Autonomic Neuroscience: Basic and Clinical 160 (2011) 82–89

Contents lists available at ScienceDirect

Autonomic Neuroscience: Basic and Clinical

journal homepage: www.elsevier.com/locate/autneu

Anti-inflammatory effect of vagus nerve stimulation in a rat model of inflammatory bowel disease

J. Meregnani [a,1], D. Clarençon [a,b,1], M. Vivier [b], A. Peinnequin [a,b], C. Mouret [b], V. Sinniger [a], C. Picq [a,b], A. Job [b], F. Canini [a,b], M. Jacquier-Sarlin [a], Bruno Bonaz [a,c,*]

[a] Stress et Interactions Neuro-Digestives (SIND, EA3744), Grenoble Institut des Neurosciences (GIN), INSERM U836 UJF-CEA-CHU, Université Joseph Fourier, Site Santé La Tronche, BP170, 38042 Grenoble Cedex 9, France
[b] Institut de Recherche Biomédicale des Armées, Antenne de La Tronche, BP87, 38702 La Tronche Cedex, France
[c] Clinique Universitaire d'Hépato-Gastroentérologie, CHU de Grenoble, BP217, 38043 Grenoble Cedex 09, France

ARTICLE INFO

Article history:
Received 30 June 2010
Received in revised form 7 October 2010
Accepted 15 October 2010

Keywords:
Vagus nerve stimulation
Trinitrobenzensulfonic acid
Colitis
Inflammation
Inflammatory bowel diseases

ABSTRACT

Vagus nerve stimulation of afferents is used as an adjunctive treatment for drug-resistant epilepsy and depression. In addition, anti-inflammatory properties of vagus nerve stimulation have been reported in various experimental models of inflammation but not in colitis. These effects are thought to be mediated via peripheral release of acetylcholine from the vagus and subsequent activation of macrophages. Our aim was to evaluate in rats the anti-inflammatory effects of chronic vagus nerve stimulation on colonic inflammation. Colitis was induced by intracolonic instillation of trinitrobenzene sulfonic acid. Vagus nerve stimulation (left cervical) was performed in freely moving animals 3 h per day for five consecutive days. Assessment of colonic inflammation was obtained using physiological (e.g. body weight, temperature and locomotor activity) parameters, macroscopical (area of lesions), histological, and biological parameters (e.g. myeloperoxidase activity, cytokine and cytokine-related mRNAs), both at the level of the damaged colon and the colon immediately above. A global multivariate index of colitis was then generated for a better characterization of colonic inflammation. Vagus nerve stimulation reduced the degree of body weight loss and inflammatory markers as observed above the lesion by histological score and myeloperoxidase quantification. This anti-inflammatory effect was also demonstrated by the improvement of the multivariate index of colitis. These data argue for an anti-inflammatory role of vagus nerve stimulation chronically performed in freely moving rats with colitis and provide potential therapeutic applications for patients with inflammatory bowel diseases.

1. Introduction

The vagus nerve contains sensory and motor components that control organ functions as varied as heart rate and digestion (Foley and DuBois, 1937). Vagus nerve stimulation (VNS) of afferent fibers is an approved treatment for epilepsy and depression, but the precise mechanism of action of VNS on the central nervous system is still unknown (Groves and Brown, 2005). In addition to controlling physiological functions through efferents fibers, a new cholinergic anti-inflammatory pathway has been described by Tracey (2002), providing a way for the brain to regulate the cytokine response in specific organs, such as the gut.

Inflammatory bowel diseases (IBD) include two main types of chronic inflammation affecting the gastrointestinal tract, ulcerative colitis and Crohn's disease. The treatment of IBD is only suspensive and represented by aminosalicylates, steroids, immunosuppressors, and biological therapies, e.g. anti–tumor necrosis factor (TNF) agents (Baumgart and Sandborn, 2007). A growing body of evidence suggests the existence of autonomic dysfunctions in IBD patients (Taylor and Keely, 2007), whatever the severity of symptoms and even during remission. Using heart rate variability as a marker of the sympatho-vagal balance, we have recently demonstrated that cardiac neural control is altered according to the disease depending on the psychological adjustment of the patients to their disease (Pellissier et al., 2010).

Neuroimmunomodulation in IBD remains a challenging theory through the influence of the brain–gut axis on intestinal inflammation and its perpetuation (Anton and Shanahan, 1998; Paschos et al., 2009). Circulating pro-inflammatory cytokines, interleukin (IL)-1β, IL-6, and TNF, released from the intestinal mucosa are able to communicate with the brain through neural and humoral pathways (Dantzer et al., 2000). The neural pathway involves vagus nerve

* Corresponding author. Stress et Interactions Neuro-Digestives (SIND, EA3744), Grenoble Institut des Neurosciences (GIN), INSERM U836 UJF-CEA-CHU, Université Joseph Fourier, Site Santé La Tronche, BP170, 38042 Grenoble Cedex 09, France. Tel.: +33 4 76 76 55 97; fax: +33 4 76 76 52 97.
E-mail address: BBonaz@chu-grenoble.fr (B. Bonaz).
[1] The first two authors contributed equally to this work.

1566-0702/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.autneu.2010.10.007

afferents locally stimulated by cytokines to activate the hypothalamic pituitary adrenal axis (Dantzer et al., 2000). Tracey's group reported an anti-inflammatory role of vagal efferents through the cholinergic anti-inflammatory pathway: acetylcholine (Ach) released at the distal end of the vagus nerve, interacts with α-7 nicotinic Ach receptor (α7nAChR) of human macrophages to inhibit the release of pro-inflammatory cytokines such as TNF (Pavlov et al., 2003; Tracey, 2009; van der Zanden et al., 2009b). Smoking (i.e. nicotine) is a risk factor for IBD, aggravating Crohn's disease while having beneficial effects on ulcerative colitis (Tobin et al., 1987).

The anti-inflammatory role of the cholinergic pathway has been demonstrated in models of high-fat enteral nutrition on hemorrhagic shock–induced TNF release (Luyer et al., 2005), pancreatitis (van Westerloo et al., 2006), dextran sulfate sodium colitis (Ghia et al., 2006), trinitrobenzene sulfonic acid (TNBS) colitis (Bai et al., 2007) and postoperative ileus (The et al., 2007). This effect is produced either pharmacologically, using selective α7nAChR agonists (de Jonge and Ulloa, 2007; The et al., 2007; van Westerloo et al., 2006), central injections of anti-inflammatory agent CNI-1493 (Borovikova et al., 2000), peripheral or central cholinesterase inhibitors (Miceli and Jacobson, 2003; Pavlov et al., 2009), by vagotomy (Ghia et al., 2006) or through acute VNS (de Jonge et al., 2005), but the effect of VNS has never been studied in experimental colitis. In addition, most of these studies were performed in anesthetized animals.

VNS has been approved in humans by the Food and Drug Administration (FDA), a U.S. government agency, as a therapy for treatment-resistant epilepsy or depression (Milby et al., 2008) through high-frequency stimulation (20–30 Hz) of vagal afferents (Milby et al., 2008) while the use of low-frequency stimulation (1–10 Hz) (Bernik et al., 2002b; Borovikova et al., 2000; de Jonge et al., 2005) in animal models is thought to activate vagal efferents i.e. the cholinergic anti-inflammatory pathway through alpha7 nicotinic acetylcholine receptors (de Jonge and Ulloa, 2007).

Our aim was to evaluate the anti-inflammatory effect of chronic VNS in a model of TNBS colitis in rats. For this purpose, we have performed VNS in freely moving animals chronically implanted with an electrode on the left cervical vagus nerve, using stimulation parameters known to activate vagal efferents, and studied the effect of VNS on TNBS colitis using classical markers of inflammation and a multivariate index of colitis.

2. Methods

2.1. Animals

Adult male Sprague–Dawley rats (Janvier, Le Genest St Isle, France) were individually housed in controlled conditions: temperature, 22 ± 1 °C and 12:12 h light–dark cycle (lights on at 08:00 and lights off at 20:00). Rats were allowed to acclimate to these conditions for at least 7 days before inclusion in experiments. Animals were weighed before induction of colitis and at the end of the VNS experiment. Procedures were carried out in accordance with the European Communities Council Directive and guidelines of the local ethical animal research committee.

2.2. Surgical procedures

Rats were anesthetized with a mixture of ketamine (125 mg/kg intramuscularly, i.m.) and acepromazine (0.15 mg/kg i.m.). A telemetric device (Physiotel TA10TAF40; Data Sciences International, MN, USA) was implanted into the abdominal cavity to record body temperature and locomotor activity (Chevrier et al., 2006). Then, the left cervical vagus nerve was identified and an electrode (Cyberonics, Lyon, France) was gently wrapped around the vagus nerve and carotid (Handforth and Krahl, 2001) and linked to a connector fixed to the rat's head with dental cement. The connector was linked to a stimulator chain (S88, SIU5, CCU1, Grass Technologies, Astro-Med, RI, US) through a slip ring (TA13EEG12F2, Air Precision, Le Plessis Robinson, France).

2.3. Induction of colitis

Colitis induction was based on the classical model of TNBS colitis, developed by Morris et al. (1989). Twelve days after surgery, rats deprived of food for at least 12 h were anesthetized (50 mg/kg ketamine and 0.05 mg/kg acepromazine i.m.). A 7.5-cm length cannula was inserted into the colon and TNBS (Fluka, St Quentin Fallavier, France) was instillated at a dose of 10 mg per rat in 50% ethanol (total volume, 0.25 ml). To ensure the retention of TNBS within the colon, rats were maintained in the head-down position following intracolonic administration. Controls received saline.

2.4. Vagus nerve stimulation

VNS was performed in freely moving animals 3 h per day (9:00 am to 12:00 am) for 5 days, with stimulation parameters (1 mA, 5 Hz, pulse width of 500 μs; 10 s ON, 90 s OFF; continuous cycle) adapted from previous studies (Bernik et al., 2002b; Naritoku et al., 1995). Stimulation frequency is in the range of normal nerve traffic in the vagus nerve (Fogel et al., 1996). The first day, VNS started 1 h before intracolonic instillation. Control rats implanted according to the same procedure were not stimulated.

2.5. Experimental groups

Rats were randomly assigned to one of four treatment groups ($n = 12$ rats per group): 1) non-stimulated/saline injected (controls), 2) stimulated/saline injected (VNS/saline), 3) non-stimulated/TNBS injected (noVNS/TNBS) and 4) stimulated/TNBS injected (VNS/TNBS).

2.6. Assessment of colonic inflammation

Five days after instillation, rats were deeply anesthetized (ketamine 150 mg/kg and acepromazine 0.15 mg/kg, i.m.) and transcardially perfused with saline. The distal colon (8 cm from the anus) was isolated, opened longitudinally and photographed for quantification of colonic damage by determining three areas (normal, moderate and severe inflammation) using colorimetric evaluation (Visilog 6 software, NOESIS, Crolles, France). Colonic samples were then separated into two parts: the damaged colon with macroscopically necrotic lesions and the part immediately above the lesion (without macroscopic lesion), i.e. a 1-cm-long piece proximal to the most anterior aspect of the macroscopically observed damage. Samples of lesion tissue and immediately adjacent tissue were cut longitudinally in equal parts for histological analysis, myeloperoxidase (MPO) assay, and mRNA quantification.

2.7. Histological analysis

Colonic sections (5 μm) were stained with H&E and blindly evaluated (Vetopath Laboratory, Sofia-Antipolis, France). Histological parameters considered for semi-quantitative grading of colonic inflammatory lesions were: epithelial damage (erosions, ulcers, necrosis), inflammatory changes (infiltration of mono and polymorphonuclear cells, vascular changes) and distribution of lesions (focal, diffuse, transmural). A score distinguishing five major grades was generated: grade 0 (normal mucosa), grade I (mild, focal increase of subacute inflammatory infiltrate), grade II (moderate, patchy, or diffuse increase of inflammatory infiltrate in lamina propria, erosions), grade III (marked inflammation with ulcerations and/or

superficial necrosis), and grade IV (severe acute inflammation with transmural necrosis) (Geboes et al., 1999).

2.8. Myeloperoxidase quantification

Colonic samples were homogenised in PBS-EDTA (5 mM) buffer (pH 7.3) using FastPrep-24 Instrument Device system (Lysing matrix D, FastPrep, Qbiogen, Illkirch, France) and quantified for MPO concentration by ELISA kit (HK210, Hycult Biotechnology, Uden, the Netherlands). MPO concentration of samples was expressed as ng/mg protein.

2.9. Quantification of cytokine (TNF, IL-1β, IL-6) and cytokine-related (ICAM1, NFκBIA, SOCS3, TBX21) mRNAs

2.9.1. mRNA isolation and reverse transcription reaction

Preconditioned colonic samples in RNALater (Qiagen, Courtaboeuf, France) were disrupted in lysis buffer (Qiagen). mRNA was isolated using MagNA Pure LC mRNA isolation kit II in a MagNA Pure LC instrument (Roche Applied Science Meylan, France). Reverse transcription was carried out using the Reverse Transcriptase Core Kit (Eurogentec, Angers, France) with 50 μM oligo (dT) 15 primer and RNAse inhibitor (2 UI).

2.9.2. Real-time quantitative PCR

PCR was carried out with LC Fast Start DNA Master SYBR Green kit (Roche Applied Science). PCR was performed using a LightCycler (Roche Applied Science) for 45 cycles at 95 °C for 20 sec, 58 °C [except T-box 21 (TBX21): 56 °C, acidic ribosomal phosphoprotein P0 (ARBP) and nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α (NFκBIA): 57 °C, hypoxantine guanine phosphoribosyltransferase (HPRT) and IL-6: 60 °C] for 5 sec, and a final step of 10 sec at 72 °C. Quantification was achieved using a pool of all the cDNA samples as calibrator according to the comparative threshold cycle method (Schmittgen and Livak, 2008) with efficiency correction (Pfaffl, 2001) using the geometric average of three internally validated control genes [Cyclophylin A (CycA), ARBP, HPRT] (Vandesompele et al., 2002). Therefore, ARBP, CycA, and HPRT were used for normalization.

2.9.3. Primer design

Primer design, optimization and specificity checking were done as described previously (Peinnequin et al., 2004). GenBank accession numbers used for the primers design were for ARBP: NM_022402; CycA: NM_017101; HPRT: NM_012583; intercellular adhesion molecule 1 (ICAM-1): NM_053565; IL-1β: NM_031512; NFκBIA: XM_343065; suppressor of cytokine signalling 3 (SOCS3): NM_053565; TBX21: XM_220914 and TNF: NM_ 012675. IL-6 (GenBank NM_012589) primers present LNA-substitutions (N^L) as described previously (Malgoyre et al., 2007).

2.10. Multivariate index of colitis

All statistical analyses were performed using Statistica software (Statsoft-France, Maison-Alfort, France).

MPO, body weight, and histological score of colitis are basic parameters currently studied for the characterization of colitis but many other parameters are thought to be markers of colitis severity and of inflammation process: means of body temperature and locomotor activity during the two dark periods, geometric means of damaged areas, major cytokines and cytokines associated mRNAs quantified in the two areas (TNFα, IL1β, IL6, ICAM1, NFκB, SOCS3, TBX21). To characterize the inflammatory profile by a unique index, we performed a discriminant analysis including all these parameters. Discriminant analysis is a multivariate analysis that allows a better characterization of the inflammatory profile in providing a weighted (canonical coefficients) linear combination of parameters.

Discriminant analysis was first performed on noVNS/saline (controls) and noVNS/TNBS groups in order to identify the parameters that best discriminated between experimental colitis and controls. Canonical coefficients obtained were then applied to VNS/saline and VNS/TNBS groups. The relative contribution of each parameter presented here in the discriminant model was ranked by partial Wilks lambda. The unstandardized coefficients of the model were used to calculate a general weighted score for colitis inflammation for each rat. Discriminant score was obtained by multiplying the coefficient by the value of measured parameters. The higher the absolute value of the coefficient was, the better was its association to colitis. The inflammatory index was then used to test the effects of VNS between noVNS/TNBS and VNS/TNBS groups.

2.11. Statistical analysis

We used ANOVA for repeated measures for analysis of telemetric data and, when appropriate, a Bonferroni post hoc test was performed for two by two comparisons.

For all others results, a Mann–Whitney test was used. $p<0.05$ was considered as statistically significant. All data are expressed as mean ± SEM.

3. Results

3.1. Effects of VNS on circadian body temperature after colitis induction

Baseline circadian temperatures were similar for the four groups of rats before instillation of TNBS or saline. Rats had higher body temperature during the dark period and a lower one at light period; this coincided with a decrease of spontaneous locomotor activity (data not shown). The same pattern was observed 12 h after instillation in both control and VNS/saline groups (Fig. 1). In contrast, rats with colitis presented a disruption in normal thermoregulation which started during the first night after TNBS instillation. During this period, noVNS/TNBS-injected rats exhibited a significant hypothermia (-0.9 ± 0.1 °C, $p<0.05$ vs. controls) while they displayed no change of body temperature during the light period. No change was observed during the night in VNS/TNBS animals compared to the noVNS/TNBS group (Fig. 1). The same effect was reported during the following day (day 2; data not shown).

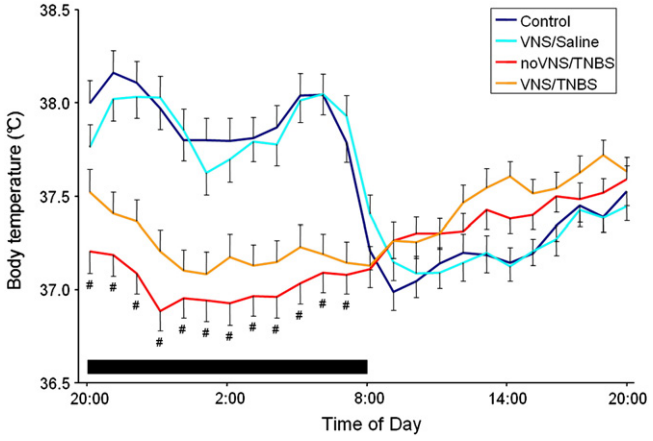

Fig. 1. Effect of VNS on 24-h body temperature evolution 12 h after colonic instillation of saline or TNBS: rats with colitis (noVNS/TNBS, red curve) presented a disruption in normal thermoregulation occurring as of the first night following TNBS instillation associated with significant hypothermia (-0.9 ± 0.1 °C) compared to controls (blue curves) (# $p<0.05$, ANOVA for repeated measures and Bonferroni post hoc test for two by two comparisons). Bar indicates the dark period ($n \geq 13$).

3.2. Effect of VNS on classical evaluation of colitis

3.2.1. Body weight loss

A significant weight loss, expressed as the percentage of body weight change between the 1st and 5th day of VNS, was observed in noVNS/TNBS (−4.1 ± 1.1%) compared to the control group (14.5 ± 1.3%) ($p<0.0001$) (Fig. 2). In the VNS/TNBS group, the decrease of body weight was significantly lower (−0.3 ± 1.5%, $p=0.04$) compared to no VNS/TNBS animals (Fig. 2).

3.2.2. Macroscopic assessment

No macroscopic damage of the recto-colon was observed after saline. In animals receiving TNBS/ethanol, either stimulated or not, macroscopic colonic damage was observed between 3 and 7 cm proximal to the anus. Quantification of colonic damages showed no change in VNS/TNBS animals (data not shown).

3.2.3. Histological assessment

No histological damage was observed in saline-injected animals with or without VNS (grade 0) (Fig. 3A, Table 1). TNBS induced mucosal lesions: one grade 0 and two grade II above the lesion (Fig. 3B, Table 1) and one grade II and two grade IV in the lesion (Fig. 3C, Table 1). Histological scores in the lesion were not modified by VNS (one grade I and two grades IV) (Fig. 3C, Table 1), while colonic tissue above the lesion was relatively protected by VNS and showed a lower degree of inflammation (two grades 0 and one grade I) (Fig. 3B, Table 1).

3.2.4. Myeloperoxidase quantification

TNBS induced a significant increase of MPO concentration both at the level of macroscopic colitis and just above the lesion (1,426.6 ± 119.4 ng/mg and 23.9 ± 6.0 ng/mg protein, respectively) when compared to control group (6.1 ± 2.5 ng/mg protein) ($p<0.0001$ and $p=0.008$, respectively) (Fig. 4). Compared to noVNS/TNBS, VNS/TNBS animals presented a decrease of MPO concentration which was significant only at the level of the colon immediately above the lesion (9.5 ± 4.2 ng/mg protein versus 23.9 ± 6.0 ng/mg protein, $p=0.03$) (Fig. 4).

3.3. Effect of VNS on levels of cytokine mRNAs in animals without colitis

A significant decrease of TNFα and IL-1β mRNAs was observed in VNS/saline group compared to control (respectively 73.5 ± 6.9% and 79.8 ± 8.8% of control group's levels, $p<0.05$) (Fig. 5).

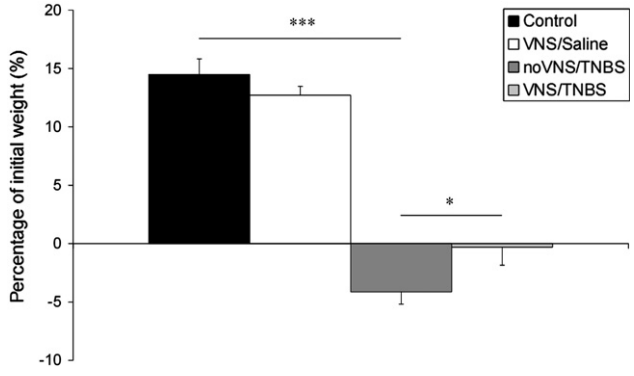

Fig. 2. Effect of VNS on body weight change 5 days after colonic instillation of saline or TNBS: stimulated saline group (VNS/saline) was not significantly different compared to the control group (12.7 ± 0.7% vs. 14.5 ± 1.3%). A significant body weight loss (−4.1 ± 1.1%, $p<0.0001$) was observed in the nonstimulated colitis group (noVNS/TNBS) compared to control group. The VNS/TNBS group showed significantly less reduction in body weight (−0.3 ± 1.5%, $p=0.04$) compared to nonstimulated animals (noVNS/TNBS). $n\geq12$; * $p<0.05$; *** $p<0.0001$; Mann–Whitney test.

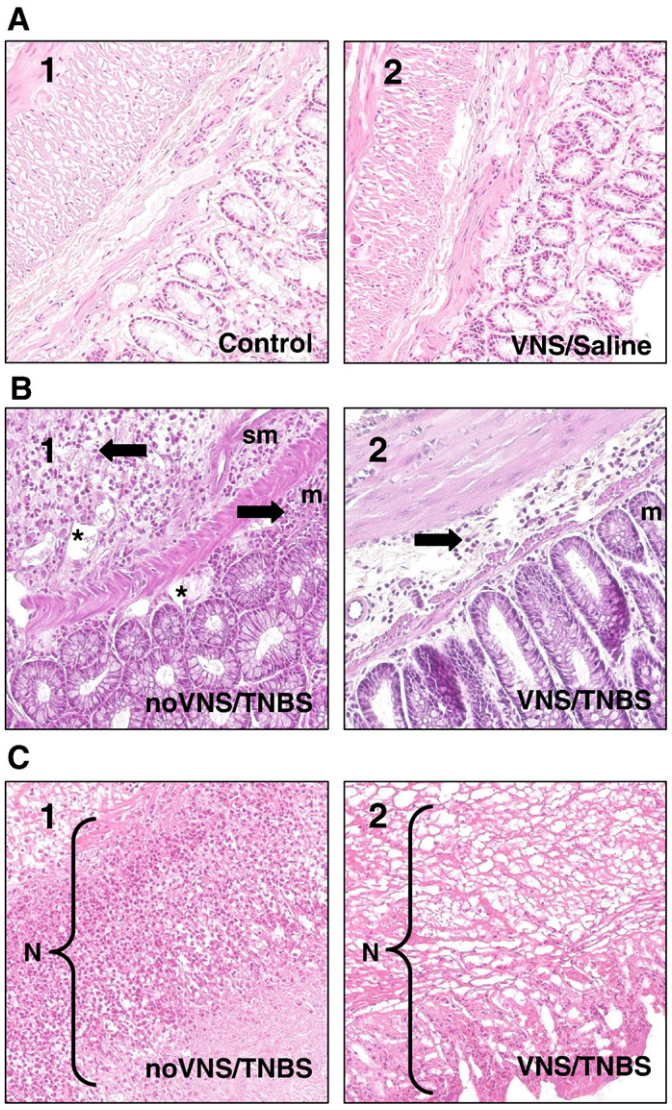

Fig. 3. Effect of VNS on histological staining of representative colons 5 days after colonic instillation of saline or TNBS. Microscopic photographs of colonic samples, (A) in normal tissues: A1 and 2; (B) above the lesion: B1) grade II [⋆: capillary congestion; →: interstitial polymorphic inflammatory infiltrate diffuse in the mucosa (m) and submucosa (sm); B2) grade I (mild, focal inflammatory infiltrate]; (C) in the lesion: C1 and 2: transmural necrosis (N).

Table 1
Grading of histological lesions on microscopic sections of the colon.

	Above the lesion	In the lesion
Control	0	0
	0	0
	0	0
VNS/saline	0	0
	0	0
	0	0
noVNS/TNBS	0	II
	II	IV
	II	IV
VNS/TNBS	0	I
	0	IV
	I	IV

Effect of VNS on histological staining of representative colons 5 days after colonic instillation of saline or TNBS: histological score from grade 0 to IV in these areas. A lower degree of inflammation was observed in the colon above the lesion in VNS/TNBS animals by comparison to noVNS/TNBS animals. No change was observed in the lesion both in noVNS/TNBS and VNS/TNBS animals ($n=3$).

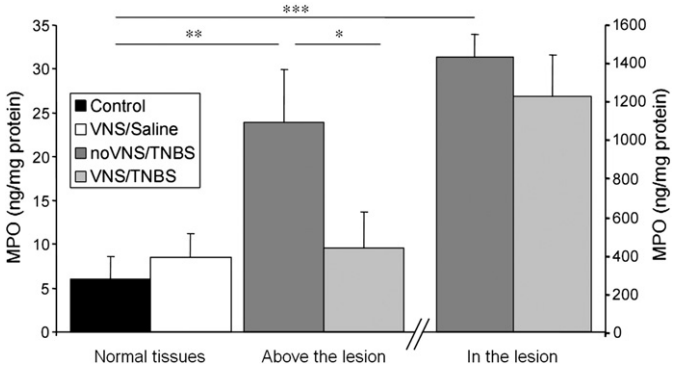

Fig. 4. Effect of VNS on MPO quantification (ng/mg protein) in the colon. TNBS induced a significant increase of MPO both at the level of the lesion and just above (*** $p<0.001$ and ** $p<0.008$, respectively). VNS/TNBS animals presented a decrease of MPO concentration which was significant above the lesion (* $p<0.05$; $n\geq 7$, Mann–Whitney test).

3.4. Effect of VNS on levels of cytokine mRNAs after colitis induction

TNBS induced a significant increase of all studied mRNAs in the lesion compared to the control group ($p<0.0001$) (Fig. 5). VNS induced no change of TNFα, IL-1β, ICAM1 and IL-6 mRNA levels in the lesion as well as TNFα and ICAM1 mRNAs above the lesion.

3.5. Effect of VNS on the multivariate index of colitis

Table 2 shows that all studied parameters contribute to the inflammation observed in TNBS colitis and that the model of multivariate analysis is significant (Lambda Wilk: 0.050; F approx (17.7) = 7.814; $p<0.0051$). According to the standardized coefficient (SC), some of these parameters were more particularly associated with the inflammatory process in the lesion, such as IL-6 (SC: 4.027) while some parameters such as the locomotor activity (SC: 0.093) were less involved.

In noVNS/TNBS animals, a significant increase of the multivariate index of colitis was observed compared to the control group (9.35 ± 0.34 RU vs. 0.99 ± 0.22 RU, $p<0.0001$) (Fig. 6). VNS induced a significant decrease ($p<0.001$) of the same multivariate index of colitis (all previous parameters included) compared to noVNS/TNBS rats (3.49 ± 1.98 RU) (Fig. 6).

4. Discussion

The role of the vagus nerve as a modulator of intestinal inflammation has been recently reviewed (Van Der Zanden et al., 2009a). So far, all studies investigating the anti-inflammatory effect of VNS were performed acutely in anesthetized animals and the frequency range commonly used for this purpose was 5–10 Hz (Bernik et al., 2002b; Naritoku et al., 1995; Tracey, 2007). The originality of our experiments was to perform VNS on unanesthethized animals and, to our knowledge, this is the first study on the effects of VNS on the digestive tract in chronically implanted freely moving animals. We chose to stimulate only the left vagus nerve to minimize the effects on heart rate (Hotta et al., 2009), this nervous branch selectively innervates the atrio-ventricular node (McDowall R.J.S. et al., 1956). For the 5 Hz frequency, we also observed no change in behaviour of awakened or sleeping rats. A low-frequency range of stimulation (1–5 Hz) was classically used to obtain a peripheral anti-inflammatory effect through vagal efferents (Bernik et al., 2002a)(Borovikova et al., 2000) but vagal afferents may also be activated. For this purpose, we have recently provided the first fMRI study of VNS performed in rodents with low-frequency stimulation (i.e. 5 Hz). Highly significant VNS-related deactivations were found in large portions of the brain and particularly in the nucleus tractus solitarius which is the first central relay of vagal afferents (Reyt et al., 2010). Consequently, both vagal afferents and efferents are involved by VNS.

The rat model of colonic inflammation was first developed by Morris et al. (1989) using intra-luminal instillation of TNBS. In our experiments, the choice to perform VNS for 5 days was based on the work of Miceli and Jacobson (2003) who used a pharmacological approach to the cholinergic anti-inflammatory pathway using acetylcholinesterase inhibitors and reported a significant increase in the macroscopic colonic damage score on day 5 following induction of colitis. This is also the time required to obtain an acute inflammatory response with transmural inflammation (Miceli and Jacobson, 2003) (Alex et al., 2009)(Morris et al., 1989).

Our results indicate that VNS has a protective effect on colitis-induced weight loss, a classical parameter of colitis in humans and animals (Ballinger et al., 2000; Morris et al., 1989; Rigaud et al., 1994). In our experimental conditions, with the lowest dose of TNBS (10 mg/rat) to induce colitis (Morris et al., 1989), a 4% weight loss was observed which was reduced significantly to only 0.3% by VNS.

Another novel aspect of our study was to perform histological, biochemical, and molecular assessments in two different areas: the macroscopic damaged colon (lesion) and the area immediately above the lesion. Indeed, it is expected that the area above the lesion may be a key target for VNS therapy, i.e. to have a preventive effect or to limit the extension of the lesion. Qualitative histological evaluation supports the different effects of colitis in both areas with previously described modifications (Miceli and Jacobson, 2003; Morris et al., 1989; Porcher et al., 2004), while no macroscopic and microscopic lesions were observed in saline-treated animals. Interestingly, VNS/TNBS rats had less inflammatory infiltrate immediately above the lesion while VNS caused only a slight reduction in inflammatory infiltrate in the lesion compared to noVNS/TNBS. This argues for a major efficiency of VNS on less damaged tissues in our experimental conditions. This could be explained by the fact that the lesion is represented by damaged and necrotic cells with a poor response to therapeutics. These histological results were further supported by MPO quantification. MPO is an enzyme predominantly found in neutrophils and is currently used as a quantitative index of intestinal inflammation (Bradley et al., 1982). Previous studies have reported a statistically significant increase of MPO in colonic tissue, but without any distinction between lesion and adjacent regions (Ghia et al., 2006; Miceli and Jacobson, 2003). In our experiments, TNBS induced a statistically significant increase of MPO not only in the lesion (>200-fold higher) but also above (4-fold higher). VNS significantly reduced MPO levels only above the lesion, indicating that it is very pertinent to analyze each area separately. This observation reinforces our hypothesis that VNS therapy could be of interest for the treatment of mild colitis but also to prevent extension of the lesion and relapse.

Concerning circadian rhythms, TNBS classically induced a sickness response characterized by a disruption of rhythms with a lower body temperature during the dark period and a mild fever during the light period (Boisse et al., 2003). We saw the same effects on non-stimulated animals, 12 h after TNBS instillation, with a significant hypothermia during the dark period. It is well known that TNF alpha is transiently increased after TNBS instillation (Tateishi et al., 1997). In our experimental conditions, an increase of TNF in colonic tissues started 90 min after TNBS instillation, with a peak at 3 h and then values remained elevated until the 4th day (data not shown). It has been reported that the first response of increased levels of TNF was hypothermia (Tollner et al., 2000). Interestingly, in our results, a partial but nonsignificant inhibition of hypothermia was observed in the stimulated group during the same period; thus this effect could be related to the anti-TNF effect of VNS.

Inflammation is a complex, multi-scale biological response required for repair and regeneration after tissue injury. Due to the complexity of delayed inflammation kinetics, a global assessment

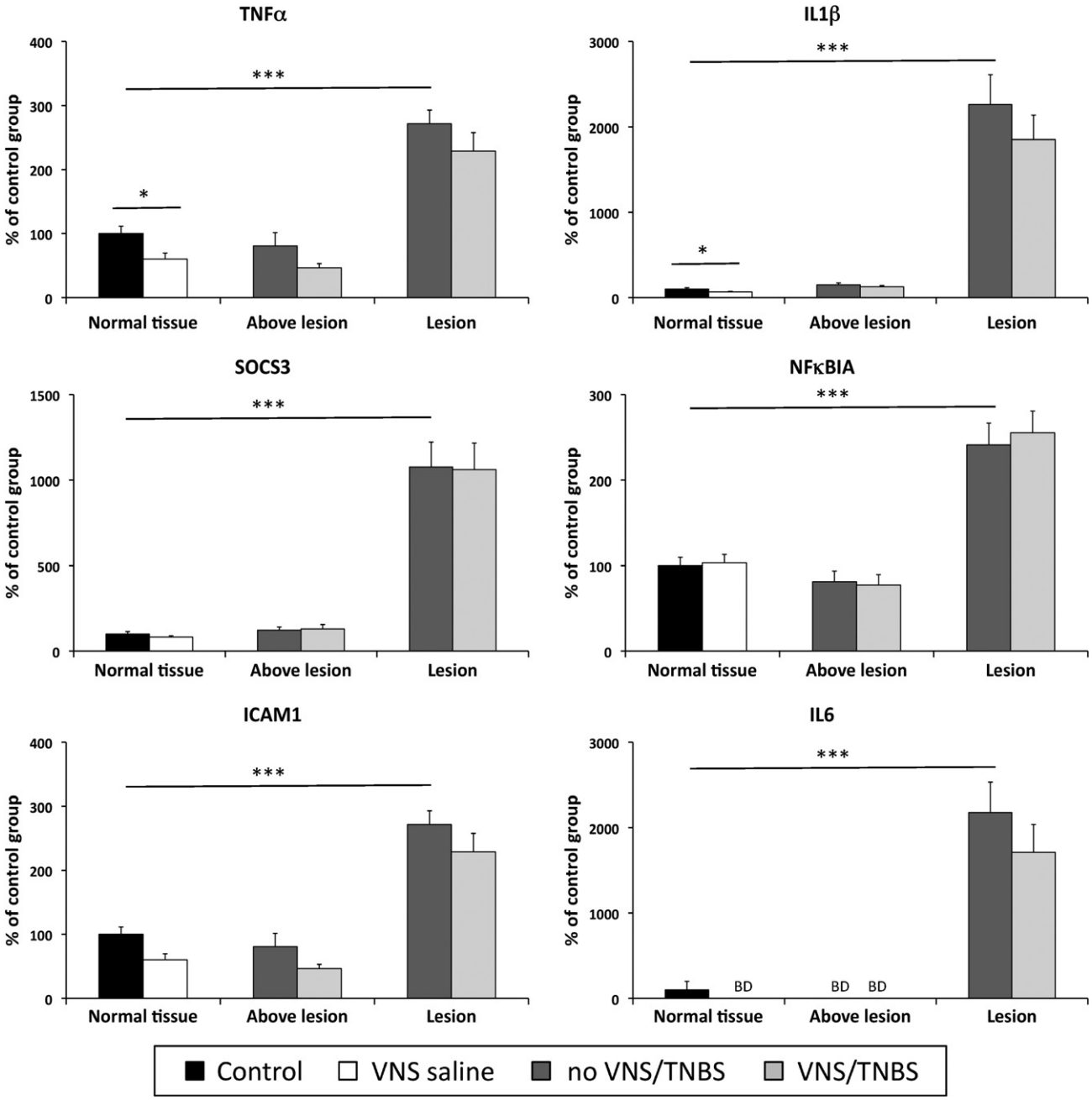

Fig. 5. Effect of VNS on TNFα, IL-1β, SOCS3, NFκBIA, ICAM1, and IL-6 mRNA levels in colonic tissues. TNBS induced a significant increase of all studied mRNAs in the lesion compared to the control group (*** $p<0.0001$). VNS induced significant decreases of both TNFα and IL-1β mRNA levels in normal tissues (* $p<0.05$, $n\geq 12$, Mann–Whitney test). BD: below the lower standard detection.

seems more appropriate to consider all biological interactions (Vodovotz et al., 2008). Hence, in addition to currently studied parameters like body weight or MPO quantification, additional physiological and molecular parameters were studied (telemetric data, areas of lesion, cytokine and cytokine-related mRNAs). These parameters allowed us to distinguish control and TNBS animals in non-stimulated conditions using a multivariate index of colitis. Using this discriminant analysis, a significant increase of this inflammatory index was observed in the noVNS/TNBS group, while VNS significantly decreased this index by approximately three times in VNS/TNBS (Fig. 6). In addition, values of standardized coefficients (range, 0.093–4.027) allowed an evaluation of the significance of each parameter in the TNBS colitis model. Among the five most important parameters (i.e. high values, Table 2) characterizing the inflammation, three of them (IL-1beta, IL-6 and TNF alpha mRNAs) were decreased by VNS treatment (Fig. 5). Although the variation of these mRNA cytokine parameters were individually nonsignificant after VNS, the effect of the stimulation on chronic inflammatory response could be related when these parameters were combined in a multivariate analysis. Expression of cytokine-related mRNA observed at day 5 is quite different than the inflammatory response as previously observed by Tateishi et al. (1997) at the protein level. This discrepancy could be explained by both differences between mRNA and protein kinetics and the higher sensitivity of mRNA assessments. TNBS colitis shares many of the clinical, histopathological, and immunological features of Crohn's disease which is characterized by an impairment of the Th1-Th17/Th2 inflammatory balance (Alex et al., 2009). TNF, a key cytokine in IBD, is necessary for both the initiation and perpetuation

of the Th1 response in TNBS colitis (Strober et al., 2002) and it is the most important parameter above the lesion in our study (Table 2).

Interestingly, the decrease of TNFα and IL1β mRNAs on normal tissue in VNS/saline animals suggests the possibility of an anti-inflammatory effect on the basal level of cytokines.

It has been proposed that VNS improvement of postoperative ileus inflammation in rats was mediated by the macrophage nAChR (for review (Van Der Zanden et al., 2009a)). It has also been described that the vagus nerve plays an important role in lymphocyte trafficking (Antonica et al., 1996). Thus, VNS could potentially affect several types of immune cells, i.e. modulating CD4+ cell activity (Karimi et al., 2010). Mechanisms involved in this anti-inflammatory effect remain to be determined as for example i) the JAK2-STAT3 signalling pathway macrophage activation which has been described to be attenuated by VNS in a mouse model of surgery-induced inflammation (The et al., 2007), and ii) the role played by the spleen reported to be necessary in the modulation of inflammation by the vagus nerve (Huston et al., 2006; Rosas-Ballina et al., 2008). VNS may also affect intestinal permeability which is known to be increased in IBD, forming the basis of lower inflammation as presented in a nonlethal rat hemorrhagic shock model (Luyer et al., 2005).

The results of this study have clinical relevance. Indeed, because of its anti-inflammatory property, notably on the low damaged tissue, VNS should be of interest in the treatment of IBD (Bonaz, 2007), particularly in the case of mild colitis and/or in the prevention of recurrence. In addition, since overall intentional non-adherence is reported by 39% of IBD patients (Cerveny et al., 2007), VNS therapy is of interest because it is independent of patient compliance.

Acknowledgements

Authors would like to acknowledge the involvement of Edgar Gentilhomme, Aurélie Faure, Jacques Mathieu (Institut de Recherche Biomédicale des Armées) and Marie-Hélène Laverrière (Département d'Anatomie et Cytologie, CHU Grenoble) for histological studies and for critical reading of the manuscript. This work was supported by Université Joseph Fourier, Direction Générale de l'Armement (DGA), INSERM, UCB Pharma, and from the Center of Medical Technology of St-Etienne, France.

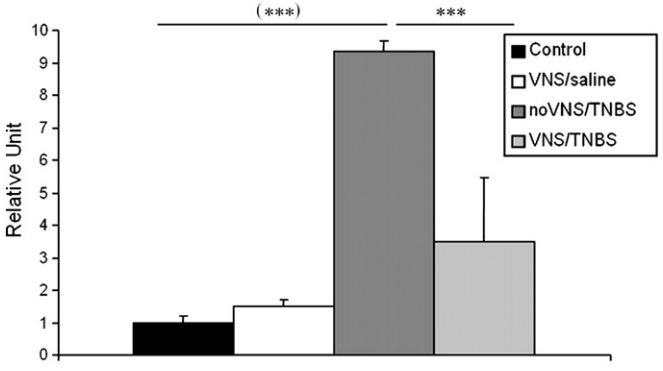

Fig. 6. Multivariate index of colitis obtained after discriminatory analysis applied on physiological parameters (locomotor activity, body temperature), quantification level of cytokine and cytokine-related mRNAs (TNF, IL-1β, IL-6, SOCS3, NFκBIA, TBX21) above and in the lesion (relative unit) and mean of lesion areas for the four groups. In noVNS/TNBS animals, a significant increase of the multivariate index of colitis was observed compared to the control group (*** $p<0.0001$). A significant decrease (*** $p<0.001$) of the multivariate index of colitis was observed in VNS/TNBS animals compared to noVNS/TNBS rats ($n\geq 12$).

Table 2
Characterization of TNBS inflammatory response.

	Parameters	Standardized coefficients
1	IL-6 in the lesion	4.027
2	SOCS3 in the lesion	3.622
3	TNFα above the lesion	2.719
4	IL-1β in the lesion	2.201
5	NFκBIA in the lesion	2.091
6	NFκBIA above the lesion	1.820
7	Body temperature at day 1	1.735
8	Body temperature at day 2	1.726
9	ICAM1 above the lesion	1.641
10	ICAM1 in the lesion	1.068
11	Mean of lesion areas	1.041
12	IL-1β above the lesion	0.964
13	SOCS3 above the lesion	0.959
14	TNFα in the lesion	0.863
15	TBX21 in the lesion	0.569
16	Locomotion at day 2	0.432
17	Locomotion at day 1	0.093

Characterization of TNBS inflammatory response: parameters in descending order are expressed by standardized coefficients; all studied parameters contribute to colitis and the model of multivariate index of colitis is significant (Lambda Wilk: 0.050; F approx $(17.7)=7.814$; $p<0.0051$).

References

Alex, P., Zachos, N.C., Nguyen, T., Gonzales, L., Chen, T.E., Conklin, L.S., Centola, M., Li, X., 2009. Distinct cytokine patterns identified from multiplex profiles of murine DSS and TNBS-induced colitis. Inflamm. Bowel Dis. 15, 341–352.
Anton, P.A., Shanahan, F., 1998. Neuroimmunomodulation in inflammatory bowel disease. How far from "bench" to "bedside"? Ann. NY Acad. Sci. 840, 723–734.
Antonica, A., Ayroldi, E., Magni, F., Paolocci, N., 1996. Lymphocyte traffic changes induced by monolateral vagal denervation in mouse thymus and peripheral lymphoid organs. J. Neuroimmunol. 64, 115–122.
Bai, A., Guo, Y., Lu, N., 2007. The effect of the cholinergic anti-inflammatory pathway on experimental colitis. Scand. J. Immunol. 66, 538–545.
Ballinger, A.B., Azooz, O., El-Haj, T., Poole, S., Farthing, M.J., 2000. Growth failure occurs through a decrease in insulin-like growth factor 1 which is independent of undernutrition in a rat model of colitis. Gut 46, 694–700.
Baumgart, D.C., Sandborn, W.J., 2007. Inflammatory bowel disease: clinical aspects and established and evolving therapies. Lancet 369, 1641–1657.
Bernik, T.R., Friedman, S.G., Ochani, M., DiRaimo, R., Susarla, S., Czura, C.J., Tracey, K.J., 2002a. Cholinergic antiinflammatory pathway inhibition of tumor necrosis factor during ischemia reperfusion. J. Vasc. Surg. 36, 1231–1236.
Bernik, T.R., Friedman, S.G., Ochani, M., DiRaimo, R., Ulloa, L., Yang, H., Sudan, S., Czura, C.J., Ivanova, S.M., Tracey, K.J., 2002b. Pharmacological stimulation of the cholinergic antiinflammatory pathway. J. Exp. Med. 195, 781–788.
Boisse, L., Van Sickle, M.D., Sharkey, K.A., Pittman, Q.J., 2003. Compromised neuroimmune status in rats with experimental colitis. J. Physiol. 548, 929–939.
Bonaz, B., 2007. The cholinergic anti-inflammatory pathway and the gastrointestinal tract. Gastroenterology 133, 1370–1373.
Borovikova, L.V., Ivanova, S., Zhang, M., Yang, H., Botchkina, G.I., Watkins, L.R., Wang, H., Abumrad, N., Eaton, J.W., Tracey, K.J., 2000. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature 405, 458–462.
Bradley, P.P., Christensen, R.D., Rothstein, G., 1982. Cellular and extracellular myeloperoxidase in pyogenic inflammation. Blood 60, 618–622.
Cerveny, P., Bortlik, M., Kubena, A., Vlcek, J., Lakatos, P.L., Lukas, M., 2007. Nonadherence in inflammatory bowel disease: results of factor analysis. Inflamm. Bowel Dis. 13, 1244–1249.
Chevrier, C., Bourdon, L., Canini, F., 2006. Cosignaling of adenosine and adenosine triphosphate in hypobaric hypoxia-induced hypothermia. Am. J. Physiol. Regul. Integr. Comp. Physiol. 290, R595–600.
Dantzer, R., Konsman, J.P., Bluthe, R.M., Kelley, K.W., 2000. Neural and humoral pathways of communication from the immune system to the brain: parallel or convergent? Auton. Neurosci. 85, 60–65.
de Jonge, W.J., Ulloa, L., 2007. The alpha7 nicotinic acetylcholine receptor as a pharmacological target for inflammation. Br. J. Pharmacol. 151, 915–929.
de Jonge, W.J., van der Zanden, E.P., The, F.O., Bijlsma, M.F., van Westerloo, D.J., Bennink, R.J., Berthoud, H.R., Uematsu, S., Akira, S., van den Wijngaard, R.M., Boeckxstaens, G.E., 2005. Stimulation of the vagus nerve attenuates macrophage activation by activating the Jak2-STAT3 signaling pathway. Nat. Immunol. 6, 844–851.
Fogel, R., Zhang, X., Renehan, W.E., 1996. Relationships between the morphology and function of gastric and intestinal distention-sensitive neurons in the dorsal motor nucleus of the vagus. J. Comp. Neurol. 364, 78–91.
Foley, J.O., DuBois, F., 1937. Quantitative studies of the vagus nerve in the cat. I.The ratio of sensory to motor fibers. J. Comp. Neurol. 67, 49–67.
Geboes, K., Desreumaux, P., Jouret, A., Ectors, N., Rutgeerts, P., Colombel, J.F., 1999. Histopathologic diagnosis of the activity of chronic inflammatory bowel disease. Evaluation of the effect of drug treatment. Use of histological scores. Gastroentérol. Clin. Biol. 23, 1062–1073.

Ghia, J.E., Blennerhassett, P., Kumar-Ondiveeran, H., Verdu, E.F., Collins, S.M., 2006. The vagus nerve: a tonic inhibitory influence associated with inflammatory bowel disease in a murine model. Gastroenterology 131, 1122–1130.
Groves, D.A., Brown, V.J., 2005. Vagal nerve stimulation: a review of its applications and potential mechanisms that mediate its clinical effects. Neurosci. Biobehav. Rev. 29, 493–500.
Handforth, A., Krahl, S.E., 2001. Suppression of harmaline-induced tremor in rats by vagus nerve stimulation. Mov. Disord. 16, 84–88.
Hotta, H., Lazar, J., Orman, R., Koizumi, K., Shiba, K., Kamran, H., Stewart, M., 2009. Vagus nerve stimulation-induced bradyarrhythmias in rats. Auton. Neurosci. 151, 98–105.
Huston, J.M., Ochani, M., Rosas-Ballina, M., Liao, H., Ochani, K., Pavlov, V.A., Gallowitsch-Puerta, M., Ashok, M., Czura, C.J., Foxwell, B., Tracey, K.J., Ulloa, L., 2006. Splenectomy inactivates the cholinergic antiinflammatory pathway during lethal endotoxemia and polymicrobial sepsis. J. Exp. Med. 203, 1623–1628.
Karimi, K., Bienenstock, J., Wang, L., Forsythe, P., 2010. The vagus nerve modulates CD4 + T cell activity. Brain Behav. Immun. 24, 316–323.
Luyer, M.D., Greve, J.W., Hadfoune, M., Jacobs, J.A., Dejong, C.H., Buurman, W.A., 2005. Nutritional stimulation of cholecystokinin receptors inhibits inflammation via the vagus nerve. J. Exp. Med. 202, 1023–1029.
Malgoyre, A., Banzet, S., Mouret, C., Bigard, A.X., Peinnequin, A., 2007. Quantification of low-expressed mRNA using 5′ LNA-containing real-time PCR primers. Biochem. Biophys. Res. Commun. 354, 246–252.
McDowall, R.J.S., Malcomson, G.E., I., M., 1956. The control of the circulation of the blood. Dawson, London, p. 619.
Miceli, P.C., Jacobson, K., 2003. Cholinergic pathways modulate experimental dinitrobenzene sulfonic acid colitis in rats. Auton. Neurosci. 105, 16–24.
Milby, A.H., Halpern, C.H., Baltuch, G.H., 2008. Vagus nerve stimulation for epilepsy and depression. Neurotherapeutics 5, 75–85.
Morris, G.P., Beck, P.L., Herridge, M.S., Depew, W.T., Szewczuk, M.R., Wallace, J.L., 1989. Hapten-induced model of chronic inflammation and ulceration in the rat colon. Gastroenterology 96, 795–803.
Naritoku, D.K., Terry, W.J., Helfert, R.H., 1995. Regional induction of fos immunoreactivity in the brain by anticonvulsant stimulation of the vagus nerve. Epilepsy Res. 22, 53–62.
Paschos, K.A., Kolios, G., Chatzaki, E., 2009. The corticotropin-releasing factor system in inflammatory bowel disease: prospects for new therapeutic approaches. Drug Discov Today 14, 713–720.
Pavlov, V.A., Wang, H., Czura, C.J., Friedman, S.G., Tracey, K.J., 2003. The cholinergic anti-inflammatory pathway: a missing link in neuroimmunomodulation. Mol. Med. 9, 125–134.
Pavlov, V.A., Parrish, W.R., Rosas-Ballina, M., Ochani, M., Puerta, M., Ochani, K., Chavan, S., Al-Abed, Y., Tracey, K.J., 2009. Brain acetylcholinesterase activity controls systemic cytokine levels through the cholinergic anti-inflammatory pathway. Brain Behav. Immun. 23, 41–45.
Peinnequin, A., Mouret, C., Birot, O., Alonso, A., Mathieu, J., Clarencon, D., Agay, D., Chancerelle, Y., Multon, E., 2004. Rat pro-inflammatory cytokine and cytokine related mRNA quantification by real-time polymerase chain reaction using SYBR green. BMC Immunol. 5, 3.
Pellissier, S., Dantzer, C., Canini, F., Mathieu, N., Bonaz, B., 2010. Psychological adjustment and autonomic disturbances in inflammatory bowel diseases and irritable bowel syndrome. Psychoneuroendocrinology 35, 653–662.
Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.
Porcher, C., Sinniger, V., Juhem, A., Mouchet, P., Bonaz, B., 2004. Neuronal activity and CRF receptor gene transcription in the brains of rats with colitis. Am. J. Physiol. Gastrointest. Liver Physiol. 287, G803–814.
Reyt, S., Picq, C., Sinniger, V., Clarençon, D., Bonaz, B., David, O., 2010. Dynamic Causal Modelling and physiological confounds: a functional MRI study of vagus nerve stimulation. Neuroimage 52, 1456–1464.
Rigaud, D., Angel, L.A., Cerf, M., Carduner, M.J., Melchior, J.C., Sautier, C., Rene, E., Apfelbaum, M., Mignon, M., 1994. Mechanisms of decreased food intake during weight loss in adult Crohn's disease patients without obvious malabsorption. Am. J. Clin. Nutr. 60, 775–781.
Rosas-Ballina, M., Ochani, M., Parrish, W.R., Ochani, K., Harris, Y.T., Huston, J.M., Chavan, S., Tracey, K.J., 2008. Splenic nerve is required for cholinergic antiinflammatory pathway control of TNF in endotoxemia. Proc. Natl Acad. Sci. USA 105, 11008–11013.
Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C (T) method. Nat. Protoc. 3, 1101–1108.
Strober, W., Fuss, I.J., Blumberg, R.S., 2002. The immunology of mucosal models of inflammation. Annu. Rev. Immunol. 20, 495–549.
Tateishi, H., Mitsuyama, K., Toyonaga, A., Tomoyose, M., Tanikawa, K., 1997. Role of cytokines in experimental colitis: relation to intestinal permeability. Digestion 58, 271–281.
Taylor, C.T., Keely, S.J., 2007. The autonomic nervous system and inflammatory bowel disease. Auton. Neurosci. 133, 104–114.
The, F.O., Boeckxstaens, G.E., Snoek, S.A., Cash, J.L., Bennink, R., Larosa, G.J., van den Wijngaard, R.M., Greaves, D.R., de Jonge, W.J., 2007. Activation of the cholinergic anti-inflammatory pathway ameliorates postoperative ileus in mice. Gastroenterology 133, 1219–1228.
Tobin, M.V., Logan, R.F., Langman, M.J., McConnell, R.B., Gilmore, I.T., 1987. Cigarette smoking and inflammatory bowel disease. Gastroenterology 93, 316–321.
Tollner, B., Roth, J., Storr, B., Martin, D., Voigt, K., Zeisberger, E., 2000. The role of tumor necrosis factor (TNF) in the febrile and metabolic responses of rats to intraperitoneal injection of a high dose of lipopolysaccharide. Pflugers Arch 440, 925–932.
Tracey, K.J., 2002. The inflammatory reflex. Nature 420, 853–859.
Tracey, K.J., 2007. Physiology and immunology of the cholinergic antiinflammatory pathway. J. Clin. Invest. 117, 289–296.
Tracey, K.J., 2009. Reflex control of immunity. Nat. Rev. Immunol. 9, 418–428.
Van Der Zanden, E.P., Boeckxstaens, G.E., de Jonge, W.J., 2009a. The vagus nerve as a modulator of intestinal inflammation. Neurogastroenterol. Motil. 21, 6–17.
van der Zanden, E.P., Snoek, S.A., Heinsbroek, S.E., Stanisor, O.I., Verseijden, C., Boeckxstaens, G.E., Peppelenbosch, M.P., Greaves, D.R., Gordon, S., De Jonge, W.J., 2009b. Vagus nerve activity augments intestinal macrophage phagocytosis via nicotinic acetylcholine receptor alpha4beta2. Gastroenterology 137, 1029–1039, 1039.e1-4.
van Westerloo, D.J., Giebelen, I.A., Florquin, S., Bruno, M.J., Larosa, G.J., Ulloa, L., Tracey, K.J., van der Poll, T., 2006. The vagus nerve and nicotinic receptors modulate experimental pancreatitis severity in mice. Gastroenterology 130, 1822–1830.
Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3 RESEARCH0034.
Vodovotz, Y., Csete, M., Bartels, J., Chang, S., An, G., 2008. Translational systems biology of inflammation. PLoS Comput. Biol. 4, e1000014.