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Vagal afferent activation suppresses systemic inflammation via the splanchnic anti-inflammatory pathway

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Abstract

Electrical stimulation of the vagus nerve (VNS) is a novel strategy used to treat inflammatory conditions. Therapeutic VNS activates both efferent and afferent fibers; however, the effects attributable to vagal afferent stimulation are unclear. Here, we tested if selective activation of afferent fibers in the abdominal vagus suppresses systemic inflammation. In urethane-anesthetized rats challenged with lipopolysaccharide (LPS, 60 μg/kg, iv.), abdominal afferent VNS (2 Hz for 20 min) reduced plasma tumor necrosis factor alpha (TNF) levels 90 min later by 88% compared with unmanipulated animals. Pre-cutting the cervical vagi blocked this anti-inflammatory action. Interestingly, the surgical procedure to expose and prepare the abdominal vagus for afferent stimulation ('Vagal manipulation') also had an anti-inflammatory action. Levels of the anti-inflammatory cytokine IL-10 were inversely related to those of TNF. Prior bilateral section of the splanchnic sympathetic nerves reversed the anti-inflammatory actions of afferent VNS and vagal manipulation. Sympathetic efferent activity in the splanchnic nerve was shown to respond reflexly to abdominal vagal afferent stimulation. These data demonstrate that experimentally activating abdominal vagal afferent fibers suppresses systemic inflammation, and that the efferent neural pathway for this action is in the splanchnic sympathetic nerves.

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Declarations of interest.

none.

Keywords

Lipopolysaccharide (LPS); Vagus nerve; Vagus nerve stimulation; Sympathetic nervous system; Splanchnic anti-inflammatory pathway; Inflammation; Tumor necrosis factor α (TNF); Interleukin 10 (IL-10); Endotoxemia; Greater splanchnic nerve

1. Introduction

Vagal nerve stimulation (VNS) is now considered a promising therapeutic tool to treat inflammatory diseases, including cases that are not responsive to classic pharmaceutical treatments (Bonaz et al., 2016b). VNS can suppress both local (Borovikova et al., 2000a) and systemic inflammation (Borovikova et al., 2000b). Selectively stimulating efferent fibers in the vagus has this action, and many studies have shown that it suppresses the levels of tumor necrosis factor α (TNF), a key mediator of the inflammatory response, in endotoxemia (intravenous lipopolysaccharide; LPS) (Pavlov and Tracey, 2015; Rosas-Ballina et al., 2008; Vida et al., 2011a). That anti-inflammatory action has been shown to depend upon a number of factors including the spleen, splenic nerves, a subset of T cells and alpha-7 nicotinic receptors (Pavlov and Tracey, 2015; Rosas-Ballina et al., 2011).

Therapeutic VNS, however, activates both efferent and afferent fibers in the vagus. At least 80% of these fibers are afferent (Prechtl and Powley, 1990). The effects of this form of stimulation have wide ranging actions, including suppression of inflammation in the gut (Bonaz et al., 2016a) and joints (Koopman et al., 2016). In these cases, the relative contributions of vagal afferent and efferent fibers are unclear. While the anti-inflammatory effects of vagal efferent stimulation are well described (see above), those of vagal afferent stimulation are only now emerging.

It is already evident that the anti-inflammatory actions of vagal afferent stimulation must be mediated by different mechanisms than those of vagal efferent stimulation. An incidental finding reported by Vida and colleagues was that stimulating the intact vagus (activating both afferent and efferent pathways) had a systemic anti-inflammatory action even in alpha-7 nicotinic receptor knockout mice (Vida et al., 2011a) in which selective vagal efferent stimulation is ineffective (Vida et al., 2011a; Wang et al., 2003). Afferent VNS has also been shown to exert a *local* anti-inflammatory action in experimental arthritis (Bassi et al., 2017). This local action was found to be due to the activation of sympathetic nerves which project to the affected joint, and could be mimicked by electrical stimulation of specific brain nuclei or of the ipsilateral sympathetic chain. The effects of afferent VNS on *systemic* inflammation remain to be clarified, however. Clarification is also needed regarding which afferent fibers produce this action. The methodology chosen in almost all studies so far has been stimulation of the vagus nerve in the neck: a nerve which includes a diverse mixture of afferent and efferent fibers, both myelinated and unmyelinated, and which supplies targets ranging from the larynx to the proximal colon.

In the present experiments, we tested whether selective stimulation of abdominal vagal afferent fibers depresses systemic inflammation elicited by LPS. These afferent fibers include inputs from the liver and the gastrointestinal tract, and are overwhelmingly

unmyelinated (Prechtl and Powley, 1990). Secondly we set out to test whether their action depends on the splanchnic anti-inflammatory pathway; previously shown to mediate the endogenous anti-inflammatory reflex in endotoxemia (Martelli et al., 2016, 2014a).

2. Methods

2.1. Animals and ethical approval

Fifty one adult male Sprague-Dawley rats (290–350 g) were obtained from Animal Resources Centre, Perth, Western Australia for use in these experiments. The rats were caged individually and housed at 22 °C on a 12:12-h light–dark cycle. They were provided with food (Barastoc) and water *ad libitum*. At the end of each experiment, animals were killed with an injection of pentobarbital sodium (> 100 mg/kg iv; Troy Laboratories, Glendenning, NSW, Australia).

All experiments were performed in accordance with guidelines of the National Health and Medical Research Council of Australia and were approved by the Animal Experimentation Ethics Committee of the Florey Institute of Neuroscience and Mental Health.

2.2. General surgical preparation

Initial anesthesia on rats was induced with pentobarbital sodium (60 mg/kg ip). After shaving their torso, the trachea was cannulated and anesthesia maintained for the duration of surgery by artificial ventilation with 2% isoflurane in pure oxygen, delivered by a rodent ventilator (Ugo Basile, Italy). A water-perfused Silastic jacket or electric homeostatic blanket was positioned under the animal to maintain its core body temperature at 37 °C. Core temperature was measured by a thermocouple inserted 5 cm into the rectum. The right femoral artery and vein were cannulated for monitoring of arterial blood pressure and intravenous administration of drugs, respectively. Blood pressure, airway pressure, rectal temperature and neural signals (described below) were recorded on a computer-based acquisition system (CED Power 1401 interface with Spike2 software; Cambridge Electronic Design, Cambridge, U.K.).

Animals were subdivided randomly into 5 experimental groups (n = 6 per group). These were: (1) Sham splanchnic nerve section (Sham), (2) sham splanchnic nerve surgery with vagal stimulation (Vagal stim), (3) sham splanchnic nerve surgery with vagal manipulation (Vagal manip), (4) splanchnic nerve section with vagal stimulation (Vagal stim + SplancX), and (5) splanchnic nerve section with vagal manipulation (Vagal manip + SplancX); see Fig. 1, surgical details are given below). When preparatory surgery was complete, isoflurane anesthesia was gradually withdrawn and replaced by urethane (1.2 g/kg iv). Artificial ventilation with oxygen was maintained for the rest of the experiment.

After obtaining a baseline blood sample (0.8 ml) from the femoral artery at time −10 min, LPS (60 μg/kg) isolated from *E. coli* (0111:B4; Sigma-Aldrich, MO, USA) was injected intravenously at time zero. The arterial blood samples were collected into EDTA-containing tubes and immediately centrifuged (15 min, 3,000g). Plasma was frozen and stored at −80 °C. A second blood sample was obtained at 90 min after LPS injection.

2.3. Splanchnic nerve section procedure

The greater splanchnic sympathetic nerves were exposed via a retroperitoneal flank incision on each side of the animal. The adrenal gland was identified, freed from the perirenal fat and gently retracted using cotton tips. The greater splanchnic nerve was followed along the greater psoas muscle in the centripetal direction from the adrenal to the diaphragm (Martelli et al., 2014b). When indicated, both greater splanchnic nerves were sectioned just centrally to the splanchnic (suprarenal) sympathetic ganglion. Sham splanchnic nerve section, omitted this last step.

2.4. Abdominal vagus nerve preparation and stimulation

Access to the abdominal vagus nerve was gained by a ventral abdominal midline incision. The stomach was retracted caudally to expose the subdiaphragmatic oesophagus. The overlying liver lobes were dissected free from the oesophagus and retracted, using saline-soaked gauze pads. The subdiaphragmatic anterior vagus nerve trunk was identified running along the oesophagus and was dissected free from the oesophagus for ~6 mm. A cuff electrode pair was then placed around the freed segment of abdominal vagus. The electrode pair consisted of two 75 μm diameter silver wires, separated by ~2 mm, threaded through a segment of silastic tubing (internal diameter 500 μm): a longitudinal slit in the tubing allowed it to be placed around the intact nerve. A sliver of thin black plastic sheeting was placed under the electrodes to insulate any exposed wire from the oesophagus, and the nerve was covered with mineral oil to prevent drying while maintaining electrical insulation. The Teflon-coated wire leads were connected to a stimulator (SD9H, Grass Instruments, USA) such that the rostral-most electrode was the cathode. The vagus was crushed below the electrode pair by tightening a ligature (0.3 mm silk suture) around it. At least 30 min later, when urethane anesthesia had been stabilized, the vagus nerve was stimulated through the electrode pair (cathode rostral), using constant voltage monopolar pulses (20 V, 0.1 ms at 2 Hz) for 20 min (from 10 min before LPS administration until 10 min after). For the vagus nerve manipulation maneuver, the same procedure described above was repeated, except that no electric pulses were delivered to the stimulating electrode.

2.5. Cytokine and corticosterone measurements

Plasma samples from all animals were assayed for TNF, interleukin 10 (IL-10) and interleukin 1β (IL-1β) by sandwich ELISA (R&D Systems, Minneapolis, MN). Quantification limits were: 12.5-800 pg/ ml for TNF; 31-2,000 for IL-10 and 31-2000 pg/ml for IL-1β. If necessary, plasma samples were diluted to fall within these quantification ranges. Another aliquot of each sample was also assayed for corticosterone by ELISA (Abnova, Jhongli, Taiwan).

We detected a possible labeling error in three cases in the IL-10 assay (raised ‘baseline’ levels but undetectable levels ‘90 min after LPS’, which we consider biologically impossible). These data were excluded, leaving n = 5 instead of n = 6 in three experimental groups (Vagal stim, Vagal stim + SplancX, Vagal manip + SplancX).

2.6. Electrophysiology recording

In 2 rats, electrophysiological recording of the vagal mass action potential was used to verify that the stimuli delivered by the cuff electrodes effectively activated vagal C fibers. The distal section of the anterior abdominal vagus was cut close to the stomach and prepared for recording. The cut end was placed over a pair of silver wire electrodes and immersed under mineral oil. Single 0.1 ms pulses were delivered to the cuff stimulating electrodes (see description above), with the distal electrode as the cathode, and the mass action potential was recorded monophasically from the distal cut end of the nerve (N102 preamplifier: Neurolog, Welwyn Garden City, U.K.) at an overall gain of 5,000 and bandpass 10–500 Hz.

To record the reflex response to VNS in the splanchnic nerve, the stimulating cuff electrode around the anterior abdominal vagus was secured in place with silicon elastomer (Kwik-Sil, World Precision Instruments, Sarasota, FL) in 5 rats. The vagus was crushed distal to the cuff. The left splanchnic nerve was approached via a flank incision, as described above, cut distally and placed over a pair of silver wire electrodes. Ongoing and reflex activity was recorded from the nerve under mineral oil. The signal was recorded with a Neurolog 102 preamplifier, amplified × 10,000 and filtered (bandpass 80–600 Hz). To demonstrate the splanchnic reflex response to vagal afferent stimulation, the signal was rectified, smoothed (time constant 20 ms) and averaged.

Finally, we investigated the possibility that the abdominal manipulation used to prepare the animal for VNS (see above) could trigger action potentials in the afferent vagus. In 2 rats, the abdomen was opened ventrally and the anterior subdiaphragmatic vagus nerve exposed as previously described. The left cervical vagus was also dissected free from the surrounding tissue and cut centrally. The peripheral end was desheathed and split into filaments for recording, using fine silver wire electrodes under a pool of mineral oil. Afferent filaments with spike activity originating from sub-diaphragmatic regions, characterized by a lack of respiratory modulation, were selected. While monitoring the ongoing activity of these fibers, the subdiaphragmatic vagus, the stomach and the duodenum were probed with a cotton bud, mimicking the abdominal manipulation used to prepare animals for VNS.

2.7. Section of the cervical vagus nerves

Finally, we wished to establish unequivocally that vagal afferent activation was the cause of the anti-inflammatory effect observed under our experimental conditions. To this end, the abdominal vagus was electrically stimulated (as described above) in 12 animals whose cervical vagus nerves had previously been bilaterally exposed, or exposed and cut. As before, blood samples were collected for assay of plasma TNF concentrations 10 min before and 90 min after the administration of LPS.

2.8. Statistical analysis

Statistical analyses were carried out using the R statistical programming environment (RStudio V1.0.143). Each data set was tested for normality using the Shapiro test for normality. Homogeneity of variance was established using Levene’s test. Data were transformed to achieve normality and homogeneity of variance where appropriate and possible. Evidence for an effect of treatment on cytokine concentrations at baseline was

assessed via one-way ANOVA or Kruskal-Wallis rank sum test before post-hoc analysis by Student's t-Test or Wilcoxon's test. Similarly, evidence for an effect of treatment on cytokine production in response to LPS was assessed by one-way ANOVA performed on the mean changes in cytokine levels from baseline 90 min after administration of LPS followed by post-hoc analysis as above. In instances where multiple comparisons were made, p values were adjusted using the Holm method. Linear regression was used to test for a correlation between the ratio of TNF to IL10 (TNF:IL10) concentrations as a function of TNF or IL10 concentrations among individual rats. The data were checked for homoscedasticity by consultation of residual diagnostic plots and transformed where necessary.

3. Results

3.1. Abdominal vagal afferent stimulation suppresses systemic inflammation in endotoxemia

In control animals (n = 6), LPS (60 μg/kg i.v.) raised plasma levels of TNF from low or undetectable levels at baseline to 11.01 ± 2.24 ng/ml at 90 mins (Fig. 2). Selective stimulation of abdominal vagal afferent fibers at a strength that maximally activates C fibers (see below) reduced the increase in TNF levels detected at 90 mins after LPS to 1.34 ± 0.40 ng/ml ($p < 0.001$, n = 6). Interestingly, the surgical manipulation to place the stimulating cuff electrodes around the abdominal vagus and ligate it distally also had an anti-inflammatory action, as judged by a reduction of plasma TNF levels 90 mins after LPS compared with those of the Sham group ($p = 0.015$; n = 6; Fig. 2). All animals in these three groups received sham splanchnic nerve surgery.

Plasma levels of the anti-inflammatory cytokine IL-10 showed an inverse pattern compared to that of TNF. Both vagal manipulation (n = 6) and vagal stimulation (n = 5) were associated with enhanced increases in plasma IL-10 levels as recorded 90 min after administration of LPS when compared to that of the sham group (Fig. 3; $p = 0.019$ and $p = 0.019$ for Vagal manip and Vagal stim vs. Sham).

Over all animals in the 3 groups receiving sham splanchnic surgery there was a clear, non-linear inverse relation between individual TNF and IL-10 levels: high TNF levels were linked to low IL-10 levels, and *vice versa* (Fig. 4A). To confirm this, the TNF:IL10 ratio measured in each animal was plotted as a function of the TNF concentration (Fig. 4B): the natural logarithms of the TNF/IL10 ratio and of the plasma TNF concentration were strongly correlated (adjusted $R^2 = 0.84$, $p < 0.001$). Similarly, the natural logarithm of the IL:10/TNF ratio correlated well with that of the plasma IL10 level (adjusted $R^2 = 0.59$, $p < 0.001$, data not shown).

The mean plasma level of IL-1β tended to be higher 90 min after treatment with LPS than at baseline, but we found no evidence for an effect on IL-1β levels by vagal afferent stimulation or vagal manipulation ($p = 0.916$, $p = 0.838$, respectively: Fig. 5). There was no consistent change in plasma corticosterone levels between baseline values and those 90 mins after LPS ($p = 0.942$: Fig. 6).

3.2. Anti-inflammatory action of abdominal vagal afferent activation depends on the splanchnic nerves

After bilateral section of the splanchnic nerves, afferent vagal nerve stimulation and vagal manipulation failed to inhibit the increase in plasma TNF induced by LPS (Fig. 2). Increases in plasma TNF in response to LPS were greater in the Vagal manip + SplancX and Vagal stim + SplancX groups than in those receiving the same treatment after sham splanchnic nerve section ($p = 0.015$ and $p = 0.014$, respectively; $n = 6$). Splanchnic nerve section also prevented the increase in plasma IL-10 after LPS in animals receiving vagal manipulation and vagal stimulation (Fig. 3). Ninety minutes after LPS, IL-10 levels were lower in the Vagal manip + SplancX group ($n = 5$) than in the Vagal manip group ($n = 6$; $p = 0.020$) and were also lower in Vagal stim + SplancX group ($n = 5$) than in the Vagal stim group ($n = 5$; $p = 0.025$).

3.3. Splanchnic nerve section unmasks a pro-inflammatory effect of surgical preparation

Interestingly, when baseline cytokine levels of animals whose splanchnic nerves were cut and those who received sham splanchnic surgery were compared, we found that the mean baseline TNF levels were raised in animals with cut splanchnic nerves (3.15 ± 0.36 ng/ml, $n = 12$) from low or undetectable levels in splanchnic-intact animals ($n = 18$; $p = 0.011$, Fig. 7). The time between splanchnic nerve section or sham section and baseline blood samples was approximately 1 h. Baseline levels of IL-10 and IL-1β were unaffected by prior splanchnic nerve section (data not shown).

3.4. Abdominal vagal afferent stimulation activates vagal C fibers and reflexly modulates splanchnic nerve efferent activity

In 2 rats, abdominal vagal afferent fibers were stimulated with the cuff electrode as above, except that the distal part of the vagus was cut and prepared for electrophysiological recording. Single shocks of 20v, 0.1 ms at 1 or 2 Hz evoked a supramaximal C-fiber volley, as shown by the mass action potential recorded distally from the cut nerve at stomach level (Fig. 8A). The mass action potential showed that the stimulus activated fibers (orthodromically-activated efferent fibers and antidromically-activated afferent fibers), all with calculated conduction velocity in the C fiber range (~1 m/s). No measurable faster-conducting fiber groups were detected (the stimulus artifact was small and would not have masked shorter latency peaks).

In 5 rats, the vagus was ligated distally to the cuff electrode (as above), and the efferent nerve response recorded electrophysiologically from the central end of the cut, left splanchnic nerve. Averaged, rectified nerve recordings showed that splanchnic nerve efferent fibers were stimulated in a time-locked fashion by the supramaximal vagal afferent shocks. The response profile was a phase of inhibition followed by a phase of activation (Fig. 8B). Control activity was averaged in response to the same time sequence of trigger pulses during a period without stimulation.

In 2 rats, the possibility that manipulating the abdomen may itself generate vagal afferent nerve traffic was investigated electophysiologically. Probing the area that connects the

duodenum and the stomach with a cotton bud produced activation of abdominal afferent nerve fibers recorded at the level of the left cervical vagus (Fig. 9).

3.5. Suppression of inflammation is mediated by vagal afferent transmission

Finally, to show unequivocally that vagal afferent activation produces an anti-inflammatory action under our experimental conditions, we repeated the abdominal nerve stimulation protocol in a cohort of rats whose cervical vagus nerves had been pre-cut, or exposed and left uncut (Fig. 10A). Ninety minutes after the administration of LPS, the plasma TNF concentration of animals whose cervical vagi were cut before electrical stimulation of the abdominal vagus nerve was 19.8 ± 7.75 ng/ml (n = 6: Fig. 10B). This was greater than that of animals whose cervical vagus nerves were exposed but not cut before electrical stimulation of the abdominal vagus nerve (2.34 ± 0.39 ng/ml; n = 6; p = 0.04995).

4. Discussion and conclusions

These results show two main new findings: first, selective activation of afferent fibers in the abdominal vagus has an anti-inflammatory action in an animal model of endotoxemia; second, that action depends upon the splanchnic sympathetic nerves. The simplest interpretation of these findings is that stimulating abdominal vagal afferent fibers reflexly activates the splanchnic anti-inflammatory pathway. This interpretation is supported by three findings: first, electrical afferent VNS or vagal manipulation failed to suppress inflammation if splanchnic nerves were previously sectioned (Figs. 2 and 3); second, vagal afferent transmission to the brain was required for the anti-inflammatory action of VNS (including preparatory vagal manipulation); third, selective electrical activation of vagal afferent C fibers reflexly modulates splanchnic sympathetic nerve activity (Fig. 8).

Therapeutic VNS invariably involves stimulating both afferent and efferent fibers. The anti-inflammatory actions of vagal efferent fibers have been well studied, and shown to depend upon the spleen, splenic nerves, alpha-7 nicotinic receptors and a subset of T cells (Pavlov and Tracey, 2015).

In the context of endotoxemia, Vida and co-workers showed that stimulating the whole intact vagus suppressed plasma TNF levels at least as effectively as selective efferent stimulation, and was able to do so in the alpha-7 receptor knockout mouse where vagal efferent stimulation was ineffective (Vida et al., 2011a). These findings clearly demonstrate that the suppression of systemic inflammation by vagal afferent fibers acts by a different mechanism. Unlike selective vagal efferent stimulation, vagal afferent + efferent stimulation has been shown to result in a rise in plasma adrenaline (Vida et al., 2011a,b). Because adrenaline comes specifically from the adrenal medulla, under the control of the splanchnic sympathetic nerves, this finding indicates that splanchnic sympathetic fibers are activated.

The anti-inflammatory mechanisms engaged by vagal *afferent* fibers have not been extensively studied. In a model of local inflammation (zymosan-induced arthritis), Bassi et al found that the anti-inflammatory action of cervical vagal afferent stimulation was dependent on the locus coeruleus and on the sympathetic nerves on the side of the affected joint (Bassi et al., 2017). Unlike vagal *efferent* stimulation, this effect did not require the

spleen. In this context, the anti-inflammatory action was evidently local rather than systemic, and was mediated directly by the sympathetic nerves to the inflamed joint (Bassi et al., 2017).

A high fat diet has been shown to suppress plasma TNF and IL-6 levels, as well as measures of gut leakiness, in response to hemorrhagic shock or to LPS in rodents (Lubbers et al., 2010; Luyer et al., 2005). This anti-inflammatory action was blocked by vagotomy and by antagonists of cholecystokinin, strongly implicating a role of vagal afferent fibers. The nicotinic antagonist, chlorisondamine, also blocked this action (Lubbers et al., 2010) While the authors interpreted these findings as showing the involvement of the cholinergic anti-inflammatory pathway (i.e. a vagal efferent pathway), they are equally compatible with efferent transmission by a sympathetic pathway such as the splanchnic anti-inflammatory pathway. This is because vagotomy would block the reflex by interrupting vagal afferents, while chlorisondamine would block both parasympathetic and sympathetic efferent transmission. All these previous data are compatible with the proposal that activation of vagal afferent fibers reflexly engages sympathetic anti-inflammatory nerves. In the case of arthritis, local sympathetic innervation of the joint appears to be the effector pathway. In the case of systemic inflammation, our present findings indicate that it is the splanchnic sympathetic nerves.

We have previously demonstrated that the splanchnic sympathetic nerves are endogenously activated by systemic endotoxemia, and these in turn have an anti-inflammatory action (Martelli et al., 2016, 2014a,b). This represents the endogenous ‘inflammatory reflex’ to endotoxemia. The present finding that baseline TNF values after splanchnic nerve section are raised (Fig. 7), indicates that this endogenous reflex also holds down the inflammatory response to surgical tissue damage. We previously found that cutting the vagi did not affect the endogenous inflammatory response to systemic LPS, indicating that neither vagal afferent nor efferent fibers were involved under those conditions (Martelli et al., 2014b). Exogenous activation of vagal afferent fibers, however, seems able to exert an additional reflex antiinflammatory action that is superimposed upon that of the endogenous reflex. Indeed, the preparatory surgery before vagal afferent stimulation (‘vagal manipulation’) was sufficient to suppress systemic inflammation via the splanchnic sympathetic pathway. We believe that mechanical stretch of the stomach and esophagus probably activated mechanoreceptive vagal afferent fibers (see Fig. 9). These and chemoreceptive vagal afferent fibers (activated by a high fat diet and blocked by cholecystokinin antagonists, (Luyer et al., 2005)) may be the substrate for the anti-inflammatory reflex driven by abdominal afferent VNS. VNS is a very powerful anti-inflammatory procedure. It has been reported that only one electric shock is sufficient to inhibit the systemic TNF production in response to intravenous LPS (Olofsson et al., 2015). We speculate that the anti-inflammatory action induced by vagal manipulation was the result of a vagal afferent activation that was maximal and could not be enhanced by further electrical stimulation of the vagus.

An alternative possibility should be considered. The stomach is also innervated by ‘sympathetic’ afferent fibers whose axons travel within the splanchnic nerves (Longhurst et al., 1981). These include mechanosensitive fibers that respond to distension of the stomach and produce reflex modulation of sympathetic outputs when activated (Longhurst et al.,

1981; Sabbatini et al., 2017; Traub et al., 1996). The possibility remained that activation of mechanosensitive splanchnic afferent nerves during preparatory surgery could have provoked reflex activation of the sympathetic anti-inflammatory pathway: a 'splanchno-splanchnic reflex'. Our final series of experiments show that, in rats subjected to abdominal vagal afferent nerve stimulation, the levels of plasma TNF after LPS challenge are higher in the group where the cervical vagi were previously cut compared to animals were the vagi were left intact (Fig. 10). This difference should be considered as a lack of the anti-inflammatory action of VNS rather than an enhanced pro-inflammatory response mediated by cervical vagal nerve section. This is because the vagus nerve does not endogenously control inflammation in LPS challenged animals (extensively reviewed in Martelli et al., 2016). Therefore, these results demonstrated unequivocally that afferent transmission through the cervical vagi was essential for producing the anti-inflammatory action evoked by exposing, manipulating and electrically stimulating the anterior abdominal vagus nerve, eliminating the possibility that the anti-inflammatory effect was due to the activation of a hypothetical 'splanchno-splanchnic reflex'.

Given the systemic nature of the endotoxemic insult and the number of tissues innervated by neurons which travel in the splanchnic sympathetic nerves, it is difficult to pinpoint the target of the sympathetic inflammatory reflex evoked by LPS. A likely candidate for involvement is the spleen. Treatment with LPS produces a sustained increase in the efferent activity of the splenic nerve; itself driven by neurons with axons in the greater splanchnic nerves (MacNeil et al., 1997; Bratton et al., 2012; Martelli et al., 2014b). Spleen tissue homogenates collected from rats 90 min after LPS possess elevated concentrations of TNF (Huston et al., 2006; Martelli et al., 2014a) and these levels are further enhanced by section of the splanchnic nerves (Martelli et al., 2014a). Presumably, secreted noradrenaline derived from sympathetic terminals located in the white pulp of the spleen will activate β2 adrenoceptors on nearby macrophages and natural killer cells, and so produce the anti-inflammatory action associated with splenic nerve activation (Katafuchi et al., 1993; Elenkov et al., 1995; Kees et al., 2003; Vida et al., 2011a; Abe et al., 2017). Interestingly, splenocytes isolated from mice after activation of C1 neurons of the ventrolateral medulla, by either physiological (restraint stress) or exogenous (optogenetic activation) means, can confer protective effects when transplanted into naive animals in a model of renal ischemia–reperfusion injury (Abe et al., 2017). While it seems likely that the spleen is important to the sympathetic inflammatory reflex, none of these data preclude the possibility that other organs supplied by the greater splanchnic nerves may also participate. For example, prior section of the splanchnic nerves has been shown to elevate TNF levels in tissue homogenates of the liver after treatment with LPS (Huston et al., 2006; Martelli et al., 2014a). The involvement of other tissues supplied in the sympathetic inflammatory reflex remains to be tested.

The key pro-inflammatory cytokine TNF is considered a necessary and sufficient mediator of acute inflammation (Tracey et al., 1986), and it was strongly suppressed by afferent vagal nerve activation in the current study. The reciprocal changes seen in levels of the anti-inflammatory IL-10 (cf. (Harden et al., 2014)) suggest that afferent vagal nerve activation triggers a coordinated anti-inflammatory response. Plasma IL-1β clearly did not participate in that response. We cannot explain this, but note that it also showed an anomalous response

for a pro-inflammatory cytokine in the endogenous inflammatory reflex, being reduced by splanchnic nerve section (Martelli et al., 2014a). Perhaps the sympathetic neural control of this cytokine is excitatory rather than inhibitory. Independent support for this suggestion is provided by the report that the IL-1β response to LPS was reduced by thoracic epidural anesthesia (Enigk et al., 2014), which would have inhibited sympathetic nerve activity.

VNS is one of the most promising therapeutic tools to treat medical conditions associated with uncontrolled inflammation, including those that are unresponsive to pharmacological treatment (Bonaz et al., 2016b). We believe that it is important to understand the disparate neural pathways engaged by VNS when both efferent and afferent vagal fibers are activated. The present data indicate that selective activation of the afferent vagus acts reflexly to suppress systemic inflammation via the splanchnic sympathetic nerves – the same effector pathway that mediates the endogenous inflammatory reflex (Martelli et al., 2014a,b) – by enhancing that endogenous reflex action. The efficacy of this action encourages investigation of new “electroceutical” strategies which target sympathetic rather than parasympathetic nerves for their antiinflammatory potential. Indeed, exploration along these lines has already begun (Willemze et al., 2017).

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Abbreviations:

VNS	vagal nerve stimulation
LPS	lipopolysaccharide
TNF	tumor necrosis factor α
IL-10	interleukin 10
IL-1β	interleukin 1β

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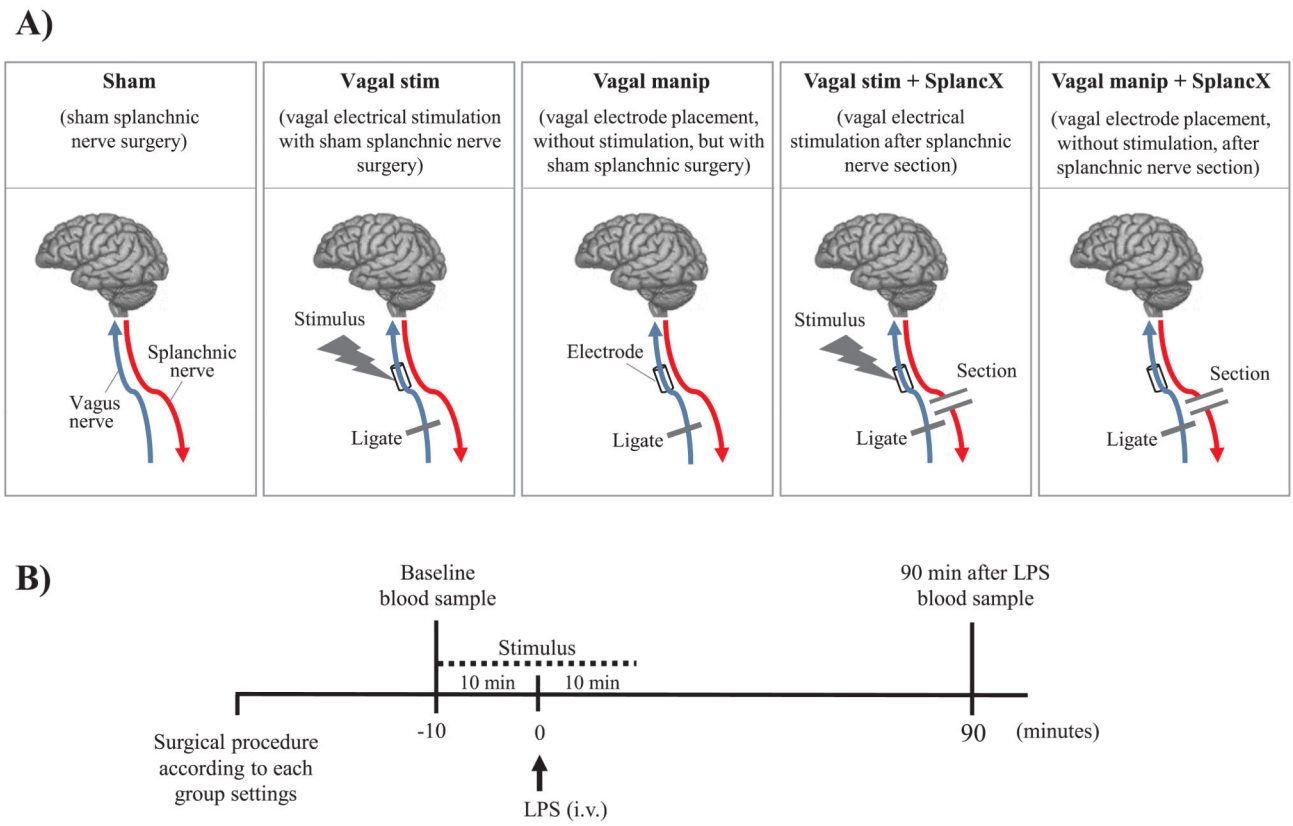

Fig. 1.

A. Schematic representation of the experimental setups that define the five experimental groups (n = 6). Sham stands for sham splanchnic surgery. Vagal electrical stimulation (Vagal stim) parameters used were 20 V, 0.1 ms (cathode rostral) at 2 Hz for 20 min. The anterior subdiaphragmatic vagus nerve was crushed below the electrode pair by tightening a ligature (ligate, in the figure) around it. Splanchnic nerve section (SplancX) and sham splanchnic surgery were performed bilaterally. B. Experimental timeline. After approximately 2 h of surgical preparation and establishment of urethane anesthesia, animals belonging to the "Vagal stim" group and the "Vagal stim + SplancX" group, were subjected to afferent vagal nerve stimulation (see above for parameters) for 20 min, 10 min before and 10 after LPS i.v. injection (60 μg/kg). Blood was collected at −10 min (baseline sample, just before vagal stimulation) and +90 min minutes from LPS injection. Plasma samples derived from these were assayed for cytokines and corticosterone content.

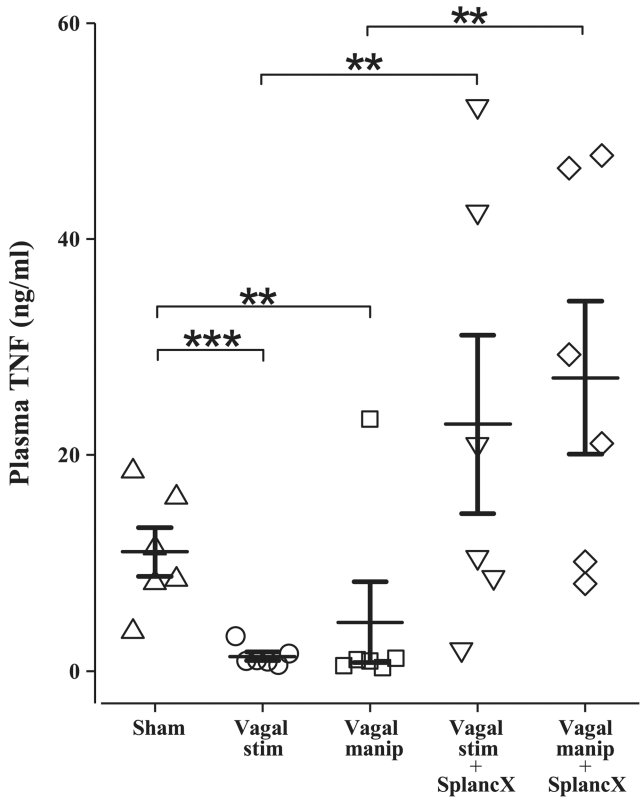

Fig. 2.

Plasma TNF levels measured 90 mins after giving LPS to animals of 5 experimental groups (n = 6 in each). These were: i) Sham (sham splanchnic nerve surgery), ii) Vagal stim (vagal stimulation with sham splanchnic nerve surgery), iii) Vagal manip (vagal electrode placement, without stimulation, but with sham splanchnic surgery), iv) Vagal stim + SplancX (as ii, but after splanchnic nerve section), v) Vagal manip + SplancX (as iii, but after splanchnic nerve section). Bars represent mean ± SEM. Data were analyzed using one-way ANOVA followed by post-hoc analysis (Student's *t*-test or Wilcoxon rank sum test). **p < 0.01; ***p < 0.001.

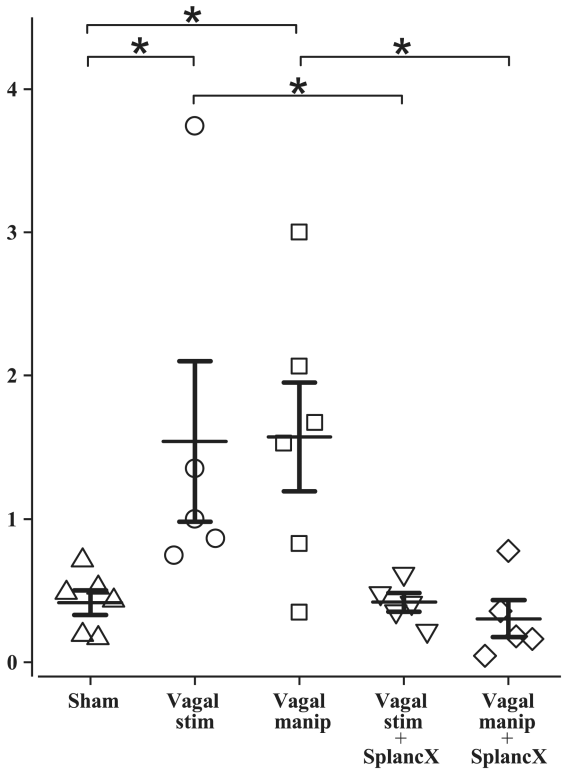

Fig. 3.

Plasma IL-10 levels 90 mins after giving LPS. Groups and details as in Fig. 2 except that Vagal stim, Vagal manip + SplancX and Vagal stim + SplancX groups are n = 5. Bars represent mean ± SEM. Data were analyzed using one-way ANOVA followed by post-hoc analysis (Student's *t*-test) *P < 0.05.

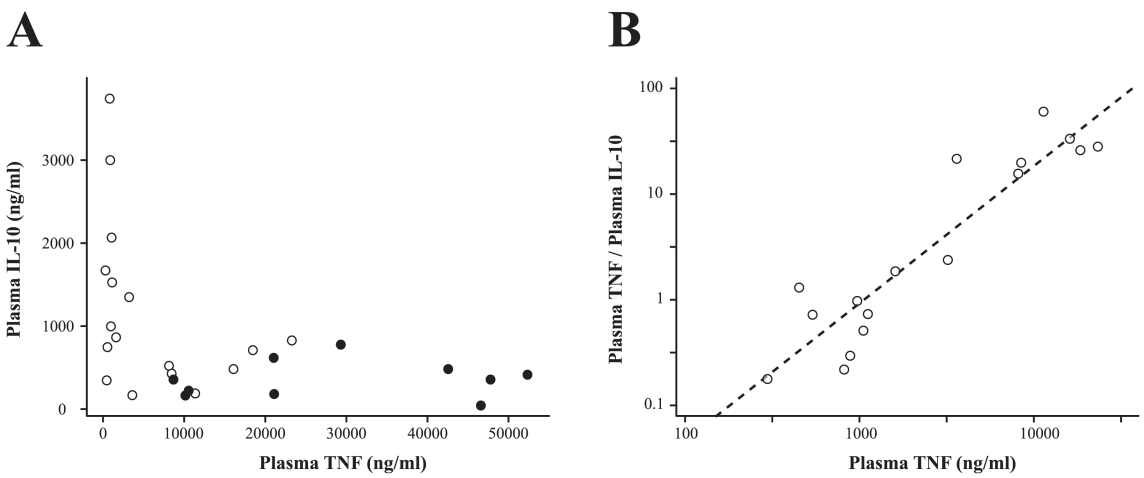

Fig. 4.

A. Non-linear inverse relation between plasma levels of TNF and IL-10 taken 90 mins after LPS in individual animals. Black and open symbols show data from animals subjected to splanchnic nerve section (n = 10) and sham splanchnic nerve surgery (n = 17), respectively.

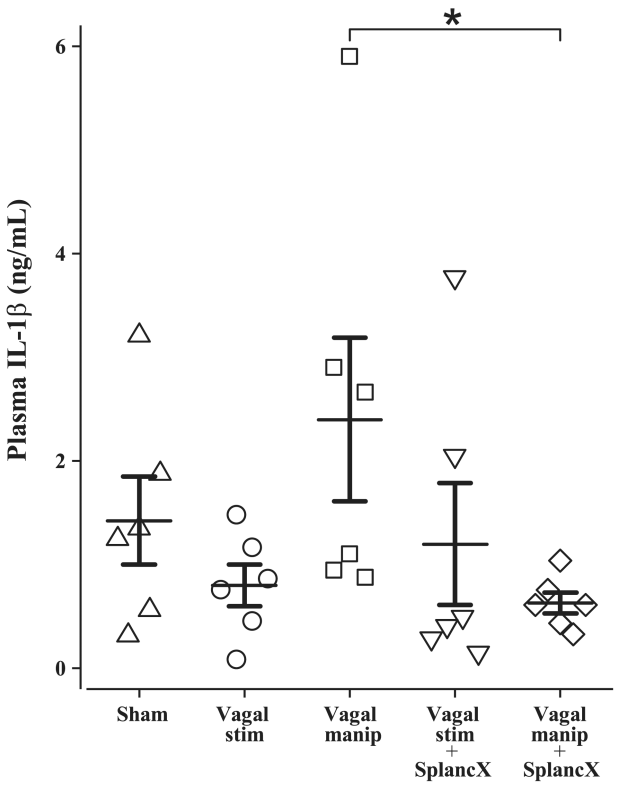

Fig. 5.

Plasma IL-1β levels 90 mins after giving LPS. Groups and details as in Fig. 2. Bars represent mean ± SEM. Data were analyzed using one-way ANOVA followed by post-hoc analysis (Student's *t*-test) *$p < 0.05$.

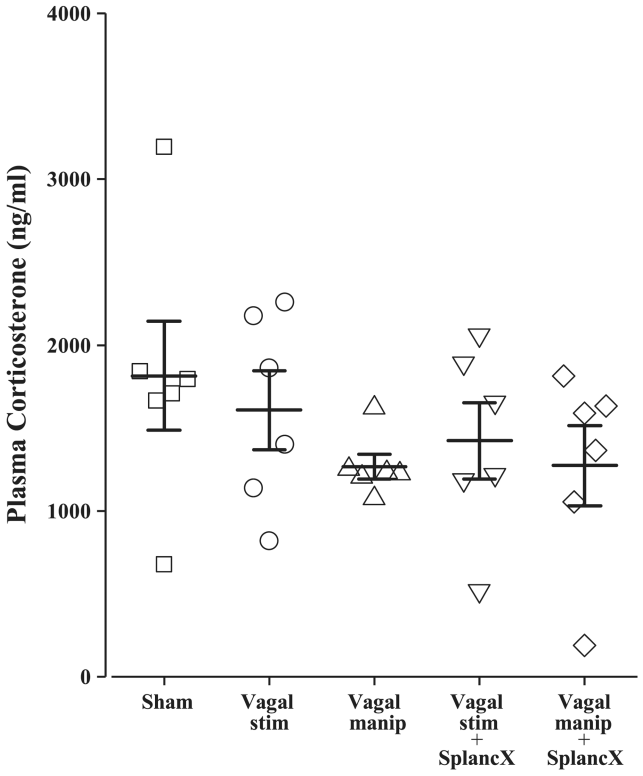

Fig. 6.

Plasma corticosterone levels 90 mins after giving LPS. Bars represent mean ± SEM. Data were analyzed using one-way ANOVA. Groups and details as in Fig. 2.

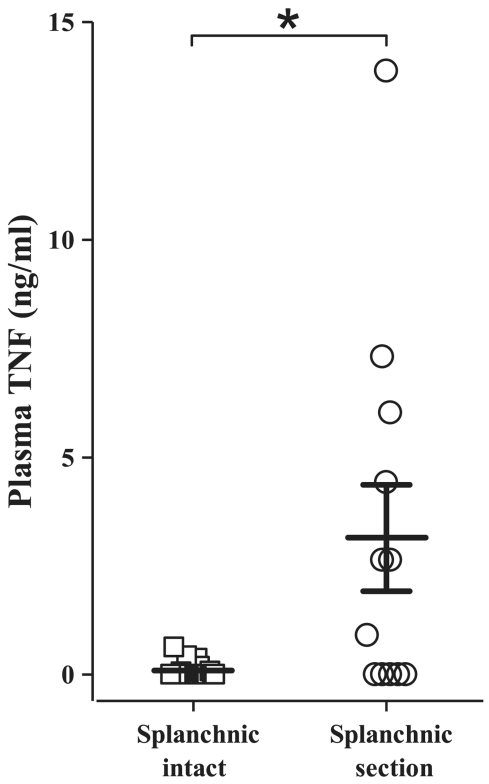

Fig. 7.

Baseline plasma TNF levels taken before giving LPS or vagal stimulation. Values from animals given sham splanchnic surgery (n = 18) are plotted on the left and those after bilateral splanchnic nerve section (n = 12) are plotted on the right. Bars represent mean ± SEM. Data were analyzed using Kruskal-Wallis test followed by post-hoc analysis (Wilcoxon rank sum test), *$p < 0.05$.

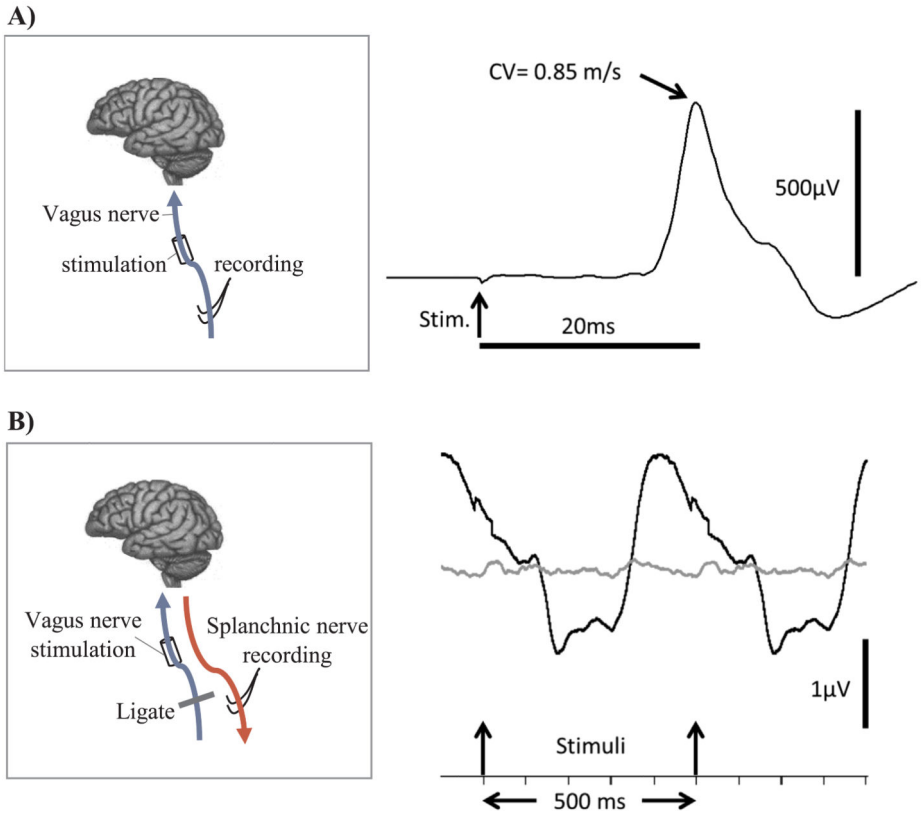

Fig. 8.

A, left: schematic diagram of experimental setup to record vagal mass action potential. Right: mass action potential (average response to 27 stimuli) following supramaximal stimuli (20v, 0.1 ms) delivered to the bipolar cuff electrode (cathode caudal) around the anterior vagal trunk. The conduction distance was 17 mm. B, left: schematic diagram of experimental setup to record the reflex splanchnic response to afferent vagal nerve stimulation. Right: black trace shows averaged (x614 sweeps) rectified, smoothed (time constant 20 ms) splanchnic efferent nerve activity in response to supramaximal (20v, 0.1 ms) stimuli delivered at 2 Hz to the vagal bipolar cuff electrode (cathode rostral). Two stimulus times (at arrows) are included in this 1 s long average. The grey trace shows averaged activity triggered by the same series of time points, but without delivering the stimulus to the nerve.

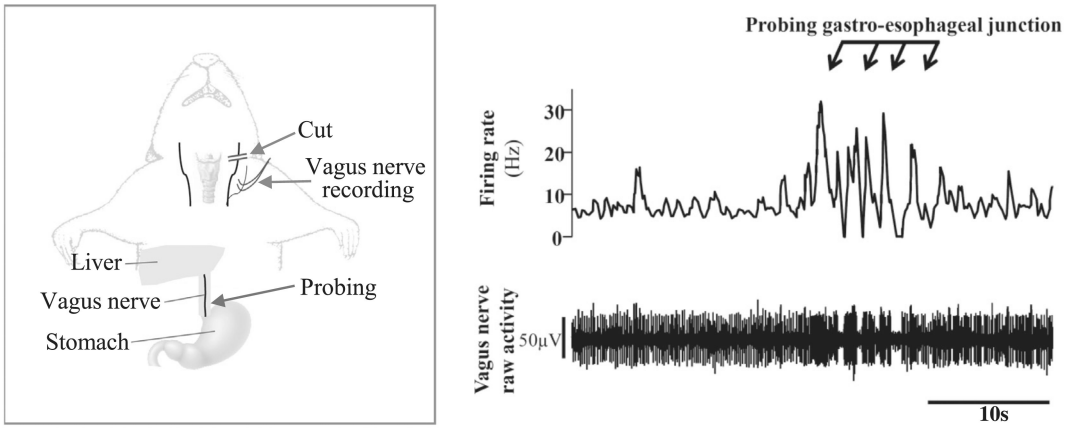

Fig. 9.

Left: schematic diagram of experimental setup to record cervical vagal nerve activity from afferent filament characterized by lack of respiratory modulation. Right: Firing rate and raw nerve activity of the selected vagal filament. The effects of probing the gastro-esophageal region are highlighted.

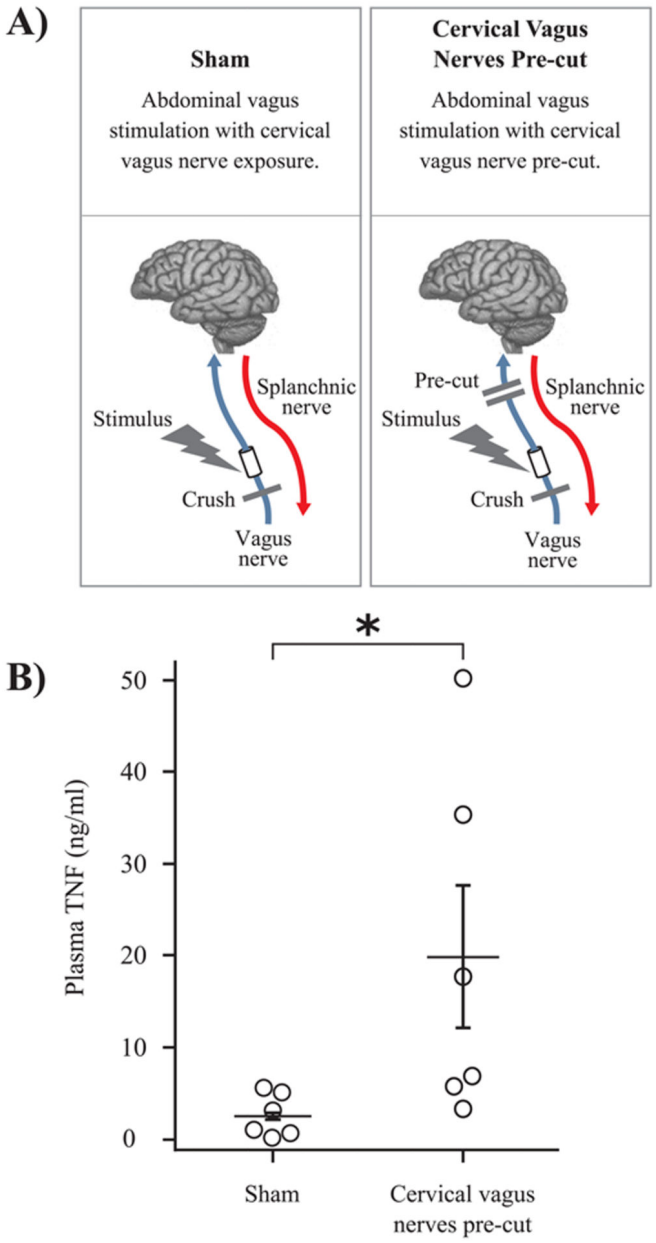

Fig. 10.

A, top: Schematic representation of the experimental setup. The cervical vagus nerves were pre-cut (Cervical Vagus Nerve Pre-cut) or exposed without being cut (Sham). As before, the anterior subdiaphragmatic vagus nerve was crushed below the electrode pair by tightening a ligature around it (crush, in the figure). Vagal electrical stimulation (Vagal stim) parameters used were 20 V, 0.1 ms (cathode rostral) at 2 Hz for 20 min. B, bottom: Plasma TNF levels measured 90 mins after giving LPS to animals in two groups. 1) Sham: animals whose cervical vagus nerves were bilaterally exposed but not cut prior to surgical preparation and electrical stimulation of the abdominal vagus (n = 6); 2) Cervical Vagus Nerve Pre-cut: animals whose cervical vagus nerves were exposed and cut prior to surgical preparation and

electrical stimulation of the abdominal vagus nerve. Bars represent mean ± SEM. Data were analyzed using one-way ANOVA followed by post-hoc analysis (Student's *t*-test), *$p < 0.05$.