK005-02, Aurora Borealis control). The time period required for a retrograde tracer to reach the brainstem was estimated to be 7 days. However, to avoid possible interference of inflammation triggered by tracer injection, we expanded the period of recovery to 15 days. To validate the specificity of the tracer injection within the small intestine, we performed injections of CTB-fluorophore 555 and 647 in the proximal and distal part of the ileum. Moreover injection of the same volume of the tracer was applied in the peritoneal cavity.

To further validate the specificity of the tracer protocol evaluating the vagal innervation of the spleen, we added an additional control in which tracer was applied on the top of the splenic hilum (i.e., the point of insertion of the splenic artery and vein). Finally, we included a group of mice that underwent vagal denervation of the spleen followed by
CTb injection at the tip of the organ. Loss of CTb positive neurons in the DMV indicated successful (i.e., complete) vagal denervation of the spleen.

Experimental protocols

Protocol 1: Neuronal circuitry and cFos expression at different time points post-surgery

Control animals (no treatment) and animals that underwent laparotomy (L) or intestinal manipulation (IM) were examined at different time points after surgery: L and IM at 2, 6 and 24 hrs (n = 24) and controls at 2 hrs (n = 12). The controls mice were divided into two groups: one group underwent standard anesthesia (Control group) whereas the other was left untouched to estimate the baseline expression of cFos proteins (Baseline (BL) group). Mice were sacrificed by transcardiac perfusion with Phosphate buffered saline (PBS) followed by 4% paraformaldehyde PFA (pH 7.4). Brains and nodose ganglia were collected, post-fixed for 4 hrs (4°C) and cryo-protected by immersion with 30% sucrose in 0.2M PBS (pH 7.4) at 4°C overnight. Intestinal tissue was collected prior to PFA perfusion. Intestinal tissue was cut along the mesentery border, washed in cold saline and directly fixed into 100% ethanol for 30 min and then kept in 70% ethanol at 4°C until analysis.

Protocol 2: The spleen as target of the endogenous vagal anti-inflammatory pathway

Intestinal inflammation leads to a consistent cFos expression in the DMV that is not restricted to the small intestine (Figure 3A, B). Recent data identified the spleen as an important player in the cholinergic anti-inflammatory mechanism. Since a direct vagal innervation of the spleen has been recently identified, we decided to investigate whether the vagal motor neurons that innervate directly the spleen express cFos IR upon intestinal inflammation, i.e. 24 hrs after IM. Mice (n = 12) were injected with CTB in the spleen. Two weeks after recovery, mice underwent either laparotomy (n = 5) or IM (n = 7). 24 hrs later, mice were sacrificed with transcardiac perfusion, brain and intestinal tissue was collected.

Protocol 3: Selective vagal denervation of the small intestine: analysis 24hrs post-surgery

To evaluate the role of the afferent vagal pathway in the activation of the anti-inflammatory efferent, the small intestine was selectively denervated (i.e., disruption of the sensory and motor nerves fibers of the vagus, IntX) and the effect on cFos expression in the brain stem nuclei involved in the “inflammatory reflex” was studied. Four groups were examined: L, IM, L+IntX and IM+IntX. In short, vagal denervation (n = 24) or sham-operation (n = 16) mice received intestinal CTB injection. Two weeks later, mice underwent IM or L and sacrificed 24hrs after surgery with transcardiac perfusion, as described above. Brain and intestinal tissue were collected.
Surgical procedures
Surgical experiments started at 8:30 am. Mice were operated at 11-13 weeks of age after one week of adaptation in the animal facilities. For all surgical procedure (tracer injection, denervation, intact, L or IM), mice were anesthetized by an intraperitoneal (i.p.) injection of a mixture of fentanyl citrate/fluanisone (Hypnorm; Janssen, Beerse, Belgium) and midozolam (Dormicum; Roche, Mijdrecht, The Netherlands). The anesthetic mixture was in a ratio of 1:1:2 of hypnorm, midazolam and water respectively. Subcutaneous injection of fynadine (0.03ml/100g, from a 10x diluted stock solution, 50mg/ml) was performed after the first surgery (i.e., denervation or injection of the tracer). The same length of the midline incision was performed in all surgical protocols (i.e. sham-operated vs denervated, L vs IM).

CTB injection: In protocols 1 & 3, mice were injected with the neuronal tracer in the small bowel. In brief, the animals were anesthetized and the abdomen was opened by a midline incision. The small bowel was carefully eviscerated and placed on a moist gauze pad to allow CTB injections. In protocol 2, mice underwent CTB injection in the spleen.

Vagal intestinal denervation (IntX): The right celiac branch of the vagus nerve supplies the jejunum, ileum and caecum and is embedded in fat/connective tissue that lies above the superior mesenteric artery. After removal of this fat/connective tissue, the nerve was cut. Vagal denervation was combined with CTB injection to evaluate the success of the denervation procedure. The absence of CTB labeled motor neurons in the DMV is considered indicative for complete vagal denervation of the bowel.

Intestinal manipulation (IM) or laparotomy (L): Mice underwent L or L followed by IM, as previously described. In brief, a midline abdominal incision was performed along the linea alba and the peritoneum was opened. The small bowel was carefully removed from the peritoneal cavity and positioned on a moist gauze pad. The entire small bowel was manipulated from the distal duodenum to the cecum with moist cotton applicators for 5 minutes. Contact or stretch on the stomach or colon was strictly avoided. The surgical procedures were performed under sterile conditions. At the end of the surgery, the abdomen was closed with Mersilene, 6-0 silk.

Histological techniques
Coronal sections of 30μm for brain/brainstem and 16μm for nodose ganglia were collected. After rinsing in 0.05M Tris-buffered saline TBS (pH 7.4), sections were incubated overnight at 4°C with goat anti-Fos (1:1500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and/or rabbit anti-CTB (1:10 000; C3062, Sigma, USA, to recognize the CTb-conjugated alexa fluorophore 555) primary antibodies.

For cFos staining, sections were first incubated 1hr in biotinylated secondary antibody and then in avidin-biotin complex (ABC, Vector) for 1hr. The reaction product was
visualized by incubation with 1% diaminobenzidine (DAB), 0.05% nickel ammonium sulfate and 0.01% hydrogen peroxide H$_2$O$_2$ for 5min. For cFos/CTB double staining, sections underwent once more 1 hr incubation in secondary antibody followed by 1hr incubation with ABC and ended with 7 min incubation in 1% DAB and 0.01% H$_2$O$_2$.

To count CTB and/or cFos immunoreactive neurons, tiled images were captured by a computerized image analysis system consisting of an Axioskop 9811-Sony XC77 color camera (Sony Corp., Tokyo, Japan). Brainstem sections, from bregma -7.20 to -7.76mm, were used for cFos and CTB counting in the NTS/DMV counted the brainstem section for the experiments. The counting of cFos in the PVN was performed on hypothalamic sections collected from Bregma -0.58 mm to -1.22 mm. The counting of cFos + cells was performed bilaterally for each nucleus. Data are represented as a mean of the relative density of c-fos positive cells counts on 9-10 and 9-11 sections (non adjacent section, at least separated 90μm) for the NTS/DMV and PVN, respectively.

Immunohistochemical staining for leukocyte infiltration of the small intestinal muscularis

Whole mounts of the small intestinal muscularis were used to determine the degree of inflammation. Myeloperoxidase staining was performed with 3-amino-9-ethyl carbazole (Sigma, St Louis, MO), 0.01% H2O2 in Sodium Acetate buffer (pH = 5) for 20 minutes, as previously described.$^{21,22}$

Random counting of whole mount sections

MPO$^+$ cells in the whole mount of the intestinal muscularis were counted using an image analysis system (ImagePro v4.5, Media Cybernetics, Silver Spring, USA) connected to a color camera (JVC KY-F55 3CCD) and a plain objective microscope (Zeiss Axioskop with Plan-NEOFLUAR Zeiss objectives). For each section, an image covering the entire sample (2.5× objective) was loaded into the IBAS and displayed on the computer monitor. The region of interest, i.e. the muscularis devoid of Peyer’s patch and damaged areas, was identified and a grid of rectangular areas (representing 20X magnification) was superimposed on the image. From this grid, 5% of the fields were selected randomly and from the selected images (20X), MPO$^+$ cells were counted.

Statistical analysis

Data from all experiments are presented as a mean ± S.E.M. Kolmogorov-Smirnov Test was used to determine whether the data set followed a normal distribution (SPSS package v 16.0). Square root transformation was applied to the non normal data set. Two way ANOVA analysis was performed to evaluate the two factors interaction: time (i.e. 2, 6 and 24hrs) and treatment (L vs IM) or vagotomy (i.e., intact vs. denervation)
and treatment (i.e., L vs. IM). When the ANOVA analysis indicated difference, One way ANOVA was performed to demonstrate a statistical significant difference among the different time point per treatment group (i.e., L vs. IM) followed by post hoc (LSD) analysis. The student T test (un-paired) was applied to estimate a significant difference between L vs. IM at a specific time point or between intact vs. denervated. Repeated-measure analysis of variance (ANOVA) was conducted to test the effect of vagal denervation on the mice body weight during the recovery period. All statistical analysis was performed with a significance set at 0.05.

Results

Distribution of CTb labeled neurons after intestinal/splenic injection

Intestine: The retrograde labeling was limited to the circumscribed region of the DMV. CTb labeled neurons were localized in the lateral part of the DMV observed from -7.32 to -7.76mm Bregma, as expected from previous anatomical studies\textsuperscript{23, 24} (Figure 1A) Given the distribution of the vagal sensory innervation of the gastrointestinal tract\textsuperscript{25-27} and the distribution of the CTb positive neurons, brainstem sections from bregma -7.20 to -7.76mm were used for cFos and CTB counting in the NTS/DMV. Injections of CTB-fluorophore 555 and 647 in the proximal and distal part of the ileum respectively labeled 2 distinct set of neuronal population (Figure 1B). No CTB\textsuperscript{*} cells were observed in the DMV after injection of the same volume of tracer in the peritoneal cavity. This observation shows a restricted uptake of the neuronal tracer by the small intestine. Moreover the remarkable differentiation of innervation of the ileum by DMV neurons illustrates the specific uptake by defined sites of the ileum.

Spleen: The retrograde tracer labeled a restricted neuronal population within the DMV (bregma -7.32mm to -7.76mm; Figure 1C), as previously reported\textsuperscript{17, 28}. Application of the tracer on splenic vessels at the level of the hilum led to faint limited amount of labeled neurons (1 or 2) that were located in the DMV more rostrally of the brainstem from bregma -6.96mm to -7.08mm (Figure 1B). These CTb positive neurons, however, were not found after injection of the CTb at the extremity of the spleen. Vagal denervation of the spleen followed by CTb injection at the tip of the organ led to a loss of CTb positive cells in the DMV (Figure 1E) excluding the possibility that CTb positivity results from leakage of tracer into the peritoneal cavity. On the other hand, sympathetic post-ganglionic neurons exhibit CTb stained cell bodies in the mesenteric ganglion (Figure 1F). We, therefore, used the brainstem sections from bregma -7.32mm to -7.76mm for cFos counting in NTS/DMV in the following experiments.
Figure 1 | Distribution of CTb labeled cells in the brainstem nuclei after tracer injection in the intestine (A). Injection of Cholera toxin b (fluorophore 555, red and 647, blue) into the proximal and distal part of the ileum labeled distinct motor neurons in the DMV (B). The distribution of the CTb+ cells in the brainstem nuclei after CTb application on splenic blood vessels differ form the CTb labeled cells (C) found in the DMV after tracer injection at the tip of the spleen (D). Vagal denervation of the spleen prevents CTb labeling of neurons in the DMV. Here we show an example of complete denervation in which no CTb positive cell body was found in the DMV (E). CTb labeled neuron population in the mesenteric ganglion (i.e., sympathetic post-ganglionic neurons) was found in denervated mice (F). The scale bars represent 100 µm and 0.50 mm for (A,B,F) and (C,D,E), respectively. Green: primary cFOS antibody and streptavidine alexa-fluo 488. Sx: vagal denervation of the spleen.
Early and late phase of postoperative ileus: role of inflammation

Intestinal inflammation was monitored by the degree of leukocyte infiltration at the different time points after surgery. MPO$^+$ cells were occasionally observed in the muscle layers of both L and IM mice early after surgery (2hrs L, 1±1 cells/mm$^2$ vs. IM, 2±1 cells/mm$^2$; 6 hrs L, 1±1 cells/mm$^2$ vs. IM, 3±1 cells/mm$^2$). In contrast, a clear infiltration of leukocytes was observed in the late phase of postoperative ileus (24hrs after surgery) (Figure 2A-B) and was significantly higher in IM mice (276 ± 64 cells/mm$^2$) compared to the L mice (1 ± 1 cells/mm$^2$, p=0.007).

Neuronal circuitry: cFos expression at the early phase (2 and 6hrs)

Two hours after surgery, all experimental groups (control, L and IM) exhibited a significant increase of cFos IR in the NTS and PVN compared to the baseline (Figure 2). Even the control mice that only received anesthesia had a comparable number of cFos$^+$ cells as L or IM, suggesting NTS/PVN activation induced by anesthesia and/or peri-operative stress. In the same line, no difference was observed in cFos IR in nodose ganglia cells bodies (i.e., vagal afferent nerve) (Figure 2D) between L and IM mice, 2 hrs after surgery.

In animals receiving L only, the number of cFos$^+$ cells in the NTS decreased in time (one way ANOVA, p =0.002) and reached basal levels at 24hrs post-surgery (L 14.5±5.5 vs BL 15.44±2.7, p=0.689), suggesting a transient effect of the surgical intervention on cFos expression (Figure 2C). In animals that underwent IM, the level of cFos expression remained high (one way ANOVA, p = 0,028) and was significantly different from L at 6 hrs post-surgery.

In the PVN (Figure 2E), cFos IR in both L and IM groups significantly decreased with time after surgery (Two way ANOVA: time effect p < 0.05; treatment or treatment*time interaction, p > 0.05), suggesting a role of the PVN only at the early phase of the POI.

The number of cFos IR motor neurons in the DMV was significantly increased in both L and IM compared to the control (anesthesia only) or basal level (p < 0.05; Figure 3A) at 2 and 6hrs post-surgery, suggesting that additional sensory afferent activation triggered by skin incision and/or intestinal handling generates vagal output. In the L group, the number of cFos$^+$ neurons returned to baseline 24hrs after surgery.
cFos IR in CTB labeled motor neurons innervating the small intestine

To evaluate whether trauma/inflammation activates neurons in the DMV targeting the inflamed intestine, we counted the amount of cFos + cells in the CTB labeled neurons, i.e., motor neurons that specifically innervate the small intestine. The quantitative cFos analysis within the CTB labeled neuronal population showed that 2hrs
and 6hrs after surgery a low percentage (below 10%) of CTB+ neurons co-express cFos IR in both L and IM mice (Figure 3C).

Neuronal circuitry: cFos expression at the late phase of POI (24hrs)

At the late phase of the POI (t=24 hrs, inflammatory phase characterized by leukocyte infiltrates in the gut muscularis), activation of the NTS and nodose ganglia after IM was still significantly increased compared to L and baseline: IM 73 ±35 vs. L 14±4 or BL 15±3, p = 0.005) (Figure 2C and D). However, no significant difference in cFos expression in the PVN was observed between L and IM (Figure 2E). In contrast, the number of cFos + neurons in the DMV further increased after IM at 24hrs (IM 24.3±8 vs L 7.7±1.2, p = 0.001) while the number of cFos + neurons after L returned to basal level (Figure 3A-B).

cFos IR in CTB labeled motor neurons innervating the small intestine

Nearly half of the CTB neurons that project to the manipulated intestine exhibited positive IR for c-Fos (p < 0.05), i.e., 42% in IM vs. 4% in L (Figure 3C-D). These data provide evidence that the late inflammatory phase is associated with cFos expression in NTS, DMV. This vagal neuronal circuit activation is based on a vagal output targeting specifically the inflamed zone, i.e., the intestine.

cFos IR in CTB labeled motor neurons innervating the spleen

Previous studies of Tracey et al., provided strong evidence that the spleen is involved in the vagal anti-inflammatory pathways 5, 16. Since the spleen is directly innervated by the vagus nerve 17, 28, we investigated whether the vagal output triggered by intestinal inflammation also targeted the spleen. To this end, we retrogradely labeled the vagal motor neurons innervating the spleen prior to intestinal manipulation. As previously reported 17, injection of the CTB in both ends of the spleen labeled a neuronal population in the DMV (-7.20 to -7.76mm Bregma) (Figure 3E). The average mean of cFos + cells counted in NTS and DMV from control mice (n = 2; injection of CTb only) was not significantly different from the mice that underwent laparotomy and euthanized 24hrs after surgery. However, twenty four hours after surgery, IM triggered an increase of cFos expression in the NTS and DMV compared to L (NTS: 82±10 vs 5±1; DMV: 15±2 vs. 1±0). Seven percent of the CTB + neurons that project to the spleen exhibited c-Fos IR (p < 0.05) in IM mice while no cFos+ CTB+ neurons were observed in L mice (Figure 3C).
Figure 3 | IM induced cFos expression at different time points after surgery in the DMV (A, B) and in a subgroup of motor neurons innervating the small intestine (C). In panel D, part of the cFos + neurons (black nucleus) is also stained with the retrograde tracer (cytoplasm), identifying vagal motor neurons innervating the intestine (black arrow). cFos IR was also detected in the motor neurons that innervate the spleen at t= 24hrs post-surgery (C, E). Data are expressed as mean ± S.E.M. for n= 5-7. The scale bars represent 200µm (B, E) and 50µm (D). # indicates significant differences (#, p <0.05; ###, p <0.01) compared to t=2hrs. Asterisks indicate significant differences: *, p < 0.05; **, p <0.01), L vs. IM. AP, Area postrema; NTS, nucleus of the solitary tract; DMV, dorsal motor nucleus of the vagus; PVN, paraventricular nucleus of the hypothalamus; IM, intestinal manipulation.
Selective vagal denervation: analysis 24 hrs post-surgery

The neuro-anatomical proof of an endogenous vagal response triggered at the late inflammatory phase of POI and restricted to the inflamed intestine suggests the existence of a vago-vagal inflammatory reflex. To confirm our hypothesis, we selectively denervated the vagal innervation of the small bowel to prove that the afferent limb of the “reflex” is indeed of vagal origin.

In the set up of the denervation procedure, we monitored the mice with sham-operation and denervation during their recovery period (sham-operated mice n = 5, denervated mice n = 7). Mice started with an average body weight of 22.7±0.2g prior surgery. At the end of the recovery period, we did not observe a difference between sham-operated and denervated mice body weight (23.7±1.5g and 23.9±0.96g, respectively). ANOVA with repeated measurement indicated a time effect (i.e., body weight measurement over days, \( p < 0.05 \)) but no interaction time*denervation effect (\( p = 0.740 \)) or denervation effect (\( p = 0.432 \)). At the time of euthanasia, the control groups (laparotomy) did not exhibit distended stomach in sham-operated or denervated mice.

Selective vagal denervation (IntX) completely abolished the presence of CTB\(^+\) cells in the DMV indicating that the vagal denervation was successful. Among the 20 mice (out of 24) successfully denervated, 11 underwent IM and the 9 left underwent laparotomy. In denervated mice, IM tended to increase the degree of intestinal inflammation compared sham operated mice, but this difference did not reach statistical significance (336±79 vs. 254±43, respectively; \( p = 0.188 \)). In both IM groups, inflammation of the intestinal muscularis 24hrs after surgery (Figure 4A) was significantly more pronounced compared to laparotomy mice (7±6 vs. 9±4 for denervated and sham respectively). Even in the presence of inflammation, cFos expression in both the NTS and DMV was significantly reduced by selective vagal denervation (Figure 4B, C and D). IM mice displayed the same levels of cFos expression as the control group (IM IntX vs L IntX; \( p = 0.289 \) for NTS and \( p = 0.043 \) for DMV). Selective vagal denervation did not affect cFos expression in laparotomy animals.

These data indicate that the vagal sensory pathway indeed transmits the inflammatory signal to the brain stem activating the vagal output to modulate the inflammatory response.
Figure 4 | MPO+ cells in whole mount preparations at 24hrs post-surgery (A). cFos expression in NTS and DMV (B, C, D) in denervated and sham-operated mice 24hrs after L or IM. Data are expressed as mean ± S.E.M. for n= 5-7. # indicates significant differences (#, p < 0.05; ##, p <0.01), L vs. IM. Asterisks indicate significant differences (*, p < 0.05; **, p <0.01), sham vs. IntX. The scale bar represents 200µm. AP, Area postrema; NTS, nucleus of the solitary tract; DMV, dorsal motor nucleus of the vagus; PVN, paraventricular nucleus of the hypothalamus; IM, intestinal manipulation; IntX, vagal intestinal denervation.

Discussion

Recently, Tracey and coworkers introduced the concept of the vagal “inflammatory reflex” as a protective mechanism to restore immune homeostasis after an immunological challenge 29. Although it is well established that pro-inflammatory cytokines and endotoxin 30 stimulate vagal afferents leading to brain stem activation, data supporting activation of motor neurons of the vagal nerve closing the anti-inflammatory loop are lacking. Here, we demonstrate that subtle intestinal inflammation leads to NTS and DMV activation that is abolished by selective intestinal vagotomy. Importantly, more than 40% of the activated DMV neurons targeted the inflamed intestine, supporting the existence of an endogenous vagal “inflammatory reflex” modulating intestinal inflammation.
During abdominal surgery, multi-synaptic neuronal pathways are activated involving mainly the NTS and the PVN. All experiments started early morning to avoid any circadian influence on cFos expression \cite{31, 32}. The effect of intestinal manipulation was compared to laparotomy group to avoid any influence of the circadian rhythm while intact mice control groups performed at ZT =2 were compared with 2 and 24hrs (ZT=2).

The NTS is the brain stem nucleus receiving somato/visceral sensory information whereas the PVN is located in the hypothalamus known to regulate stress-related events and endocrine response \cite{14, 33}. The PVN, most specifically the parvocellular part, plays an essential role in mediating the immediate and early (up to 3hrs) hypomotility of the GI tract after abdominal surgery \cite{14, 15}, most likely in response to surgery-induced stress and activation of visceral sensory afferents \cite{34-36} by skin incision and surgery-induced noxious stimuli. In the present study, we confirmed activation of the PVN in the peri-operative phase (2hrs post-surgery). Interestingly, cFos expression was similar in animals that underwent anesthesia, laparotomy only or intestinal handling suggesting that factors such as stress induced by pain, anesthesia and others largely contribute to PVN activation and even appear to overrule the effect of intestinal handling. Alternatively, the PVN is maximally activated by anesthesia and peri-operative stress thereby obscuring the effect of adding intestinal handling to the surgical protocol. The fact that PVN activation returned to baseline levels at 6 hrs in control and laparotomy animals suggests that peri-operative stress is indeed a major trigger in the early phase. In contrast, the degree of activation in NTS and PVN remained elevated in animals that underwent IM up to 6 hours after surgery. As the effect of peri-operative stress and anesthesia has disappeared by then, as shown in the L mice, these data suggest that NTS and PVN activation at this stage is rather related to the procedure of surgery and intestinal manipulation. As shown in Figure 3, both L and IM activated the DMV, but this activation was not specifically directed towards the intestine as less than 10% these neurons were labeled with the retrograde tracer injected in the intestine. These data thus suggest that in the early phase of postoperative ileus, activation of the brain stem nuclei is triggered by a generalized stress response combined with activation of mechano/nociceptive pathways, most likely to control systemic homeostasis of the organism.

In the second inflammatory stage of postoperative ileus, however, different mechanisms come into play. It is well recognized that intestinal handling during surgery triggers the influx of inflammatory cells, mainly neutrophils and monocytes. This inflammatory response is observed from 6 hours post-surgery onwards and is well established by 24 hours \cite{11, 12}. Twenty four hours after surgery, infiltration of leukocytes within the muscle layers of the small intestine was observed in mice that underwent IM, associated with activation of the NTS and a subset of vagal motor neurons in the DMV.
targeting the inflamed zone. Indeed, we showed a significant increase of cFos IR in those neurons specifically innervating the intestine in IM mice (42%) compared to laparotomy mice (3.8%). Subsequently, we demonstrated that these motor neurons were activated by inflammation detected by vagal afferents. IM, but not laparotomy, resulted in cFos IR in the nodose ganglia. We are aware that the illustration of the presence of cFos + cells in IM in contrast to laparotomy is not sufficient to draw such statement. However the selective vagal denervation of the inflamed intestine abolished both NTS and DMV activation, confirming the importance of the vagal afferent activation in mediating the motor vagal neurons activation in response to IM. Although the latter could result from neuronal degeneration triggered by axotomy, studies demonstrate that neuronal degeneration is rather induced by vagotomy performed at the mid cervical level, i.e. close to the cell body of the neurons 37. In our study, vagotomy was performed distally close to the nerve endings, reported to lead to neuronal regeneration rather than degeneration 38-40. This makes the explanation of neuronal degeneration as explanation for the loss of cFos expression in the DMV/NTS after vagotomy less likely. Therefore, our data indicate that vagal afferents and not splanchnic afferent nerves (through the spino-solitary tract) triggered NTS activation, subsequently generating DMV activation and thereby closing the inflammatory reflex. Most likely, the vagal sensory limb of the inflammatory reflex is triggered by the release of pro-inflammatory cytokines 11, 12, 41, 42.

Vagal afferents indeed express IL1 and PGE2 receptors 43, two pro-inflammatory mediators known to be elevated 24hrs after intestinal manipulation 42, 44. However, further studies are needed to identify the exact underlying mechanisms. Nevertheless, our data clearly demonstrate that intestinal inflammation triggers vagal afferents resulting in the activation of a vagal efferent feedback loop targeting the inflamed area. Although our data suggest a direct input of the vagal anti-inflammatory pathway to the gut, recent studies identified the spleen as a major player in the cholinergic anti-inflammatory effect in a model of sepsis 5 but also in a model of local inflammation such as the carrageenan air pouch model 16. To evaluate whether intestinal inflammation would lead to increased vagal output to the spleen, CTB was injected in both ends of the spleen to retrogradely label the DMV neurons innervating the spleen 17. Only seven percent of the labeled motor neurons expressed cFos IR after IM, indicating that the inflammatory reflex is mainly targeted to the inflamed area. Nevertheless, we cannot exclude that the spleen may contribute, albeit partially to the vagally mediated modulation of the inflammatory response.

In our previous studies, we demonstrated that perioperative electrical stimulation of the vagus nerve decreases intestinal inflammation most likely by inhibiting macrophage activation 10, 22. Here we provide evidence that this cholinergic inflammatory pathway (i.e. DMV activation) mainly occurs 24hrs after surgery, i.e. once
leukocytes have infiltrated the gut muscularis, but not at the earlier stage where chemokines and cytokines are secreted by the resident immune cells. This would indicate that the endogenous anti-inflammatory pathway is most likely involved in restoring homeostasis once the inflammatory response is established and has fulfilled its task of attacking micro-organisms and clearing tissue damage. Compatible with this hypothesis is the observation that vagal denervation of the small intestine did not significantly enhance the inflammatory response (nor prostaglandin E2 release, data not shown) 24hrs after surgery. Further studies are however required to confirm this assumption.

In summary, we provide the first neuro-anatomical evidence illustrating the existence of an “inflammatory reflex” triggered by inflammation. Subtle intestinal inflammation is detected by vagal afferents triggering NTS activation and generating a specific vagal outflow previously shown to modulate the inflammatory response. These data provide solid evidence to accept the immune-modulatory role of the vagus nerve providing new opportunities to identify targets for the development of new anti-inflammatory strategies.

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