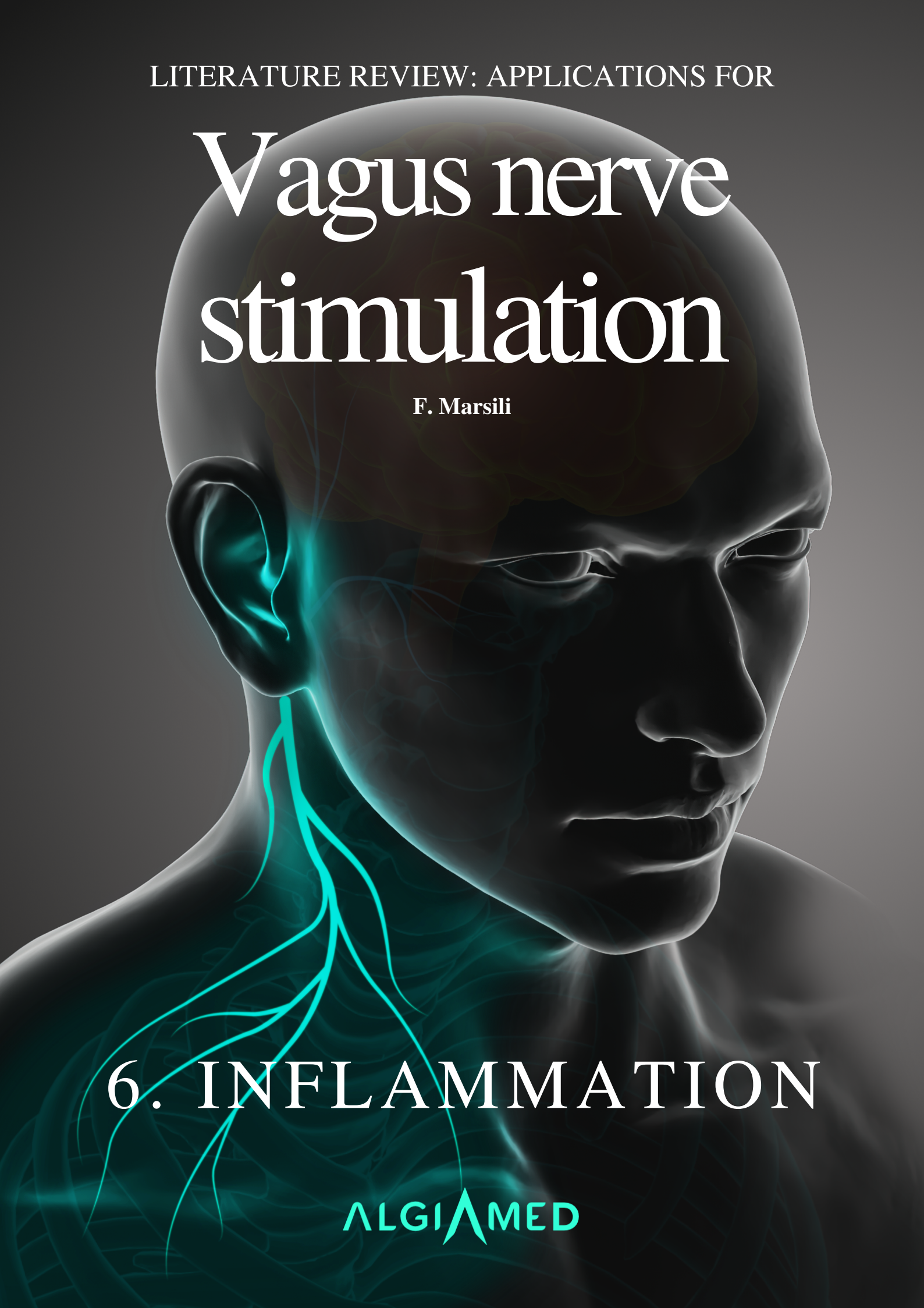
LITERATURE REVIEW: APPLICATIONS FOR

Vagus nerve stimulation

F. Marsili



6. INFLAMMATION

ALGIAMED

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The papers in this collection focus on the application of Vagus Nerve Stimulation (VNS) as established therapeutic solution for difficult-to-treat conditions.

The vagus nerve is the longest cranial nerve and is widely distributed throughout the body, traversing the neck, thorax and abdomen. It is composed by motor fibres and sensory fibres from sympathetic and parasympathetic branches. [1], [2]. Afferent branches of the vagus nerve innervate brain behavioural areas involved in depressive states, and it desynchronises cortical activity with anti epileptic effects [3], [4]. Efferent branches of the vagus nerve regulate gastrointestinal secretory and motor function [5]. Recent advances in the field, have unraveled an anti-inflammatory role of the efferent vagus nerve via the Cholinergic Anti-inflammatory Pathway (CAP), a known mechanism for neural inhibition of inflammation linked to the activation of the autonomic nervous system (ANS) [6], [7].

Electrical stimulation of the VN modulates the nervous system at central, peripheral, and autonomic levels without the need for pharmacological interventions. For decades, invasive techniques of VNS have demonstrated their clinical efficacy in VN-related diseases and, to these days, efforts have been made to create a more safe, effective, and non-invasive solution to VNS.

The auricular branch is the only peripheral branch of the VN on the human body, it is part of the afferent portion of the VN that directly connects to the brainstem. Thus, auricular VN has become the most favourable access point for non-invasive VNS. Neuroimaging studies on animal models and humans have confirmed the modulatory efficacy of auricular VNS (aVNS). For examples, fMRI studies show identical activation patterns in the brain between invasive and aVNS, with significant inhibitory and anti-inflammatory effects. Due to the existence of different control systems, the anti-inflammatory effects of aVNS (i.e., release of norepinephrine and noradrenaline, and neurotrophic factors) seem to occur immediately after intervention, while neuroplastic changes only occur as a consequence of sustained regenerative efforts [7].

Colleciton 1 and collection 2 are the most extensive selections, since VNS has been standard-of-care for epilepsy and depression for decades. Collection 3 explores the possibility of using VNS for the treatment of posttraumatic stress disorders. Collection 4 focuses on fibromyalgia and collection 5 on multiple sclerosis. Collection 6 and 7 corroborates the hypothesis that VNS can be used to activate the cholinergic anti-inflammatory pathway to treat inflammatory diseases, such as inflammatory bowel disease or rheumatoid arthritis. Collection 8 and 9 focus on the use of VNS for ameliorating pain sensitivity in chronic pain conditions and for rehabilitating upper limb motor fibres after ischemic strokes, respectively. In conclusion, collection 10 opens up other possibilities for clinical applications of VNS, ranging from cardiovascular diseases, through ADHD disorders, to tinnitus.

To summarise, VNS is a novel technology and its non-invasive configuration is still under investigation. Further clinical examinations are mandatory in order to understand the underlying mechanism of VNS and to open the door to new possible therapeutic applications. However, being a non-invasive, safe, and efficient therapeutic solution, VNS is an attractive tool for further implementation and new creative clinical applications.

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6. VNS and inflammation

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2012, Article ID 627023, 10 pages
doi:10.1155/2012/627023

Research Article

Transcutaneous Auricular Vagus Nerve Stimulation Protects Endotoxemic Rat from Lipopolysaccharide-Induced Inflammation

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Received 21 August 2012; Revised 5 December 2012; Accepted 6 December 2012

Academic Editor: Ying Xia

Background. Transcutaneous auricular vagus nerve stimulation (ta-VNS) could evoke parasympathetic activities via activating the brainstem autonomic nuclei, similar to the effects that are produced after vagus nerve stimulation (VNS). VNS modulates immune function through activating the cholinergic anti-inflammatory pathway. *Methods*. VNS, ta-VNS, or transcutaneous electrical acupoint stimulation (TEAS) on ST36 was performed to modulate the inflammatory response. The concentration of serum proinflammatory cytokines and tissue NF-kappa B p65 (NF-κB p65) were detected in endotoxaemia affected anesthetized rats. *Results*. Similar to the effect of VNS, ta-VNS suppressed the serum proinflammatory cytokines levels, such as tumour necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) as well as NF-kappa B p65 expressions of lung tissues. ST36 stimulation also decreases LPS-induced high TNF-α level and NF-κB signal, but it did not restrain proinflammatory cytokine IL-1β and IL-6. Neither ta-VNS nor ST36 stimulation could suppress LPS-induced TNF-α and NF-κB after vagotomy or with α7nAChR antagonist injection. *Conclusions*. The present paper demonstrated that ta-VNS could be utilized to suppress LPS-induced inflammatory responses via α7nAChR-mediated cholinergic anti-inflammatory pathway.

1. Introduction

Inflammation is a local, protective response to microbial invasion or injury, which must be fine-tuned and regulated precisely [1]. Several potent cytokines including tumour necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), and transforming growth factor-beta (TGF-β) are produced by activated macrophages and other immune cells, as necessary and sufficient mediators involved in local and systemic inflammation [2, 3]. Overproduction of cytokines leads to systematic inflammation and tissue injury. If the overproduced cytokines spread into the bloodstream, dangerous inflammatory responses will be induced. In the past ten years, abundant studies have been focused on "the cholinergic anti-inflammatory pathway," namely, the efferent vagus nerve which inhibits proinflammatory cytokine production and protects against systemic inflammation via a α7nAChR-dependent pathway [4]. Vagus nerve stimulation (VNS) prevents the occurrence and development of inflammation effectively via activating the cholinergic anti-inflammatory pathway. VNS and acetylcholine (Ach) attenuated the release of cytokines significantly and improved survival in lethal endotoxemia or sepsis models [5, 6]. For instance, in a rat model of lethal endotoxamia, electrical stimulation of the efferent vagus nerve decreases serum and hepatic TNF levels [6]. Moreover, VNS inhibited all lipopolysaccharide- (LPS-) induced procoagulant responses strongly, attenuated the fibrinolytic response more modestly, and improved hepatic Ach levels significantly in endotoxemia rats [7]. VNS attenuated the LPS-induced increases of the plasma and splenic proinflammatory cytokines, rather than influencing the anti-inflammatory cytokine IL-10 [8]. In addition, activation of this neural immune-modulatory pathway by electrical

stimulation of vagus nerve also protects animals from various circumstances, such as ischemia-reperfusion injury, hypovolemic hemorrhagic shock, heart failure, and myocardial ischemia/reperfusion [9–12].

It was demonstrated that the transcutaneous auricular vagus nerve stimulation (ta-VNS) induced a series of parasympathetic activities [13–17]. Auricular branch of vagus nerve is a special vagal branch that innervates the body surface which could not be found on the other parts of the body [18], mainly innervating the cymba conchae and cavum conchae within the auricle. Our previous studies indicated that there is an intimate connection between auricular concha, the nucleus tractus solitarius (NTS), dorsal motor nucleus of the vagus nerve (DMN), and vagus nerve, which constructs the pathway of the auricular-vagal reflex. Accordingly, there might be some kinds of connection between auricular concha and efferent vagus nerve. In the present study, we reasoned that ta-VNS may have a role in activating the vagus nerve-based cholinergic anti-inflammatory pathway. Here, the effect of ta-VNS on proinflammatory cytokines and NF-kappa B p65 was explored to clarify the mechanism of ta-VNS underlying regulating inflammatory diseases.

2. Materials and Methods

2.1. Animals. Male Sprague Dawley rats (12 weeks old) were used in the present study, weighing 275–350 g, supplied by China Academy of Military Science. Rats were housed in groups of 5-6 in standard polycarbonate cages at ambient temperature (22°C) and allowed access to food and water *ad libitum*. Lights were set to an automated 07:00 on and 19:00 off light-dark cycle, and all animal experiments were done between 08:00 and 11:00 a.m. Rats received care consistent with *the National Institutes of Health Guide for the Care and use of Laboratory Animals*, and the experiments were conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee of China Academy of Chinese Medical Sciences.

2.2. Experimental Protocols. The present study consisted of 4 main parts. (1) The first detects the effect of ta-VNS, VNS, or transcutaneous electrical acupoint stimulation (TEAS) on ST36 on LPS-induced serum cytokine response. In this part, rats were randomly divided into 5 groups of twelve each: (a) saline-treated animals (NS), (b) endotoxemia model rats (LPS), (c) endotoxemia model rats receiving treatment of ta-VNS (ta-VNS), (d) endotoxemia model rats receiving treatment of VNS (VNS) and (e) endotoxemia model rats received treatment of TEAS on ST36 (ST36). (2) The second detects the effect of ta-VNS, VNS, or TEAS on ST36 on LPS-induced pulmonary NF-κB p65 expression. In this part, rats were randomly divided into 6 groups, which consisted of the 5 groups aforementioned, and a group of saline-treated animals received treatment of ta-VNS (NS+ta-VNS). (3) The third observes the effect after vagotomy (VGX). In this part, rats were randomly divided into 4 groups: (a) saline-treated animals (NS), (b) endotoxemia model rats (LPS), (c) ta-VNS-treated animals following vagotomy (VGX+LPS+ta-VNS), and (d) TEAS-treated animals following vagotomy (VGX+LPS+ST36). (4) The fourth observes the effect after α7nAChR antagonist injection. Rats were randomly divided into 4 groups: (a) saline-treated animals (NS), (b) endotoxemia model rats (LPS), (c) ta-VNS-treated animals following α-bungarotoxin (α-BGT+LPS+ta-VNS), and (d) TEAS-treated animals following α-bungarotoxin (α-BGT+LPS+ST36).

2.3. Endotoxemia Model. LPS is an endotoxin derived from cell wall of gram-negative bacteria, and systemic injection of LPS results in various symptoms of bacterial infection including fever and inflammation [19]. Rats were injected intravenously with lipopolysaccharide (LPS, *Escherichia coli* 0111:B4; Sigma, 5 mg/kg), dissolved in sterile, pyrogen-free saline that was sonicated for 30 minutes immediately before use. Rats ($n = 12$ per group) were killed 2 hours after LPS injection (Figure 1), and the blood was collected from abdominal aorta, allowed to clot for 2 hours at room temperature, and then centrifuged at room temperature for 15 minutes at 2000 rpm. Serum samples were stored at −20°C before cytokine analysis. Lung samples were rapidly excised, rinsed of blood with normal saline, placed into liquid nitrogen immediately and then frozen and stored at −80°C till measurement of NF-κB p65 expression.

2.4. Electrical Vagus Nerve Stimulation. Rats were anaesthetized with urethane (1 g/kg, intraperitoneally). A midline cervical incision was made to expose the left cervical branch of the vagus nerve. The left carotid sheath was isolated. After blunt preparation, the left vagus nerve trunk was carefully freed from surrounding tissue, separated from the carotid artery trunks, and placed on a custom-made bipolar platinum electrode connected via an isolation unit to a stimulator (SEN-7203, Nihon Kohden). All the exposed nerves were protected from dehydration by covering warm paraffin mineral oil tampons [6]. One and a half hour after LPS administration, constant electrical current stimuli with parameter of 1 mA, 10 Hz, 1 ms were turned on for 20 min (Figure 1(a)).

2.5. Transcutaneous Electrical Acupoint Stimulation (TEAS) on ST36. Transcutaneous surface electrodes were placed bilaterally on the depilatory rat skin at Zusanli (ST36). The ST36 points are located at 5 mm lateral to the anterior tubercle of the tibia and 10 mm below the knee joints. Bilateral surface electrodes at hind limbs were connected via an isolation unit to a stimulator (SEN-7203, Nihon Kohden), and the points were stimulated with the same parameter aforementioned. One and a half hour after LPS administration, the stimulation started and lasted for 20 min (Figure 1). Stimulation intensity was adjusted to a level that elicited a slight muscle twitch at the stimulated site and was limited to a maximum of 1 mA to minimize animal discomfort.

2.6. Transcutaneous Auricular Vagus Nerve Stimulation (ta-VNS). Transcutaneous surface electrodes were placed bilaterally on the auricular concha, which mainly includes cymba conchae and cavum conchae of the auricular. Bilateral surface electrodes at auricular concha were connected via an

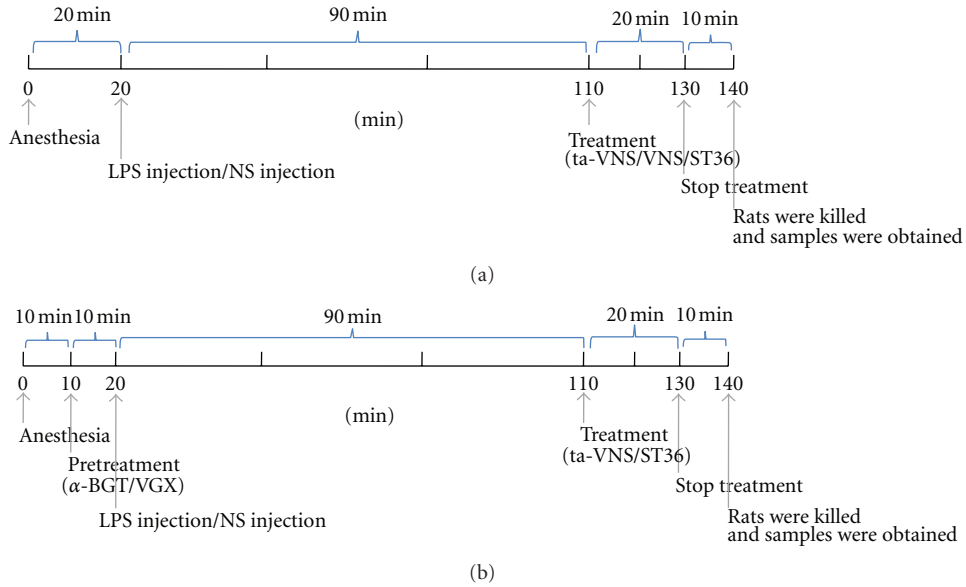

Figure 1: The time flow chart indicates the precise time for various operations in the present study, from the time point of anesthetic injection to the time point for sampling. (a) Twenty minutes after anesthesia, rats were injected intravenously with LPS or NS. One and a half hour after modeling, treatment (ta-VNS, VNS, or TEAS on ST36) was performed for twenty minutes. Two hours after LPS injection, rats were killed, and samples were collected. (b) Ten minutes after anesthesia, administration of α-BGT or vagotomy was performed. The rest of the operations were the same with the time flow in (a).

isolation unit to a stimulator (SEN-7203, Nihon Kohden), and the auricular conchae on both sides were stimulated with the same parameter. One and a half hour after LPS administration, the stimulation started and lasted for 20 min (Figure 1). Stimulation intensity was adjusted to a level that elicited a slight twitch of the auricle and was limited to maximum of 1 mA to minimize animal discomfort.

2.7. Vagotomy. Vagotomy was performed before LPS administration (Figure 1(b)). In vagotomized animals, following a ventral cervical midline incision, bilateral vagus trunks were exposed and separated from the common carotid artery, ligated with a 4-0 silk suture.

2.8. Administration of α7nAChR Antagonist. The specific α7nAChR antagonist α-bungarotoxin (α-BGT) was obtained from Alexis Biochemicals Corporation (San Diego, CA, USA). The drug was administered intravenously at a dose of 1 μg/kg before LPS administration [20] (Figure 1(b)).

2.9. Cytokine Analysis. Abdominal aortic blood was collected two hours after LPS administration, allowed to clot for 2 h at room temperature, and centrifuged for 20 min at 2500 rpm. Serum TNF-α, IL-1β, and IL-6 concentrations were analyzed, respectively, by TNF-α, IL-1β, and IL-6 ELISA kits (R&D Systems) following the manufacturer's instructions.

2.10. Western Blot. Procedures of western blot analysis were followed as described previously [21]. Protein samples denatured in SDS sample buffer (125 mmol/L Tris-HCl, pH 6.8, 50% glycerol, 2% SDS, 5% mercaptoethanol, and 0.01% bromophenol blue) were subjected to SDS-PAGE and blotted onto Immobilon-FL transfer membrane (Millipore). Blotted membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.05% Tween-20 for 2 hours and were subsequently incubated with rabbit anti-human-NF-κB (p65) (diluted 1/200; Santa Cruz Biotechnology Inc., CA, USA) overnight at 4°C. After three washes in Tris-buffered saline containing 0.05% Tween 20, the membranes were incubated with an anti-rabbit IgG antibody-HRP (diluted 1/4000; Santa Cruz Biotechnology Inc., CA, USA) for 1 hour. Quantification of western blots was performed by the Odyssey infrared imaging system (Li-Cor Biosciences) to detect protein expression.

2.11. NF-κB Immune-Histochemistry. NF-κB p65 immunohistochemistry staining was performed as described previously [22] to evaluate the lung tissues inflammatory response. Briefly, tissue sections were deparaffinized with xylene and rehydrated through graded series of alcohols. Tissue sections were rinsed in PBS, pretreated with citrate buffer at 93°C, blocked with PBS containing 2% BSA, and then incubated with a primary antibody reactive against rabbit-activated p65 subunit of NF-κB (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA). Washed sections were incubated for 10 min with secondary goat anti-rabbit IgG biotin. The reaction product was visualized with DAB chromogenic agent. The sections were counterstained with hematoxylin stain. Slides were analysed on a light microscope (Olympus BX60) using an ImagePro Plus Imaging System (Universal Imaging).

2.12. Statistical Analysis. All the data in the present study were expressed as means ± SEM and analyzed by one-way

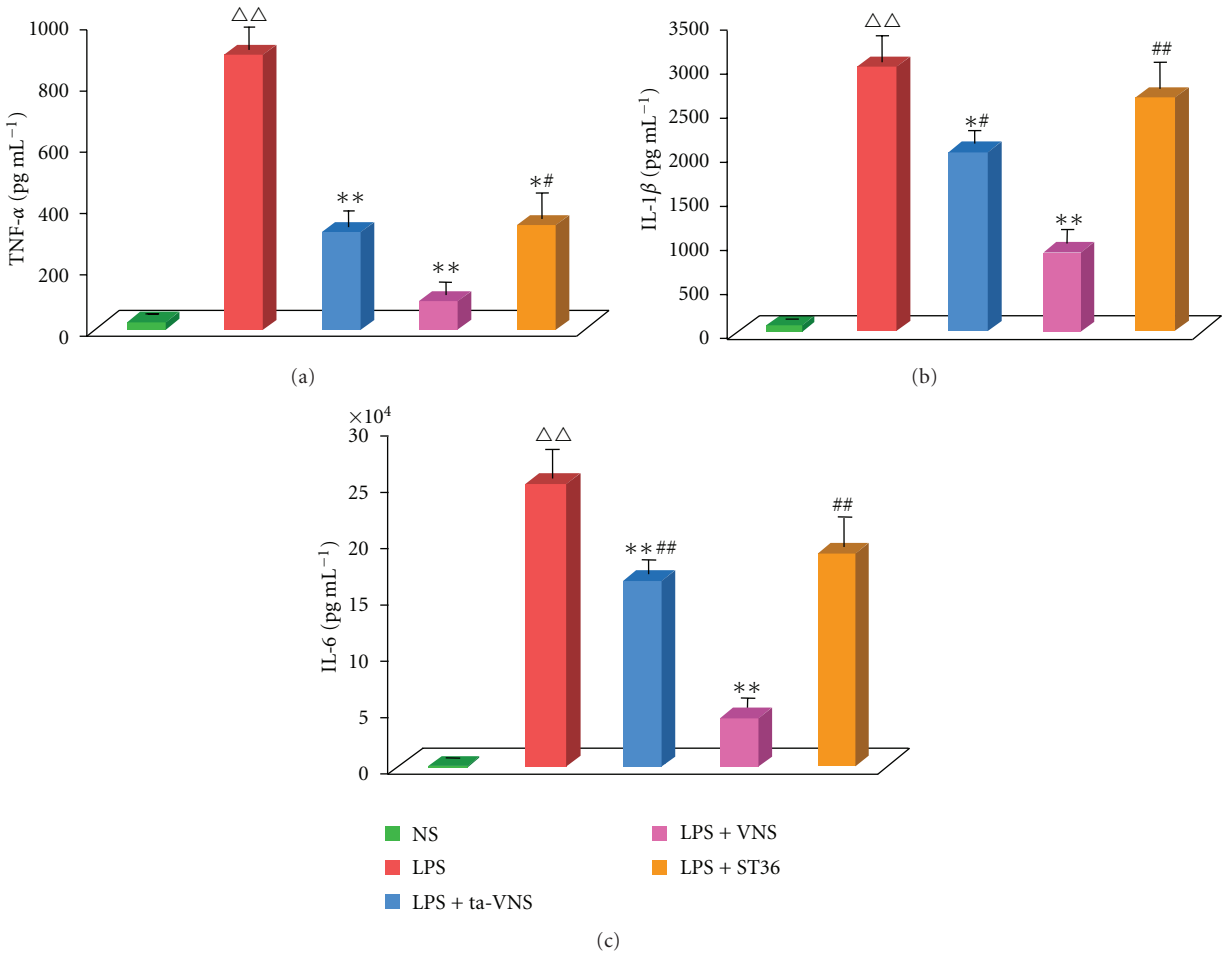

Figure 2: Vagus nerve stimulation (VNS) or transcutaneous auricular vagus nerve stimulation (ta-VNS) attenuates the LPS-induced serum cytokine (TNF-α, IL-1β, and IL-6) response. TEAS on ST36 inhibited TNF-α level significantly. Serum TNF-α (a), IL-1β (b), and IL-6 (c) contents were measured by ELISA. The columns represent mean ± SEM for 12 animals in each group. $^{\triangle\triangle}P < 0.01$ versus the normal saline (NS) group; $^{*}P < 0.05$ versus LPS group (LPS); $^{**}P < 0.01$ versus LPS group (LPS); $^{\#}P < 0.05$ versus LPS+VNS group; $^{\#\#}P < 0.01$ versus LPS+VNS group.

ANOVA with SPSS software. The two-tailed Student's t-test was used to compare mean values between two groups. Pvalues < 0.05 were considered significant.

3. Results

3.1. Cytokine Levels in the Serum. LPS evoked an inflammatory response characterized by the upregulation of cytokine expressions. After systemic administration of LPS (5 mg/kg, i.v.), TNF-α (Figure 2(a)), IL-1β (Figure 2(b)), and IL-6 (Figure 2(c)) increased significantly in sera. Both electrical VNS and ta-VNS strongly inhibited LPS-induced proinflammatory cytokine concentrations including TNF-α, IL-1β, and IL-6 ($n = 12$, $P < 0.01$, $P < 0.05$, resp.). TEAS of ST36 lowered serum TNF-α level ($n = 12$, $P < 0.05$) in endotoxemic rats but failed to significantly alter serum IL-1β and serum IL-6 levels.

3.2. The Effect of ta-VNS or TEAS on ST36 on Serum TNF-α Level Was Blocked by α-BGT Administration. The above results showed that ta-VNS has similar effects to VNS on cytokine levels. Previous studies show that VNS-activated "cholinergic anti-inflammatory pathway" regulates systemic inflammatory responses via α7nAChR, hereby ta-VNS may have the same effect. To test this hypothesis, we pretreated animals with the α7nAChR antagonist α-BGT. LPS injection induced profound rise in the concentration of serum TNF-α. Either ta-VNS or TEAS on ST36 failed to inhibit TNF-α level after α-BGT administration (Figure 3).

3.3. Effect of ta-VNS or TEAS on ST36 on Serum TNF-α Was Blunted by Vagotomy. To examine the mechanism of ta-VNS in "cholinergic anti-inflammatory pathway," we pretreated animals with vagotomy. The result indicated that intravenous injection of LPS elicited a rapid raise of TNF-α level. Neither ta-VNS nor TEAS on ST36 was effective on inhibiting TNF-α level after vagotomy (Figure 4).

3.4. NF-Kappa B p65 Expressions in Lung Tissues. The systemic administration of LPS was followed with a significantly

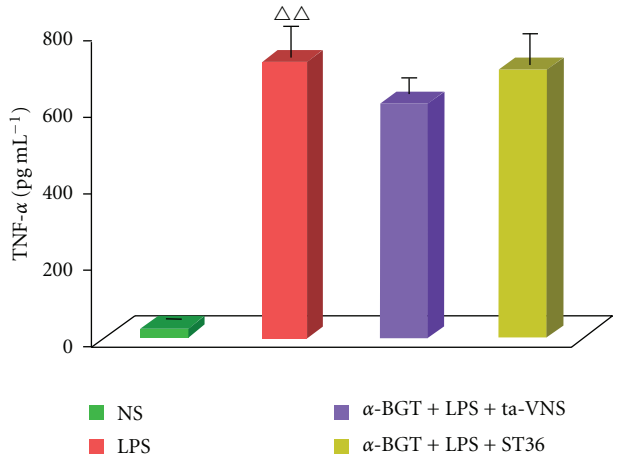

Figure 3: ta-VNS or TEAS on ST36 with α-bungarotoxin (α-BGT) administration fails to inhibit the LPS-induced serum TNF-α response. Serum TNF-α concentrations were measured by ELISA. Data are expressed as mean ± SEM ($n = 12$ per group). $^{\triangle\triangle}P < 0.01$ versus the normal saline (NS) group.

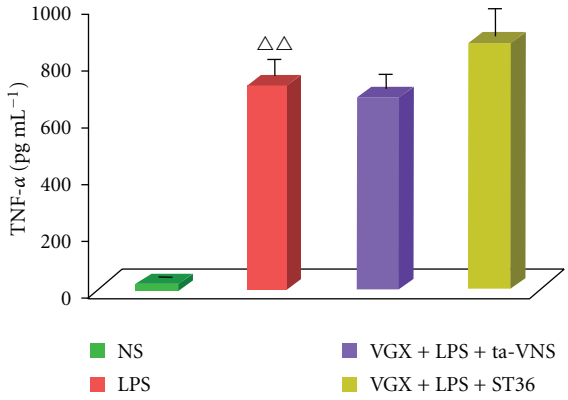

Figure 4: ta-VNS or ST36 stimulation with bilateral cervical vagotomy (VGX) fails to inhibit the LPS-induced serum TNF-α response. TNF-α amounts were measured by ELISA. Data are expressed as mean ± SEM ($n = 12$ per group). $^{\triangle\triangle}P < 0.01$ versus the normal saline (NS) group.

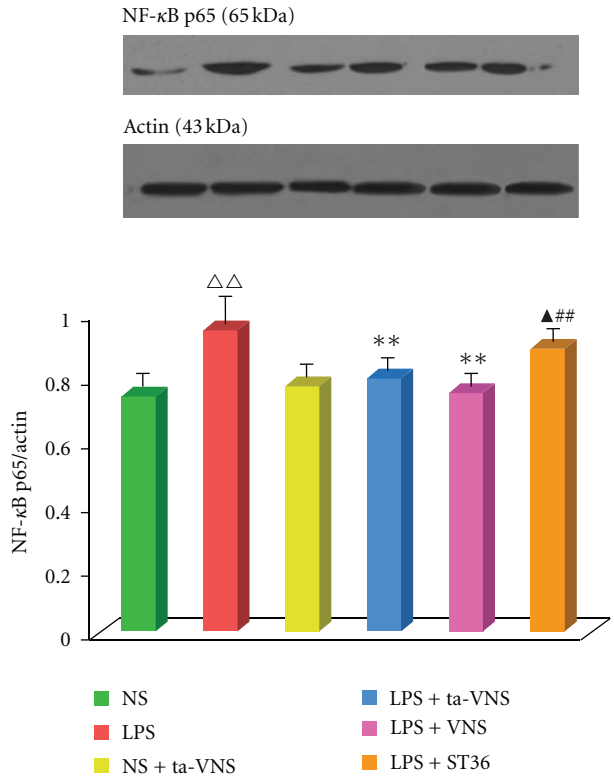

Figure 5: VNS or ta-VNS suppresses LPS-induced NF-κB expression; ST36 stimulation did not affect NF-κB in endotoxemia animals significantly. ta-VNS did not significantly affect pulmonary NF-κB expression with normal saline administration. NF-κB expressions were measured by western blot technique. Data are shown by mean ± SEM ($n = 12$ per group). $^{\triangle\triangle}P < 0.01$ versus normal saline (NS) group; $^{**}P < 0.01$ versus LPS group (LPS); $^{\#\#}P < 0.01$ versus LPS+VNS group; $^{\blacktriangle}P < 0.05$ versus LPS+ ta-VNS.

increased expression of NF-κB p65 in lung tissues (Figures 4 and 5). Both electrical VNS and ta-VNS strongly inhibited LPS-induced NF-κB p65 ($n = 10$, $P < 0.01$, Figures 4 and 5). TEAS on ST36 did not have the same effect (Figures 5 and 6).

3.5. Effect of ta-VNS or TEAS on ST36 on NF-κB p65 Was Blunted by Vagotomy. We pretreated animals with vagotomy. The result indicated that ta-VNS or ST36 failed to inhibit the expressions of NF-κB p65 after vagotomy (Figure 7).

4. Discussion

Here, we reported our original study that auricular concha stimulation is also a potent anti-inflammatory stimulus that can modulate immune factors in endotoxemia rat model. The present study demonstrates that ta-VNS may have an important role in suppressing inflammatory responses, and this contributes to the involvement of the cholinergic anti-inflammatory pathway in the mechanism.

Previous study demonstrated that the cholinergic anti-inflammatory pathway is a α7nAChR-dependent, vagus nerve-mediated pathway [1]. It can inhibit macrophage activation through parasympathetic outflow, which functions as an anti-inflammatory pathway in systemic and local inflammation. Inflammatory signals stimulate sensory fibers that ascend in the vagus nerve to synapse in the NTS and then activate efferent fibers in the vagus nerve to suppress peripheral cytokine release through alpha7nAChR.

The most important cytokine involved is TNF-α, which activates other proinflammatory cytokines such as IL-1β, IL-6, and high mobility group B1 (HMGB1) and amplifies other inflammatory mediators. VNS has been demonstrated to inhibit proinflammatory cytokine production [23–25], especially the release and synthesis of TNF-α. The present study indicates that VNS decreases LPS-induced TNF-α, IL-1, and IL-6 in circulation. And ta-VNS reduced the levels of proinflammatory cytokines TNF-α, IL-1β, and IL-6, which is similar to the effect of VNS. After administration of α7nAChR antagonist α-BGT, ta-VNS failed to attenuate

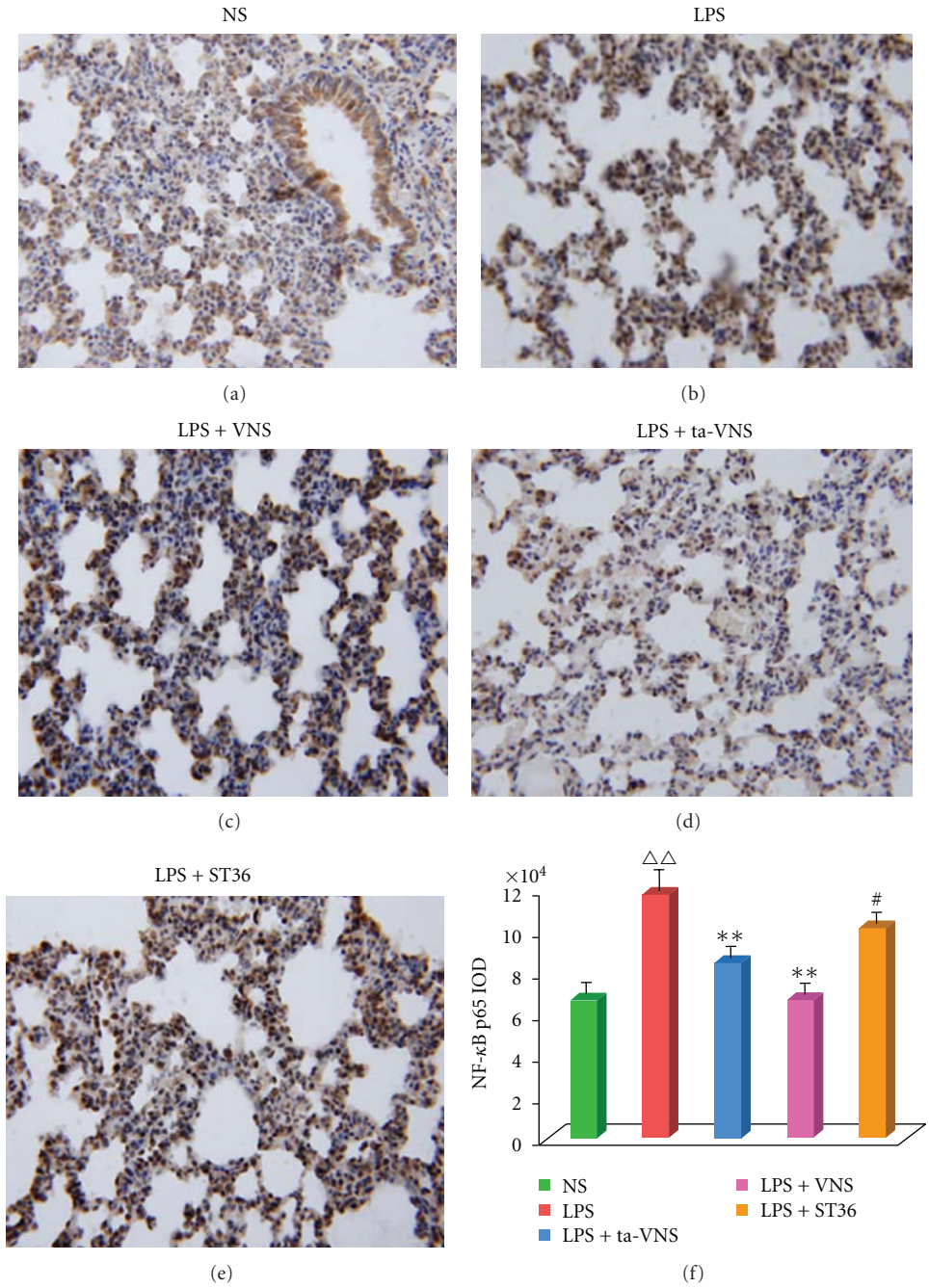

Figure 6: Immunohistochemical staining with anti-NF-κB antibodies reveals significant decrease in LPS-induced NF-κB immunoreactivity evoked by interventions as of VNS, ta-VNS, and TEAS on ST36. Data are expressed as mean ± SEM ($n = 12$ per group). $^{\triangle\triangle}P < 0.01$ versus the normal saline (NS) group; $^{**}P < 0.01$ versus LPS group (LPS); $^{\#}P < 0.05$ versus LPS+VNS group. Original magnification: ×400.

serum TNF-α level, which is consistent with previous reports [6–10, 20, 23]. This result indicated that α7nAChR played a critical role in anti-inflammatory effect of ta-VNS. The present study also demonstrated that vagotomy exacerbated serum TNF responses to inflammatory stimulation, sensitized animals to the lethal effects of endotoxin, and abolished the anti-inflammatory effect of ta-VNS. The results indicated that ta-VNS fails to suppress excessive cytokine response characterized by exaggerated TNF-α level if there is deficiency in either the α7nAChR subunit or vagus nerve.

NF-κB is a master transcription factor controlling the expression of a wide range of proinflammatory genes [26–28]. Previous studies reported that NF-κB is involved in TNF-α genetic activation and TNF-α production [29, 30]. In the present study, both western blot data and immunehistochemical results indicated that ta-VNS suppresses the

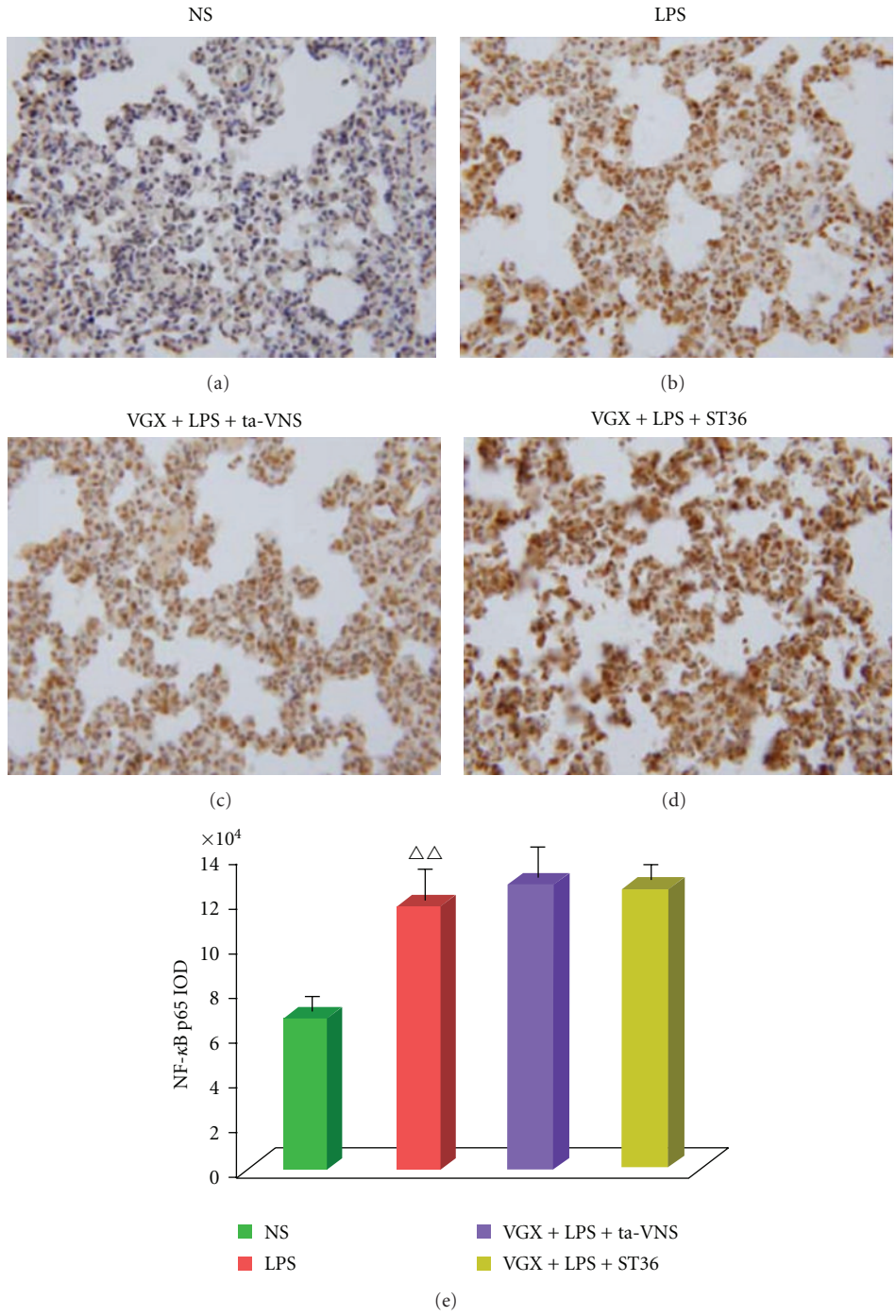

Figure 7: ta-VNS or ST36 stimulation with bilateral cervical vagotomy (VGX) fails to inhibit the LPS-induced overexpression of NF-κB. NF-κB distribution was measured by immunohistochemical staining. Data are shown as mean ± SEM ($n = 12$ per group). $^{\triangle\triangle}P < 0.01$ versus the normal saline (NS) group. Original magnification: ×400.

LPS-induced NF-κB expression in rat lung tissue (Figures 4 and 5), which mimicking the effects of VNS [25, 31]. In vagotomy animals, ta-VNS failed to inhibit increased NF-κB expression, suggesting ta-VNS functions in situations that require intact vagus nerve.

Some investigators demonstrated that electroacupuncture (EA, on ST36, PC6, and GV20) could increase the vagal activity of experimental animals and human subjects [13–17, 32–34]. The present study demonstrated that TEAS on ST36 decreased serum TNF-α level in endotoxemia rats. After pretreatment with vagotomy or α7nAChR antagonist α-BGT, TEAS failed to inhibit serum proinflammatory level in LPS-induced endotoxemia animals.

Auricular acupuncture, as a special form of acupuncture, has been used for the treatment of different disorders for centuries in China. Our research group previously demonstrated

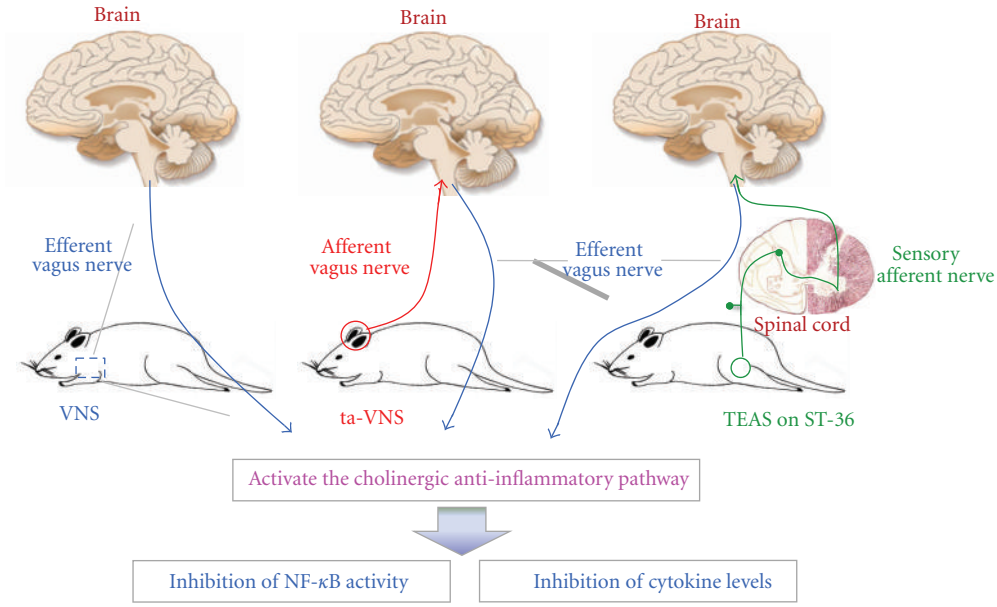

Figure 8: The anti-inflammatory mechanisms of the three interventions used in the present study might be as follows: (1) VNS directly activates the cholinergic anti-inflammatory pathway via stimulating efferent vagus nerve. (2) ta-VNS evoked the activity of the auricular branch of vagus nerve (ABVN). The activated signals ascending within afferent vagus nerve are transmitted to the nucleus tractus solitarius. The integrated output is carried by the efferent vagus nerve to inhibit inflammatory responses. (3) TEAS on ST36 activates the somatic fiber endings of the skin around ST36 point, sending signals to the spinal cord via somatic sensory nerve fibers. The nerve impulses were relayed and integrated by NTS by the secondary order neurons in the spinal cord, and the cholinergic anti-inflammatory pathways are activated by the increased efferent vagal output.

that auricular acupuncture stimulation could activate neurons of NTS and upregulate vagal tune, to decrease MAP and HR [35], to trigger gastric motility [36]. Our previous studies also demonstrated that TEAS of auricular concha could activate the parasympathetic nervous system and mimic the effect of VNS to suppress epileptic seizures [18]. In the present study, the results showed that ta-VNS inhibited proinflammatory cytokine levels and suppressed NF-κB expressions in endotoxaemia rats (Figures 1, 4, and 5), which is similar to the effect of VNS. However, vagotomy or α7nAChR antagonist α-BGT could diminish the effect of ta-VNS on the anti-inflammatory responses, suggesting that auricular acupuncture may perform an anti-inflammatory effect via cholinergic anti-inflammatory pathway.

In general (Figure 8), VNS directly activates the cholinergic anti-inflammatory pathway via stimulating efferent vagus nerve. As the peripheral branch [18], auricular branch of vagus nerve (ABVN) innervates the auricular concha and the external auditory meatus. Stimulation of the ABVN region could evoke parasympathetic excitation [37–39]. Acupuncture in the area of auricular concha may increase discharge of NTS [18], as the central terminal nuclear for afferent vagal fibers, which primarily transmit signals from local inflammation lesion [4, 40]. Thus, we hypothesize that ABVN could be evoked by ta-VNS, and the activated signals ascend with vagal input to the NTS. The signals are processed within the NTS, and the integrated output signal is carried by efferent vagus nerve to inhibit inflammatory responses. TEAS on the acupoint of ST36 activates the somatic fiber endings around ST36 point, which send the acupuncture signals to the spinal cord via somatic sensory nerve fibers. In the spinal cord, the nerve impulses are delivered to the NTS by the secondary order neurons, where the signals were processed. Ultimately, the increased efferent vagal output activates the cholinergic anti-inflammatory pathway.

5. Conclusions

The results presented here demonstrate that ta-VNS plays an important role in immuneregulation, through the activation of the cholinergic anti-inflammatory pathway and the down-regulation of proinflammatory cytokine expressions and NF-κB activities. VNS and TEAS on ST36 might suppress the inflammatory responses via different mechanisms.

Abbreviations

ta-VNS:	Transcutaneous auricular vagus nerve stimulation
LPS:	lipopolysaccharide
NTS:	Nucleus tractus solitarius
DMN:	Dorsal motor nucleus of the vagus
VNS:	Vagus nerve stimulation
TEAS:	Transcutaneous electric acupoint stimulation
Ach:	Acetylcholine
α7nAChR:	Nicotinic acetylcholine receptors
NF-κB p65:	NF-kappa B p65
TNF-α:	Tumour necrosis factor-alpha
IL-1β:	Interleukin-1 beta

IL-6: Interleukin-6
α-BGT: α-Bungarotoxin
NS: Normal saline
VGX: Vagotomy
ABVN: Auricular branch of vagus nerve.

Acknowledgments

The authors thank Dr. Gao Xinyan for her excellent editorial assistance. They thank Dr. Qiao Haifa for his critical reading of the paper. This work was supported by the National Basic Research Program (973 Program, no. 2011CB505201), the National Natural Science Foundation of China Research Grants (nos. 30973798 and 30901931), and the Beijing Natural Science Foundation (no. 7111007).

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Bioelectron Med. Author manuscript; available in PMC 2018 January 05.

Published in final edited form as:
Bioelectron Med. 2016 January 1; 3: 1–6.

Vagus Nerve Stimulation for Treatment of Inflammation: Systematic Review of Animal Models and Clinical Studies

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Abstract

Vagus nerve stimulation (VNS) has been used since 1997 for treatment of drug-resistant epilepsy. More recently, an off-label use of VNS has been explored in animal models and clinical trials for treatment of a number of conditions involving the innate immune system. The underlying premise has been the notion of the cholinergic antiinflammatory pathway (CAP), mediated by the vagus nerves. While the macroanatomic substrate – the vagus nerve – is understood, the physiology of the pleiotropic VNS effects and the “language” of the vagus nerve, mediated brain-body communication, remain an enigma. Tackling this kind of enigma is precisely the challenge for and promise of bioelectronic medicine. We review the state of the art of this emerging field as it pertains to developing strategies for use of the endogenous CAP to treat inflammation and infection in various animal models and human clinical trials. This is a systematic PubMed review for the MeSH terms “vagus nerve stimulation AND inflammation.” We report the diverse profile of currently used VNS antiinflammatory strategies in animal studies and human clinical trials. This review provides a foundation and calls for devising systematic and comparable VNS strategies in animal and human studies for treatment of inflammation. We discuss species-specific differences in the molecular genetics of cholinergic signaling as a framework to understand the divergence in VNS effects between species. Brain-mapping initiatives are needed to decode vagus-carried brain-body communication before hypothesis-driven treatment approaches can be devised.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Bioelectronic Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

INTRODUCTION

Rationale

Vagus nerve stimulation (VNS) has been used for treatment of drug-resistant epilepsy since 1997, when the US Food and Drug Administration approved it (1–3). More recently, an off-label use of this well-tolerated treatment modality has been explored in multiple animal experimental models and clinical trials for treatment of a number of conditions involving the innate immune system (4,5). The underlying systemic antiinflammatory mechanism is mediated by the vagus nerves relaying onto the spleen's α7 nicotinic acetylcholine receptor (α7nAChR) expressing macrophages as part of the cholinergic antiinflammatory pathway (CAP) (6). While the macroanatomic wiring through the vagus nerve seems clear, the physiology of the pleiotropic VNS effects and the "language" of the vagus nerve–mediated brain-body communication remain an enigma. Tackling this kind of enigma is precisely the challenge for and promise of bioelectronic medicine (7).

Objectives

We review the state of the art of this emerging field as it pertains to developing strategies to harness the endogenous CAP via VNS for treatment of inflammation and infection in various animal models and human clinical trials.

METHODS

Protocol and registration are available online at PROSPERO (http://www.crd.york.ac.uk/PROSPERO/display_record.asp?ID=CRD42016035733) under the registration number CRD42016035733.

Eligibility Criteria, Information Sources and Search Strategy

We included any studies listed on PubMed in the English language that met the search term criteria (vagus nerve stimulation [MeSH terms] AND inflammation [MeSH terms]). All years up to June 27, 2016, were considered. The results are depicted in Figure 1 (PRISMA flow diagram). One article was found through Google Scholar when searching for a full-text version of another paper, Shi *et al.,* Effects of efferent vagus nerve excitation on inflammatory response in heart tissue in rats with endotoxemia (article in Chinese).

Study selection—All study designs were considered.

Data collection process and data items—We extracted data on animal model used, location and site of VNS, frequency, intensity, pulse and stimulation durations.

Risk of bias in individual studies—Our inclusion criteria were very broad. As such, while the review has high precision, there is also potential for bias from combining studies in various animal and human trials with acute and chronic experimental designs and somewhat variable readouts. Aside from actual VNS settings, its effects on inflammation may vary by species, gender, age, anatomical site, duration of treatment application and time horizon of readouts (acute versus chronic).

Summary measures—We addressed the risk of bias by not only providing the VNS settings used throughout the literature, but also organizing the results by animal species, including gender whenever possible, VNS treatment duration and resulting effect on inflammation.

Synthesis of results—All PubMed hits were imported into EndNote software and reviewed based on the following criteria: animal model, gender, age, VNS site and duration, frequency, amplitude, intensity and duration of the impulse. If the study was conducted during a single day (<24 h), it was noted as acute; otherwise it was deemed chronic. Lastly, the outcome of the VNS was noted as decreasing or increasing inflammation. All data have been summarized in Table 1.

RESULTS

Study selection and characteristics are summarized in Figure 1 (PRISMA flow diagram).

We identified 290 records, of which 36 were deemed eligible and reviewed.

All studies were conducted in adult subjects (Table 1). In total, 80% of the studies were conducted in rodents, 19 in rats and nine in mice; 69% of the studies were done in male adult subjects, and left or right cervical vagus nerve was stimulated. We found a large variance in VNS settings, with approximately one-third of the studies not reporting the intensity of the stimulus. A total of 77% of the studies were designed as acute protocols (less than 24 h VNS treatment) and observed the inflammatory profile for less than 24 h. All rodent VNS studies except for one resulted in reduction of inflammation (8). Meanwhile, two of the three human studies did not show an antiinflammatory effect (9,10), while one, a recent study by Koopman *et al.*, demonstrated *in vitro* antiinflammatory programming effects in peripheral blood monocytes due to VNS in adult human subjects with no known immune system dysfunction and in patients with diagnosed rheumatoid arthritis (42). In the latter, chronic improvement in disease severity was also observed. All studies except two used VNS treatment before inducing inflammatory response rather than after, to mimic a clinical scenario.

DISCUSSION

We report the diverse VNS methodological profile reviewing the currently used VNS antiinflammatory strategies in animal studies and human clinical trials. While tolerance for VNS is good, apart from its successful use in refractory epilepsy in humans, its antiinflammatory effects are not supported by the two human studies included in this review (9,10). Notably, as of July 10, 2016, a search for “vagus nerve stimulation AND antiinflammatory effects” on clinicaltrials.gov turned up 11 registered studies, four of them actively recruiting. Seventy studies are listed for “vagus nerve stimulation,” excluding epilepsy-related research. This is a testament to the high interest and hopes this nonpharmacologic treatment modality elicits among various fields of medicine and the relevance of creating and maintaining a uniform reporting standard for VNS.

The discrepancy between the promising antiinflammatory effects of VNS in animal and human studies may be due to a lack of basic physiological understanding of the "vagus code," that is, how the efferent and afferent signals are encoded in the vagus nerve and how the information about the various stimuli is represented within the efferent and afferent vagal pathways. Evidence exists from VNS studies that frequency coding may be an approach by which to discretely stimulate its antiepileptic (~25 Hz activating vagal afferents) or antiinflammatory (~5 Hz activating vagal efferents) effects (43). Recently, as proof of principle, temporal patterning of VNS has been applied to selectively stimulate vagus nerve fibers, inducing bradycardia (44). A systematic effort to decode communication via the vagus nerve is needed to devise more hypothesis-driven VNS paradigms that are likely to lead to dedicated immune-modulatory effects in humans. This represents the promise and mission of bioelectronic medicine and the National Institutes of Health Brain Research through Advancing Innovative Neurotechnologies (BRAIN) initiative.

The first step should be a consistent reporting framework for VNS studies. We propose that such framework should include the variables presented in Table 1 of this review. The lack of consistency in reporting the VNS paradigms makes it difficult to validate and develop some of the pioneering work done in this field.

Another reason for the discouraging results in human studies is the male gender bias, which became apparent when reviewing the animal, mostly rodent, literature.

Furthermore, the myelinated part of the vagus nerve is phylogenetically more recent than the nonmyelinated; differences among species and during development exist as to the degree of myelination (45–47). Such functional anatomical differences should have an impact on VNS results.

Recent work in molecular genetics provides another rich dimension to the complexity of the vagus code: pre- and post-transcriptional and epigenetic modifications govern the bioavailability of acetylcholine, the carrier of vagus code within the central and peripheral nervous systems, as well as in the neuroimmunological synapse, via the species-specific variants of microRNA (miRNA or miR) and alternative splicing, ultimately resulting in the complex spatiotemporal landscape of acetylcholine esterase variants (48–51).

Some of these miRNA, for example miR-608, are primate-specific. SNP variants in the miR-608 binding region modify miR-608–mediated suppression of acetylcholine esterase, and consequently activity of the autonomic and central nervous systems in humans (52–54). This explains our finding that rodent VNS models may not be good predictors of VNS treatment effects in humans.

Lastly, we found no studies on developing organisms, from perinatal to juvenile age, where putative salutary effects of VNS would be investigated, although CAP is active as early as in the late-gestation fetus (55,56). There is a continued need to provide better treatments for sepsis, severe infection and cardiovascular compromise in early life (55,57).

CONCLUSION

Overall, this review reveals the nascent stage in which the field of VNS treatment of inflammation finds itself 16 years since its inception (6). The results of the animal studies are very promising and call for a theoretical modeling of vagus code accounting for all levels of organization, from systems biology to systems physiology; a more systematic approach to experimental design and reporting; consideration of the gender effect on inflammation (58) developmental stages; and more diverse animal models (to better gauge the putative species diversity in the vagus code) to ultimately harness the salutary potential of this treatment modality. Such framework has the potential to lead to the development of truly personalized VNS regimens. Lastly, concerted and well-funded efforts are required to devise noninvasive alternatives to VNS to translate this treatment approach into widely used clinical experimentation, and eventually practice, to benefit patients.

Acknowledgments

Supported by grants from the Canadian Institute of Health Research (MGF) and Fonds de la recherche en santé du Québec (MGF).

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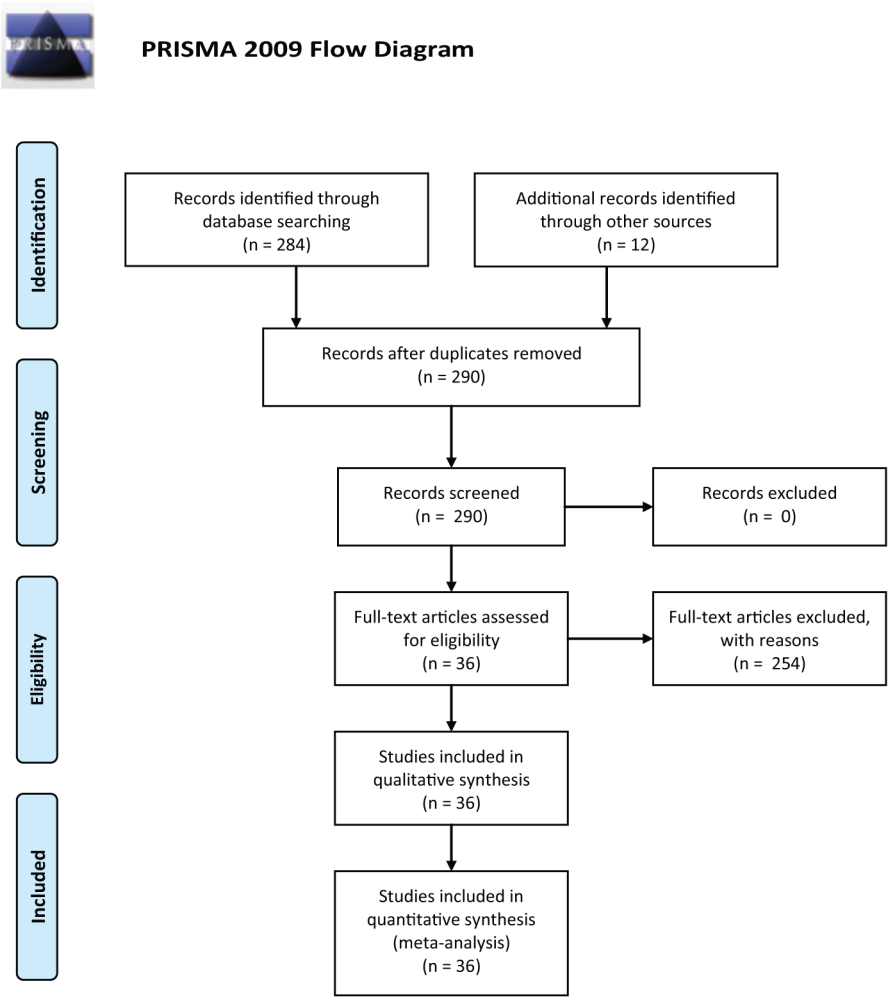

Figure 1.

Approach to systematic review of studies on vagus nerve stimulation to treat inflammation. Based on template provided by Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement at http://prisma-statement.org.

Table 1

Summary of the systematic literature review on antiinflammatory effects of VNS treatment in animal models and human trials.

	Ref	Sex	VNS site	Design	F (*Hz*)	A (*V*)	I (*mA*)	Pulse (*ms*)	Duration (*min*)	Effect	Persistence
Mice	[11]	F	LCV	C	20		0.8	0.5	0.5	↓	A
	[12]		LCV	C	5	1		2	20 (10 b/a LPS)	↓	C*$$*
		M		A	5	1		2	2 (1 b/a LPS)		
					5	1		2	0.5 (5 a/ LPS)		
					30	1		0.5	0.5 (5 a/ LPS)		
	[13]	M	RCV	A	1		2	?	10 b/	↓	A
	[14]										
	[15]	M	RCV	A			2		Intermittently for 10	↓	A
	[16]	M	RCV	A			2		10	↓	A
	[17]	M	RCV	A		5			15 or 20	↓	A
	[18]	M	LCV	C	5		0.05	1	10	↓	C*$$$*
Rats	[6]	M	R/LCV	A	1	5		2	20 (10 b/ and 10 a/ LPS)	↓	A
	[19]	M	LCV	A	2		2	0.3	10 q45 b/ LPS	↓	A
	[8]	M	LCV	A	5	5		2	3	=	A
	[20]	M	LCV	A	1	5		2	20 (10 b/ and 10 a/ LPS)	↓	A

Ref	Sex	VNS site	Design	F (*Hz*)	A (*V*)	I (*mA*)	Pulse (*ms*)	Duration (*min*)	Effect	Persistence
[21]	M & F	LCV	C	20		0.25	500	0.5 q5 for 3 h per d from d 1 to d 6	↓	A
[22]	M	LCV	A	1	5		2	20 a/ LPS	↓	A
[23]	M	R heart	A	5	2–6		1	15 b/ and 120/a	↓	A
[24]	M	RCV	A	8–10	2.5		0.5	40	↓	A
[25]	M	R/LCV	A	1	5		2	15 b/ and a/ 6 h	↓	C[$]
[26]	M	R/LCV	A	1	5		2	20 a/ LPS	↓	A
[27]	M	LCV	A	5	10		2	20 a/	↓	C
[28]	M	RCV	A	5	15		2	20	↓	A
[29]	M	RCV	A	1	5		2	Either 20 starting 5 b/ LPS or 2 (1 b/ and 1 a/ LPS)	↓	A
[30]	-	LCV	A	10		0.0005	1s	0.3 b/ 45 a/LPS	↓	A
[31]	M	LCV	A	2		2	0.3	10 q45, starting right b/ LPS, until 4.5 h a/ LPS	↓	A
[32]	M	RCV	A	20		0.5	0.5	0.5 train q5 for 30	↓	**C**
[33]	-	LCV	A	10		0.5	1000	0.02 with 500 μs bipolar stimul q0.3 for 10; repeat 10 a/ LPS for 45 b/ break of 3 h	↓	A

	Ref	Sex	VNS site	Design	F (*Hz*)	A (*V*)	I (*mA*)	Pulse (*ms*)	Duration (*min*)	Effect	Persistence
	[34]	M	RCV	A	5	5			10	↓ *§*	C
	[35]	F	LCV	C	20		1	10	3 h at same time every other day, beginning 1 wk after operation for 12 wks	↓	A
	[36]	M	RCV[#]	A	25	7.2		1	2 q10 for 60	↓	A
	[37]	M	RCV	A	10	1.0–7.0	N/A	2	N/A	↓	A
Pigs	[38]	M	R/LCV	A	3		1	0.2		n/a	
Dogs	[39]	M and F	RCV	C	20		0.75–2.5	0.5	14 s on 12 s off continuously for 8 wks	↓	A
	[40]	F	RCV	A	1 or 2		0.02–50	0.3	0.006	n/a	
Humans	[41]	M and F	LCV	C	1–145	-	0.25–4 in 0.25 steps	130–1000	7–270 s in 30 s steps	↓	C
	[9]	M and F	LCV	C	30		0.75–1.75	500	0.5 on 5 s off for 3 months	=	A
	[10]	M	LCV[*]	A	20	0–10		1	Continuously for 30	=	C
	[42]	M and F	LCV	A[**]	20		1	0.5	0.5	↓	A
				C[*** §§]	10		0.25–2	0.25	1	↓	C[§§]

Abbreviations: F, frequency, Hz; A, amplitude, volt; I, intensity, milliampere; Pulse, duration of a VNS pulse; Duration, duration of VNS stimulation treatment; A, acute effect <24 h (on inflammation); C, chronic >24 h (on the experimental endpoint); ↓, R of inflammation due to VNS; =, no effect; RCV, right cervical vagus; LCV, left cervical vagus

[*] Catheter inserted in the left internal jugular vein at spinal level 5-C7, adjacent to the vagus nerve

[**] Epilepsy cohort

[***] Rheumatoid arthritis cohort

[#] Skin overlying the right cervical vagus nerve

$ Improvement of renal function at three days, return to control at five days

§ Reduced (except in α7nAChR knockout mice)

§§ Acute design for epilepsy cohort; chronic design for rheumatoid arthritis cohort

$$ 48 h but not 72 h

$$$ VNS 10 min b/ischemia resulted in no protection; protective effects for at least two days

Journal of Inflammation Research

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REVIEW

A review of vagus nerve stimulation as a therapeutic intervention

This article was published in the following Dove Press journal:
Journal of Inflammation Research

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Abstract: In this review, we provide an overview of the US Food and Drug Administration (FDA)-approved clinical uses of vagus nerve stimulation (VNS) as well as information about the ongoing studies and preclinical research to expand the use of VNS to additional applications. VNS is currently FDA approved for therapeutic use in patients aged >12 years with drug-resistant epilepsy and depression. Recent studies of VNS in in vivo systems have shown that it has anti-inflammatory properties which has led to more preclinical research aimed at expanding VNS treatment across a wider range of inflammatory disorders. Although the signaling pathway and mechanism by which VNS affects inflammation remain unknown, VNS has shown promising results in treating chronic inflammatory disorders such as sepsis, lung injury, rheumatoid arthritis (RA), and diabetes. It is also being used to control pain in fibromyalgia and migraines. This new preclinical research shows that VNS bears the promise of being applied to a wider range of therapeutic applications.

Keywords: vagus nerve stimulation, pediatrics, inflammation, peripheral nerve stimulation, autonomic circuits

Introduction

Vagus nerve stimulation (VNS) is US Food and Drug Administration (FDA) approved for use in the treatment of epilepsy and depression in patients aged >12 years and is currently being explored as treatment for a variety of other autoimmune and chronic inflammatory disorders, due to its demonstrated anti-inflammatory properties.[1–6] In this review, we provide an overview of the evidence and diverse applications of VNS in clinical practice, clinical trials, and preclinical research. In addition, we provide a rationale for expanding the use of VNS to a wider range of patients across a wider range of diseases, including sepsis, lung injury, rheumatoid arthritis (RA), stroke, traumatic brain injury (TBI), obesity, diabetes, cardiovascular control, and pain management.

The vagus nerve provides an extensive afferent and efferent network of innervation for the viscera and plays a key role as an interface between higher central nervous system (CNS) circuits and the autonomic control circuitry of the brain stem. It is a mixed autonomic nerve originating at the medulla oblongata and projecting from the brain stem bilaterally along the neck (bundled with the carotid artery rostrally) and esophagus before branching diffusely to innervate the viscera. Most information about the anatomy of the vagus nerve and its projections has been discovered through tract tracing of the rat vagus and is generally assumed to be similar to humans.[7] The complete innervation extent of the vagus remains incompletely known, but we have included a brief, simplistic overview of the primary branches and targets of the vagus.

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http://dx.doi.org/10.2147/JIR.S163248

The branches that extend off the cervical vagus innervate the bronchi, lungs, heart, and esophagus.[7] The subdiaphragmatic vagus has five primary branches, including the dorsal and ventral gastric branches (innervating the stomach) as well as the dorsal and ventral celiac branches (innervating the proximal and descending colon). The hepatic branch divides into the hepatic branch proper (innervating the liver) and the gastroduodenal branch (innervating the duodenum and pancreas).[1] The ventral trunk branches into the common hepatic, ventral gastric, and ventral celiac branches. The vagus consists of ~80% sensory afferent and 20% motor efferent fibers.[8] Further branching and tertiary targets for the vagus nerve are largely unknown. A fine wire electrode extends from the device and is typically wrapped around the left cervical vagus. Case reports suggest that the right vagus can be used in circumstances where approaching the left vagus is inadvisable. Since the right vagus innervates the sinoatrial node, stimulating on the right is best done with ECG monitoring.

The afferent projections of the vagus nerve are integrated at the level of the autonomic brain stem within the nucleus tractus solitarii (NTS) before projecting to other regions of the CNS. We have incomplete knowledge of how VNS modulates the CNS but the brain stem plays a critical role in integrating and gating signals between the CNS and peripheral organs (Figure 1). Descending efferents from these regions are responsible for driving cardiorespiratory and gastrointestinal autonomic tone as well as other autonomic functions.[1,9] Stimulation of the vagus nerve provides a way to regulate the autonomic tone. Because the vagus nerve is easily accessible at the neck, it is a convenient access point for the implantation of stimulating or surface electrodes for chronic or acute stimulation.

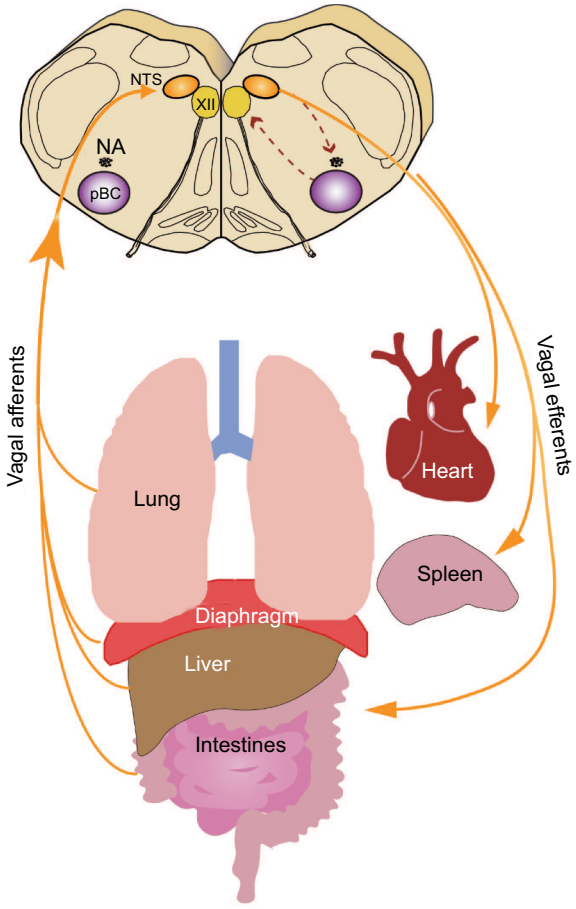

Figure 1 Overview of vagal circuitry linking the central and peripheral nervous system.
Notes: Visceral afferents converge on the NTS in the brain stem, the first point of integration between the peripheral autonomic nervous system and the central nervous system. Visceral afferents project from the dorsal motor nucleus of the vagus and are key to exerting autonomic control in the periphery.
Abbreviations: NA, nucleus ambiguus; NTS, nucleus tractus solitarii; pBC, preBötzinger complex.

Current clinical uses of VNS

Treatment of epilepsy

Epilepsy affects 1% of the US population but costs $12 billion US dollars to treat (figures from 2008).[10] VNS to treat epilepsy was first used in the early 1880s by JL Corning, who believed that seizures were caused by changing cerebral blood flow.[11] In 1988, the first chronic implantable stimulator was used to treat drug-resistant epilepsy.[1] The stimulator was approved by the FDA in 1997 to treat partial onset seizures that were resistant to pharmacological control.[1] The current Livanova© (formerly Cyberonics) implantable treatment device consists of a small battery-powered stimulator that requires battery removal and replacement approximately every 6 years.[12] A fine wire electrode extends from the device and is typically wrapped around the left cervical vagus. Case reports suggest that the right vagus can be used in circumstances where approaching the left vagus is inadvisable. Since the right vagus innervates the sinoatrial node, stimulating on the right is best done with ECG monitoring.[13–15] The device can be turned on for 30–90 seconds to provide a brief stimulus to the vagus.[16] Once the device is implanted, it is programmed by a physician using a microcomputer, but patients can alter the stimulus program as needed when they feel the onset of a seizure.[17] Over 100,000 VNS devices have been implanted in patients worldwide (as of 2015).[18] The most common side effects reported are dysphonia, hoarseness, and cough, all of which may be mitigated by changing stimulus parameters.[19] Stimulation parameters vary widely, but typical treatment for epilepsy and depression uses a range of stimulation of 20–30 Hz, a pulse duration of up to 500 microseconds, and stimulation on-time of 30–90 seconds followed by off-time

of 5 minutes, although stimulus intensity is usually decreased over time.[16]

Data gathered over the first decade of clinical VNS showed efficacy in patients who had pharmacoresistant seizures. Approximately 40% of patients using VNS showed a 50% reduction in seizures after 2–3 years of treatment.[20] The mechanisms by which VNS causes changes in neurochemistry and prevents epileptic seizures are not yet known, although some evidence suggests the vagus nerve plays a role in quenching kindling of seizures in regions susceptible to heightened excitability. These regions include the limbic system, thalamus, and thalamocortical projections.[1] VNS may also affect structures in the midbrain and hindbrain, which can contribute to seizure suppression, although the specific changes in these cortical circuits remain unknown. VNS also increases activity in the locus coeruleus and the raphe nuclei and moderates the downstream release of norepinephrine and serotonin, both of which have been shown to have antiepileptic effects.[21] VNS success in treating refractory epilepsy with few side effects provides justification for its expansion to both additional conditions and wider populations. Figure 2 shows an overview of the CNS regions likely affected by VNS.

VNS may also be useful as a treatment for expecting mothers with treatment-resistant epilepsy. One study showed that women with epilepsy had a significantly higher risk of mortality during delivery when compared to women without epilepsy.[22] The goal of current epilepsy treatment is to optimize seizure control and minimize in utero fetal exposure to antiepileptic drugs which, during the perinatal period, are associated with major congenital malformation, growth retardation, and neurocognitive developmental deficits.[23] VNS has been used successfully as a treatment for medically refractory epilepsy in pregnant women, and physicians have concluded that VNS is a viable option for treatment during

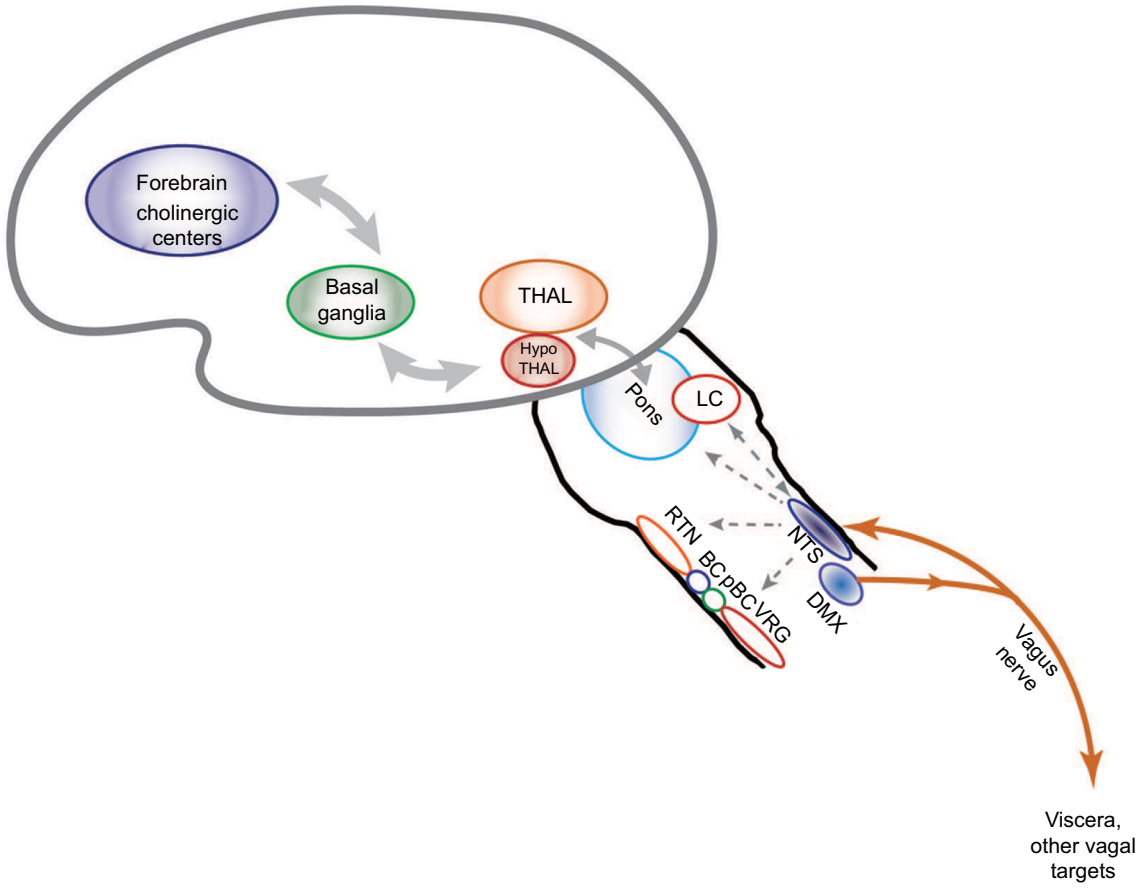

Figure 2 Putative pathways involved in vagus nerve stimulation.
Notes: Stimulation of the vagus activates ascending pathways that alter neural circuits in the brain stem, midbrain, and cortex. Regions that are impacted by vagus nerve stimulation based on past research are included in this diagram.
Abbreviations: NTS, nucleus tractus solitarii; DMX, dorsal motor nucleus of the vagus; LC, locus coeruleus; THAL, thalamus; HypoTHAL, hypothalamus; RTN, retrotrapezoid nucleus; BC, Bötzinger complex; pBC, preBötzinger complex; VRG, ventral respiratory group.

pregnancy.[24,25] As a non-pharmacological treatment, VNS seems to be beneficial for seizure control in the expecting mother, and there is no evidence of harm to the developing fetus. To date, no large clinical trials have been performed to assess whether VNS has a long-term effect on the developing fetus.

Epilepsy affects 0.5%–1% of the pediatric population[19] or ~470,000 children.[26] Chronic epileptic seizures can have a profound impact on children's long-term neurodevelopmental and social outcome, as well as a lasting impact upon their families.[27] Finding an effective treatment can improve the children's quality of life since children with epilepsy often experience psychiatric and cognitive difficulties and have poor social outcomes as adults.[27] Antiepileptic medications have high side effect profiles and can adversely affect the behavior in susceptible children.[27] This means that even in children with more easily controlled epilepsy, there is a high risk of psychological and psychiatric disturbance. Children with benign rolandic epilepsy and absence epilepsy have exhibited more aggressive behavior, depression, and anxiety than children without epilepsy.[28] Ongoing studies are focused on noninvasive methods to treat epilepsy in pediatric patients. This includes a study by the Chinese Academy of Chinese Medical Sciences testing a non-implant, less invasive transcutaneous auricular vagus nerve stimulator as an effective treatment of pediatric epilepsy.[29] The study examines the change in frequency of seizures as well as heart rate variability, quality of life, and electroencephalogram recordings at 2, 4, and 6 months after the start of stimulation.[29] In a retrospective cohort study by Elliott et al,[19] the effects of VNS on 141 children were analyzed, 61% of whom were <12 years of age. They concluded that VNS was just as effective and relatively complication free in children aged <12 years as it was in older pediatric patients. This study also showed a significant decline in the frequency of seizures in these children, from an average of 10 per week down to three per week. In 41% of patients, there was a 75% reduction in the frequency of seizures.[19] Side effects of VNS occurred in a small percentage of children and included hoarseness (0.7%), cough (0.7%), and minor arm pain (0.5%).[19] Hallböök et al[30] found that pediatric responses to VNS were similar to those of adults. In 40% of children implanted with VNS stimulators, there was a 50% decrease in seizure frequency.[30] A retrospective study on 75 children with epilepsy showed that side effects such as hoarseness, cough, and drooling were reported in only 5.4% of patients, in which all were reversible with adjustments to the stimulation parameters.[31]

Neonates could also benefit from a non-pharmacological approach to controlling epilepsy. Current pharmaceutical treatment options for epilepsy in neonates include phenobarbital and levetiracetam, although each can have detrimental side effects. Phenobarbital is the most widely used antiepileptic in neonates but can have respiratory and cognitive side effects.[32] Levetiracetam has psychological side effects.[33] Although VNS is only FDA approved in children aged >12 years, it has been used, along with antiepileptic medication, in children as young as 1 year of age.[34] A study conducted by Fernandez et al on children aged <3 years showed that VNS was effective in children with medically intractable epilepsy.[34] Their study showed that VNS led to decreased seizure frequency in 33% of patients, and status epilepticus was no longer a symptom after 1 year of treatment. In addition, normal MRIs were associated with decreased seizure incidence.[34]

Children with developmental disabilities or autism are more likely to exhibit medically refractory epilepsy. One study estimated that between 5% and 38% of children with autism have epilepsy.[35] A study by Kirchberger et al[85] reported that VNS caused a 50% reduction in seizure frequency in 61% of patients with developmental delays.[35] Levy et al also found that there was no statistically significant difference in seizure reduction benefits and quality of life improvements between patients with refractory epilepsy and autism and those without autism.[35] Based on these results, VNS seems to be a safe and effective treatment for epilepsy in pediatric patients.

Treatment of depression

Chronic or severe depression affects up to 1.5% of the general population,[8] and many of these patients obtain little relief from pharmaceutical treatment. In 2000, depression was estimated to cost $83.1 billion in the United States. Of this cost, $26.1 billion was in direct medical costs, $51.5 billion in indirect workplace costs, and $5.4 billion in suicide-related mortality costs.[36] Although VNS was not originally developed to treat depression, patients using VNS to treat epilepsy experienced mood improvement; thus, VNS was expanded to include the treatment of depression. The FDA approved VNS for the treatment of chronic or recurring depression in 2005.[37] VNS treatment has been approved for patients aged ≥18 years who have experienced at least one major depressive episode and did not respond well to any of four different pharmaceutical antidepressant treatments.[8] A major depressive episode is defined by the *Diagnostic and*

Statistician Manual of Mental Disorders (DSM-IV)[38] as having five out of the nine depressive symptoms, including depressed mood or lack of interest in normal day-to-day activities occurring almost daily for at least 2 weeks.[39] The goal of treatment is to restore day-to-day function and prevent relapses and remission, as well as alleviate current symptoms, in which VNS has been proven effective in a wide range of patients.[8] In a study conducted by Bajbouj et al, patients suffering from chronic "treatment refractory depression" received VNS, in which 53.1% of patients met the response criteria of a 50% reduction in the Hamilton Rating Scale for Depression (HRSD28) (the most commonly used symptom severity scale).[37,40] In addition, 38.9% fulfilled the remission criteria with HRSD scores <10.[37]

Depression is often difficult to treat because patients experiencing recurrent depressive episodes treated with conventional pharmaceuticals often experience relapses or do not experience full remission. A study conducted by Nahas et al showed patients with chronic or recurrent major depressive disorder receiving VNS may have beneficial long-term outcomes.[41] In their study, 42% of their patients experienced a positive effect and 22% saw remission after 2 years. Both Bajbouj et al and Nahas et al use the same criteria for clinically significant remission, defined as the absence of clinically significant depressive symptoms.[42] Results from neuroimaging studies suggest that the mood-enhancing benefits are caused by VNS stimulation altering medial and prefrontal cortical transmission (Figure 2). These regions comprise neurons that release neurotransmitters such as serotonin and norepinephrine which have both anticonvulsive and antidepressant effects.[37] Although VNS will likely never be recommended as a "first-order" or sole treatment for depression, current clinical evidence shows success with its use as a supplemental treatment for chronic refractory depression.

Between 6% and 13% of pregnant women report symptoms of depression during and post pregnancy.[43] The most commonly prescribed drug family for pregnant women with depression is selective serotonin reuptake inhibitors (SSRIs), although there are still unanswered questions about the safety of SSRI treatment for the fetus.[43] Antidepressant use during pregnancy may lead to low birth weight and preterm delivery, since they can pass through the placenta.[44] A case report by Husain et al showed that VNS was an effective treatment for depression during pregnancy and delivery with no adverse outcomes for the mother or fetus.[45] A recent work in a rat model of VNS shows no significant effect of VNS on pups born to a dam with an implanted VNS stimulator.[46] Preliminary research suggests that VNS can be a beneficial treatment for both mother and fetus, although more research is required for a clearer picture of the outcome.

Although depression also affects many adolescents, treatment options for pediatric patients are limited. Longitudinal studies on children with major depressive disorders have also shown that the relapse rate is 40% within 2 years and 70% within 5 years.[47] Many children with major depression are treated using psychotherapy, but if their depressive symptoms persist, they are typically prescribed antidepressant medications in addition to therapy.[48] As with pregnant mothers, the most common antidepressant medications prescribed in the pediatric population are SSRIs.[48] As in adults treated with VNS, studies of pediatric patients with VNS implants to treat epilepsy have shown mood improvement. A study by Hallböök et al[30] showed that, in children with epilepsy treated using VNS, not only were seizures reduced, but behavior and mood improved while depressive symptoms decreased. Twelve of the 15 children examined had improvement in their quality of life.[30] Further studies are needed to examine the impact of VNS on pediatric depression, but the preliminary data show that it remains a promising treatment option and may provide long-term benefit for children with depression.

Potential uses and mechanisms of VNS

An exciting new application of VNS is as an anti-inflammatory treatment. Inflammation is implicated in many chronic diseases including cardiovascular disease, arthritis, and Alzheimer's disease. Preliminary preclinical evidence suggests that VNS may attenuate the inflammatory response through activation of the cholinergic anti-inflammatory pathway (CAP) – a long loop from the vagus afferents, through the autonomic brain stem and forebrain cortical structures, and then back through the descending vagus efferents (Figure 2). The CAP upregulates HMGB1, which may regulate cytokine expression, leading to anti-inflammatory effects. In recent years, Tracey et al have devoted significant efforts to quantifying the role that VNS plays as an anti-inflammatory regulator primarily through altered regulation of acetylcholine.[2–6] These findings provide strong evidence that stimulation of the vagus nerve plays a key role in peripheral cholinergic release and its putative role in suppressing inflammation. The CAP also affects the levels of acetylcholine through nicotinic acetylcholine receptors (nAChRs).[2] Several recently completed and ongoing studies are focused on the effects of VNS on inflammatory disorders such as RA, Crohn's disease, irritable bowel syndrome, and fibromyalgia. Other studies are focused on how VNS affects brain trauma and stroke. Because these

are ongoing studies, the efficacy of VNS treatment for these disorders is currently unknown. Many of the inflammatory disorders that VNS may potentially treat also affect the pediatric and neonatal population. Since VNS has been shown to be effective for adult and pediatric populations for epilepsy and depression, it stands to reason that VNS treatment may be beneficial to younger patients for a variety of disorders; however, there is limited data on pediatric applications. We summarize the results of studies related to these disorders below; however, these preliminary data provide a strong rationale for expanding research on the applications of VNS as an anti-inflammatory treatment across a range of different inflammatory diseases.

Sepsis

Sepsis is a multibillion dollar health care burden typically due to systemic bacterial infection and chronic activation of the pro-inflammatory cytokine cascade. Sepsis is estimated to cost $22,000 per patient and affects up to 18 million individuals each year.[49] Kessler et al used vagotomized mice to show that lack of vagus input to the CNS can lead to adverse outcomes in a murine model of colon ascendens stent peritonitis.[50] In an ex vivo culture of Kupffer cells, tumor necrosis factor α (TNFα) levels were decreased in vagotomized mice compared to controls, even when stimulated with lipopolysaccharide (LPS).[50] Huang et al showed that VNS helped to attenuate inflammation by restoring the balance between parasympathetic and sympathetic tone and thus arresting the progression to sepsis.[51] These investigators used an intravenous LPS injection model of inflammation to induce sepsis and found that in addition to a decrease in ACh release, heart rate variability was decreased back to baseline levels in the LPS + VNS treatment group, compared to the elevated levels in the LPS-only group.[51] In a similar model of LPS-induced endotoxemia, Borovikova et al used VNS and found decreased mortality that they attributed to vagally induced release of acetylcholine.[3]

Limiting inflammation in pediatric patients without the use of pharmaceuticals is important because neonates, particularly preterm infants, are more susceptible to developing sepsis due to their underdeveloped immune systems and susceptibility to perinatal infection (chorioamnionitis, etc). Since VNS seems to regulate inflammation by modulating the cytokine cascade, our laboratory is looking at the effect of VNS on the early pro-inflammatory cytokines, interleukin-6 (IL-6), TNFα, and IL-1β, in respiratory control regions of the brain stem. We looked at the NTS and hypoglossal motor nucleus (XII), which are regions of critical importance for the control of breathing and implicated in breathing problems in neonatal rats as a model for preterm infants. In the present study, we show that VNS reduces the expression of IL-6 and TNFα in response to a brief (30 minute) bout of high-frequency VNS stimuli.[52] Our hope for this preclinical translational work is that it will eventually lead to minimally invasive VNS treatment to provide early intervention and reduce the likelihood of sepsis in preterm infants.

Two major concerns in extending VNS to neonatal practice are the length of time that VNS takes to be effective and the invasive nature of the implantation procedure. Although VNS as currently used for epilepsy and depression can take months to show dramatic effect, short-term stimulation has been used to reduce inflammation quickly, and our experiments have shown that a single bout of high-frequency stimulation (30 minutes) can be effective as an anti-inflammatory treatment.[52] In addition, transcutaneous stimulation has been used to treat depression and headaches showing efficacy for even short-term applications using surface electrodes.[29,53] A study by He et al uses transcutaneous stimulation at the cervical or auricular vagus to effectively treat epilepsy.[29] Further research will be needed to determine if high-frequency stimulation can be paired with transcutaneous stimulation to be an effective treatment for neonatal inflammatory disorders. Preliminary work performed by the Wilson laboratory and others suggests that this may be possible and useful for implementation of VNS treatment in neonates.[52,54,55]

Pain management

The applications of VNS also extend to widespread inflammatory disorders associated with chronic or intermittent bouts of pain such as fibromyalgia and migraines. Lange et al conducted a Phase I/II clinical trial to assess VNS as an adjunct treatment for patients with fibromyalgia due to its effects on serotonergic and noradrenergic neural circuits – both of which are implicated in pain sensation.[56] Their theory was based on results from patients with depression treated with VNS who reported decreased sensation of pain.[57] Lange et al's study included 12 women with fibromyalgia and used the same stimulus parameters as for treatment of epilepsy. After 11 months, 7 of the patients had the minimum clinical difference (MCID+) in their pain symptoms for VNS to be considered effective.[56]

Another chronic pain ailment that VNS shows promise in treating is migraine headaches. In a study conducted by Barbanti et al, 50 patients with migraine were given VNS treatment applied externally at the neck in two 120-second intervals with 3 minutes in-between. Of those patients, 56%

reported pain relief at 1 hour and 64% reported pain relief at 2 hours.[53] Silberstein et al performed the ACT1 study (NCT01792817), a clinical trial to use a noninvasive VNS treatment at the neck to treat cluster headaches.[58] Their findings suggest that noninvasive VNS can be successfully used to treat episodic cluster headaches.[58] Although these results need further research and larger multicenter randomized trials, they present hopeful evidence that VNS can be used to control fibromyalgia and migraines.

Obesity

Although VNS would not likely be recommended as a first-line defense against obesity, research on the effects of VNS on diet and weight have been performed to evaluate VNS for its use as an adjunct treatment in controlling obesity. Finding alternative treatments to obesity is especially important considering 69% of adults and 20% of adolescents in the United States are overweight or obese.[59] Burneo et al found that in patients implanted with VNS to control epilepsy, 62% experienced significant weight loss.[60] Bodenlos et al[61] conducted a study looking at the association between VNS and food cravings in depressed adults and found that left cervical VNS resulted in attenuated food craving.[61] Recent work by Val-Laillet et al has shown that bilateral constant current stimulation of the vagus nerve led to lower food intake and sweet cravings in obese minipigs.[62] This study did not suggest that VNS actually caused weight loss but rather that VNS helped prevent excess weight gain. The implications of these studies are compelling, but the mechanism by which VNS influences weight loss is still unknown. Some hypotheses include changes in metabolism, decreases in fat stores, or changes in satiety signaling.[61] Another potential proposed mechanism for the effect of VNS on weight is reduced intestinal caloric absorption, which can be hypothesized based on the findings that vagal tone can alter peptides that can change gut motility and absorption.[63] These preliminary results encourage more extensive research into VNS, and the impact of autonomic control modulation, and hypothalamic signaling and its interactions with the enteric nervous system.

A study performed by Ikramuddin et al showed the effect of VNS on morbid obesity. Since bariatric surgery presents some major risks, investigators are looking for alternative, less-invasive methods of controlling obesity. Their trial showed that weight loss was higher by a statistically significant margin in patients who underwent VNS when compared to sham patients.[64] More research is needed, but implantation of VNS devices may be an attractive option for controlling weight and managing obesity for patients who have not had success with more traditional methods of weight control.

Cardiovascular disease

VNS must alter cardiovascular control due to the convergence of inputs in the autonomic control centers of the brain stem, but for how long and to what extent is unknown. The descending cardiac branch of the vagus is key for normal cardiac function.[65] Atherosclerosis, which often predisposes one to coronary heart disease, is believed to be due to low-grade systemic inflammation.[66] Since there is growing evidence that VNS is anti-inflammatory, it may provide another avenue for treating cardiac dysfunction and atherosclerosis. In the 2007 CARDIA study, Sloan et al showed that there was an inverse relationship between inflammatory markers and vagus nerve activity, measured by heart rate variability, suggesting that VNS is key to anti-inflammatory tone. They also suggest that high levels of pro-inflammatory markers such as IL-6 and C-reactive protein may indicate a predisposition to coronary artery disease.[67] VNS may also provide a therapeutic application for preventing heart failure. Zhang et al used a canine model to show that chronic VNS helps to regulate heart rate and improves heart function in a high-rate ventricular pacing model.[68] In a rat ischemia/reperfusion (I/R) model, Zhao et al showed that VNS improved cardiac function and reduced infarct size.[69] Their results also showed that VNS reduced mesenteric artery pathology and vasodilation caused by the I/R model and that lower levels of TNFα and IL-1β were found in serum. This is likely due to VNS effects on acetylcholine release and systemic levels and upregulation of M3AChR/a7nAChr expression[69] which have been implicated in inflammatory modulation (see "Potential uses and mechanisms of VNS" section), but the precise mechanism is still unknown and needs further work. Chapleau et al used a high salt, spontaneously hypertensive rat model to show that right VNS prevented aortic stiffening and slowed the progression of endothelial dysfunction.[70] In addition, they saw significantly higher serum IL-6 levels in VNS rats, which may indicate that VNS modulates inflammatory function in this severe hypertension model.[70] Based on these studies, there is a relationship between cardiovascular disease, inflammation, and vagal activity that may be altered by VNS.

Lung injury

VNS is being considered as a treatment for ventilator-induced lung injury (VILI) caused by pressure-induced damage to lung alveoli. Inflammation has been shown to increase the

likelihood of VILI, which is often the result of severe lung infection. Other respiratory disorders such as acute lung injury and acute respiratory distress syndrome, both of which can be complicated by sepsis, can also result in pronounced pulmonary inflammation.[71–73] Experiments by dos Santos et al showed that the vagus nerve plays an important role in pulmonary inflammation. Interruption of the CAP by vagotomy leads to worsening VILI.[71] Vagotomized animals with mechanical ventilation had increased alveolar damage and levels of IL-6 and hemorrhage compared to control animals.[71] Later experiments showed that VNS, both electrical and pharmacological, attenuated the lung injury in a "two-hit" model of VILI (I/R injury followed by high tidal volume ventilation, which can be further injurious to the lung) by decreasing the pro-inflammatory and pro-apoptotic responses.[71]

VNS may also be useful in treating gut and lung injuries together. In a study of lung injury caused by hemorrhagic shock, Reys et al showed that VNS prevents intestinal barrier failure and protects against lung injury.[74] In addition, pharmacologic blockade of nicotinic cholinergic receptors in an in vitro culture model of pulmonary endothelial cells suggests that VNS acts through the CAP to prevent lung injury and gut–barrier breakdown.[73] A study by Levy et al also showed that VNS alleviated lung injury caused by trauma hemorrhagic shock through the reduction of gut permeability.[72] These studies suggest that vagus nerve activity is crucial for normal lung function, and the possibility of using VNS to improve outcome in lung injury has great promise for further research. In addition, preventing gut–barrier interaction that contributes to visceral inflammation also deserves further study.

Stroke and TBI

Stroke and TBI are also causes widespread neural inflammation which VNS may be able to alleviate. In a study conducted by Bansal et al, the effect of VNS on TBI was evaluated by measuring tissue and serum ghrelin and serum TNFα.[75] Their study was based on the hypothesis that preventing the inflammatory surge after TBI could prevent sepsis, multiorgan failure, and other adverse effects. Since ghrelin is mediated through acetylcholine levels, it is reasonable to assume that VNS may have an application in treating TBI through a ghrelin or other hypothalamic-gated mechanism.[75] VNS decreased the serum levels of TNFα, an early cytokine marker for damage in trauma and ischemic injury. Regulation of cytokine expression by VNS may provide significant therapeutic value in these patients. Because VNS is known to have anti-inflammatory properties and affect the levels of acetylcholine, these changes in cytokine upregulation and rebalancing of neurotransmitter release may provide an immediate and controllable way to modulate injury due to stroke, ischemia, or trauma.

Diabetes

Diabetes is another inflammatory-related disorder that may benefit from treatment with VNS. Recent work has shown the role of the vagus nerve in the pathophysiology of diabetes and other related diseases, which may, in turn, suggest that VNS could be useful in treating such disorders. Cardiovascular risk has long been associated with diabetes, but the exact mechanism by which increased risk and diabetes synergize to exacerbate morbidity is not known. Pal et al found that relatives of type 2 diabetics had increased risk for cardiovascular diseases, which they attributed to sympathovagal imbalance.[76] Changes in sympathovagal tone may underlie the increased autoinflammation that could be the foundation of this increased cardiovascular risk. Woie and Reed showed a relationship between changes in tracheal edema in control, diabetic, and insulin-treated diabetic rats which suggests that barrier breakdown was significantly greater in control animals but was attenuated in diabetic rats.[77] Changes in airway secretion are vagally mediated, and the altered vagus tone in diabetes may exacerbate susceptibility to chronic inflammation. A broader concern is the role vagal tone plays in metabolic disease and obesity. Vagal afferents and projections to the hypothalamus play a significant role in satiety and feeding behavior, and disruption of vagal afferent traffic may contribute to obesity and downregulation of cholinergic descending tone to arrest inflammation.[77] Meyers et al used selective efferent stimulation to significantly lower blood glucose, which may be a potential treatment for type 2 diabetes.[78] While the interaction of inflammation and metabolic disorders is becoming clear, the potential role of VNS in treating diabetes needs further investigation. This study by Meyers et al uses a crude method of selective efferent stimulation, by cutting the vagus nerve above the stimulating electrode. However, selective blockade of vagal fibers using different electrical stimulation parameters may provide the answer for using VNS to affect metabolism. Since cutting the vagus nerve is not an ideal solution for human patients, similar results may be obtained by using different parameters that can selectively stimulate fiber types or afferent/efferent traffic. Patel and Butera have achieved such results by using high-frequency stimulation in both the vagus and the sciatic nerves in rats.[54,79] Whether parameters

for selective stimulation such as used by Patel et al could be used to replicate Meyers et al's results without the need to sever the vagus nerve remains to be determined.

RA

RA is a chronic, inflammatory autoimmune disease of unknown origin, which results in chronic synovial inflammation and damage to cartilage and bone due to release of cytokines and progressive inflammatory damage.[80,81] Alpha 7 (α7) nicotinic receptors are present in cells recovered from synovial fluid and from synovial tissue, particularly in cells with a macrophage-like morphology.[82] The general suppression of anti-inflammatory cholinergic pathways plays a critical role in RA. Das' review summarizes the current literature on the role that vagus tone plays in modulating RA and, as in so many of these inflammatory disorders, the preliminary evidence justifies further experiments.[83] In a recently completed study by SetPoint Medical Corporation, VNS devices were implanted in patients with RA to test the safety and efficacy of treatment. Patients were assessed after 6 weeks of treatment and showed a 20% improvement in symptoms.[84] Further experiments are needed to determine the role that VNS can play in the treatment of RA, in particular, the potential role of VNS in the modulation of cytokine cascade through α7 receptors.

Conclusion

VNS has been proven to be a useful treatment across a number of domains and has been used effectively to treat epilepsy and depression in adults. There is accumulating evidence to suggest that it can be used to help quell inflammation in a number of other autonomic or inflammatory disorders, which would make it useful for a wider range of pediatric patients as well. Preliminary studies have shown promise for VNS being used for stroke, autoimmune diseases, heart and lung failure, obesity, and pain management, but further studies are needed to fully elucidate the mechanistic actions that explain VNS's potential role in treating these disorders. Many of these studies are not mechanistic in nature, and further pathway analysis and studies focused on the mechanisms by which VNS alters autonomic tone are key to further our understanding of vagus nerve modification. VNS interacts with the body's immune system to modify inflammatory tone by altering the release of pro- and anti-inflammatory cytokines. We have summarized some of these key inflammatory markers in Figure 3. There is an overwhelming evidence to suggest that vagus nerve is an important component of the immune response and manipulating vagal tone is a way to modulate the immune system. Using VNS to manipulate vagal tone provides an exciting new opportunity for minimally invasive therapeutic intervention in adult and pediatric patients.

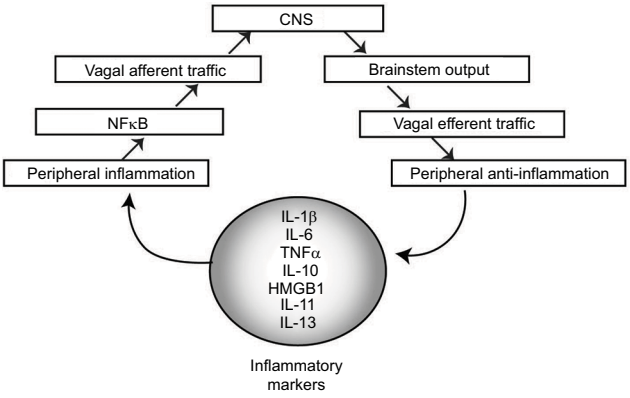

Figure 3 Flow diagram showing the inflammatory neural circuit.
Notes: Inflammatory markers including IL-1β, IL-6, TNFα, IL-10, HMGB1, IL-11, and IL-13 alter peripheral inflammatory tone, which stimulates NFκB, resulting in increased inflammatory signaling to the CNS via the brain stem. This results in output that generates an inflammatory signal. VNS can be used to alter the input/output of this autonomic control circuitry.
Abbreviations: IL, interleukin; TNF, tumor necrosis factor; NFκB, nuclear factor κB; CNS, central nervous system; VNS, vagus nerve stimulation.

Acknowledgment

Funding for this work was provided by intramural funding through the GRASP and Pediatric Research Initiatives (Loma Linda University) to CGW.

Disclosure

The authors report no conflicts of interest in this work.

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frontiers in Neuroscience

ORIGINAL RESEARCH
published: 08 May 2019
doi: 10.3389/fnins.2019.00418

Anti-inflammatory Effects of Abdominal Vagus Nerve Stimulation on Experimental Intestinal Inflammation

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Neural Technology, a section of the journal Frontiers in Neuroscience

Received: *05 February 2019*
Accepted: *11 April 2019*
Published: *08 May 2019*

Citation:
Payne SC, Furness JB, Burns O, Sedo A, Hyakumura T, Shepherd RK and Fallon JB (2019) Anti-inflammatory Effects of Abdominal Vagus Nerve Stimulation on Experimental Intestinal Inflammation. Front. Neurosci. 13:418. doi: 10.3389/fnins.2019.00418

Electrical stimulation of the cervical vagus nerve is an emerging treatment for inflammatory bowel disease (IBD). However, side effects from cervical vagal nerve stimulation (VNS) are often reported by patients. Here we hypothesized that stimulating the vagus nerve closer to the end organ will have fewer off-target effects and will effectively reduce intestinal inflammation. Specifically, we aimed to: (i) compare off-target effects during abdominal and cervical VNS; (ii) verify that VNS levels were suprathreshold; and (iii) determine whether abdominal VNS reduces chemically-induced intestinal inflammation in rats. An electrode array was developed in-house to stimulate and record vagal neural responses. In a non-recovery experiment, stimulation-induced off-target effects were measured by implanting the cervical and abdominal vagus nerves of anaesthetized rats (n = 5) and recording changes to heart rate, respiration and blood pressure during stimulation (10 Hz; symmetric biphasic current pulse; 320 nC per phase). In a chronic experiment, the efficacy of VNS treatment was assessed by implanting an electrode array onto the abdominal vagus nerve and recording *in vivo* electrically-evoked neural responses during the implantation period. After 14 days, the intestine was inflamed with TNBS (2.5% 2,4,6-trinitrobenzene sulphonic acid) and rats received therapeutic VNS (n = 7; 10 Hz; 320 nC per phase; 3 h/day) or no stimulation (n = 8) for 4.5 days. Stool quality, plasma C-reactive protein and histology of the inflamed intestine were assessed. Data show that abdominal VNS had no effect (two-way RM-ANOVA: $P \geq 0.05$) on cardiac, respiratory and blood pressure parameters. However, during cervical VNS heart rate decreased by 31 ± 9 beats/minute ($P \geq 0.05$), respiration was inhibited and blood pressure decreased. Data addressing efficacy of VNS treatment show that electrically-evoked neural response thresholds remained stable (one-way RM ANOVA: $P \geq 0.05$) and therapeutic stimulation remained above threshold. Chronically stimulated rats, compared to unstimulated rats, had improved stool quality (two-way RM ANOVA: $P < 0.0001$), no blood in feces ($P < 0.0001$), reduced plasma C-reactive

protein (two-way RM ANOVA: $P < 0.05$) and a reduction in resident inflammatory cell populations within the intestine (Kruskal–Wallis: $P < 0.05$). In conclusion, abdominal VNS did not evoke off-target effects, is an effective treatment of TNBS-induced inflammation, and may be an effective treatment of IBD in humans.

Keywords: vagus nerve stimulation, peripheral nerve stimulation, inflammatory bowel disease, medical device, bioelectric neuromodulation

INTRODUCTION

Inflammatory bowel diseases (IBDs), encompassing Crohn's disease and ulcerative colitis, are progressive debilitating immune-mediated disorders of the gastrointestinal tract (Ananthakrishnan, 2015b). The impact of the disease on patient quality of life is substantial due to its onset in young adulthood, fluctuating periods in which the disease is active (relapse and remission) and the lack of a cure (Abraham and Cho, 2009). The incidence of IBD is on the increase worldwide, with the prevalence of the disease highest in North America with an estimated 1.5 million people affected (Ananthakrishnan, 2015b).

The etiology of IBD is unknown, however, interactions between an individual's genetic makeup and external environment (i.e., diet, stress) play key roles in the emergence of IBD (Ananthakrishnan, 2015a). IBD is characterized by the over production of the key upstream pro-inflammatory mediator tumor-necrosis-factor-alpha (TNF-α) from macrophages, monocytes and differentiated T cells within the gastrointestinal tissue (Sanchez-Munoz et al., 2008). The production of TNF-α leads to the infiltration of inflammatory cells, which themselves further release pro-inflammatory cytokines, such as interleukin-1β (IL-1 β), IL-6, and interferon-gamma (IFN-γ) (Sanchez-Munoz et al., 2008; Neurath, 2014). Current gold standard immunosuppressant pharmacological therapies and anti-TNF-α biologicals, suppress the immune reaction and reduce the cascade of cytokine release by targeting TNF-α production (Rutgeerts et al., 2004; Nielsen and Munck, 2007). Although clinical management of such combined therapies has led to advancements in the control and prediction of the disease (Caprilli et al., 2006; De Cruz et al., 2015), adverse events in response to medication can be experienced in up to 20% of IBD patients when using these therapies (Chaparro et al., 2013; Gecse et al., 2016). Furthermore, despite the development of new anti-TNF-α therapies and clinical management strategies, surgical resection of the inflamed area of the gastrointestinal tract is necessary in 80% of ileocecal Crohn's disease patients (Caprilli et al., 2006). Therefore, an alternative therapy that keeps patients in remission is needed to more effectively treat IBD over the long-term.

A growing body of evidence has demonstrated that unilateral electrical stimulation of the left cervical vagus nerve is a feasible treatment in a rodent model of colitis (Meregnani et al., 2011; Sun et al., 2013). Following chemically-induced colitis in rats, cervical vagus nerve stimulation (VNS) improved the disease activity index (DAI: weight and stool quality), decreased histological damage and reduced inflammatory molecular markers expressed in colonic tissue (Meregnani et al., 2011). However, the effects of VNS therapy on histology and molecular markers were only seen in areas adjacent to, but not within, the inflammatory lesion. In a subsequent colitis study, inflammatory markers were only modestly reduced by cervical VNS, and inflammatory disease parameters (DAI, histology, inflammatory cytokine production) in VNS treated tissue did not return to control levels (Sun et al., 2013).

A first, small clinical trial demonstrated efficacy cervical VNS in ileocecal Crohn's disease patients (Bonaz et al., 2016) The majority of patients (5 of 7) responded to treatment and showed a reduction in clinical symptoms (Crohn's DAI), improvements in molecular markers (C-reactive protein and fecal calprotectin) and endoscopy DAI score. However, two patients experienced worsening of clinical symptoms and were removed from the study (Bonaz et al., 2016). Additionally, patients reported voice alterations (dysphonia) during stimulation (Bonaz et al., 2016). Other side effects, such as coughing, pain and labored/shortness of breath (dyspnea) are frequently reported following cervical VNS in patients treated with VNS for drug resistant epilepsy (Ben-Menachem et al., 2015). Studies in epileptic children fitted with a cervical VNS report more serious complications during sleep. Stimulation-induced effects on respiration and a reduction in overall oxygen saturation was seen in the majority of patients (87.5%, 8 patients; 100%, 10 patients, respectively) (Nagarajan et al., 2003; Zaaimi et al., 2005), while stimulus-induced changes to heart rate variability are reported in 50% of patients (10 patients) (Pruvost et al., 2006; Zaaimi et al., 2007). Another study reports stimulus-induced obstructive sleep apnea (15%; 26 patients) (Khurana et al., 2007). Such off-target affects are due to the activation of low threshold cervical vagal fibers to the larynx, pharynx, heart, and lungs (discussed in detail in the discussion), while the abdominal vagus nerve is at a site below these branches and its stimulation is predicted to results in fewer off-target effects.

Although evidence for VNS therapy to treat IBD is promising, the current approach of stimulating the cervical vagus nerve has a number of clinical limitations, including potentially an undesirable side-effect profile. To overcome the limitations of cervical VNS, we hypothesized that stimulating the sub-diaphragmatic abdominal vagus nerve (**Figure 1A**), which is below vagal branches to the lungs and heart and closer to the end organ, will improve the therapeutic effect of VNS and have fewer off-target effects. To address this hypothesis, in this study we first developed an electrode array (in house) that was able to stimulate and record neural responses from the vagus nerve of the rats. Using this electrode array in a non-recovery experiment, referred to as the "*VNS off-target experiment*," off-target effects to cardiac and respiratory rate were assessed during abdominal and cervical

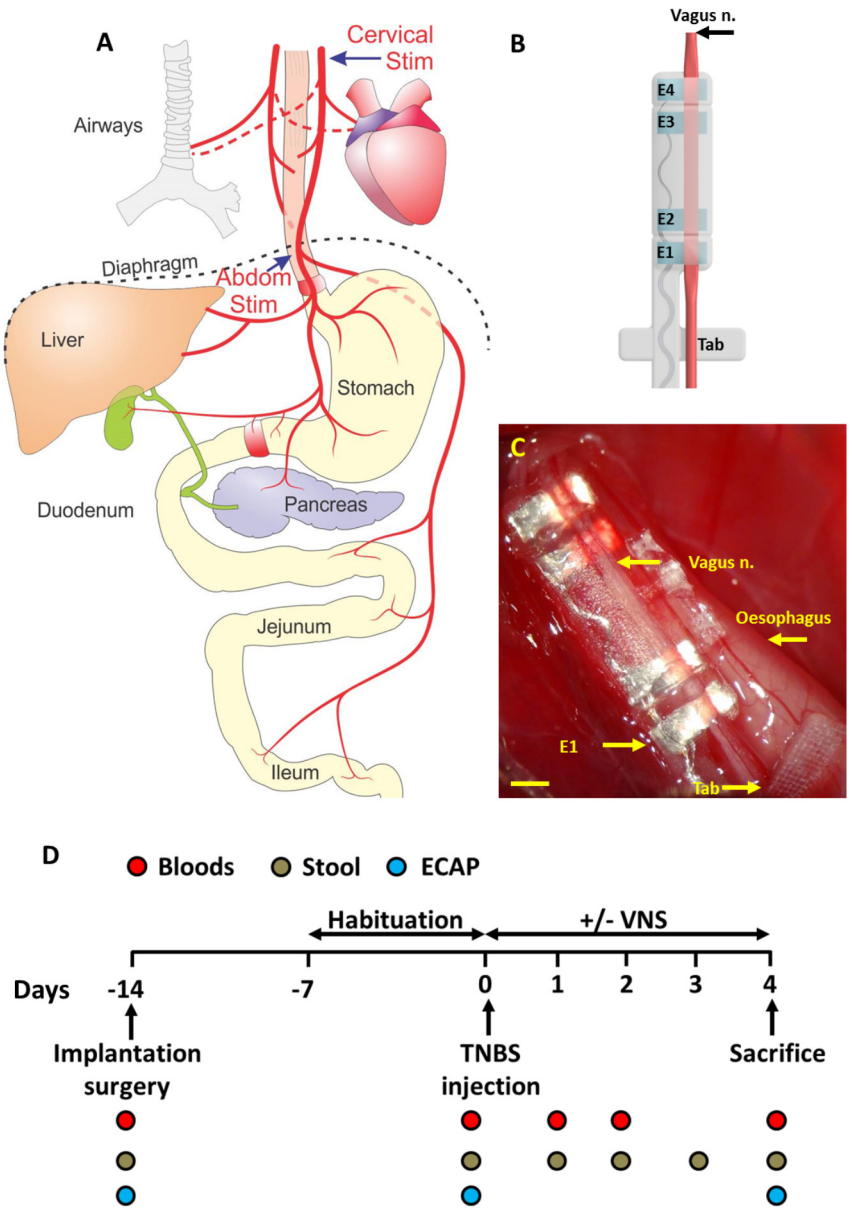

FIGURE 1 | Abdominal vagus nerve anatomy, electrode design and experimental schedule. **(A)** Schematic anatomical diagram shows the cervical and abdominal branches of the vagus nerve. Off-target effects in response to cervical stimulation (Cervical Stim indicated by arrow) and abdominal stimulation (Abdom Stim indicated by arrrow) were evaluated. For the VNS efficacy experiment, the electrode array was implanted onto the anterior abdominal vagus nerve, below the diaphragm and above the hepatic and celiac vagal branches. **(B)** The cuff electrode array had two platinum electrode pairs (E1–E2; E3–E4) that stimulated and recorded evoked neural responses. The array was anchored by suturing the Dacron tab to the esophagus. **(C)** Image of the abdominal vagus nerve electrode array *in vivo*. **(D)** Experimental schedule for the VNS efficacy experiment. Scale bar in **(C)** 1 mm.

VNS. In a recovery experiment, referred to as the "*VNS efficacy experiment,*" the efficacy of abdominal VNS was assessed using a rodent model of chemically-induced intestinal inflammation (**Figure 1A**). Behavioral, molecular and histological markers of inflammation were evaluated to determine the efficacy of abdominal VNS, and the histology of the implanted vagus nerve examined to confirm the safety of electrode array and stimulation delivered.

MATERIALS AND METHODS

This paper describes: (i) an acute (non-recovery) experiment that evaluated off-target effects during cervical and abdominal VNS. This is referred to as the "*VNS off-target experiment*"; and (ii) a chronic VNS efficacy experiment that evaluated the efficacy of abdominal VNS following chemically-induced intestinal inflammation, which is referred to as the "*VNS efficacy*

experiment" The methods section describes general procedures common to all experiments, followed by techniques specific to the VNS off-target and efficacy experiments.

General Methods

Animals and Anaesthesia

Sprague-Dawley rats (10–12 weeks old, Animal Resource Centre, Western Australia) were used, and all animal procedures were approved by the Animal Research and Ethics Committee of the Bionics Institute and complied with the Australian Code for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council of Australia). Approval was also obtained from the United States Army Medical Research and Material Command Animal Care and Use Review Office, protocol SSC-7486.02. Animals were kept on a 12 h light (7 a.m.–7 p.m.)/dark cycle (7 p.m.–7 a.m.) and allowed *ad libitum* access to fresh food, standard chow and water. For all surgical interventions, rats were anaesthetized (2–3% isoflurane using an oxygen flow rate of 1–1.5 L/min) and breathing rate remained between 45 and 60 breaths per minute (Payne et al., 2018c). All procedures were performed under aseptic conditions and following recovery surgery, rats were monitored carefully, given an analgesic (Carprofen 5 mg/kg sub-cutaneous) and housed separately. At the conclusion of the experiment, rats were anaesthetized (2% isoflurane using an oxygen flow rate of 1–1.5 L/min), then euthanized (300 mg/kg Lethabarb, intracardial injection).

Design of the Vagus Nerve Electrode Array

The vagus nerve electrode array consisted of four platinum (99.95%) electrodes embedded in a medical grade silicone elastomer cuff. Each platinum electrode had an exposed surface area of 0.3 mm^2. The distance between adjacent electrodes (E1–E2, or E3–E4, center to center) was 1.2 mm, while the distance between electrode pairs (E1–E2 to E3–E4, center to center) was 4.7 mm (**Figure 1B**). A channel (0.55 mm wide × 0.2 mm deep) traversed the length of the array to position the vagus nerve in close contact with the electrodes without damaging the nerve. A silicone 'lid' completes the cuff, preventing the nerve from migrating from the channel. A Dacron embedded silicone tab adjacent to the electrode array was used to suture the array to the esophagus, when chronically implanted onto the abdominal vagus nerve, in order to provide mechanical stability (**Figure 1B**). Individually insulated 50 μm diameter platinum/iridium (90/10) wires were welded to each electrode and formed a helical cable which traversed to a percutaneous connector mounted on the lumbar region of the rat.

Abdominal Vagus Nerve Electrode Array Implantation Surgery

Rats were anaesthetized, the skin incised on the ventral abdominal midline and along the dorsal-lumbar aspect of the spine. The vagus nerve electrode array was tunneled subcutaneously from the dorsal-lumbar incision to exit through the ventral abdominal incision. The abdominal cavity was exposed and the liver retracted gently using sterile saline soaked gauze. Abdominal tissue was kept moist at all times using warm sterile saline. The sub-diaphragmatic anterior abdominal branch of the vagus nerve was dissected away from the esophagus and the array implanted rostral to the hepatic and celiac branches of the vagus (**Figures 1A,C**). The array was sutured (7-0 silk, Ethicon) to the esophagus to provide stabilization and the abdominal cavity and skin sutured closed. The rat was rotated to expose the dorsal aspect of the spine, the percutaneous connector was anchored to the connective tissue of the lumbar region of the spine, and the skin closed around it.

Electrode Impedance Testing and Electrophysiological Recordings

To test the functionality of electrodes, the impedance of electrodes was measured using biphasic current pulses passed between the electrode of interest and all other implanted electrodes (Fallon et al., 2009). The peak voltage at the end of the first phase (V_{total}) of the current pulse was measured following delivery of a 25 μs per phase current pulse and current of 931 μA (Richardson et al., 2009). The V_{total} value was then used to calculate total impedance (Z_{total}) using Ohm's law (Z = voltage/current).

Electrically-evoked compound action potentials (ECAPs) were recorded in anaesthetized rats using bipolar vagus nerve electrodes. Either pair of electrodes (E1–E2 or E3–E4) could be used to stimulate or record neural responses. Two sets of evoked electrophysiological recordings (averaged from a total of 50 responses) were made at currents from 0 to 2 mA in 0.1 mA steps using a biphasic pulse (width = 200 μs, 50 μs interphase gap) presented at a rate of 10 pulses per second. Recordings were sampled at a rate of 100 kHz and filtered (high pass: 200 Hz; low pass: 2000 Hz; voltage gain 10^3) (Payne et al., 2018a). The electrically-evoked neural response threshold was defined as the minimum stimulus intensity producing a response amplitude of at least 0.1 μV within a post-stimulus latency window of 4.0–7.0 ms (Payne et al., 2018a). All recorded neural responses had conduction velocities within the range of a C-fiber response (Castoro et al., 2011).

VNS Off-Target Effects Experiment

Acute experiments (n = 5) were performed in normal, isoflurane anaesthetized, freely respiring rats to assess changes to heart rate, respiration rate and blood pressure during cervical and abdominal VNS. *Vagus nerve implantation surgery*: The left cervical vagus nerve was exposed and identified (Childs et al., 2015), and a vagus nerve electrode array (**Figure 1B**) implanted around the nerve. In the same rat, a second vagus nerve electrode array was implanted onto the anterior abdominal vagus nerve (see section above for details). *Femoral artery cannulation and measurements*: To measure arterial blood pressure changes, the femoral artery was exposed and cannulated. The cannula was connected to a calibrated blood pressure transducer (World Precision Instruments (WPI), Canada), the signal amplified and waveforms recorded (Cerebus System 128 Channel Neural Stimulator, Blackrock Microsystems, Massachusetts). *Heart and respiration rate measurements*: Heart rate was measured by recording electrocardiograms (ECG) by placing needles (26 Gauge) across the thorax and a return in the left leg.

The ECG was amplified using a WPI Iso-80 bioamp (Gain: $\times 10^3$; high pass: 5 Hz; low pass 10 kHz) before being recorded via the Cerebus system. Respiration rate was measured by placing a PolyPower®stretch sensor (Danfoss PolyPower, Denmark) around the upper thorax. Care was taken to place the respiratory band sensor over the largest excursion point during respiration. *Stimulus-induced off-target testing*: Baseline recordings of heart rate, respiration rate or blood pressure were generated for 30 s during which no (cervical or abdominal) stimulation was applied. After baseline recordings were taken, the cervical or abdominal vagus nerve was stimulated (10 Hz, 50 repetitions) at 0 or 1.6 mA (200 μs pulse width) for 20 s. To confirm that 1.6 mA stimulation applied to the cervical or abdominal vagus nerve was suprathreshold, electrically-evoked neural responses were recorded (1.6 mA, 200 μs pulse width). Following this stimulation period, 30 s of recordings were taken to monitor the return of measurements to baseline. Heart rate, respiration rate and blood pressure changes from baseline were calculated from the waveforms using a detection algorithm in IGOR8 software. At the conclusion of the experiment, rats were euthanized. No tissue was taken for histology.

VNS Efficacy Experiment

Chronic experiments (n = 15) assessed the efficacy of abdominal VNS in reducing inflammatory markers following TNBS-induced inflammation of the small intestine (experimental overview shown in **Figure 1D**).

Experimental Groups

The primary experimental groups consisted of rats that were implanted with an electrode array onto the abdominal vagus, allowed to recover for 7 days, habituated for 6 days and injected with TNBS to inflame the small intestine (see below). At 4 h following the TNBS injection, rats were randomly selected to receive abdominal VNS (TNBS+VNS: n = 7) or no stimulation (TNBS: n = 8). Blood and stool samples were collected, and ECAPs were recorded on days -14, 0, and 4 (**Figure 1D**). Rats were euthanized 4.5 days after TNBS injection (T = 4) and tissue taken for histological analysis. Similar to previous experiments (Pontell et al., 2009), control tissue (n = 9) was taken 5 cm oral to the ligation for the TNBS injection from animals in the TNBS (n = 5) and TNBS+VNS (n = 4) groups. An additional cohort of animals were euthanized 4 h after TNBS injection (4 H TNBS; n = 3) in order to evaluate the degree of intestinal inflammation at the onset of stimulation (**Table 1**).

Inflammation of the Small Intestine

At 14 days following implantation of the electrode array onto the abdominal vagus nerve, rats were anaesthetized and under aseptic conditions the abdominal midline incised and an 8 cm segment of jejunum, clear of intra-luminal content, was selected and ligated between two sutures (2-0 silk, Ethicon; Nurgali et al., 2007). Inflammation was induced within this ligated area by slowly injecting 1 mL of TNBS (2.5% dilution in 50% ethanol, Sigma) at the oral end of the ligated small intestine over a course of 2 min. After 5 min the ligatures were removed, the intestine returned to the abdominal cavity and the skin and abdominal wall muscle sutured closed in two layers (Pontell et al., 2009). The small intestine was kept moist with sterile saline solution during the whole procedure.

Habituation and Vagus Nerve Stimulation

Rats were habituated for 6 consecutive days prior to the TNBS injection (T = -7 to -1) to ensure no additional stress to the animal during the testing period (T = 0–4). Animals were housed individually in Perspex boxes and percutaneous plugs connected to an external stimulator (Fallon et al., 2018), but no stimulation was delivered. Given that routine laboratory procedures can cause stress to the animal, every attempt to handle animals equally and as little as possible was made (Balcombe et al., 2004). Immediately prior to the TNBS injection (T = 0), ECAPs were generated in all implanted animals. At 4 h following TNBS injection, awake animals were randomly selected to receive VNS delivered at 1.6 mA and 200 μs/phase (i.e., 320 nC per phase) using a stimulus rate of 10 pulses/s with a 30 s ON 5 min OFF duty cycle for 3 h/day (1:30–4:30 p.m.) for 5 consecutive days (T = 0 to T = 4; 5 stimulation sessions in total). Unstimulated rats (TNBS group) were subjected to the same procedures as stimulated rats, but did not receive VNS. ECAPs were recorded on days -14, 0, and 4 (**Figure 1D**). Electrical stimulation was delivered using an external battery operated stimulator (Fallon et al., 2018) connected to the percutaneous connector. The stimulator delivered charge-balanced biphasic current pulses to the selected bipolar electrodes located on the nerve. Charge recovery was achieved via electrode shorting on completion of each current pulse.

Quantification of Disease Activity Index

Stool produced from implanted rats while being weighed (between 9 and 10 a.m. each day (T = 0 to T = 4) was assessed for consistency and signs of bleeding (**Table 2** and **Figure 1D**) (Sun et al., 2013).

TABLE 1 | Experimental cohorts in the efficacy of VNS experiment.

Experimental group	TNBS	VNS	Sample size
TNBS only (4H TNBS)	Yes	N/A	3
Unstimulated (TNBS)	Yes	No	8
Stimulated (TNBS+VNS)	Yes	Yes	7

Control tissue was taken from an oral segment of gut from implanted animals (control tissue from TNBS rats: n = 5 of 8; Control tissue from TNBS+VNS rats: n = 4 of 7).

TABLE 2 | Scoring system of stool quality following TNBS injection.

Variable	0	1	2
Stool consistency	Normal stool: hard pellet shaped form	Loose stools: Pellet is sticky and deforms under pressure	Diarrhea: No form; fecal matter adherent to fur
Signs of blood	No blood: Stool is a medium brown color	Mild bleeding: Stool is dark brown or black in appearance	Gross bleeding: Blood is visible on fur and bedding

Quantification of C-Reactive Protein

Blood was taken from the tail vein of implanted rats (**Figure 1D**). Blood taken prior to TNBS injection on day 0 served as a control. The final bleed was taken after the final round of stimulation. Whole blood (300 μL) was collected in K2-EDTA tubes (Starstedt) centrifuged (2000 g for 10 min) and plasma aliquoted and stored at −80°C. On the day of the assay, aliquots were thawed on ice and the C-reactive protein (CRP) ELISA conducted according to manufacturer instructions (Cusabio CSB-E07922r) and CRP levels determined via absorbance measurements using a Biorad BenchMark Plus microplate spectrophotometer.

Dissection, Histology, and Immunohistochemistry of Small Intestine Tissue

At the conclusion of the experiment, implanted rats were euthanized and the TNBS-inflamed segment of small intestine tissue dissected and processed as previously described (Payne et al., 2018c). In brief, a 2 cm segment of control tissue was removed spanning 5–7 cm oral to where the ligation limiting the inflamed site had been. As TNBS-induced inflammation is patchy, the 8 cm length of inflamed intestine was divided equally into four 2 cm long segments, and cut longitudinally along the mesenteric border and pinned out onto balsa boards (mucosa side up). One half was placed in fixative (2% formaldehyde plus 0.2% picric acid in 0.1 M sodium phosphate buffer, pH 7.4) overnight, embedded in paraffin, sectioned (5 μm) and stained with hematoxylin and eosin (H&E) (Pontell et al., 2009) or immunohistochemically stained with anti-CD3, a cytotoxic T cell marker (1:200; Cytomation, Dako E0432) (Payne et al., 2018c). The other half of the tissue was processed for frozen sections (14 μm) and myeloperoxidase (MPO) staining (Payne et al., 2018c). All sections were mounted with DPX.

Histopathology Scoring of Inflamed Tissue

Histopathologist (J.B.Furness), blinded to procedures, used H&E stained sections to evaluate the degree of inflammation in each segment of intestine. Histological changes were on a scale of 0–3 for the assessment of damage to the mucosa (villi architecture changes, including loss of height, pinching, clubbing, venous engorgement), and assessment of inflammatory changes (leukocyte infiltration) on a scale of 0–2 for assessment of the numbers of leukocytes within venules (adapted from Payne et al., 2018c; **Table 3**). Scores were out of a total of 9.

Inflammatory Cell Quantification in Transmural Small Intestine Tissue

Eosinophils, T cells (CD3+ cells) and neutrophils (MPO+ cells) were quantified using a Zeiss Axioplan II microscope, positive cells were counted (×40 objective) across three consecutive fields of view across the external smooth muscle layers, submucosal and mucosal layers. Cells were counted within the most inflamed area of the tissue. Images of the total field of view were generated (Axiovision, Zeiss, Germany). For MPO, eosinophil and T cell counts, cells per mm length of intestine were quantified.

Vagal Nerve Tissue Processing and Analysis

Immediately following dissection of small intestine tissue, implanted animals were perfused intracardially with 0.9% saline followed by fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, room temperature). The esophagus and implanted vagus nerve array were dissected from the carcass. At the implanted region, the vagus nerve was dissected from the array and the region of the nerve adjacent to the electrodes (E1–E4) labeled using tissue dye (Davidson's Marking system, Bradley Products, MN, United States) (Villalobos et al., 2013). Tissue proximal to the implanted site was also taken and processed as an intra-animal, non-implanted control. The esophagus and attached vagus nerve were embedded in paraffin and serial sections (5 μm) taken. The tissue dye marked the area of vagus nerve that was adjacent to electrodes. Sections were stained for H&E and mounted with DPX. Sections were examined by an observer (S.C Payne), blinded to procedures, for signs of histopathological damage. At each location light microscope images were taken using a Zeiss Axioplan II microscope and Axiovision software (Zeiss, Germany). Using ImageJ, total fascicle area was quantified across one section per electrode position, per animal. The cross-sectional area of the vagus nerve was not measured as the boundary of the epineurium was difficult to define.

Statistics

Differences between normally distributed data were tested using a one- or two-way repeated measures (RM) ANOVA with Sidak or Tukey *post hoc* tests as appropriate. Differences between data that was not normally distributed was analyzed using a non-parametric Kruskal Wallis one-way ANOVA and Dunn's *post hoc* test. Details of each statistical test are stated in the relevant results section. Statistically significant differences were accepted

TABLE 3 | Histological parameters used to score tissue taken from the small intestine following TNBS injection.

	Variable	0	1	2	3
Mucosal damage	Extent of mucosal damage (including loss of height, pinching, clubbing, venous engorgement)	No damage	Damage affects less than 1/3 of villi	Damage affects between 1/3 and 2/3 of villi	Damage affects more than 2/3 of villi
	Shortening of villi	0–20% shortening	20–60% shortening	60–100% shortening	N/A
	Pinching of villi	Absent	Affecting < 50% of villi	Affecting > 50% of villi	N/A
Inflammatory changes	Leukocyte presence in large venules (avoiding capillaries and small venules or lymphatics)	<4 adherent leukocytes in venules	4–10 adherent leukocytes in venules	>10 adherent leukocytes in venules	N/A

Adapted from Payne et al. (2018c).

as P-values of < 0.05 and GraphPad Prism 4 (GraphPad Software, United States) was used for all analysis.

RESULTS

VNS Off-Target Effects Experiment

No Measurable Off Target Affects During Abdominal Vagus Nerve Stimulation

The average (standard error of mean, SEM) threshold for activation of C-fibers by cervical VNS was 0.25 ± 0.07 mA and abdominal VNS was 0.43 ± 0.11 mA, indicating the test stimulation of 1.6 mA was substantially suprathreshold for C-fibers at both stimulation sites in all animals. In the example shown in **Figure 2A**, cervical VNS resulted in an average heart rate drop of 43 beats per minute (bpm) from baseline (370 ± 15.4 bpm), a maximum decrease in blood pressure of 8.2 mmHg and an almost complete cessation of breathing (baseline respiration rate: 52 ± 7.0 cycles per minute, cpm). In contrast, the same level of abdominal VNS produced no change in heart rate (400 ± 31.4 bpm), repiration rate (54 ± 5.0 cpm) or blood pressure (**Figure 2B**).

Statistical analysis [two-way (Current × Location) RM ANOVA; $n = 5$] of the effects of VNS on heart rate revealed a significant effect of Current ($P = 0.06$), Location ($P = 0.04$) and a significant Interaction ($P = 0.02$). Tukey's *post hoc* analysis showed the average 31 ± 9 bpm (mean ± SEM) drop in heart rate during suprathreshold cervical VNS was significantly ($P = 0.02$) greater than suprathreshold abdominal VNS, which was not different to no stimulation ($P = 0.9$). Respiration recordings were noisy and difficult to quantify, however, severe disruptions to the regular respiration pattern were observed in 4 of 5 rats during the suprathreshold cervical VNS, while no changes in respiration were observed with abdominal VNS. Blood pressure changes were only assessed in $n = 2$ animals and therefore no statistical comparisons of the data were performed.

VNS Efficacy Experiment

Thresholds for Electrically-Evoked Neural Responses Remained Below Stimulation Levels

ECAPs were recorded to ensure stimulation levels were above neural threshold. The mean threshold of recorded ECAPs remained unchanged (one-way RM ANOVA (Time): $P = 0.8$) between day -14 (393 ± 74 μA; **Figure 3A**), day 0 (357 ± 65 μA; **Figure 3B**) and day 4 (325 ± 75 μA; **Figure 3C**) and substantially below the levels used to delivery therapeutic stimulation (1.6 mA, 200 μs/phase). The latency of ECAPs also remained unchanged (one-way RM ANOVA; $P = 0.16$) during the implantation period (T = -14: 5.43 ± 0.35 ms; T = 0: 6.69 ± 0.58 ms; T = 4: 5.52 ± 0.35 ms).

Impedance of Implanted Abdominal Vagus Nerve Electrodes

The mean electrode impedance (±SEM) in saline (prior to implantation) was 2308 ± 96 Ω (range: 1880–3340 Ω). Immediately following implantation, common ground impedance of electrodes increased to 5070 ± 246 Ω (range

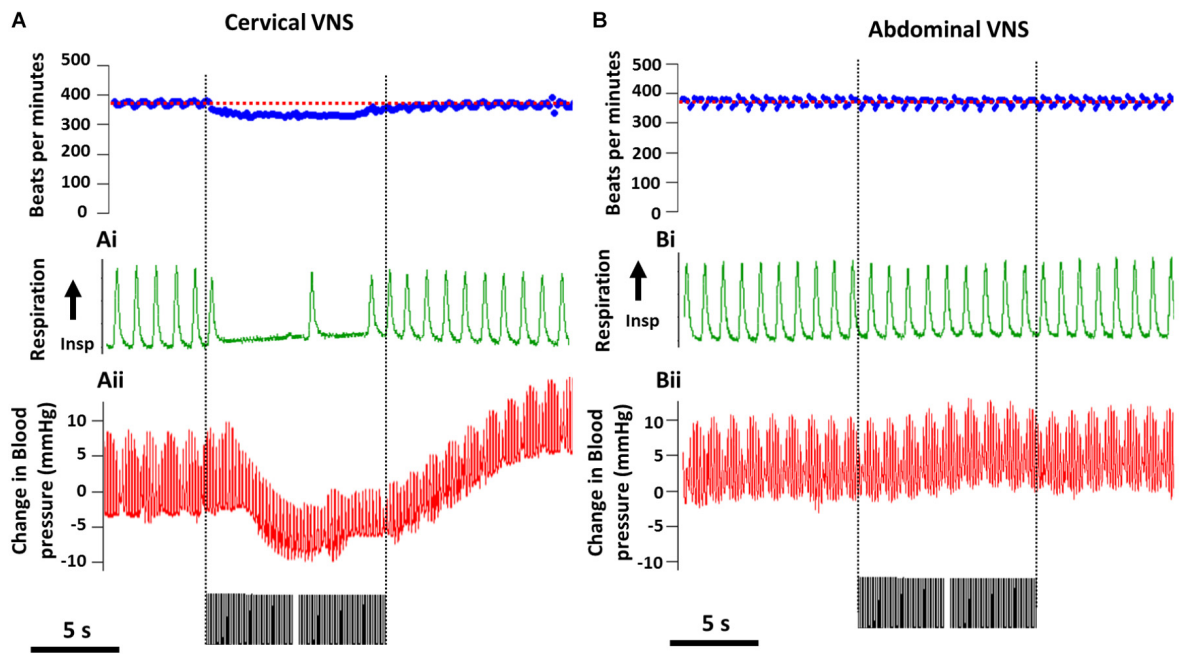

FIGURE 2 | Off-target effects during cervical and abdominal VNS. Representative recordings from from one animal show a decrease in heart rate (baseline heart rate indicated by red dotted line) **(A)**, respiratory rate **(Ai)** and blood pressure **(Aii)** during cervical VNS (1.6 mA, 200 μs/phase). **(B–Bii)** No changes in heart rate (baseline heart rate indicated by red dotted line) **(B)**, respiratory rate **(Bi)** and blood pressure **(Bii)** were seen during abdominal VNS (1.6 mA, 200 μs/phase). Scale bar in **(A,B)** represents 5 s.

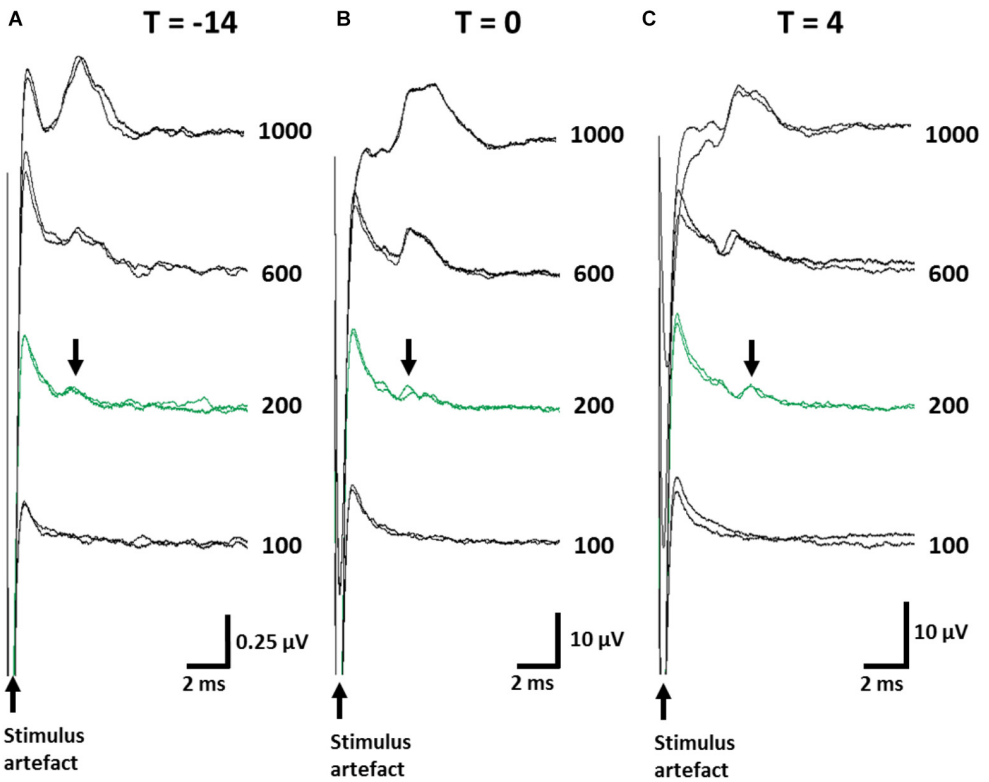

FIGURE 3 | Electrically-evoked C-fiber responses from the abdominal vagus nerve were recorded over the implantation period. Representative ECAPs from one animal are shown when taken immediately following implantation surgery (**A**, $T = -14$), prior to the injection of TNBS (**B**, $T = 0$) and at the conclusion of the experiment (**C**, $T = 4$). Note, the amplitude of the neural response recorded immediately post-implantation ($T = -14$) were consistently smaller than those at later time points green traces indicate the neural threshold (200 µA) and arrows indicate the elecrically evoked C-fiber response (Latency range: 5.3–5.6 ms). Current amplitude (in µA) is indicated to the right of each set of ECAPs.

between 3485 and 7194 Ω). At the conclusion of the experiment the *in vivo* impedances had increased to 8379 ± 269 Ω (5807–9650 Ω). During the implantation period, there were no short circuits, and only 4 out of 60 electrodes ($n = 4$ electrodes per rat; $n = 15$ implanted rats in total) became open circuit. These short circuits did not compromise the delivery of VNS.

Vagus Nerve Stimulation Improved Clinical Measurements of Inflammation

On day 0 (referred to as the "control"), prior to the TNBS injection, all implanted rats ($n = 15$) produced stools that were solid, dry pellets, with no signs of blood. Following TNBS injection, unstimulated rats (TNBS, $n = 8$) had significantly worse stool quality [two-way RM ANOVA (Time × Treatment): Time: $P < 0.0001$; Treatment $P = 0.0009$; Interaction: $P = 0.02$] than control (day 0) on day 1 (*Sidak post hoc* test: $P < 0.0001$), day 2 ($P < 0.0001$), day 3 ($P = 0.002$) and day 4 ($P = 0.015$; differences indicated by "*" in **Figure 4A**). The stool quality of stimulated rats (TNBS+VNS, $n = 7$) remained similar to control following TNBS injection ($P \geq 0.05$). Furthermore, stimulated rats (TNBS+VNS) had significantly better stool quality than unstimulated rats (TNBS) on day 1 ($P = 0.0002$), day 2 ($P = 0.012$), day 3 ($P = 0.04$), and day 4 ($P = 0.03$; differences indicated by a 'circle' in **Figure 4A**).

Following TNBS injection, the presence of blood in feces was observed (two-way RM ANOVA: Time: $P = 0.058$; Treatment $P < 0.0001$; Interaction: $P = 0.058$) in unstimulated rats (TNBS; $n = 5$) significantly more often, compared to control (day 0), on days 1 ($P = 0.0002$) and day 2 ($P = 0.006$; differences indicated by "*" in **Figure 4B**).

Unstimulated rats (TNBS; $n = 5$) were observed to have higher presence of blood in feces than stimulated rats (TNBS+VNS) on day 1 ($P = 0.001$) and day 2 ($P = 0.02$; differences indicated by a "circle" in **Figure 4B**). Stimulated rats (TNBS+VNS; $n = 4$) were not observed to have blood in feces at any time point following TNBS injection (**Figure 4B**).

Following TNBS treatment, plasma CRP levels in unstimulated rats (TNBS) were significantly increased [two-way RM ANOVA (Stimulation × Time); Treatment: $P = 0.005$; Time, $P < 0.0001$, Interaction, $P < 0.0001$] at day 1 (Sidak *post hoc*: $P < 0.0001$) and day 2 ($P = 0.0004$), compared to day 0 (indicated by "*" in **Figure 4C**). By day 4, CRP levels returned to baseline levels. CRP levels of stimulated rats (TNBS+VNS) were significantly higher than baseline only at day 1 ($P = 0.006$), and no different from control (day 0) on days 2 and 4 ($P \geq 0.05$). However, on day 1 CRP levels were still significantly lower ($P < 0.0001$) than unstimulated rats (TNBS, indicated by a "circle," **Figure 4C**).

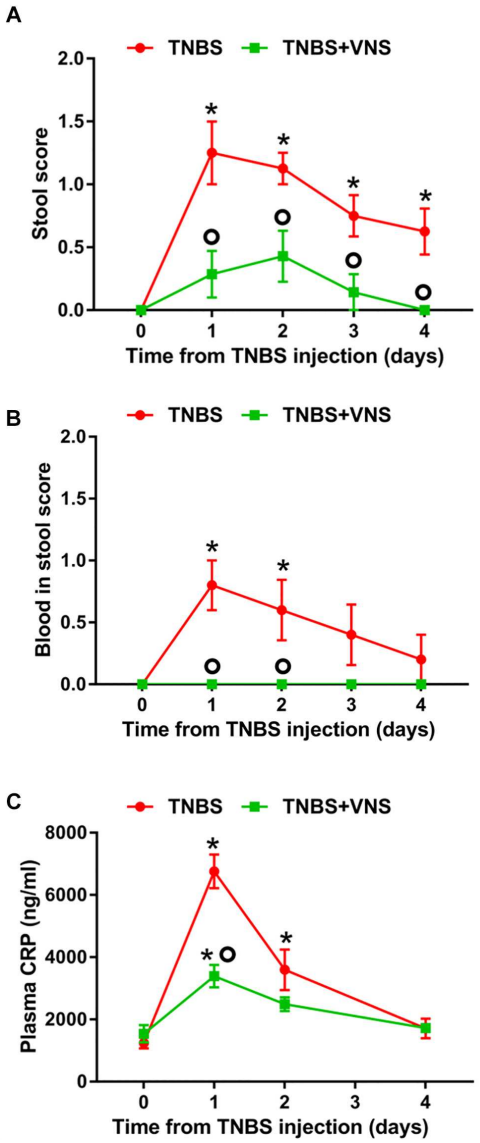

FIGURE 4 | (A) Vagus nerve stimulation improves clinical measurements of inflammation following TNBS injection. **(A)** The quality of stool produced from unstimulated (TNBS; $n = 8$) and stimulated (TNBS+VNS; $n = 7$) rats were scored following the TNBS injection. **(B)** Blood in stool produced from unstimulated (TNBS; $n = 5$) and stimulated (TNBS + VNS; $n = 4$) rats was scored following TNBS injection. **(C)** CRP content in blood plasma was measured in unstimulated (TNBS; $n = 6$) and stimulated (TNBS+VNS; $n = 6$) on day 0 (pre-TNBS control) and days 1, 2, and 4 following TNBS injection. All data show mean ± SEM. Significant differences from control (day 0, pre-TNBS) are indicated by "*", while significant differences between unstimulated (TNBS) and stimulated (TNBS+VNS) groups are indicated by "O".

TNBS-Induced Inflammatory Damage Improved Following VNS

In small intestine tissue 5–7 cm oral to the site of TNBS induced inflammation (i.e., control tissue), there was no damage to the epithelial cells of the villi along the length of the analyzed tissue, with villi exhibiting the normal long finger-like projections (**Figures 5A,Ai**). Few leukocytes (less than 4) were observed within venules of the lamina propria and within the circular and longitudinal muscle layers, while there was mild infiltration of leukocyte populations within the mucosa and submucosa layers. In acute TNBS tissue (4 h post-injection, at the time that active VNS was applied) extensive epithelial cell loss (**Figures 5B,Bi** indicated by arrows) and leukocyte infiltration (**Figures 5B,Bi**, indicated by circle) was observed. Leukocyte infiltration into the mucosa/submucosal layers was moderate. At 4 days following TNBS injection, tissue from unstimulated (TNBS) rats had extensive irregularities to villus epithelial cells (**Figures 5C,Ci**, indicated by arrows). Villi were shorter and had a blunted appearance, and leukocyte infiltration was severe within the mucosa, submucosa and venules (**Figures 5C,Ci**). Tissue from stimulated (TNBS+VNS) animals exhibited reduced inflammation-induced damage. Less damage or abnormalities to villi surface epithelium was observed (**Figures 5D,Di**), while villi architecture was sometimes indistinguishable from control small intestine tissue. However, some inflammation-induced irregularities, such as shortening of villi remained within tissue from stimulated animals (TNBS+VNS; **Figure 5D**).

The histological score measured from control tissue of unstimulated (TNBS; $n = 5$) and stimulated (TNBS+VNS; $n = 4$) rats were no different from each other ($P \geq 0.05$; unpaired T-test). Therefore the control groups were combined ($n = 9$). Statistical analysis of the histological score showed there was significant (Kruskal–Wallis one-way ANOVA: $P = 0.001$) histological damage in tissue from 4 h post-TNBS injection (Dunn's *post hoc*: $P = 0.002$) and in unstimulated rats (TNBS; $P = 0.0006$; **Figure 5E**). However, the histological score of tissue taken from stimulated animals (TNBS+VNS) was no different from control ($P = 0.17$), suggesting that VNS improved recovery from inflammation-induced damage (**Figure 5E**).

Numbers of Leukocytes in Mucosal and Submucosal Layers Was Reduced Following VNS

The numbers of leukocytes measured from the control mucosal, submucosal and muscle layers were no different from each other ($P \geq 0.05$; unpaired T-test). Thus the control groups were combined ($n = 9$). Tissue taken 4 h (4 H TNBS; $n = 3$) following TNBS is shown in **Figures 5B,Bi**, but was not included in the statistical cell count analysis, nor included in subsequent graphs (**Figure 6**), due to the low number of animals in this group. Four days following the TNBS injection, there was a significant increase in eosinophils within the mucosal layer (Kruskall–Wallis: one-way ANOVA: Treatment $P = 0.0002$), and a significant increase in the number of CD3+ cells (T cells) within both the mucosa ($P = 0.001$) and submucosa ($P = 0.01$) in unstimulated animals (TNBS, **Figures 6A–C**). In contrast, there was no significant elevation in the number of eosinophils, MPO+ cells or CD3+ cells in stimulated animals (TNBS+VNS; **Figures 6A–C**). Furthermore, eosinophil (Dunn's *post hoc*: $P = 0.03$) and T cells ($P = 0.02$) populations were significantly reduced in the mucosal layer of stimulated

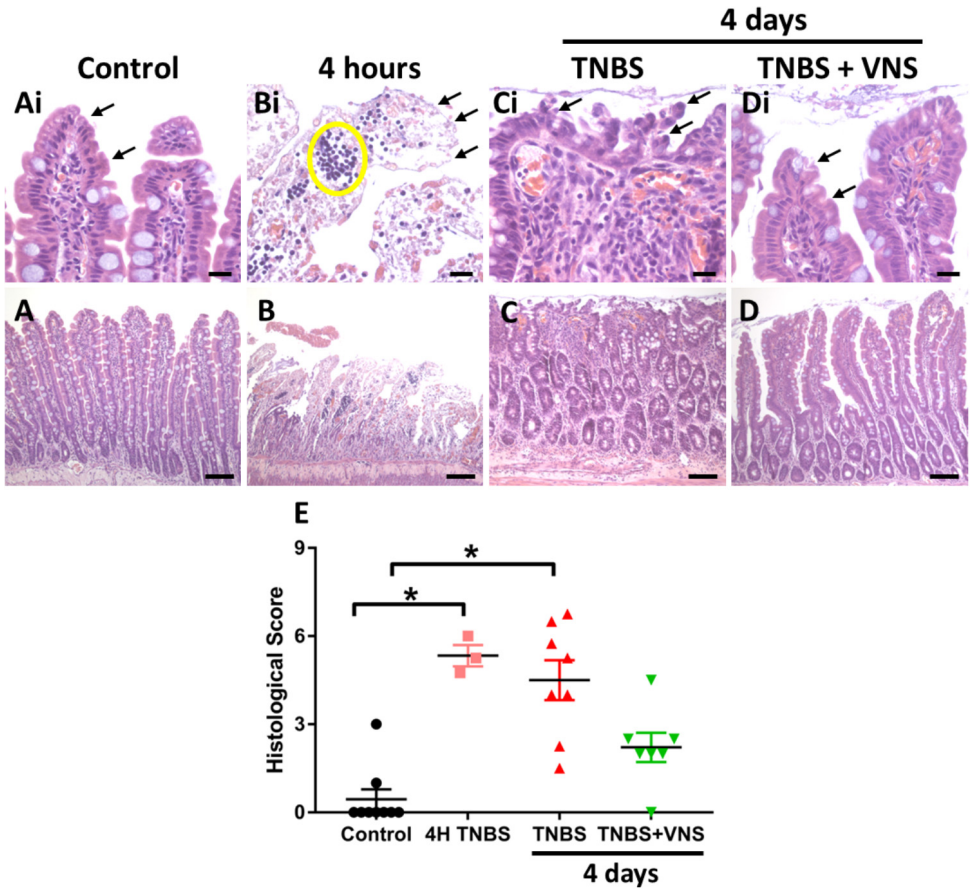

FIGURE 5 | Vagal nerve stimulation reduced histological damage of small intestine tissue following TNBS injection. **(A)** Control small intestine **(A,Ai)** had undamaged, intact surface epithelium (arrows, **Ai**). **(B,Bi)** At 4 h following TNBS injection (the time that VNS was applied), extensive epithelial cell loss (arrow, **Bi**) and leukocyte infiltration (yellow circle) was observed. **(C,Ci)** At 4 days following TNBS injection, there was extensive damage to villus architecture (**Ci**, arrows) and leukocyte infiltration in venules (circle) in tissue from unstimulated rats (TNBS). **(D,Di)** In tissue from stimulated rats (TNBS+VNS), histological damage was less severe than tissue taken from unstimulated (TNBS) rats. Surface epithelial cells were intact (arrows), and villi were mostly undamaged, although some inflammation-induced irregularities remained. **(E)** The histological score was quantified in tissue taken from control animals, at 4 h following TNBS injection, and from unstimulated (TNBS) and stimulated animals (TNBS+VNS). Data show raw histological score (0–9) of each animal, median and interquartile range. Significant differences of $P < 0.05$ are indicated by "*." Scale bars in **(A–D)**: 100 μm; in **(Ai–Di)**: 20 μm.

animals (TNBS+VNS), compared to unstimulated animals (TNBS; **Figures 6A,C**).

Leukocyte Infiltration Into External Muscle Layers Was Reduced Following VNS

Tissue taken 4 h (4 H TNBS; $n = 3$) following TNBS was not included in the statistical cell count analysis, nor included in subsequent graphs (**Figure 7**), due to the low number of animals in this group. In the circular and longitudinal muscle layers, there was an increase in eosinophils within tissue taken from unstimulated (TNBS; Kruskal–Wallis: $P = 0.001$) and stimulated rats (TNBS+VNS; $P = 0.012$), compared to control (**Figure 7D**). Numbers of MPO+ cells (representative images for control, TNBS and TNBS+VNS tissue shown in **Figures 7A–C**) (neutrophils) increased in tissue taken from unstimulated rats (TNBS; $P < 0.0001$), but was significantly less in tissue taken from stimulated rats (TNBS+VNS; $P = 0.048$; **Figure 7E**). Numbers of CD3+ cells (T cells) within the muscle layers of tissue taken from TNBS treated rats were greater than control ($P < 0.0001$). However, there were significantly fewer T cells in the muscle layer of tissue taken from stimulated rats ($P = 0.04$), compared to unstimulated rats treated with TNBS (**Figure 7F**).

Assessment of the Implanted Vagus Nerve

Chronically implanted abdominal vagus nerves ($n = 9$) were assessed for histopathological changes (**Figures 8A,Ai**) and changes in fascicle area (**Figure 8B**). We observed no tissue granulation or infiltration of acute inflammatory cells, however, a foreign body tissue response (**Figure 8Ai**, indicated by **) was seen within the electrode channel, surrounding the nerve (**Figure 8Ai**). No inflammatory cells were observed suggesting the foreign body response was likely benign. Furthermore, blood vessels (**Figure 8Ai**, indicated as "BV") were observed within the nerve. There were no observed differences between stimulated and unstimulated nerve histology.

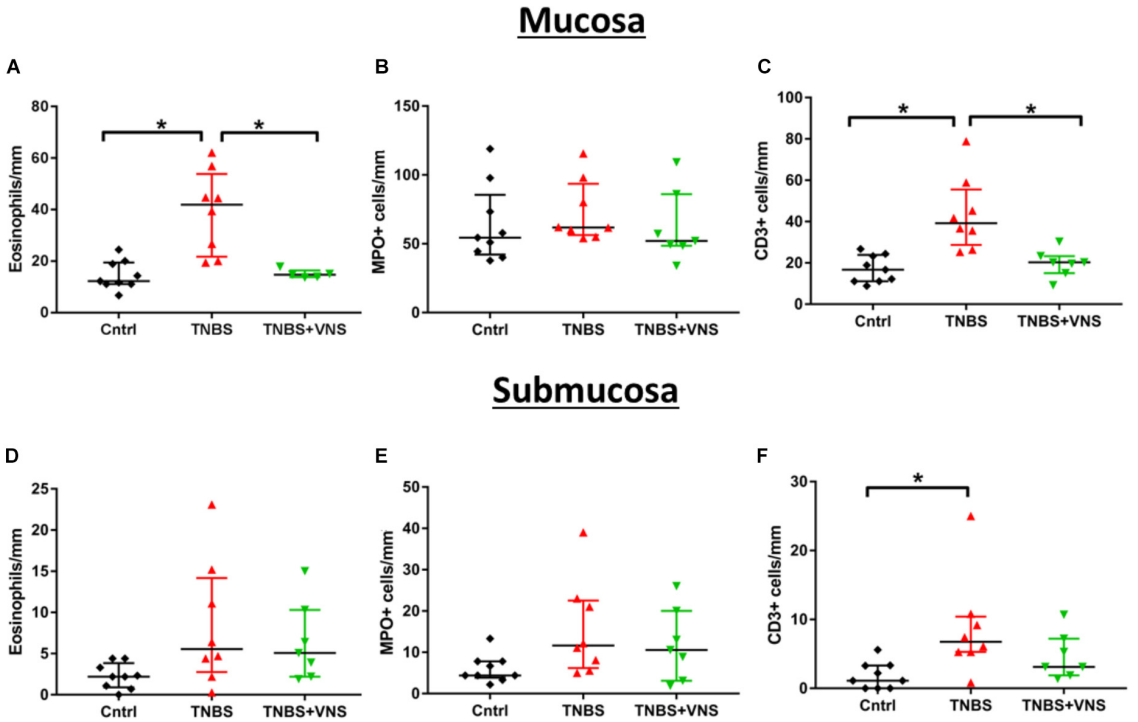

FIGURE 6 | VNS reduces resident leukocyte populations in mucosal and submucosal layers following TNBS injection. **(A–C)** Quantification of eosinophils **(A)**, MPO+ cells (neutrophils, **B**) and CD3+ cells (T cells; **C**) within the mucosal layer. **(D–F)** Quantification of eosinophils **(D)**, MPO+ cells **(E)** and CD3+ cells **(F)** within the submucosal layer. Data show raw data from each animal, median and interquartile range. Significant differences of $P < 0.05$ are indicated by "*."

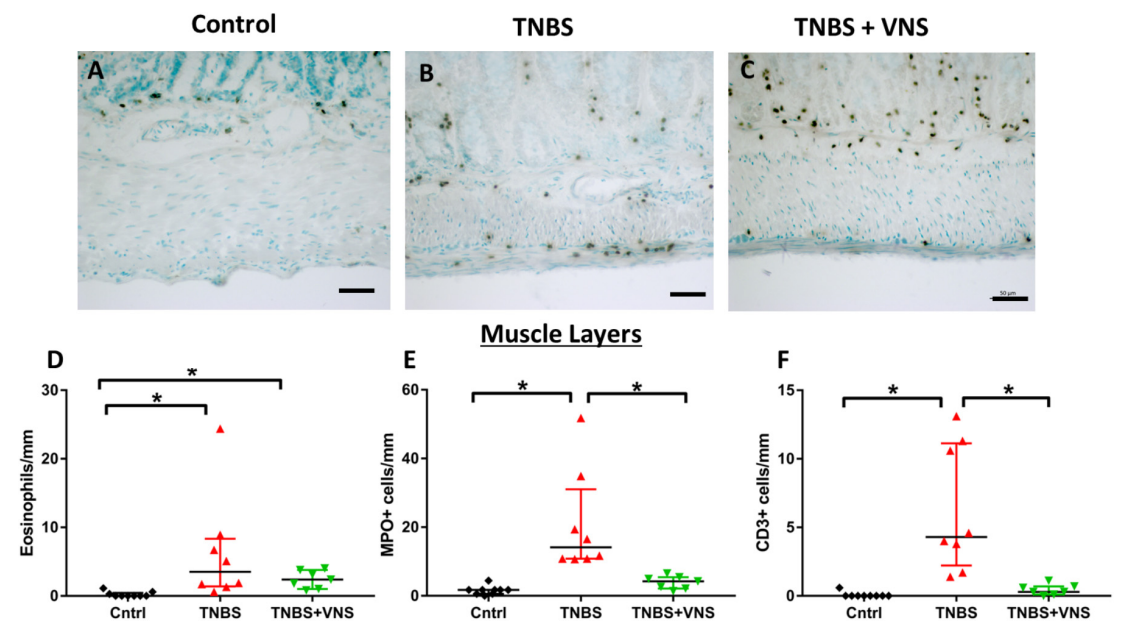

FIGURE 7 | VNS reduces resident leukocyte populations in muscle layers following TNBS injection. **(A–C)** Images show myeloperoxidase (MPO) positive cells (neutrophils) within circular and longitudinal muscle layers of tissue taken from control **(A)**, TNBS rats **(B)** and TNBS+VNS rats **(C)**. **(D–F)** Quantification of eosinophils **(D)**, MPO+ cells **(E),** and CD3+ cells **(F)** within the circular and longitudinal muscle layers. Data show mean count/animal, median and interquartile range. Significant differences of $P < 0.05$ are indicated by "*."

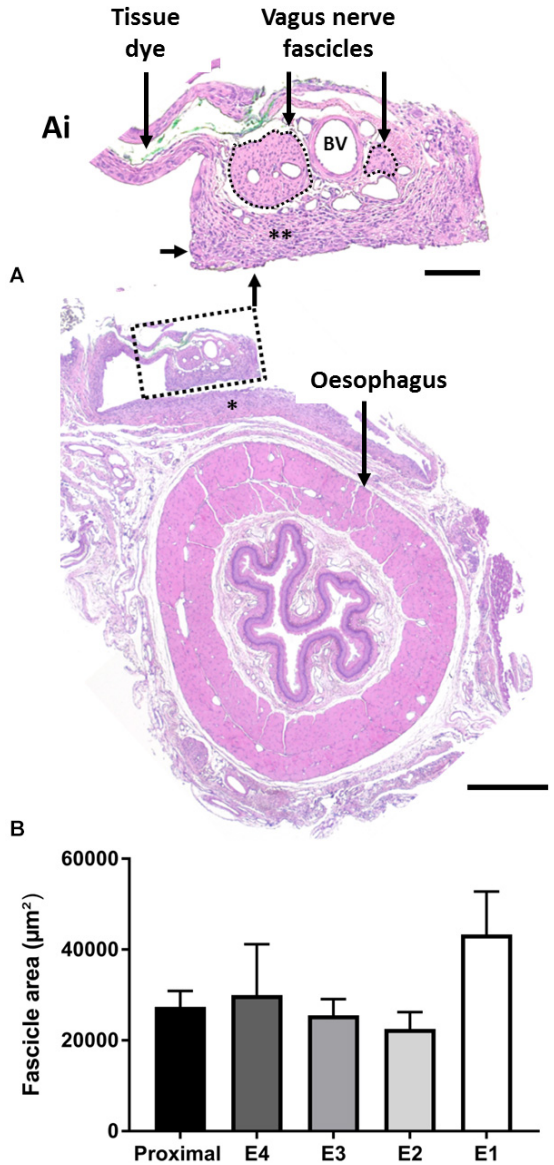

FIGURE 8 | Histopathology of the anterior abdominal vagus nerve following implantation. **(A,Ai)** H&E stained vagus nerve that was stimulated for 4 days shows a benign foreign body tissue response (**A**, indicated by *), which extended into the electrode channel (**Ai**, indicated by **). There were no acute inflammatory cells within the epineurium, the two fascicles of the vagus nerve (outlined by a dotted line) appeared undamaged, and blood vessels (example indicated by "BV") appeared patent. Tissue dye was used to identify the implanted nerve upon dissection. **(B)** Quantification of total fascicle area (example indicated by dotted lines in **Ai**) between non-implanted neural tissue, taken proximal to implantation site, and implanted tissue adjacent to electrodes E1–E4. Data show mean fascicle area (μm^2) + SEM, and there were no significant differences between locations. Scale bar in **(A)** 500 μm Scale bar in **(Ai)** 100 μm.

The fascicle area (indicated by dotted lines, **Figure 8Ai**) of the nerve adjacent to the electrodes E1-E4 was analyzed and compared to non-implanted vagus nerve tissue (taken proximal to the implantation site). There were no significant changes in fascicle area between implanted (E1–E4) and non-implanted (proximal) tissue (one-way RM ANOVA: $P = 0.9$; $n = 9$) (**Figure 8B**).

DISCUSSION

VNS was effective in improving a number of markers of inflammation, including stool quality, systemic inflammation and leukocyte infiltration within the small intestine. Furthermore, significant off-target cardiac and respiratory events occurred during suprathreshold cervical VNS, while in contrast, no measured off-target changes were seen during abdominal VNS. The absence of off-target effects and efficacy in reducing inflammation suggests that abdominal VNS is a suitable alternative to cervical VNS. Taken together, these findings support the use of this novel peripheral nerve array for abdominal VNS as a potential treatment for IBDs, such as Crohn's disease.

Stimulation of the cervical vagus nerve at higher frequencies (30 Hz) or lower frequencies (10 Hz) has the potential to activate fibers to the larynx, heart and lungs, in addition to the gastrointestinal tract and liver (Bonaz et al., 2013). In this study, cervical VNS (10 Hz, 1.6 mA, 200 μs) caused a decrease in heart rate and respiration. These changes can be attributed to the activation of large pulmonary stretch A-fibers and cardio-inhibitory vagal B fibers (McAllen et al., 2018). In a retrospective clinical study, a significant proportion of epileptic patients ($n = 95$) receiving cervical VNS (30 Hz, 0.25–3.5 mA, 500 μs) treatment reported stimulation-induced hoarseness of voice (63%), coughing (44%), and pain (37%) (Handforth et al., 1998; DeGiorgio et al., 2000). Similarly, ileocecal Crohn's disease patients treated with cervical VNS (10 Hz, 500 μs, 1.25 mA) also reported dysphonia during stimulation, although symptoms resolved over time (Bonaz et al., 2016). In humans, the effects on heart rate are less pronounced than that reported in rats, nevertheless, some clinical studies show disruptive effects to heart contractility and heart rate during stimulation of the left cervical and thoracic vagus nerve, i.e., above the vagal cardiac branches (Frei and Osorio, 2001; Lewis et al., 2001). Taken together, cervical VNS has a number of unwanted, albeit often mild, off-target effects. Such off-target effects potentially limit the intensity and duration of therapeutic stimulation delivered, thereby potentially compromising or limiting the effectiveness of the bioelectric neuromodulation treatment. In contrast abdominal VNS did not evoke changes in heart rate, respiration rate or blood pressure. These findings are in agreement with previous reports of abdominal VNS in anaesthetized pigs and humans for the treatment of post-operative ileus (Stakenborg et al., 2017), and is consistent with the known functional anatomy of the vagus.

Engaging the optimal nerve fiber population is essential for an effective bioelectric neuromodulation therapy (Payne et al., 2018b). The cervical vagus nerve consists of a mixed population of large, myelinated A-fibers, smaller myelinated B-fibers and small, unmyelinated C-fibers, which have different electrical activation thresholds (Castoro et al., 2011). This is problematic for cervical VNS for the treatment of intestinal

inflammation as activation of vagal C-fibers, thought to be involved in driving anti-inflammatory effects in the intestine (Martelli et al., 2014; Payne et al., 2018b), typically also activates A-fibers (Castoro et al., 2011; McAllen et al., 2018). Selective activation of C-fibers, while minimizing the activation of A- and B-fiber populations is very difficult to achieve long-term *in vivo* (reviewed in Guiraud et al., 2016). Some studies have demonstrated a partial preferential activation of C-fibers while suppressing A-fibers activation in the vagus nerve of pigs by delivering a non-rectangular pulse waveform (Tosato et al., 2007). However, this stimulation strategy requires high, often unsafe levels of charge to be delivered in order to be effective (Vuckovic et al., 2008; Qing et al., 2015). Targeting the abdominal vagus nerve overcomes this issue as the nerve consists primarily of C-fibers (rats: 99%; humans: 97%) (Hoffman and Schnitzlein, 1961; Prechtl and Powley, 1990). Furthermore, targeting this C-fiber dense segment of the vagus nerve might increase the clinical efficacy of stimulation in electrically-activating C-fibers. We measured the conduction velocity of the electrically-evoked neural responses elicited by abdominal VNS and they ranged between 0.55 and 0.84 m/s, which fits within the range of C-fiber conduction velocities (Castoro et al., 2011).

Abdominal VNS was effective in reducing TNBS-induced inflammation, as indicated by changes in several markers, including the stool quality, systemic CRP, histology and leukocyte infiltration into transmural layers. In contrast, previous studies on experimental colitis showed cervical VNS had no protective effects within the inflammatory lesion site (Meregnani et al., 2011), and all measured inflammatory markers of colitis (mucosal index damage index, disease activity index, histological score and colonic cytokine content) remained significantly higher in tissue taken from cervical VNS rats, compared to control ($P \geq 0.05$) (Sun et al., 2013). One explanation for this disparity is the differences in TNBS injections between studies. The concentration of TNBS administered here was 2.5% w/v, while others used higher concentrations of 40% (Sun et al., 2013) and ∼180% (Meregnani et al., 2011). Additionally, we injected TNBS into the small intestine, rather than the colon. Vagal innervation of the small intestine, specifically the jejunum, is denser than in the distal colon (Berthoud et al., 1990); therefore the therapeutic action of VNS may have had an increased potency.

The stimulation regime used in this study involved a charge density of ∼1 $\mu C/cm^2$/phase, delivered for 3 h at the same time each day (1:30–4:30 p.m.), with a duty cycle of 30 s ON, followed by 5 min OFF. This stimulation is well below the safety limit for platinum electrodes (Cogan et al., 2016). The stimulation timing and duty cycle were chosen to be similar to those used in the treatment of colitis (Sun et al., 2013) and ileocecal Crohn's disease (Bonaz et al., 2016), which in turn were based on VNS to treat patients with drug resistant epilepsy and depression (Sackeim et al., 2001; Cukiert et al., 2013). Therefore, it is quite likely that the timing and duty cycle used may not be optimal for the treatment of IBD; the optimization of stimulation parameters to those most appropriate for therapy has been poorly explored and remains a significant challenge for bioelectric neuromodulation therapies (Payne et al., 2018b). Testing of parameters, such as pulse durations, stimulation rate, duty cycles, and the duration and time of day that stimulation is applied, are essential to explore in appropriate animal models of the disease if bioelectric neuromodulation is to be successful long-term clinical treatment.

CONCLUSION

We have developed a stimulating and recording electrode array that effectively activates C-fibers in the abdominal vagus nerve, allowing for VNS to treat inflammation following TNBS injection to be applied closer to the end organ with no measurable off-target effects. Our work supports abdominal VNS as an effective approach to the treatment of IBD in humans.

ETHICS STATEMENT

All animal procedures were approved by the Animal Research and Ethics Committee of the Bionics Institute and complied with the Australian Code for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council of Australia). Approval was also obtained from the United States Army Medical Research and Material Command Animal Care and Use Review Office, protocol SSC-7486.02.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was funded by the Defense Advanced Research Projects Agency (DARPA) BTO, under the auspices of Dr. Doug Weber and Dr. Eric Van Gieson through the Space and Naval Warfare Systems Center (Contract No. N66001-15-2-4060). The Bionics Institute and the Florey Institute of Neuroscience and Mental Health acknowledge the support they receive from the Victorian Government through its Operational Infrastructural Support Program.

ACKNOWLEDGMENTS

We are grateful to Dr. Alex Thompson for his biomedical technical assistance and Ross Thomas for his assistance in designing the rat pedestals and Angel De Silva for manufacture of the electrode arrays. We also thank Dr. Martin J. Stebbing and Dr. Tyler Best for helpful comments on the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Neuroscience

REVIEW
published: 22 March 2021
doi: 10.3389/fnins.2021.650971

Therapeutic Potential of Vagus Nerve Stimulation for Inflammatory Bowel Diseases

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The vagus nerve is a mixed nerve, comprising 80% afferent fibers and 20% efferent fibers. It allows a bidirectional communication between the central nervous system and the digestive tract. It has a dual anti-inflammatory properties via activation of the hypothalamic pituitary adrenal axis, by its afferents, but also through a vago-vagal inflammatory reflex involving an afferent (vagal) and an efferent (vagal) arm, called the cholinergic anti-inflammatory pathway. Indeed, the release of acetylcholine at the end of its efferent fibers is able to inhibit the release of tumor necrosis factor (TNF) alpha by macrophages via an interneuron of the enteric nervous system synapsing between the efferent vagal endings and the macrophages and releasing acetylcholine. The vagus nerve also synapses with the splenic sympathetic nerve to inhibit the release of TNF-alpha by splenic macrophages. It can also activate the spinal sympathetic system after central integration of its afferents. This anti-TNF-alpha effect of the vagus nerve can be used in the treatment of chronic inflammatory bowel diseases, represented by Crohn's disease and ulcerative colitis where this cytokine plays a key role. Bioelectronic medicine, via vagus nerve stimulation, may have an interest in this non-drug therapeutic approach as an alternative to conventional anti-TNF-alpha drugs, which are not devoid of side effects feared by patients.

Keywords: cholinergic anti-inflammatory pathway, heart rate variability, inflammatory bowel diseases, TNF, vagus nerve, vagus nerve stimulation

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Autonomic Neuroscience, a section of the journal Frontiers in Neuroscience

Received: *08 January 2021*
Accepted: *01 March 2021*
Published: *22 March 2021*

Citation:
Bonaz B, Sinniger V and Pellissier S (2021) Therapeutic Potential of Vagus Nerve Stimulation for Inflammatory Bowel Diseases. Front. Neurosci. 15:650971. doi: 10.3389/fnins.2021.650971

INTRODUCTION

The vagus nerve, cited as the pneumogastric nerve or 10th cranial nerve, although referred in the singular is paired (right and left VN). It is the longest nerve in the body, extending from the

Abbreviations: ACh, acetylcholine; α7nAChR, alpha7 nicotinic ACh receptor; ACTH, adrenocorticotropic hormone; ANS, autonomic nervous system; CAN, central autonomic network; CAP, cholinergic anti-inflammatory pathway; CD, Crohn's disease; CNS, central nervous system; CRF, corticotrophin-releasing factor; CRP, C-reactive protein; DMNV, dorsal motor nucleus of the vagus; DSS, dextran sulfate sodium; HPA, hypothalamic pituitary adrenal; HRV, heart rate variability; IL, interleukin; IBD, inflammatory bowel diseases; LC, locus coeruleus; LPS, lipopolysaccharides; MDD, major depressive disorder; NE, norepinephrine; NTS, nucleus of the solitary tract; PVH, paraventricular nucleus of the hypothalamus; ta-VNS, transcutaneous auricular vagus nerve stimulation; TNBS, trinitrobenzensulfonic; TNF, tumor necrosis factor; UC, ulcerative colitis; VN, vagus nerve; VNS, vagus nerve stimulation.

medulla oblongata to the digestive tract. The VN is a mixed nerve containing afferent (sensory) and efferent (motor) nerve fibers. It ensures the innervation of many organs such as the pharynx, larynx, thoracic viscera (heart and lungs) and the digestive tract from the esophagus to the recto-colon. The VN is the main component of the cranial parasympathetic nervous system. The other parasympathetic component is represented by the sacral parasympathetic nucleus (S2–S4) at the origin of the pelvic nerves that provide innervation to the pelvic organs such as the bladder, genitals, and left recto-colon. These two components are part of the ANS (Langley, 1921) comprising the sympathetic and parasympathetic systems, which are classically antagonistic. Due to its mixed character, the VN ensures a bidirectional communication between the CNS and the viscera, in particular the digestive tract in the context of the brain–gut axis (Bonaz et al., 2017a). This reciprocal relationship ensures an integrated and coordinated functioning of digestive functions such as motility, sensitivity, secretion, permeability, immunity. The functioning of the digestive tract is most often unconscious (i.e., not perceived) but can, under certain conditions, become pathological (i.e., perceived as painful). Therapies targeting the VN, whether drugs, nutritional, complementary medicines, or using VN stimulation (VNS), known as Bioelectronic Medicine, could be used in the management of gastrointestinal disorders (Bonaz et al., 2017b). Bioelectronic medicine is based on neuromodulation of the nervous system restoring organ functions and health with less adverse effects than drugs, thus minimizing adherence issues (Olofsson and Tracey, 2017). In particular, due to its anti-inflammatory role, the VN could be used as a non-drug therapy in chronic IBD represented by CD and UC (Bonaz et al., 2016b). Indeed, the VN exerts a dual anti-inflammatory effect: both through its afferents, by stimulating the HPA axis and the release of glucocorticoids from the adrenal glands, and its efferents, through the CAP, more recently described.

FUNCTIONAL NEUROANATOMY OF THE VAGUS NERVE

The VN runs from the brainstem through the neck to many peripheral organs, including the lungs, heart, liver, stomach, intestines. The VN is a mixed nerve consisting of 80% afferent fibers, carrying information from the digestive tract to the CNS, and 20% efferent fibers involved in the control of gastrointestinal functions (Prechtl and Powley, 1990), as well as heart and lungs. Thus, the VN is a major component of the bidirectional communication between the brain and the gut through the brain–gut axis. We will now discuss only the GI functions of the VN.

Vagal Afferent Fibers

Vagal afferents inform the CNS, usually unconsciously, of the functional state of the gastrointestinal tract. These afferents originate from free endings in the different layers of the gut wall, including in the external muscle layers, myenteric plexus, and mucosal lamina propria and travel through the VN to the nucleus tractus solitarius (NTS) according to a viscerotopic distribution (Powley et al., 2019). The NTS, the main entry point of the digestive tract into the brain, is located in the medulla, just above the DMNV which is at the origin of vagal efferent fibers with the nucleus ambiguus (Jean, 1991). Thus, the NTS and the DMNV are closely connected. In fact, the dendrites of vagal motor neurons are connected with vagal afferents ending in the NTS, at the origin of vago-vagal reflex loops (**Figure 1**) (Travagli et al., 2006; Bonaz et al., 2019). Vagal afferent cell bodies are located in the nodose ganglia or jugular ganglia, at the base of the skull. Peripheral stimuli, via vagal afferents ending in the NTS, are transmitted to many regions of the CNS through projections of the NTS onto structures such as the parabrachial nucleus, an important sensory relay of the NTS, the hypothalamus, in particular the PVH, the limbic system including the amygdala, thalamus, hippocampus, and cerebral cortex including the insula and the prefrontal cortex (Norgren, 1978; Sawchenko, 1983; Cechetto, 1987). These different structures are part of the CAN described by Benarroch (1993). The CAN is at the origin of autonomic, behavioral, cognitive, and endocrine responses. It is capable of modulating the functioning of the ANS via descending pathways projecting onto sympathetic pre-ganglionic neurons in the spinal cord and onto the DMNV at the origin of vagal efferents. Vagal afferents are involved in detecting the presence of nutrients and their chemical composition in the digestive tract in the post-prandial period. They contain chemoreceptors, thermoreceptors, osmoreceptors, mechanoreceptors as opposed to afferent spinal fibers which essentially vehiculates pathways of visceral pain of digestive origin to the spinal cord (Berthoud and Neuhuber, 2000). Most of the nervous information coming from the viscera is not conscious but can become so in pathological conditions, particularly inflammatory. The VN is a major component of the pathways of interoception which is the sense of the body's internal physiological state (Craig, 2002), and interoceptive abnormalities are implicated in the pathophysiology of psychiatric disorders, neurodegenerative and neurological disorders, as well as in somatic disorders of brain-body interactions, including functional digestive disorders and IBD (Fournier et al., 2020; Bonaz et al., 2021).

Vagal Efferent Fibers

These fibers originate at the level of the medulla oblongata, from pre-ganglionic neurons located in the DMNV and travel through the VN toward the viscera synapsing with a second post-ganglionic neuron located in the target organ, namely the digestive wall. In the digestive tract, this second order neuron is an integral part of the enteric (or intrinsic) nervous system, a real "second brain" or "gut brain," able to ensure motor and secretory autonomy of the digestive tract (Furness et al., 2014). It is classically stated that the VN innervates the entire digestive tract up to the splenic angle (Netter, 1989). However, for others, it innervates the entire digestive tract in humans (Delmas and Laux, 1933) as well as in rats (Altschuler et al., 1993). The pelvic nerves classically innervate the left colon and the rectum as well as the bladder and genital organs. The sacral (S2–S4) parasympathetic nucleus is under the control of the Barrington's nucleus, also called the pontine micturition center (Valentino et al., 1999), which lies adjacent to, and interacts with the LC,

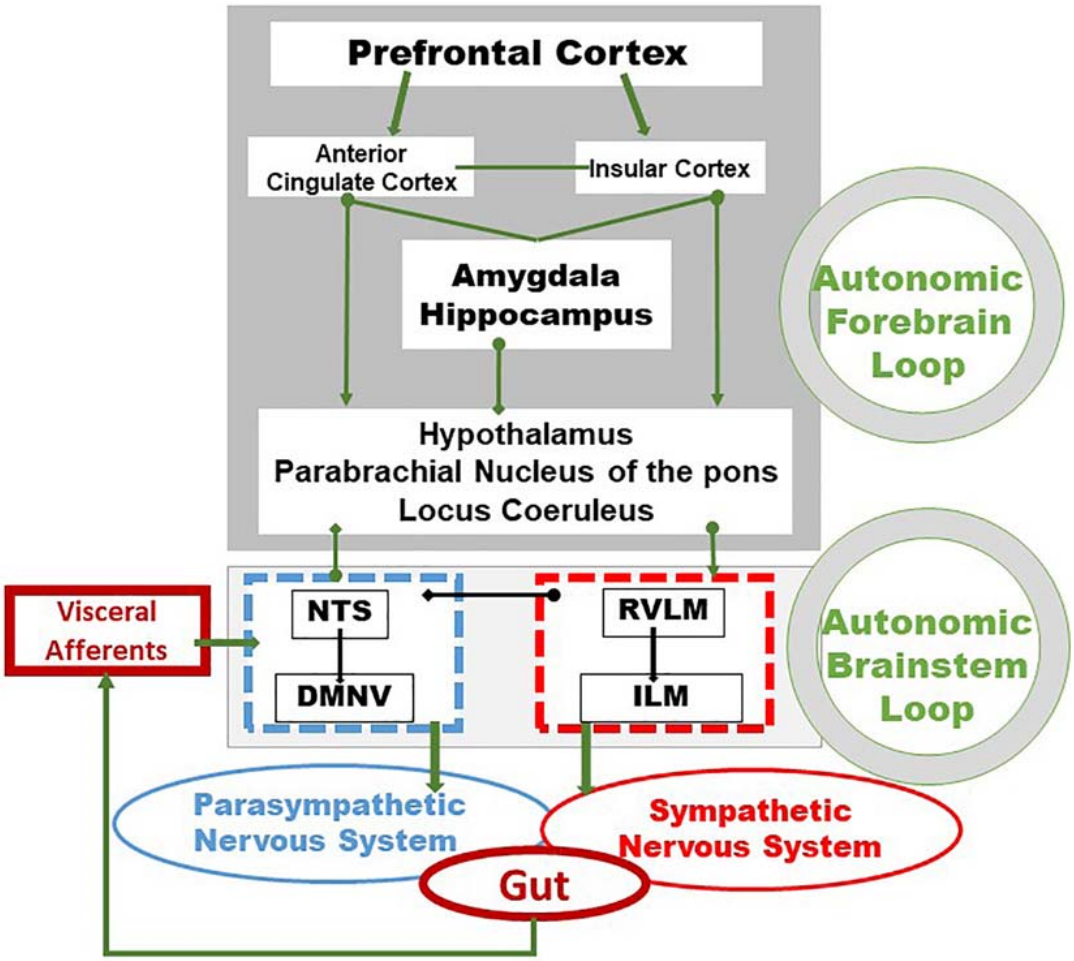

FIGURE 1 | Integrative pathways of the brain–gut axis. Vagal and splanchnic digestive afferents connect to the nucleus tractus solitarius, in close relationship with the dorsal motor nucleus of the vagus, from which vagal efferents originate, thus composing an autonomic loop of the brainstem, involved in the regulation of instinctual motility, acid secretion, food intake and satiety. This loop is modulated by the autonomic loop comprising the hypothalamus, the hippocampus, the amygdala, the anterior cingulate and the insular and prefrontal cortex. This last loop receives, coordinates and integrates visceral information enabling neuroendocrine, emotional, cognitive and behavioral responses. These two central loops explain how stress, sensations and thoughts can influence the functioning of the intestine and vice versa. Adapted from Bonaz et al. (2019). DMNV, dorsal motor nucleus of the vagus nerve; ILM, intermediolateralis nucleus; NTS, nucleus tractus solitarius; RVLM, rostral ventrolateral medulla.

the brain's principle noradrenergic projection center, involved in arousal, stress, autonomic function, cognition, and behavior (Benarroch, 2018). The neurotransmitter of the vagal and pelvic parasympathetic system is ACh for pre- and post-ganglionic neurons acting on nicotinic receptors at the pre-ganglionic level and muscarinic receptors at the post-ganglionic level.

Parasympathetic innervation of the gut is involved in the neuroimmune regulation of intestinal barrier through the recruitment of α7 nicotinic ACh receptor (α7nAChRs). It acts on enteroglial cells, interacting with innate immune cells (Fornai et al., 2018), myenteric neurons, making synaptic contacts with resident macrophage expressing α7nAChR. Vagal afferent fibers penetrate to the tips of jejunal villi and some of these nerves make intimate contact with intestinal mucosal mast cells. These data provide the microanatomical basis for direct neural communication between the CNS and mast cells in the gastrointestinal mucosa (Williams et al., 1997). Overall, the stimulation of vagal efferents could obviously "activate" these different gut cells and be one of the component of the CAP.

INFLAMMATORY BOWEL DISEASES

Inflammatory bowel diseases are organic diseases classically divided in CD and UC. CD can involve all the digestive tract, from the mouth to the anus, while UC involves the recto-colon only. IBD start early in life (between 15 and 30 years) and evolve by periods of flares alternating with periods of remission of variable duration (Chang, 2020). Symptoms are represented by abdominal pain, diarrhea, fever, weight loss, and extra-intestinal (skin, eyes, joints) manifestations. About 1.5 million Americans and 2.2 million people in Europe are

affected by IBD. There is a rising incidence of IBD in Western countries, supporting the hypothesis that "Westernization" of our lifestyle has led to the increased incidence and prevalence of IBD (Molodecky et al., 2012).

The pathophysiology of IBD is multifactorial involving genetic, immunologic, infectious and environmental factors (Chang, 2020). The theory is that genetically susceptible individuals experience an environmental trigger(s), resulting in an inappropriate immune response, potentially against gut microbes. Stress, through brain–gut interactions, as well as environmental factors, based on experimental and clinical data (Bonaz and Bernstein, 2013), has been proposed as a contributor. An imbalance of the ANS is observed in IBD, represented by a sympathetic dysfunction in CD (Lindgren et al., 1991) and a vagal dysfunction in UC (Lindgren et al., 1993). We have recently reported that this dysautonomia may be dependent on psychological adjustment in IBD. Indeed, the equilibrium of the ANS is differentially adapted according to the disease. This equilibrium is conjugated with positive affective and cognitive adjustment in IBD (Pellissier et al., 2010). Presently, there is no treatment to cure IBD. Current treatments suppress disease activity and there is generally a relapse of the disease after discontinuation of the treatment. TNF is a key cytokine that is involved in IBD and anti-TNF therapies have transformed the management of IBD (Peyrin-Biroulet, 2010). New compounds targeting other pro-inflammatory cytokines, such as IL-12, IL-23, anti-integrin therapies, and anti-Janus kinase (JAK) are available (Pagnini et al., 2019; D'Amico et al., 2020). Surgery is an alternative to a failure of treatment or a complication of IBD (perforation, abscess, stenosis) but the disease recurs after surgery. Anti-TNF therapies used in IBD have been shown to be effective but there is a 20–30% primary non-response rate (Ford et al., 2011) and the annual risk of loss of response to anti-TNF is 13% per patient-year for Infliximab (Gisbert and Panes, 2009) and 20% per patient-year for Adalimumab (Billioud et al., 2011). This secondary non-response is due to (i) the development of autoantibodies, in particular for Infliximab which is a chimeric molecule (75% human and 25% mouse), but also for Adalimumab (100% human) to a lesser degree, or (ii) a secondary failure of a well-dosed or under-dosed treatment using therapeutic drug monitoring (Ben-Horin and Chowers, 2011; Chaparro et al., 2012). Anti-TNF treatment currently represents the bulk of the cost of IBD treatment (van der Valk et al., 2014). Indeed, the median cost of a 1-year anti-TNF therapy raises up to $40,000 for CD patients (Targownik et al., 2019). In addition, biological therapies are not devoid of numerous side effects with a major impact on the patient's quality of life (Kerbleski and Gottlieb, 2009; Pereira et al., 2015; Murdaca et al., 2016). As a result, by fear of these side effects and the need for chronic treatment of these pathologies, patients are increasingly reluctant to take these treatments and to continue them once they are in remission. This explains, in particular, the 30–50% of non-adherence (Chan et al., 2017) and the growing interest of the patients for complementary medicines (Torres et al., 2019).

Consequently, a treatment targeting pro-inflammatory cytokines such as TNF-alpha and others, exploiting the CAP, with few side effects, devoid of problem of compliance, and cheaper than biologicals (i.e., anti-TNF-alpha) would be of great value. In this context, targeting the anti-inflammatory properties of the VN would be of interest (**Figure 2**). In particular, VNS, as a non-drug therapy could serve as an alternative to classical biological therapies. We have shown recently that there is a specific homeostatic link between vagal tone and TNF-alpha in CD patients since a low vagal tone was associated with a high level of TNF in the plasma (Pellissier et al., 2014). In addition, since stress is classically known to stimulate the sympathetic nervous system, which has a pro-inflammatory effect, and to inhibit the VN (Wood and Woods, 2007), and thus the CAP, VNS may help to restore an equilibrium of the sympatho-vagal balance.

ANTI-INFLAMMATORY PROPERTIES OF THE VAGUS NERVE

The VN has a double anti-inflammatory effect both via its afferents and efferents (Bonaz and Bernstein, 2013; Bonaz et al., 2017a).

Anti-inflammatory Properties of Vagal Afferents

The VN is a key element of the neuro-endocrine immune axis whose purpose is to ensure a homeostasis balance. The peripheral release of pro-inflammatory cytokines such as IL-1beta, IL-6, and TNF, activates vagal afferents via their interaction with receptors on the para-nodes (Goehler et al., 1997) (**Figure 2**). Zanos et al. (2018) have very recently shown that electrical signals recorded on the cervical VN can be decoded to discriminate between cytokine-specific signals. In animals, the induction of a septic shock by systemic or intraperitoneal injection of LPS, components of the wall of Gram-negative bacteria, leads to the release of these cytokines by blood and/or tissue macrophages, which in turn activates vagal afferents leading to fever, sleep and aphagia (Dantzer et al., 1998). This signal activates the HPA axis after being integrated at the level of the NTS and then transmitted to the hypothalamus via projections to the PVH, more particularly on CRF neurons, the main neuro-mediator of stress, located in the parvocellular zone of the PVH (Rivest et al., 2000) (**Figure 2**). These CRF neurons project themselves into the pituitary gland, whose activation will release ACTH that will stimulate the release of glucocorticoids (cortisol) by the adrenal glands with well-known anti-inflammatory properties. Most of neuromodulation studies focus on vagal regulation of inflammation via the peripheral efferent pathway toward the viscera. However, abdominal vagal afferent neurostimulation suppresses systemic inflammation in rats, and the efferent neural pathway for this action is in the splanchnic sympathetic nerves (Komegae et al., 2018). Vagal stimulation also modulates arthritic joint inflammation through an afferent pathway mediated by the LC where central stimulation is followed by activation of joint sympathetic nerve terminals releasing NE. The vagal control of arthritic joint inflammation is dampened by selective adrenergic beta-blockers. These results reveals a novel neuro-immune brain map with afferent vagal signals controlling side-specific articular

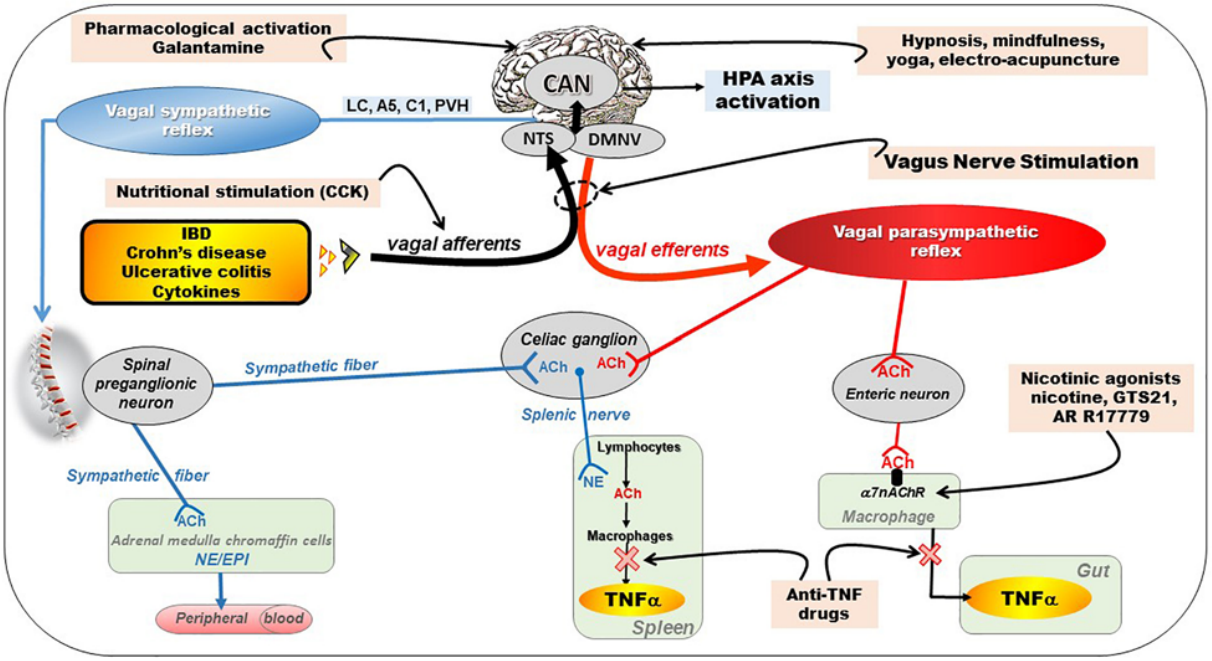

FIGURE 2 | Different pathways of the anti-inflammatory properties of the vagus nerve: (1) through activation of the HPA axis via vagal afferents and through vago-parasympathetic efferents (red), (2) through sympathetic efferents (blue) arising from thoraco-lumbar spinal preganglionic neurons through the vago-sympathetic pathway where vagal afferents activate central descending pathways (e.g., LC, A5, C1, PVH) targeting spinal pre-ganglionic neurons. Targeting the VN for its anti-inflammatory properties (pink) in chronic inflammatory diseases (orange) such as inflammatory bowel diseases appears as potentially effective therapeutics. Adapted from Bonaz et al. (2017a). A5, A5 noradrenergic group (in the brainstem); ACh, acetylcholine; C1, C1 adrenergic group (in the brainstem); CAN, central autonomic network; CCK, cholecystokinin; DMNV, dorsal motor nucleus of the vagus nerve; EPI, epinephrine; HPA, hypothalamic–pituitary–adrenal; IBD, inflammatory bowel diseases; LC, locus coeruleus; NE, norepinephrine; NTS, nucleus tractus solitarius; PVH, paraventricular nucleus of the hypothalamus; TNFα, tumor necrosis factor-alpha; α7nAChR, alpha7nicotinic acetylcholine receptor.

inflammation through specific inflammatory-processing brain centers and joint sympathetic innervations (Bassi et al., 2017).

Anti-inflammatory Properties of Vagal Efferents

In 2000, Kevin Tracey's team described, for the first time, the CAP (Borovikova et al., 2000; Pavlov et al., 2003). This group showed that a septic shock in rats, induced by iv injection of LPS, was prevented by low frequency neurostimulation of the distal end cut of the VN thus stimulating vagal efferents (Borovikova et al., 2000). The authors concluded that there was an inflammatory reflex in which stimulation of vagal afferents by pro-inflammatory cytokines resulted in activation of vagal efferents that inhibited the release of these cytokines by tissue macrophages, in particular TNF but also other proinflammatory cytokines such as IL6, IL1β but not the anti-inflammatory cytokine IL-10. The VN has therefore anti-inflammatory properties through the inhibition of pro-inflammatory cytokines (**Figure 2**). This group also characterized the cholinergic receptor of macrophages involved in this effect, which was not muscarinic but α7nAChR. Indeed, the effect of VNS was abolished in animals knock out for this receptor (Wang et al., 2003). Intracellular mechanisms downstream of α7nAChR activate the JAK2–signal transducer and activator of transcription 3 pathway, sequester Nuclear Factor-kB (NF-κB), and inhibit activation of the inflammasome (Guarini et al., 2003; de Jonge et al., 2005; Lu et al., 2014). This anti-TNF effect of the VN has, of course, therapeutic applications in pathologies where this cytokine is involved such as IBD. However, this effect of the VN is not direct via vagal efferent endings on macrophages but indirect through the interaction of the VN with nNOS, VIP and ChAT enteric neurons located within the gut muscularis with nerve endings in close proximity of the resident macrophages (Cailotto et al., 2014). Tracey's group also showed that the spleen, an important source of ACh, where it was first isolated, and TNF in the body, was also involved in the anti-inflammatory effect of the VN. This effect involves a connection between vagal efferent endings and the spleen (Rosas-Ballina et al., 2008), through the celiac sympathetic ganglion, inducing the release of NE by noradrenergic endings in the spleen (**Figure 2**). They recently showed that cholinergic neurons in the DMNV, which project to the celiac superior mesenteric ganglia, significantly increase splenic nerve activity and inhibit TNF production (Kressel et al., 2020). So there is a vago-sympathetic excitatory pathway while the VN and the sympathetic nervous system have generally antagonistic effects. Indeed, NE acts on beta2 receptors of splenic lymphocytes to release ACh by these lymphocytes: this is the non-neuronal cholinergic pathway by opposition to the neuronal (i.e., vagal) cholinergic pathway (**Figure 2**). ACh

then binds to α7nAChR of splenic macrophages to inhibit the release of TNF by these macrophages (Olofsson et al., 2012). These activated T lymphocytes do not just inhibit macrophages locally. They may also move and behave like "mobile neurons" and downregulate inflammation in areas not innervated by the VN. The characteristics of these CHAT-containing T cells have been well outlined by the Tracey's group (Rosas-Ballina et al., 2011; Olofsson et al., 2016). However, the innervation of the spleen by the VN via this interaction with the splenic nerve is questioned by some authors, notably by the work of Martelli et al. (2014). For this author, the efferent anti-inflammatory pathway is not the VN but the sympathetic system, notably the splanchnic sympathetic nerve and its anti-inflammatory effect is distributed across abdominal organs (Martelli et al., 2019). The sympathetic nervous system originates in the thoracolumbar spinal pre-ganglionic neurons (T1-L2) and synapses with post-ganglionic neurons at the origin of splanchnic sympathetic nerves distributed to the viscera, with a mirror distribution of the parasympathetic system (**Figure 2**). These pre-ganglionic spinal sympathetic neurons are under the control of central nuclei such as the PVH, Kölliker-Fuse nucleus, pontine noradrenergic groups A5, the LC (A6), the chemosensitive region of the ventral medulla, and possibly the region of the A1 catecholamine cell group (Loewy, 1981). These neural groups are part of the CAN and send descending projections to modulate these pre-ganglionic sympathetic neurons. In particular, Abe et al. (2017) showed, in an experimental model of renal inflammation, that C1 was involved in an anti-inflammatory reflex involving only the splanchnic nerve.

In fact, contrasting the vagal anti-inflammatory pathway with the splanchnic pathway is a reductive view since both pathways can act in concert to play a pivotal role in the crosstalk with the immune system, a fortiori if they are activated by VNS (Brinkman et al., 2019). In particular, because of its mixed, essentially afferent, character, the VN via its central projections on the NTS and then on the CAN is able to activate A5, A6, and C1 which will secondarily activate the splanchnic sympathetic nerves via their descending projections to the spinal cord. Our group has shown in particular that low-frequency (5Hz) VNS in rats, supposed to activate only vagal efferents, also activated vagal afferents (Reyt et al., 2010). Lomarev et al. (2002) have also shown that, compared to 5 Hz, 20 Hz VNS produced more acute activity changes from rest in regions similar to our initial VNS synchronized fMRI feasibility study in depression. There is therefore a bidirectional activation of vagal fibers by VNS, even at low frequency, involving a peripheral vagal and sympathetic action via a vago-vagal and vago-sympathetic reflex through the CNS (Bonaz et al., 2019).

THE VAGUS NERVE AT THE INTERFACE OF THE MICROBIOTA–GUT–BRAIN AXIS

The human intestine contains 10^{13} to 10^{14} microorganisms, which is much more than the cells in our body and 100 times more genes than our genome. The weight of the microbiota is about 1 kg in adults, approximately the weight of the human brain. In healthy subjects, two species of bacteria, Bacteroides and Firmicutes, dominate the bacterial composition, with smaller amounts of actinobacteria, proteobacteria and verrucomicrobia. At the species level, each individual presents a very specific signature. In addition to bacteria, the intestinal microbiota contains methanogenic archaea, eukaryotes (mainly yeasts) and numerous phages (Eckburg et al., 2005; Reyes et al., 2010). Recently, it has been shown that the microbiota, the gut, and the brain communicate via the microbiota–gut–brain axis (Cryan et al., 2019) and that a disruption of this axis is involved in the pathophysiology of neurodegenerative diseases (Cryan and Dinan, 2012; Forsythe et al., 2016). A dysbiosis is observed in various pathological conditions of the gastrointestinal tract, such as IBD (Sundin et al., 2017), and fecal transplantation is presently under investigation in clinical trials (Levy and Allegretti, 2019). However, the question is whether it is a cause or consequence of an abnormal communication between the gut and the brain. The VN is a key component of this microbiota–gut–brain axis (Bonaz et al., 2018) (**Figure 3**). Indeed, it is able to detect metabolites of the microbiota through its afferents and to transfer this intestinal information to the CAN, then generating an adapted or inappropriate response from the CAN to the intestine and the microbiota (Bonaz et al., 2018). It can also be activated indirectly by the microbiota via the interaction of the microbiota with digestive endocrine cells that will release serotonin acting on 5-HT3 receptors of vagal afferents. The VN, via the CAP, could modulate the intestinal microbiota by decreasing intestinal permeability and modulating local immunity (Bonaz et al., 2018). One can imagine that, an indicator such as HRV, easy to assess through the detection of heartbeat intervals, could be used to explore the microbiota–gut–brain axis homeostasis. Indeed, HRV is probably not a direct index of "the true" vagal tone (Marmerstein et al., 2021) since it is an indirect assessment of the parasympathetic modulation on the heart so that the metrics of the HRV would rather reflect different aspects of the neurophysiologic regulation of the heart rhythm (Reyes del Paso et al., 2013; Thomas et al., 2019). Presently, HRV is the final output of several regulatory loops resulting from afferent signals integrated at the level of the CNS influencing the efferent vagally mediated modulations on the heart (Thayer and Lane, 2009). In that way, HRV can be used to mark and follow the activity of the neurophysiological pathways involved in adaptation, homeostasis and health (Thayer et al., 2012). A recent meta-analysis underlines that HRV metrics such as the high frequency band (HF-HRV) and the standard deviation of RR intervals (SDNN) are the most robustly associated with inflammation (Williams et al., 2019). Finally, targeting the VN through VNS, even if the mechanism is not clear yet, could have therapeutic implications in the modulation of the microbiota–gut–brain axis.

STRESS AND THE VAGUS NERVE

Besides its well-known effects on gastrointestinal motility (Tache et al., 2001), stress increases intestinal permeability, modifies the intestinal flora, and intestinal immunity (Tache and Bonaz,

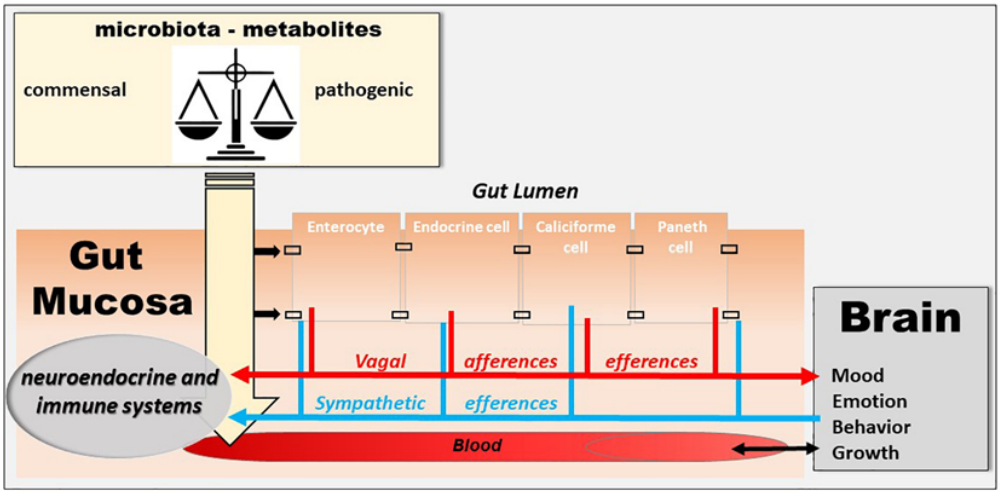

FIGURE 3 | The microbiota–gut–brain axis. The microbiota exerts an effect on the gut-brain axis, impacting the biochemistry of the peripheral and central nervous system. Commensal and/or pathogenic bacteria and their metabolites are translocated across the intestinal barrier and act on both the digestive immune system and vagal afferents. Similarly, the brain acts on the various organs, including the digestive tract, and can thus regulate the survival and proliferation of the intestinal microbiota.

2007; Larauche et al., 2009a,b). If the role of stress is well known in functional digestive disorders such as irritable bowel syndrome (Pellissier and Bonaz, 2017), recent data have shown its role in IBD (Bonaz and Bernstein, 2013). Furthermore, stress modifies the activity of the ANS. Classically, stress leads to vagal inhibition and activation of the sympathetic system, thus favoring the body's ability to cope with a "threat to its homeostasis" and in fact strengthens the defenses through the pro-inflammatory reaction (Tache and Bonaz, 2007). Any vagal hypotonia with or without sympathetic hypertonia can therefore promote an inflammatory process. In patients at the pre-rheumatoid arthritis stage, those who had vagal hypotonia developed rheumatoid arthritis more easily afterward (Roura et al., 2016). Moreover, several arguments are in favor with such a causal association in IBD. First, a low level of HRV as indexed by the RMSSD metric, at onset of newly diagnosed UC patients predicts the systemic inflammatory response over 3 years of follow-up (Gunterberg et al., 2016). Second, there is a positive association between vagotomy and later IBD, and this particularly for CD revealing the role of VN integrity in the prevention of IBD (Liu et al., 2020). Thus, we can assume that the level of vagal tone is predictive of the development of an inflammatory disorder in people at risk. We also showed in patients with CD in remission, that a low resting vagal tone correlated with elevated circulating TNF (Pellissier et al., 2014) (**Figure 4**). The systemic inflammation observed during IBD or other chronic inflammatory pathologies is capable of leading to vagal hypotonia, which in turn maintains this inflammatory state. Moreover, chronic inflammation, by its central impact, can lead to a depressive state which itself can promote an inflammatory flare-up of the disease (Mikocka-Walus et al., 2016a,b; Bullmore, 2018). Two meta-analysis have shown that the levels of proinflammatory cytokines, such as TNF, IL-6, IL-1, and CRP, increase during depressive episodes (Hiles et al., 2012; Kohler et al., 2017). Chronic infection or stress may inhibit the negative feedback of the HPA axis, triggers the activation of microglia in the brain, and increases the permeability of the blood–brain barrier, resulting in excessive activation of proinflammatory cytokines (Song and Wang, 2011). Increased proinflammatory cytokines may cause MDD by activating the HPA axis, which results in a depletion of serotonin with an increased activity of the indoleamine-2,3-dioxygenase enzyme in the tryptophan–kynurenine system (Schiepers et al., 2005). Anti-inflammatory agents improve depressive symptoms compared to placebo as add-on in MDD patients and as monotherapy (Köhler-Forsberg et al., 2019). There is a link between depression and inflammation as a vicious circle: depression promotes inflammation and vice-versa. Depression increases the risk of IBD, which may be mitigated by the use of anti-depressants in the treatment of depression (Frolkis et al., 2019). Recently, it has been shown that individuals with IBD have a higher prevalence of depression than matched controls as early as 9 years before diagnosis (Blackwell et al., 2020). Depression in the absence of prior gastrointestinal symptoms was not associated with a future diagnosis of IBD but those patients with depression diagnosed after already experiencing gastrointestinal symptoms are at increased risk of later being diagnosed with IBD. The excess prevalence of depression prior to a diagnosis of IBD may be a consequence of diagnostic delay and untreated gastrointestinal symptoms. Antidepressant drugs have also shown a definite interest in the management of IBD (Macer et al., 2017). Therefore, any therapy, whether drug-based or not such as VNS, capable of restoring vagal activity, has a potential interest in IBD, but also in other chronic non-digestive inflammatory pathologies such as depression. There is a reduced HRV in depression with a high risk of cardiovascular disease (Sgoifo et al., 2015). Recently, it has been shown that transcranial direct current stimulation as an adjunct therapy is effective in alleviating unipolar and bipolar depression.

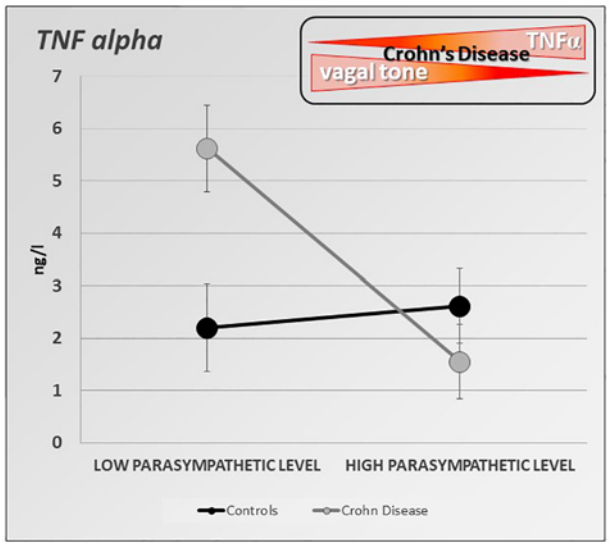

FIGURE 4 | Specific inverse relationship between the resting parasympathetic vagal tone and TNF-alpha plasma level in Crohn's disease (CD) patients. CD patients with high parasympathetic vagal tone exhibit a lower level of TNF-alpha than those with low parasympathetic vagal tone. Parasympathetic vagal tone was assessed by power spectral analysis of HRV and TNF-alpha level was assessed by ELISA-based technic. Data expressed as mean ± sem (adapted from Pellissier et al., 2014).

Moreover, the amplitude of the increase in parasympathetic signaling, as indexed by the RR interval lengthening, during the first session, predicts the clinical response after 10-sessions (Lin et al., 2021).

HOW TO USE THE ANTI-INFLAMMATORY PROPERTIES OF THE VAGUS NERVE FOR THERAPEUTIC PURPOSES?

Targeting the VN for anti-inflammatory purposes can be done in different ways (Bonaz and Bernstein, 2013; Bonaz et al., 2017a) (**Figure 2**).

Pharmacological Stimulation of Alpha7 Nicotinic Receptors

Pharmacologic stimulation can be performed with α7nAChR agonists such as GTS-21, AR-R17779 which have been used in post-operative ileus models, following intestinal macrophagic activation at the origin of ileus, as well as in experimental pancreatitis (van Westerloo et al., 2006; The et al., 2007). GTS-21, is able to inhibit proinflammatory cytokines *in vitro* and *in vivo* and improve survival in murine endotoxemia and severe sepsis (Pavlov et al., 2007). Hyperoxia-induced acute lung injury is attenuated by GTS-21 by inhibiting extracellular high mobility group box 1-mediated inflammatory responses (Sitapara et al., 2020).

Nutritional Stimulation

Cholecystokinin, a satiety hormone released at the duodenal level by I cells by the arrival of fat during the meal, acts on vagal afferents receptors. Activation of these receptors by cholecystokinin stimulates vagal afferents and an inflammatory reflex via activation of the CAP. This has been demonstrated in a model of hemorrhagic shock resulting in systemic release of proinflammatory cytokines such as TNF and IL-6 and inducing intestinal permeability through a loss of intestinal barrier function. The ingestion of high-fat enteral nutrition inhibited the release of these cytokines and reduced intestinal permeability. This effect was prevented either by vagotomy or by antagonists targeting cholecystokinin receptors or α7nAChR (Luyer et al., 2005).

Fasting

Fasting has a well-known anti-inflammatory effect, especially in IBD. This effect could be conveyed by ghrelin, an orexigenic peptide released during fasting by P/D1 cells of the gastric fundus, considered to be a leptin antagonist and also known for its gastric pro-kinetic properties (Mao et al., 2015). Plasma ghrelin levels increase during fasting and decrease after ingesting glucose and lipid, but not protein. The efferent VN contributes to the fasting-induced increase in ghrelin secretion. Ghrelin secreted by the stomach stimulates vagal afferents and promotes food intake (Nonogaki, 2008). As an illustration, mice invalidated for ghrelin have a suppression of the CAP demonstrated by a reduction in VN activity and an increase in plasma levels of the pro-inflammatory cytokines IL-1beta and IL-6. This effect is prevented by the administration of ghrelin or nicotine, which activate this anti-inflammatory pathway (Mao et al., 2015). Ghrelin stimulates the VN since vagotomy dampens its effects (Zhou et al., 2020). Ghrelin protects animals from renal ischemia-reperfusion injury through the VN (Rajan et al., 2012).

Stimulation of Central Cholinergic Pathways

Galantamine, a selective acetylcholinesterase inhibitor which has shown potential for the treatment of Alzheimer's disease, is able to cross the blood–brain barrier, after peripheral administration (Metz and Pavlov, 2020). Galantamine stimulates the central cholinergic system, activates the VN and inhibits the peripheral release of TNF during endotoxic shock in animals. This effect is prevented by a centrally acting muscarinic antagonist, or in α7nAChR knockout mice (Pavlov et al., 2009). These findings show that inhibition of brain acetylcholinesterase suppresses systemic inflammation through a central muscarinic receptor-mediated and vagal- and α7nAChR-dependent mechanism. Galantamine also improves experimental colitis in mice; this effect is suppressed by vagotomy, splenectomy or splenic denervation (Ji et al., 2014). Using forebrain-selective genetic ablation of ACh release and vagotomy, selective stimulation of ACh action on the M1 muscarinic ACh receptor (M1 mAChR), as well as selective optogenetic stimulation of basal forebrain cholinergic neurons innervating brain regions with abundant M1 mAChR localization, Lehner et al. (2019) have

shown that forebrain cholinergic neurons regulate innate immune responses and inflammation. Thus, targeting forebrain cholinergic signaling should be of interest in cholinergic dysfunction diseases.

Physical Activity

Exercise reduces systemic inflammatory activity (Heffernan et al., 2009). Regular physical activity reduces the risk of developing chronic diseases involving inflammation. Its mechanism of action is not well known but it may involve the CAP, as high levels of physical activity are associated with increased vagal tone and low levels of CRP, a systemic inflammatory marker (Lujan and DiCarlo, 2013). Physical activity therefore has a potential anti-inflammatory effect in inflammatory pathologies whether used in isolation or in combination with treatment. Physical activity, by enhancing parasympathetic tone and activating the CAP, is a therapeutic strategy to restrain chronic inflammation and prevent many chronic diseases (Lujan and DiCarlo, 2013). The anti-inflammatory effect of physical activity has also been associated with an enhancement of the sympathetic output (Weippert et al., 2013).

Complementary Techniques

Hypnosis stimulates the VN as shown in the study of HRV under hypnosis; HRV decreased during hypnosis but increased post-hypnosis (Yuksel et al., 2013). The efficacy of hypnosis is well known in patients with irritable bowel syndrome and some data are available in IBD, where its use is believed to improve patients with UC (Mawdsley et al., 2008) and prolong clinical remission (Keefer et al., 2013). *Mindfulness meditation ("mindfulness")* is able of activating the VN and may have anti-inflammatory as well as anti-stress properties by increasing HRV (Azam et al., 2015; Lumma et al., 2015). There are possible effects of mindfulness meditation on specific markers of inflammation, cell-mediated immunity, and biological aging, but these results are tentative and require further replication (Black and Slavich, 2016). Regular practice of *yoga* also increases vagal tone (Tyagi and Cohen, 2016). In a model of chronic obstructive pulmonary disease, *electro-acupuncture* can reduce the lung inflammatory response and improve lung function in this model, which may be related to its involvement in the regulation of the CAP (Zhang et al., 2018).

Vagus Nerve Stimulation for Anti-inflammatory Purposes in Chronic Inflammatory Bowel Diseases

Vagus nerve stimulation is a new therapeutic pathway for TNF-mediated chronic inflammatory diseases (Bonaz et al., 2016a,b) within the framework of Bioelectronic Medicine, whose goal is to use miniaturized stimulators to administer electrical nerve signals for non-drug therapeutic purposes (Olofsson and Tracey, 2017). VNS is already approved for the treatment of drug-refractory epilepsy and depression (Bonaz et al., 2013).

Experimental Data

The first data on the anti-inflammatory effect of the VN during digestive inflammation was reported by Miceli and Jacobson (2003). They showed that prior administration of anticholinesterase drugs such as neostigmine, which does not cross the blood–brain barrier, or physostigmine, which does, improved an experimental 2,4,6-trinitrobenzene sulfonic acid (TNBS)-colitis in rats in a model mimicking CD. This effect was more convincing for physostigmine, thus in favor of a predominant central mechanism. Vagotomy aggravated experimental colitis in mice, which is in favor of the protective role of the VN (Ghia et al., 2007). However, intestine-specific vagal nerve denervation had no effect in DSS-induced colitis (Willemze et al., 2018). Our group showed, for the first time, in the non-vagotomized vigilant rat, that low frequency (5 Hz) VNS, 3 h per day for five consecutive days, resulted in an improvement of TNBS-colitis in rats (Meregnani et al., 2011). VNS 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 a multivariate index of colitis (including body weight, temperature and locomotor activity, macroscopic area of lesions, histological, and biological parameters such as myeloperoxidase activity, cytokine and cytokine-related mRNAs). We have also shown that low-frequency stimulation (5 Hz), supposed to stimulate vagal efferents, also stimulated vagal afferents, as demonstrated in a brain imaging (MRI) study in rats where low-frequency neurostimulation led to deactivation in the NTS, the gateway to vagal afferents, and its projection sites (Reyt et al., 2010). Low-frequency VNS therefore stimulates both vagal efferents and afferents, which implies that it stimulates the two anti-inflammatory pathways of the VN: the CAP and the HPA axis, and also probably the anti-inflammatory sympathetic pathway (Martelli et al., 2016; Komegae et al., 2018). Our data were confirmed later by the study of Sun et al. (2013) who also performed chronic VNS *in vivo* in rats with TNBS-colitis, as well as LPS-induced inflammatory response in human epithelial colorectal adenocarcinoma cells (Caco-2) by ACh *in vitro*. They showed that clinical activity index, histological scores, biological inflammation using myeloperoxydase activity, iNOS, TNF and IL-6 levels were significantly decreased by chronic VNS and that vagal activity, measured by HRV, was improved. In addition, both VNS and ACh reduced the phosphorylation of MAPKs and prevented the nuclear translocation of NF-κB p65. Jin et al. (2017) also found an improvement of TNBS-colitis in rats both at the clinical, biological (MPOA, TNF, IL1-beta, IL-6) and histological level. In addition, they showed that the addition of electroacupuncture to VNS may enhanced the anti-inflammatory effect of VNS. Both VNS plus electroacupuncture and VNS alone substantially increased vagal activity, measured by HRV, and decreased sympathetic activity compared with sham ($P < 0.001$, $P < 0.001$, respectively). More recently, Meroni et al. (2018) performed VNS in mice following intracolonic oxazolone administration. This model represents a model of sepsis and may resemble a severe type of UC, resulting in a severe destruction of the colonic mucosa and a rapid drop in body temperature leading to a 65% mortality rate at day 5. Severe infiltration of neutrophils and monocytes was detected 6h after oxazolone administration which was associated with a Th2-type

inflammatory response. VNS significantly improved survival rate which correlated with decreased levels of HMGB1 and reduced colonic (IL-6 and CXCL1 mRNA) and serum cytokine levels (IL-6, TNF, and CXCL1) compared to sham treated mice. Chen et al. (2018) performed a dextran sodium sulfate (DSS) colitis in mice mimicking UC. They showed that VNS improved DSS-colitis but also alleviated cerebral cortical microinfarct induced by a two-photon laser and this neuroprotection was associated with the suppression of blood–brain barrier permeability, neuroinflammation and oxidative stress.

Clinical Data

In a translational approach from bench to bedside, we conducted, for the first time, a pilot study of VNS in patients with moderate to severe CD as an alternative to anti-TNF drug therapy or in treatment-naïve patients (ClinicalTrials.gov Identifier: NCT01569503). Nine patients were implanted with a VNS device and electrode (Cyberonics, Houston, TX, United States). Two patients were in failure of immunosuppressant (azathioprine) at the time of implantation and the other seven patients were naïve of treatment. Under general anesthesia, an electrode (Model 302) was wrapped around the left VN in the neck and connected to a bipolar pulse generator (Model 102) subcutaneously implanted in the chest wall. The day of the surgery, the device was switched on at 0.25 mA (duty cycle 30 s ON/5 min OFF, pulse width 250–500 μs -depending on patient tolerance-, 10 Hz frequency) and progressively increased up to 1.25 mA as patient tolerance permitted. VNS was continuously performed for 12 months. The first patient was implanted in April 2012 and the last in March 2016. Two patients were removed from the study after 3 months of neurostimulation for a worsening of their disease: the first patient underwent an ileo-cecal resection but, because of an initially beneficial effect and a drug treatment rejection, chose to continue neurostimulation until the end of the study. The second patient was treated with a combination of Infliximab and azathioprine and also wanted to keep on an active VNS. Six patients were in remission only under neurostimulation with a 1-year follow-up, the last patient was in flare. The first patient implanted in April 2012, was in relapse under azathioprine for an ileal CD with a history of ileo-cecal resection. We reported the results of this pilot study at 6-months follow-up in seven implanted patients (Bonaz et al., 2016a) and at 1 year in the all (n = 9) implanted (Sinniger et al., 2020). Briefly, of the seven patients who completed the 12-month VNS, five achieved clinical remission (CD activity index, CDAI < 150) and all the patients reached the CDAI-70 response (CDAI decrease from baseline by 70 points). Similarly the Crohn's disease endoscopic score of severity (CDEIS) decreased in five patients from 60 to 100%. No adverse events related to the device were observed except discomfort to the intensity/output current levels. Our results are in accordance with the preliminary results of D'Haens study (D'Haens et al., 2018) who observed clinical and endoscopic improvement for half of the 16 CD patients under either VNS monotherapy (biologics refractory patients) or VNS adjunctive therapy for 4 months.

A 12-month VNS could reduce inflammatory markers like CRP (in four patients whose three reaching normal value), fecal calprotectin (in three patients), and cytokines like TNF, IL6, IL12, and IL23, all being archetypal pro-inflammatory cytokines implicated in CD (**Figure 5**) (Sinniger et al., 2020). VNS was also able to increase or sustain plasma anti-inflammatory TGF-β1 in six patients, probably through its active regulatory role on Th17/Treg balance as demonstrated by Bettelli et al. (2006) and Tanaka and Kishimoto (2012) and also through its regulatory role in monocyte-driven inflammatory responses resulting in a reduction in TNF and a production of IL10 (Lee et al., 2013). Some plasma inflammatory markers have been correlated with gut mucosa metabolic markers; this is the case for CRP, which correlated with taurine, a metabolite produced by CRP-activated leucocytes and involved in the cytoprotection and homeostatic maintenance of cells during inflammatory/oxidative processes. We also showed a correlation between TNF and lactate, alanine and β-hydroxybutyrate that could reflect a metabolite shift occurring within the gut mucosa during the 12-months VNS, both being either involved in activation/deactivation and in the redox state of the immune cells, or as an alternative source of energy.

This pilot study requires of course confirmation in a larger randomized double-blinded control study and, overall, a

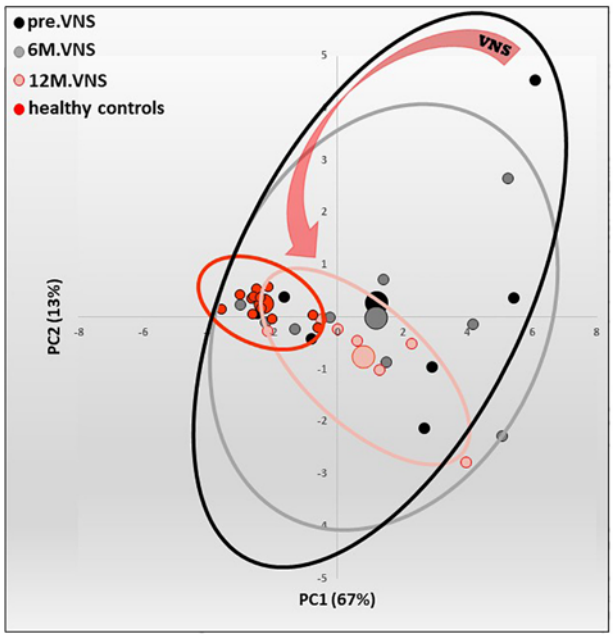

FIGURE 5 | Pilot study of vagus nerve stimulation (VNS) in patients with moderate to severe Crohn's Disease (CD). Twelve-month-VNS effect on cytokinergic profile. A plasma cytokinergic profile for controls (red), before (black), 6-month (gray) and 12-month (pink) VNS has been assessed using PCA analysis of plasma multicytokines assay for all CD patients [IL1b, IL2, IL6, IL10, IL12(p70), IL17A, IL21, IL23, MIP1α, IFNγ, GM-CSF, TNFα, TGFβ1 and MCP1]. The control values are well grouped, while profiles before VNS are very scattered, indicating that CD patients have their own cytokinergic profile. After 6 months, and even more after 12 months of VNS, the points are tightened, indicating that cytokine levels evolve through a more "common" profile. Ellipses are centered on the barycenter (big dots) of each group. Adapted from Sinniger et al. (2020).

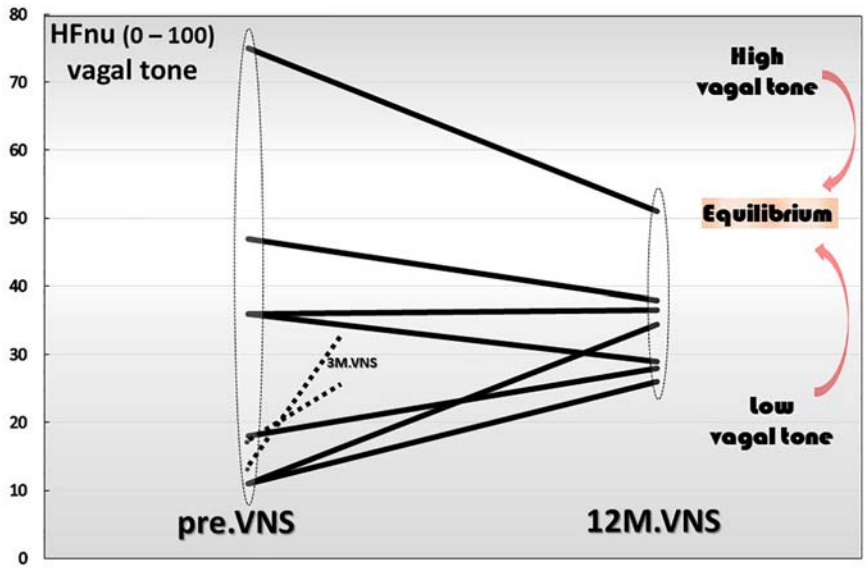

FIGURE 6 | Twelve-month-vagus nerve stimulation (VNS) effect on vagal tone. High frequencies are expressed in normal units (HFnu) and are extracted from heart rate variability analysis.

long-lasting follow-up of the patients to confirm these promising results (Cheng et al., 2020).

The Question of the Regulatory Role of VNS

Finally yet importantly, we have also shown that a 1-year chronic VNS exerts a modulatory role on vagal tone (**Figure 6**). Indeed, the trajectory toward the return to vagal equilibrium under VNS is dependent of the initial level of the HF-HRV. Interestingly, we observed in this clinical trial, that a very low HF-HRV on inclusion, increases until the equilibrium under VNS, a moderate level of HF-HRV was stabilized while an abnormally high resting HF-HRV on inclusion was decreased and brought back to equilibrium. Consequently, we can see that chronic VNS, on the long term, bring the autonomic regulation to homeostasis. At this stage, the question that arises is that of the mechanism by which this regulatory effect occurs, which has so far, never been observed before. A central mechanism through a change in the network balance within the CAN is most likely. There are several arguments in favor of this hypothesis. First, if we look at the kinetics of the evolution of the HRV over time, we can see that the return to equilibrium began at the third month of VNS (Sinniger et al., 2020). Second, we must also keep in mind that the assessment of HRV is mainly an evaluation of the ability of the central loops to regulate the functioning of the ANS, the HRV being the output (**Figure 1**). Hence, VNS logically must imply a mechanism that takes time to set up. VNS, used in the treatment of drug-refractory epilepsy, drives a 50% reduction frequency in 40–60% of the patients, with an increasing efficacy up to 10 years, showing that this treatment is a slow-acting therapy (Elliott et al., 2011). Third, electroencephalographic studies performed along with VNS over 1-year follow-up, revealed differences on power spectral bands between acute and chronic VNS. Acute VNS increased delta and theta bands on frontal, temporal and occipital sites while 1 year chronic VNS decreased power in the alpha band in correlation with the improvement of bowel mucosal inflammation, anxiety state and vagal tone. This suggests that chronic VNS has a regulatory action through the CNS and probably the CAN via afferent vagal fibers (Kibleur et al., 2018). This regulation-modulation mechanism of VNS on the return to equilibrium of the ANS but also that of the cytokines is quite original and rare in therapy outside of the example of thymoregulatory drugs like lithium. This requires serious consideration of this fundamental question by continuing investigations in this field.

CONCLUSION

Targeting the anti-inflammatory properties of the VN with VNS could be of interest in the management of patients with CD through a non-drug therapy. VNS is an alternative to biologics such as anti-TNF but also other pro-inflammatory cytokines such as IL-6, IL-12, IL-23, as observed in our study, or even as an alternative to any drug treatment: this was the case in five of our first seven patients who were naïve of treatment on inclusion. In addition, the CAP is an intrinsic anti-inflammatory non-drug pathway, which protects against the potential iatrogenic effects of treatments. VNS, on the other hand, is devoid of major side effects and cheaper than biologics (the electrode and neurostimulator cost ∼ 11,000 euros).

Non-invasive neurostimulation by the transcutaneous auricular route (ta-VNS) is an alternative to invasive neurostimulation, as used in our pilot study (Butt et al., 2020). The aim of ta-VNS is to stimulate the ear *concha* (*concha auriculae*), part of the ear which is 100% innervated by the auricular branch of the VN (Peuker and Filler, 2002) whose

stimulation would activate the "inflammatory reflex." The tragus and the cavity of the concha are 45% innervated by the auricular branch of the VN. A recent functional brain imaging study showed that neurostimulation of this region of the ear induced brain activation of the NTS and its numerous projection sites, as observed with invasive VNS (Badran et al., 2018). Ta-VNS is under clinical investigation in a double blind placebo-controlled study in adult patients with UC (ClinicalTrials.gov Identifier: NCT03908073) and pediatric patients with IBD (CD and UC) (ClinicalTrials.gov Identifier: NCT03863704). It is also possible to stimulate the VN at the left cervical level with the Gammacore device marketed by Electrocore LLC (Basking Ridge, NJ, United States) represented by two round stainless steel disks serving as a contact surface with the skin. This device, recommended in the treatment of headaches, epilepsy, and depression (Ben-Menachem et al., 2015), delivers a stimulation lasting 2 min with a frequency of 20 Hz. There is presently no clinical trial registered with this technique in Clinical.Trial.gov.

The optimal parameters of VNS to achieve efficacious inflammation-related symptomatic relief by recruiting the appropriate fibers within the VN are still unknown. Specific combinations of pulse width, pulse amplitude, and frequency produced significant increases of the proinflammatory cytokine TNF, while other parameters selectively lowered serum TNF levels, as compared to sham-stimulated mice (Bonaz, 2020; Tsaava et al., 2020). VN morphology influences fiber responses to electrical stimulation. Specifically, nerve diameter (and thus, electrode-fiber distance), fascicle diameter, fascicular organization, and perineurium thickness all significantly affect the responses of nerve fibers to electrical signals delivered through a cuff electrode (Pelot et al., 2020). Miniaturization of the VNS device is also warranted. In the same way, instead of an electrode, a VNS device which would act as an electrode by clipping it around the VN would be of interest (see setpointmedical.com; MØ1-ØØ1123). Another important progress would be a device able to record HRV and trigger VNS in case of low HRV to restore a normal tone. A VNS system, AspireSRTM, already approved in Europe, and created by Cyberonics Inc. analyzes relative changes of heart rate, particularly ictal tachycardia, and responds to seizures automatically. Consequently, all the technical and anatomical points developed above should be taken into consideration in future clinical studies and may influence the results of these studies.

AUTHOR CONTRIBUTIONS

BB wrote the first draft of the manuscript. VS and SP completed the writing of the manuscript and built the figures. All authors contributed to the article and approved the submitted version.

FUNDING

This review was supported by INSERM and DGOS ("Appel à Projet Translationnel 2011") and the DRCI from the Grenoble Hospital, France.

ACKNOWLEDGMENTS

The pilot study of VNS in patients with CD was supported by the French National Institute of Health and Medical Research (INSERM), the French Ministry of Health (DGOS call for Translational Project 2011) and the Grenoble University Hospital [Department of Clinical Research and Innovation (DRCI)]. The electroencephalographic study was performed on the IRMaGe technical platform in Grenoble (Dr. Olivier David, head of the EEG technical platform). The authors would like to thank (1) Mr. Nicolas Gonnet, Mr. David Tartry and Mrs. Mélanie Arnaud (clinical research associates) for organizing the clinical trial, (2) Mrs. Astrid Kibleur, Ph.D., for the implementation and analysis of the electroencephalograms, (3) Mrs. Florence Fauvelle, Ph.D., for the metabolomic data acquisition and spectra processing (INSERM, US17, MRI facility IRMaGe), (4) Mrs. Françoise Bardin for the formatting of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Namgung *et al.*
Journal of Neuroinflammation 2022, **19**(1):33
https://doi.org/10.1186/s12974-022-02396-z

Journal of Neuroinflammation

RESEARCH Open Access

Vagus nerve stimulation modulates hippocampal inflammation caused by continuous stress in rats

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Abstract

Background: Previous studies have shown that vagus nerve stimulation (VNS) can attenuate inflammatory responses in peripheral tissues and also improve some neurological disorders and cognitive function in the brain. However, it is not clear how VNS is involved in neuropathological processes in brain tissues. Here, we investigated the regulatory effects of VNS on the production of proinflammatory cytokines in the hippocampus of an animal model of continuous stress (CS).

Methods: CS was induced by placing rats in cages immersed with water, and acute or chronic electrical stimulation was applied to the cervical vagus nerve of CS animals. Protein levels in the gastric and hippocampal tissues were measured by western blotting and protein signals analyzed by immunofluorescence staining. von Frey test and forced swimming test were performed to assess pain sensitivity and depressive-like behavior in rats, respectively.

Results: Levels of TNF-α, IL-1β, and IL-6 in the gastric and hippocampal tissues were significantly increased in CS animals compared to the untreated control and downregulated by acute VNS (aVNS). Iba-1-labeled microglial cells in the hippocampus of CS animals revealed morphological features of activated inflammatory cells and then changed to a normal shape by VNS. VNS elevated hippocampal expression of α7 nicotinic acetylcholine receptors (α7 nAChR) in CS animals, and pharmacological blockade of α7 nAChR increased the production of TNF-α, IL-1β, and IL-6, thus suppressing cholinergic anti-inflammatory activity that was mediated by VNS. Chronic VNS (cVNS) down-regulated the hippocampal production of active form of caspase 3 and 5-HT1A receptors and also decreased levels of TNF-α, IL-1β, and IL-6 in the gastric and hippocampal tissues of CS animals. Pain sensitivity and depressive-like behavior, which were increased by CS, were improved by cVNS.

Conclusions: Our data suggest that VNS may be involved in modulating pathophysiological processes caused by CS in the brain.

Keywords: Continuous stress, Vagus nerve stimulation, Inflammatory cytokines, Neuroinflammation, Hippocampus, Cholinergic anti-inflammation

Background

Continuous stress (CS) can be induced in experimental animals by maintaining them in water-immersed cages for several days, and activation of microglial cells in the spinal cord of CS animals was shown to be related to chronic pain such as hyperalgesia and mechanical allodynia [1–3]. Studies further showed that CS alters endocrine function (e.g., downregulation of growth hormone

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production from atrophied somatotrophs in the pituitary gland and increased production of α-melanocyte stimulating hormone in the intermediate lobe of the pituitary gland) [4, 5]. Having noted that the symptoms observed from CS animals displayed the similarity partially with those of the patients suffering from myalgic encephalomyelitis/chronic fatigues syndromes (ME/CFS), CS animal was proposed as a model of ME/CFS [1, 2].

Previous studies have provided convincing evidence that vagus nerve activity is involved in regulating inflammation and several diseases in visceral organs such as chronic bowel disease, irritable bowel syndrome, obesity, diabetes and others [6, 7]. Here, acetylcholine neurotransmitters released from the efferent fibers of the vagus nerve interact with α7 nicotinic acetylcholine receptors (α7 nAChR) in target cells (e.g., splenic macrophages) and subsequently induce the JAK2 activation of STAT3 and NF-kB leading to the inhibition of the expression of proinflammatory cytokine genes [8, 9]. Since VNS is given to the exposed nerve in experimental animals, anti-inflammatory effects can also be influenced by afferent nerve activity through feedback loop in the dorsal vagal complex [10–12]. Moreover, VNS acting on the ascending fibers that transmit signals to the forebrain was reported to enhance memory formation and improve mental illnesses such as epilepsy and depression [13–15]. Cell-specific transneuronal labeling has identified the neural circuits connecting the guts and forebrain that activate the reward system, thus demonstrating the vagus nerve activity as a modulator of brain–gut axis [16]. Ascending vagus nerve activity may be involved in activating brainstem nuclei for neuromodulation and affect the limbic system and cerebral cortex, modulating emotional and cognitive function in the brain [7]. We have recently reported that the afferent component of VNS increased cholinergic nerve activity in the dorsal vagal complex and resulted in the upregulation of α7 nAChR levels in the liver [12]. α7 nAChR activity in the brain was shown to participate in the regulation of NMDA and GABA receptor activation and correlate with the occurrence of schizophrenia [17–19]. There is a report showing microglial activation of α7 nAChR receptors which is related to the suppression of neuroinflammation [20]. However, direct evidence verifying VNS-modulated neuroinflammation in the brain via α7 nAChR has not been reported so far.

Continuous stress deteriorates mental and physical activities in experimental animals and may cause a broad spectrum of neuroimmunopsychological abnormalities. As an initial step of exploring the potential effects of VNS on the regulation of neuroimmune disorders such as CFS, we investigated VNS-mediated changes of pathophysiological and behavioral responses in the hippocampus of CS animals. The present study provides evidence that VNS plays a role in regulating hippocampal production of inflammatory cytokines and behavioral abnormalities associated with CS.

Materials and methods

Animals

Sprague–Dawley rats (male and female, 7–8 weeks, 200–250 g) were purchased (Samtako Inc., Seoul, Korea) and acclimatized for 7 days before the use for the experiments in ventilated animal room with 22–23 °C of temperature and 60% humidity under a standard 12 h light and 12 h dark cycle (lights on from 7:00 am to 7:00 pm). All animals were freely accessed to commercial chow (Samyang Co., Eumseong, Korea) and drink water. In this study, a total of 111 rats were used and their use in the present study categorized into the acute VNS (aVNS) and the chronic VNS (cVNS) experiments. As for aVNS experiments, 51 rats were randomly assigned with an equal number into CS plus sham (CS+Sham), CS plus aVNS (CS+aVNS) and untreated control (CTL) groups. As for cVNS experiments, a total of 60 rats were assigned into CS+Sham, CS+cVNS, and CTL groups, distributing 20 animals equally to each experimental group. All animals were survived during and after the surgery, and thus, no animal was excluded for data analysis. All protocols involving live and postoperative animal care were approved by the Daejeon University Institutional Animal Use and Care Committee and were in accordance with the Animal-Use Statement and Ethics Committee Approval Statement for Animal Experiments provided by Daejeon University (Protocol number: DJUARB2019-029, Daejeon, Korea).

CS animal model

An experimental procedure for CS in rats was essentially the same as described previously [2]. Briefly, rats were randomly designated to CS or untreated control group (CTL). For the performance of CS, each rat was placed in a single cage filled with 23 °C of water with a height of 1.5 cm and was transferred every 24 h to a new cage filled with the same amount of water. Animals were given CS for 5 consecutive days during which food and water were provided. Twenty four hours after the final CS, animals were subjected to VNS or sham surgery, as described below.

Acute and chronic vagus nerve stimulation

After the CS, rats were anesthetized with ketamine (80 mg/kg; Yuhan, Seoul, Korea) and xylazine (5 mg/kg; Bayer, Leverkusen, Germany). VNS in rats was given as described in our previous study [12, 21]. Briefly, the anterior surface of the neck and the muscles were incised and the larynx was lifted. The lower muscle of the larynx was

cut to expose the right cervical vagus nerve and carotid artery, and the nerve was carefully separated from the carotid artery. For aVNS, the exposed vagus nerve was placed into a U-shape of a bipolar electrode of tungsten wire (250 μm diameter, A-M System Inc., Sequim, WA, USA). The electrical current (10 mA, 5 Hz, 5 ms of pulse duration) was applied for 5 min using the electrical stimulator (Isolated Pulse Stimulator Model 2100, A-M Systems Inc.). For cVNS, rats were implanted with a microelectrode cuff (Inner diameter 0.75 mm; Microprobes, Gaithersburg, MD, USA) as described previously [21] and, after the recovery from anesthesia, returned to an animal room. The first electrical stimulation was given 24 h after the implantation of the microelectrode with the same stimulation condition as the acute stimulation, and the same intensities of stimulation were applied on a daily basis for 7 consecutive days. cVNS was applied to individual animals under the awake state. For the sham treatments, CS animals were anesthetized by ketamine and xylazine, the right vagus nerves were exposed, and the skins were suture. All experimental groups of animals underwent the same procedures of recovery and maintenance and were sacrificed with an overdose of ketamine (150 mg/kg, i.p.).

Intracerebroventricular injection of drug

Rats were anesthetized with ketamine and xylazine with the same dose as above and placed in the stereotaxic apparatus for drug injection. The skull of the forebrain was perforated using a drill. An α7 nAChR antagonist methyllycaconitine citrate salt (MLA; 1 μg/μl in 0.9% NaCl; Sigma-Aldrich, St. Louis, MO, USA) or an equivalent volume of saline solution was injected into the lateral ventricle (coordinate AP: 0.8 mm; L: 1.5 mm; DV: 3.5 mm) [22] using a micropump (Pump 11 Elite, Harvard Apparatus) with a flow rate of 5 μl/min for 2 min [23] and the injection needle remained penetrated for 3 min to prevent drug reflux and also allow drug to diffuse into the surrounding area.

Western blot analysis

The hippocampal tissue was dissected from rats and sonicated in RIPA buffer (150 mM NaCl, 1.0% CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0; Thermo Fisher Scientific, MA, USA) supplemented with protease inhibitor and phosphatase inhibitor cocktails (Roche Diagnostics, Canton, Switzerland). The lysate was centrifuged at 12,000 rpm for 15 min at 4 °C and the supernatant was collected. Protein (20 μg) was resolved by SDS polyacrylamide gel electrophoresis and transferred to a PVDF membrane. The membrane was blocked with 5% BSA in 1X TBST (0.1% tween 20 in Tris-buffered saline) and incubated with the primary antibody followed by reaction with secondary antibody. Immunoblotting was performed using primary antibodies including anti-TNF-α (Rabbit-polyclonal, 1:2000; Abcam, Cambridge, UK), anti-IL-1β (Rabbit-polyclonal, 1:2000; Abcam), anti-IL-6 (Mouse-monoclonal, 1:2000; Abcam), anti-α7 nicotinic acetylcholine receptor (Rabbit-polyclonal, 1:200, Alomone Labs, Jerusalem, Israel), anti-choline acetyltransferase (Rabbit-monoclonal, 1:2000; Abcam), anti-5HT1A receptor (Rabbit-polyclonal, 1:1000, Abcam), anti-Iba-1 (Mouse-monoclonal, 1:1000; Fujifilm, Minato, Japan), anti-cleaved Caspase-3 (Rabbit-polyclonal, 1:1000; Cell Signaling Technology, Beverly, MA, USA) and anti-β-actin (Mouse-monoclonal, 1:50,000; Sigma-Aldrich) antibodies. Secondary antibodies were anti-rabbit HRP (1:5000; Cell Signaling Technology) and anti-mouse HRP (1:5000; Cell Signaling Technology) antibodies. The intensity of each protein band was analyzed using the i-Solution software (version 21.1, Image & Microscope Technology, Daejeon, Korea) and compared with that of β-actin protein band.

Reverse transcription polymerase chain reaction

Total RNA was extracted from the hippocampus using trizol reagent (Thermo Fisher Scientific). cDNA was synthesized by incubating isolated RNA in the reaction containing 50 mM Tris–HCl, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 104 μM dNTP, RNasin (30 U), random primers (16 μM, Promega, Madison, Wisconsin, USA), and MMLV reverse transcriptase (200 U, Promega, Wisconsin, USA) for 2 h at 37 °C. The primer sequences for RT-PCR were the forward primer (5′-CCTGCTCCC CAACACATGAT-3′) and the reverse primer (5′-GAC ATGAGGATGCCGATGGT-3′) for α7 nAChR mRNA and the forward primer (5′-CACACTGTGCCCATCTAT GA-3′) and the reverse primer (5′-CCATCTCTTGCT CGAAGTCT-3′) for actin mRNA.

Gastric tissue histology

Animals were deprived of food pellets for 24 h before sacrifice. The stomachs were dissected, washed with 1× PBS, and filled with 1× PBS. After freezing at − 20 °C for 10 min, the stomachs were cut from the pylorus to the cardiac orifice, and the gastric mucosal lesion was evaluated by spreading the cut stomach and digitally photographed (Nikon E-600, Tokyo, Japan). For hemotoxylin and eosin staining, rats were transcardially perfused with 4% paraformaldehyde in 1× PBS, and the gastric tissue was isolated and postfixed for 2 h with 4% paraformaldehyde and subsequently immersed with 30% sucrose solution at 4 °C for 2 days. Then, the tissue was rapidly frozen by dipping into − 80 °C of 2-methylbutane (Sigma-Aldrich) and the transverse sections (5 μm thickness) were prepared using a cryostat (Leica CM1850, Wetzlar,

Germany) and mounted on the superfrost plus microscope slide (Thermo Scientific, Waltham, MA, USA). Slides were dipped into hematoxylin solution (HEMH-OT-1L, BioGnost, Zagreb, Croatia) for 40 s and washed with distilled water. Slides were also stained with Eosin Y solution (EOYA-05-OT-1L, BioGnost) for 10 min and dehydrated gradually through 50%, 60%, 75%, 90%, and 100% ethanol for 5 min each. The sections were cleared with xylene (Sigma-Aldrich) for 5 min and mounted with xylene-based mounting medium.

Immunofluorescence staining

Rats were anesthetized with an overdose of ketamine and xylazine and perfused with 1X phosphate buffered saline (PBS) and 4% paraformaldehyde in PBS. The whole brain was dissected and immersed overnight in 30% sucrose in PBS solution. After rapid freezing at −80 °C with 2-methylbutane (Sigma-Aldrich), tissues were cut using a cryostat (Leica CM1850, Wetzlar, Germany) and thaw-mounted on the slide (16 μm thickness). Sections were fixed, permeabilized, treated with blocking solution (2.5% BSA, 2.5% horse serum, 0.1% Triton X-100 in 1× PBS), and incubated with primary antibodies for 24 h at 4 °C, washed three times with 1× PBST and incubated with secondary antibodies for 2 h at room temperature in a dark room. The primary antibodies used were anti-IL-1β (Rabbit-polyclonal, 1:2000; Abcam), anti-5HT1AR (Rabbit-polyclonal, 1:200; Abcam), anti-Iba-1 (Rabbit-polyclonal, 1:1000; Fujifilm), anti-cleaved caspase 3 (Rabbit-polyclonal, 1:1000; Cell Signaling Technology), anti-α7 nAChR (Rabbit-polyclonal, 1:200; Alomone Labs) and anti-NeuN (Mouse-monoclonal, 1:1000; BD Biosciences, Franklin Lakes, NJ, USA) antibodies. Rhodamine-goat anti-rabbit IgG (1:400, Molecular Probes, Eugene, OR, USA) and fluorescein-goat anti-mouse IgG (1:400, Molecular Probe) were used as secondary antibodies. For nuclear staining, sections were incubated with 2.5 μg/ml of Hoechst 33258 (bis-benzimide, Sigma-Aldrich) for 10 min before the final washing with 1× PBST. Branch lengths of microglial cells were quantified by i-Solutions of software program (Image & Microscope). The distances between the surface of cell body and the tip of each branch were measured and averaged per each microglial cell. In each experimental group, randomly selected 15–20 cells per animal were analyzed and the data were averaged from four independent animal preparations. Then, the statistical comparisons were made among experimental groups.

Behavioral tests

For pain sensitivity test, rats were placed in the metal mesh cuboid-shaped acrylic plastic chambers and allowed to adapt to the environment for 30 min before the test. The filament was applied to force the mid-plantar of the hind paw for intervals of 5 s. The von Frey threshold in *g* values was determined by the observation of withdrawal response to filament force (Almemo 2450, Ahlborn equipment Inc., Sayner, WI, USA) and the value per animal was determined by averaging 20 or 40 different measures. For the forced swimming test, each rat was examined in a transparent cylindrical chamber (height 50 cm, diameter 25 cm) filled with water at 27 °C (depth 35 cm). Twenty-four hours before the test, animals were adjusted in water for 15 min. Following 1 min adjustment in water, animals were placed in a water chamber for 5 min and their swimming behaviors were monitored and analyzed using a Smart version 2.5 video tracking system (Panlab, Barcelona, Spain). Immobility scores were determined by measuring the time periods that animals kept maintaining the head above the water without any additional actions, and swimming scores were determined by measuring the time period that the animals were moving actively above the water.

Statistical analysis

Data were analyzed as mean ± standard deviation (SD). The mean values of the data in individual groups were compared using one-way ANOVA and Tukey's test for multiple comparisons or two-way ANOVA repeated measures with Sidak multiple comparison test (GraphPad Prism 7.00). Statistically significant differences were set at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Results

Gastric inflammation in CS animals is ameliorated by aVNS

It was previously reported that CS in experimental animals causes severe inflammation in the stomach [24]. Here, we investigated the pathological responses of gastric tissues in male rats given CS for 5 days and further examined the effects of VNS on gastric inflammation (Fig. 1A). The gastric lumen of CS animals was swollen and showed mucosal membrane lesion such as erosion and ulcerations accompanied by erythematous gastric mucosa, compared to the control animal, and the lesion was ameliorated by VNS (marked by white arrows in Fig. 1B). Hemotoxylin and eosin staining of transverse sections revealed traces of hemorrhage and tissue disruption in the mucosal membrane of CS animals (marked red arrowheads in Fig. 1C) and tissue morphology was largely improved by aVNS. Gastric levels of proinflammatory cytokines IL-1β, TNF-α, and IL-6 were greatly elevated in CS animals, and the levels were significantly reduced by aVNS (Fig. 1D–F). Considering the possible difference in inflammatory responses between male and female rats given CS, a model of diseases such as CFS affecting more women than men [25], we also examined

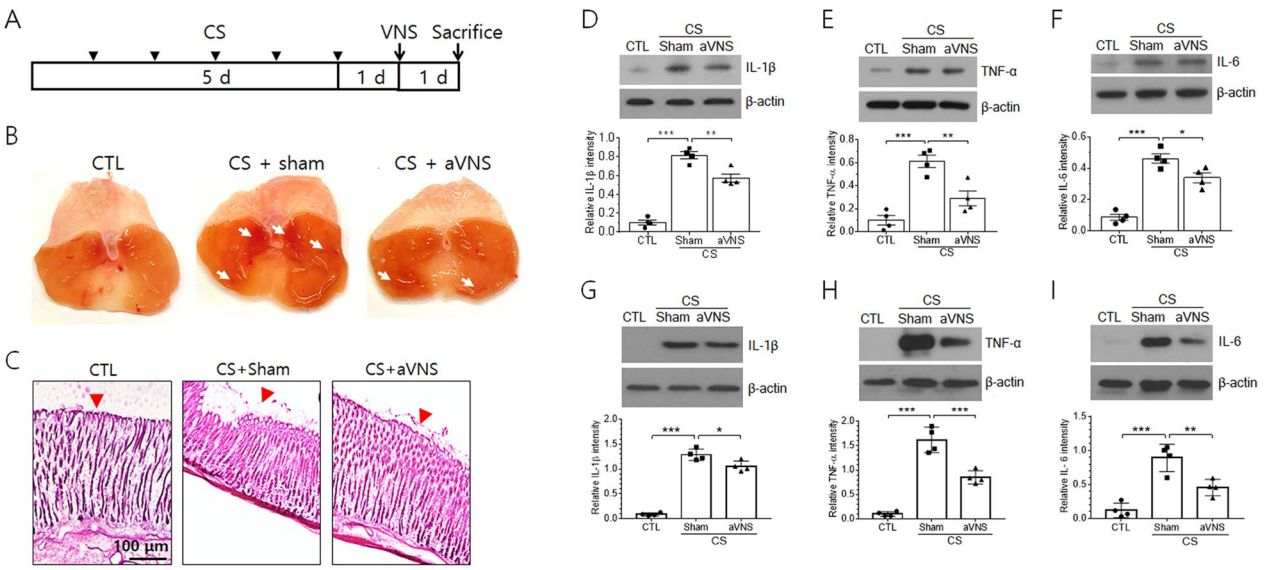

Fig. 1 Regulation of gastric mucosal lesion and production of inflammatory cytokines in CS animals by aVNS. **A** Scheme showing the time course of animal treatments before the preparation of gastric tissues. **B** Representative photographic images of the inner surface of gastric mucosal tissues. **C** Microphotographs of hemotoxylin and eosin stained gastric tissues. The surface of mucosa layer in the stomach is marked by arrowheads. **D**–**I** Western blot analysis of IL-1β, TNF-α, and IL-6 proteins from the lysates of gastric tissues in male rats (**D**–**F**) and in female rats (**G**–**I**). Representative images of four independent experiments are shown in upper panels, and quantification of the band intensity for individual proteins are plotted in lower panels. In **D**–**I**, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (One-way ANOVA)

the effects of aVNS on the production of inflammatory cytokines in female rats. As shown in Fig. 1G–I, levels of IL-1β, TNF-α, and IL-6 in the gastric tissue were significantly increased by CS and decreased by aVNS, suggesting similar anti-inflammatory effects of VNS in both male and female animals.

Production of inflammatory cytokines and microglial activation in the hippocampus of CS animals are alleviated by aVNS

We set out the experiment examining the effects of VNS on pathological responses in the hippocampus of CS animals. The levels of IL-1β, IL-6, and TNF-α in male rats were very low in the hippocampus of the control group and strongly induced by CS (Fig. 2A–C). Then, the application of aVNS significantly reduced IL-1β and IL-6 protein levels. Mean level of TNF-α was decreased by 31% after VNS but failed to show statistical significance ($p = 0.23$, One-way ANOVA). Examination of hippocampal IL-1β, IL-6, and TNF-α levels in female rats showed similar increases after CS and significant reduction by aVNS (Fig. 2D–F). We also investigated the distribution of inflammatory cytokine signals in the hippocampal subfields by immunofluorescence staining. IL-1β signals were clearly induced after CS in hippocampal neurons in the granule cell layer (GCL) and CA3 and CA1 pyramidal cell layers while showing much weak signals in non-neuronal hippocampal areas (Fig. 2G). IL-1β signals in CA3 and CA1 pyramidal cell layers were notably attenuated by VNS.

To investigate whether the regulation of inflammatory cytokine production involved the activation of microglial cells, we analyzed Iba-1, a marker protein of microglial cells in the hippocampus. Similar to inflammatory cytokines, Iba-1 protein level was elevated in the hippocampus of CS animals and downregulated by aVNS (Fig. 3A). Immunofluorescence labeling of microglial cells with Iba-1 showed that the cell morphology was highly branched and elongated in hippocampal subfields such as hilus region and dendritic zones of CA3

(See figure on next page.)

Fig. 2 Hippocampal induction of inflammatory cytokines IL-1β, IL-6, and TNF-α in CS animals is downregulated by aVNS. Western blot analysis of hippocampal IL-1β (**A**), IL-6 (**B**) and TNF-α (**C**) proteins in male rats and of the same proteins in female rats (**D**–**F**). Images in (**A**–**F**) are the representatives from four independent experiments (upper panels). Quantification of band intensity of each protein relative to actin is plotted (lower panels). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (One-way ANOVA). **G** Immunofluorescence images of IL-1β signals (red) in the granule cell layer (GCL) of the dentate gyrus (DG), CA3 and CA1 pyramidal cell layers. The nuclei of neurons were visualized by staining with Hoechst 33258 (blue)

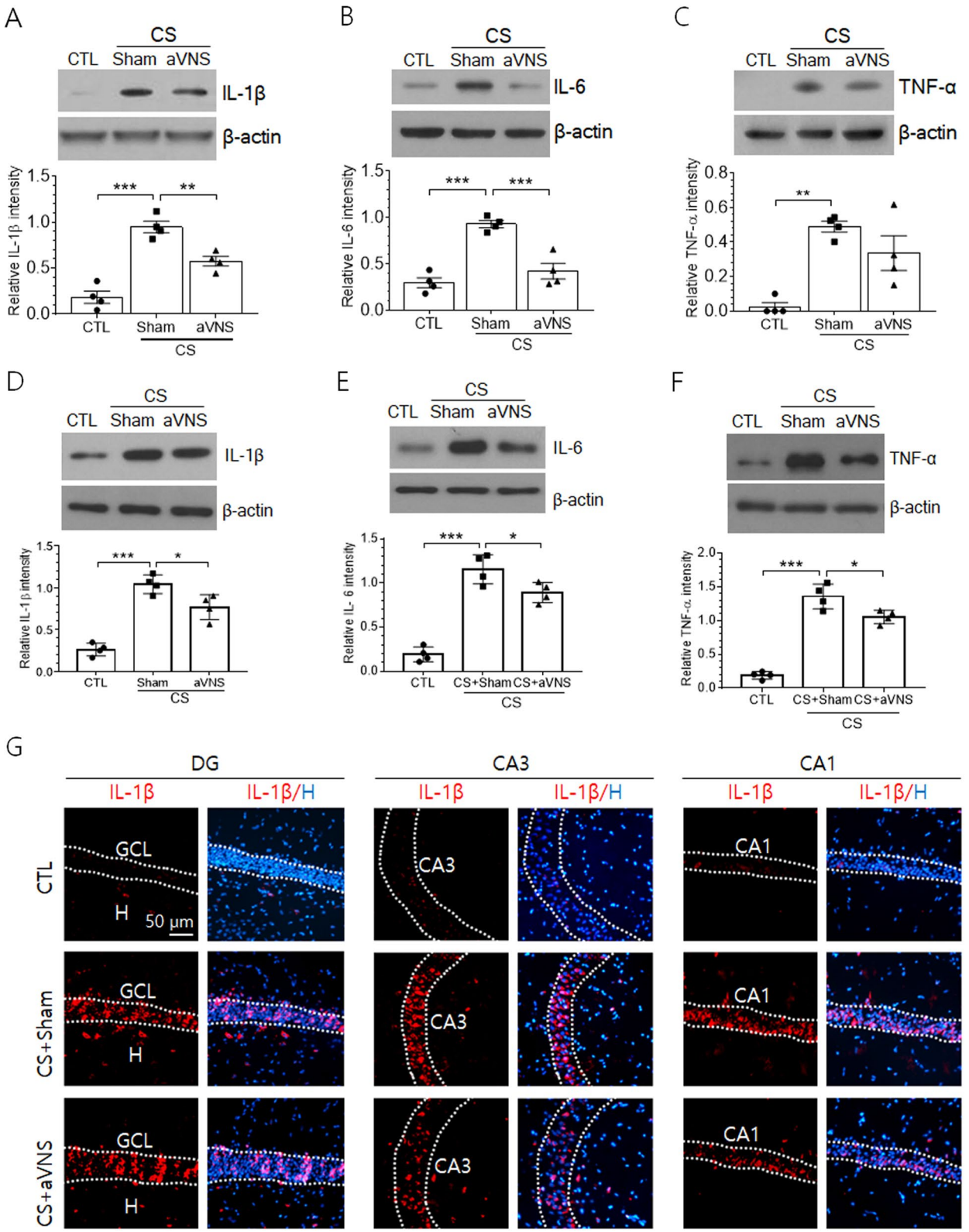

Fig. 2 (See legend on previous page.)

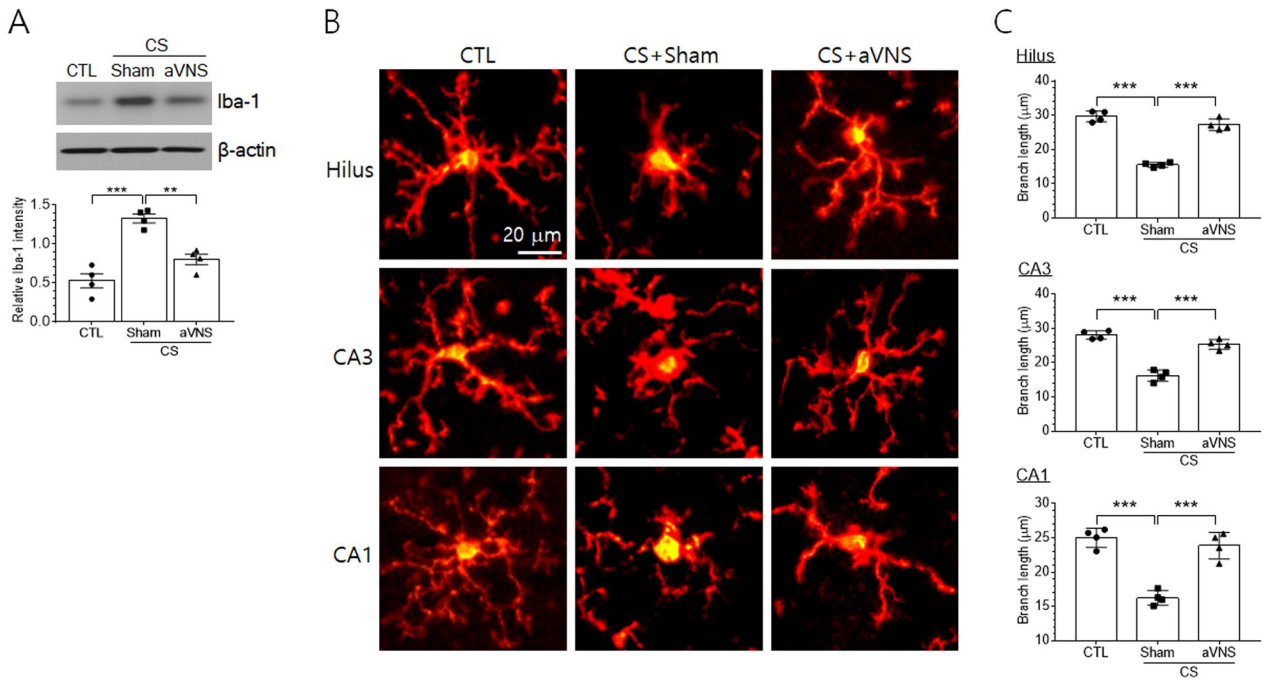

Fig. 3 Microglial activation in the hippocampus of CS animals is controlled by aVNS. **A** Western blot analysis of Iba-1 protein. Upper images are the representatives from four independent experiments. Quantification of protein band intensity relative to actin is plotted (lower panel). **B** Immunofluorescence view of Iba-1-labeled microglial cells. For three experimental groups, representative cell images were taken from the hilus region and the striatum radiatum areas of CA3 and CA1 pyramidal cells. **C** Quantification of branch length of microglial cells. Branch length was averaged by measuring randomly selected 15–20 cells per animal and the data from 4 independent animal preparations were compared among experimental groups. $^{**}p < 0.01$, $^{***}p < 0.001$ (One-way ANOVA)

and CA1 pyramidal cells in the control animals and changed to a less ramified shape with expanded cell body in CS animals (Fig. 3B). In CS+VNS group, the microglial processes were clearly extended, showing a similar morphology as in the control group (Fig. 3B). Quantitative assessment of the mean length of microglial branch showed significant decreases in all three hippocampal subfields of CS group animals and increases by VNS to similar levels as those in the control group (Fig. 3C).

Cholinergic synaptic transmissions are augmented by aVNS in the hippocampus

While cholinergic anti-inflammatory pathway via the activation of α7 nAChR has been well demonstrated in several visceral organs of experimental animals given VNS, its effects in the central nervous system remain largely unknown. Given that α7 nAChR is expressed in several brain tissues including hippocampus and innervated by the projection of cholinergic afferents from the septo-diagonal band of Broca [26–28], here we investigated possible involvement of α7 nAChR in the process of VNS-mediated regulation on the hippocampal production of inflammatory cytokines. Levels of hippocampal α7 nAChR mRNA were not changed by CS but significantly increased by VNS (Fig. 4A). Likewise, α7 nAChR protein was similarly regulated by VNS, showing no change by CS and elevation by VNS (Fig. 4B). α7 nAChR signals were detected in CA3 and CA1 pyramidal cell layers in control and CS groups of animals. α7 nAChR signals were induced clearly in the granule cell layer of CS+VNS group (Fig. 4C). It was also noted that the protein signals were detected in the hilus region of CS+VNS group of animals. To further investigate whether VNS affected cholinergic inputs into the hippocampus, we examined the production of cholinergic acetyltransferase (ChAT) in the hippocampus. ChAT was expressed at moderate level in the hippocampus of control group and slightly elevated in CS group without showing statistical significance ($p = 0.15$, One-way ANOVA). ChAT level was further increased by VNS compared to the control or CS group of animals (Fig. 4D).

To further examine whether the activity of α7 nAChR is required for VNS-mediated anti-inflammation, we analyzed inflammatory cytokine levels after the intraventricular injection of MLA, an α7 nAChR antagonist. Levels of IL-1β, IL-6, and TNF-α, which had been markedly decreased by VNS in CS animals, were significantly

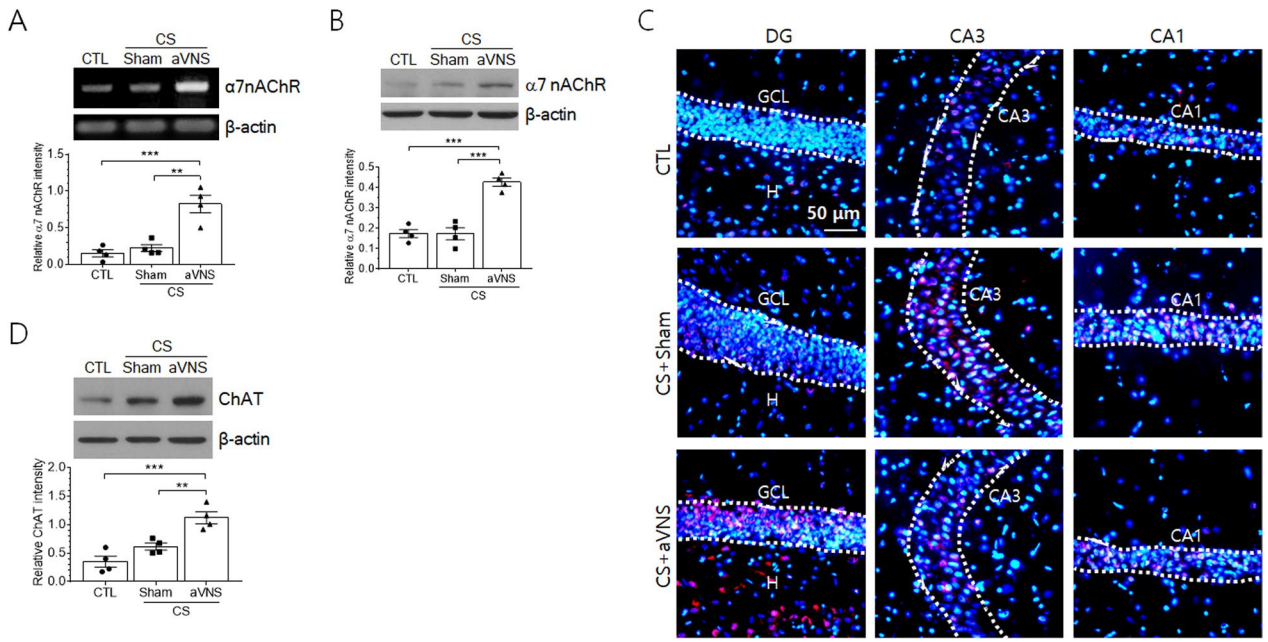

Fig. 4 aVNS upregulates the expression of α7 nAChR and ChAT in the hippocampus of CS animals. **A** RT-PCR analysis of α7 nAChR mRNA expression in the hippocampus. Upper images of agarose gel electrophoresis are the representatives of four independent experiments. Quantification of DNA band intensity relative to actin is plotted in lower panel. **B** Western blot analysis of α7 nAChR in the hippocampal tissues. **C** Immunofluorescence images of α7 nAChR signals in the granule cell layer (GCL) of the dentate gyrus (DG) and CA3 and CA1 pyramidal cell layers. The nuclei of neurons were visualized by staining with Hoechst 33258 (blue). **D** Western blot analysis of ChAT protein in the hippocampal tissue. In **B** and **D**, upper images are the representatives from four independent experiments. Quantification of band intensity relative to actin is plotted (lower panel). $^{**}p < 0.01$, $^{***}p < 0.001$ (One-way ANOVA)

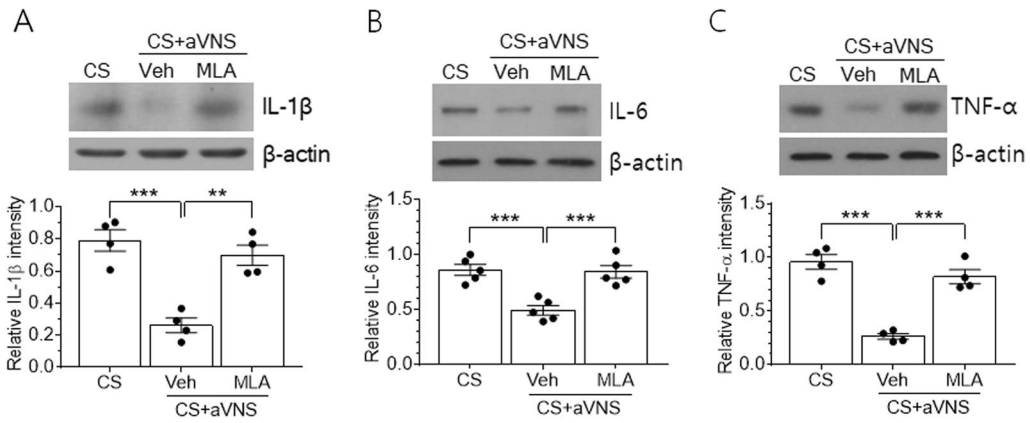

Fig. 5 Effects of α7 nAChR inhibitors on the hippocampal production of inflammatory cytokines. An α7 nAChR inhibitor MLA or saline vehicle solution (Veh) was administered 20 min prior to aVNS in CS animals, and proteins were extracted from hippocampal tissues for western blot analysis. Upper images in **A**–**C** are the representatives of 4–5 independent experiments for IL-1β, IL-6, and TNF-α proteins. Quantification of protein band intensity relative to actin is plotted in lower panels. $^{**}p < 0.01$, $^{***}p < 0.001$ (One-way ANOVA)

elevated by MLA treatment, leading to similar levels as those of the corresponding CS group of animals in all three proteins ($p = 0.54$, 0.54, and 0.97 for IL-1β, IL-6, and TNF-α, respectively) (Fig. 5A–C).

Regulation of cell death and serotonergic receptor production by chronic VNS

To determine whether the chronic application of VNS affects hippocampal neuronal activity and behavioral

consequences in CS animals, we implanted the microelectrode and stimulated the vagus nerve for 7 days. Cleaved, active form of caspase 3 was very low in the intact hippocampal tissue and was strongly induced by CS. Then, the chronic application of VNS resulted in significant reduction of caspase 3 levels (Fig. 6A). Immunofluorescence staining showed that cleaved caspase 3 signals, which were induced from the granule cells in the dentate gyrus and CA3 and CA1 pyramidal cells of CS animals as identified by colocalization with NeuN, were attenuated by chronic VNS (Fig. 6B). Cleaved caspase 3-positive cells that were detected from the outside of neuronal cell layers in CS group were also downregulated in CS+VNS group. We further investigated the effects of cVNS on the production of 5-HT1AR in hippocampal neurons. Moderate level of 5-HT1AR protein was detected in the hippocampal tissue of the control group and the protein level was significantly reduced in CS group (Fig. 6C). Then, the application of cVNS to CS animal significantly elevated 5-HT1AR level in the hippocampus. Immunofluorescence localization clearly showed the presence

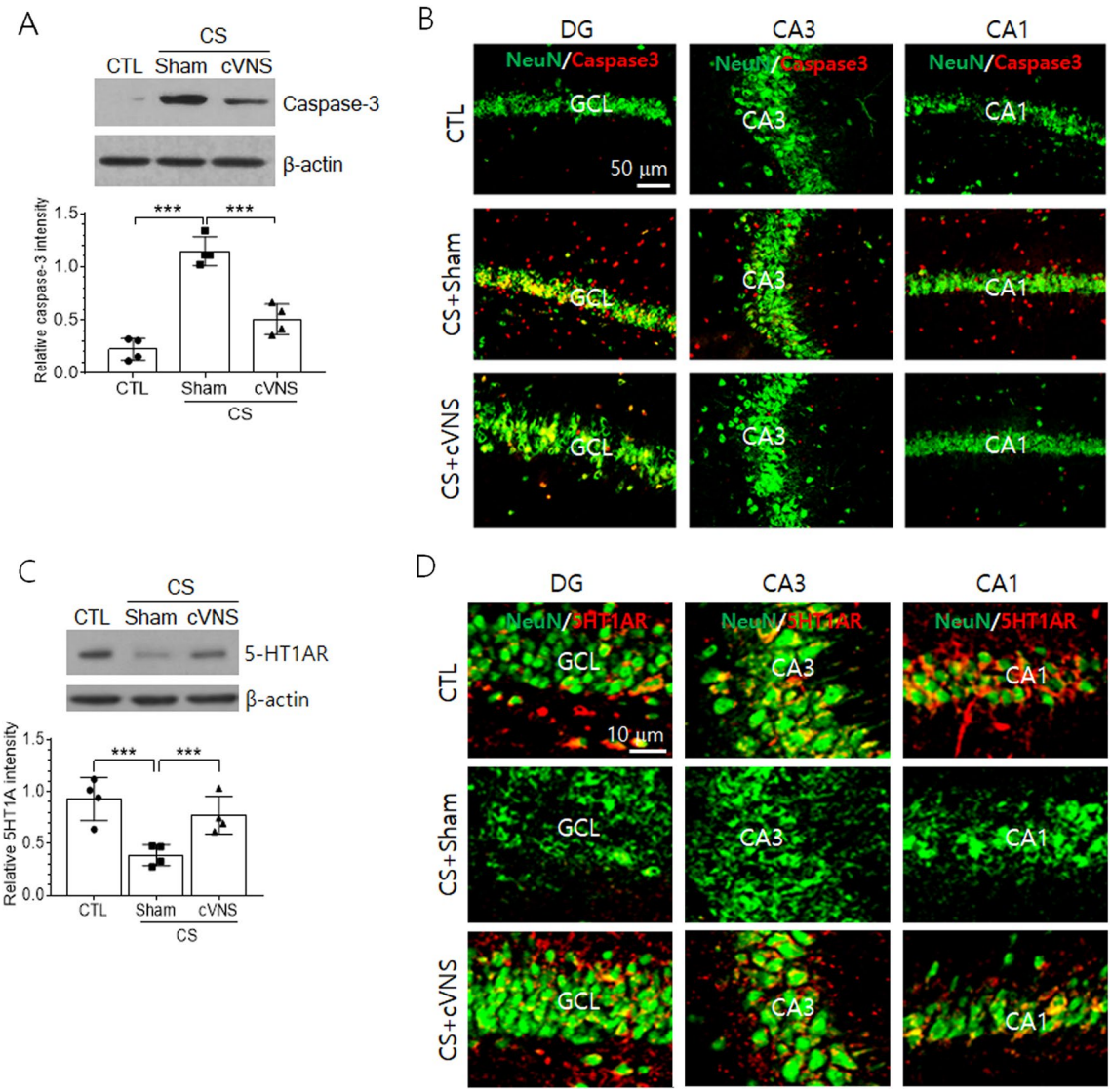

Fig. 6 Effects of cVNS on the production of active form of caspase 3 and 5-HT1A receptors in the hippocampus of CS animals. **A**, **C** Western blot analysis of cleaved active form of caspase 3 (**A**) and 5-HT1A receptors (**C**) in the hippocampus. Images in **A** and **C** are the representatives from four independent experiments. Quantification of band intensity relative to β-actin is plotted in lower panels. ***$p < 0.001$ (One-way ANOVA). **B**, **D** Immunofluorescence staining of active caspase 3 (red in **B**) and 5-HT1AR (red in **D**) merged with NeuN (green) in the granule cell layer (GCL) of dentate gyrus (DG) and CA3 and CA1 pyramidal cell layers

of 5-HT1AR signals in neurons of granule cell layer and CA1 pyramidal cell layers in the control group (Fig. 6D). Reduction and re-induction of 5-HR1AR signals were clearly observed in the granule cell layer and CA3 and CA1 pyramidal cell layers in CS and CS+VNS groups of animals, respectively.

cVNS is involved in regulating pain sensitivity and depressive-like behavior

To examine the effects of cVNS on animal behaviors, we analyzed pain sensitivity in the hind limb by von Frey test and immobility response as an indicator of depressive-like behavior by forced swimming test on a daily basis for 8 days after CS (vertical arrows in Fig. 7A). Pain responses were similar between the left and right hind limbs in each experimental group ($p=0.2$ for CTL-L and CTL-R, $p>0.99$ for CS-sham-L and CS-sham-R, $p=0.99$ for CS-VNS-L and CS-VNS-R; Two-way ANOVA, $df=1$, $n=4$). Pain sensitivity, as measured by paw withdrawal threshold, was significantly increased in CS animals compared to the control group and remained at the similar level during the period of VNS application (CTL-L vs. CS+Sham-L; $p<0.0001$, CTL-R vs. CS+Sham-R; $p<0.0001$, two-way ANOVA $df=1$, $n=4$; Fig. 7B). Pain sensitivities were then gradually recovered 5 days after applying chronic VNS (CS+Sham-L vs. CS+VNS-L; $p=0.0022$, CS+Sham-R vs. CS+VNS-R; $p=0.0005$, two-way ANOVA $df=1$, $n=4$). In the forced swimming test, immobility time duration was greatly increased in CS group of animals and maintained the similar level when measured 1 day after the last CS with a single bout of VNS (left plot in Fig. 7C). Immobility time remained elevated 7 days after CS, but the score was significantly decreased in CS+cVNS animals after 7 bouts of VNS when analyzed 8 days after CS (right plot in Fig. 7C).

Behavioral effects of cVNS on CS animals are linked to gastric and hippocampal inflammations

To further examine whether behavioral improvements by cVNS were related to the regulation of inflammatory responses, we analyzed levels of IL-1β, TNF-α and IL-6 in the gastric and hippocampal tissues. Gastric levels of these inflammatory cytokines were strongly induced in CS+Sham group of animals and then significantly decreased by cVNS (Fig. 8A–C). Similarly, IL-1β, TNF-α and IL-6 protein levels in the hippocampus were highly induced by CS and significantly decreased by cVNS (Fig. 8D–F). To examine whether the cVNS-mediated regulation of inflammation is related to animals' behavior, we performed behavioral tests for the same set of animals that were subsequently subjected to the investigation of gastric and hippocampal inflammation. Pain sensitivity as measured by paw withdrawal threshold was greatly increased by CS and reduced by cVNS (Fig. 8G). In the forced swimming test, CS-induced increase of immobility time was significantly reduced by cVNS, whereas

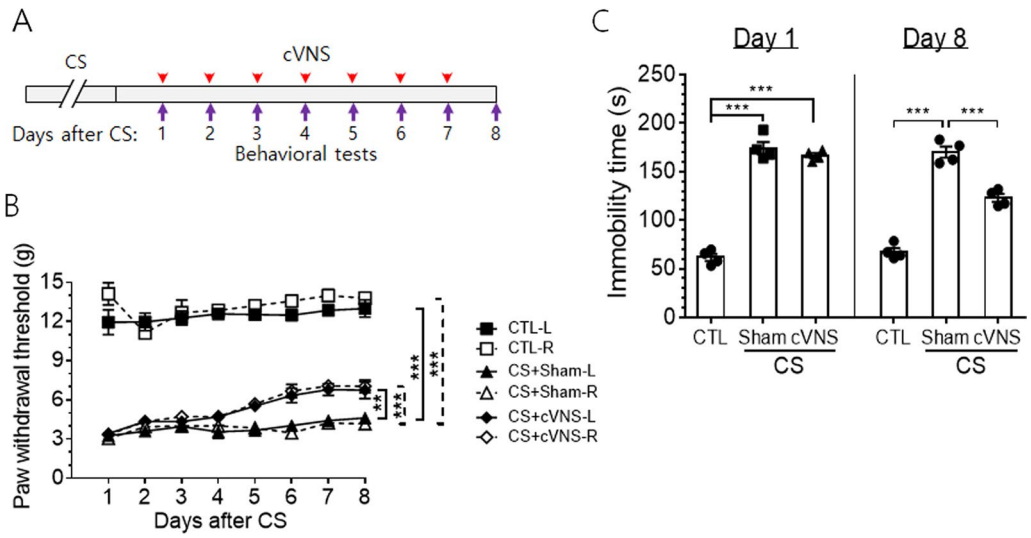

Fig. 7 cVNS improves pain response and depressive-like behavior in CS animals. **A** A schematic diagram showing animal treatments including behavioral tests. VNS was initiated 24 h after final CS and given for 7 consecutive days, Behavioral tests were performed immediately after cVNS and continued each day for 8 days. **B** Von Frey hair test. Values of withdrawal threshold to stimulations on the left (L) and right (R) hind limbs were measured 20 times for individual animals as indicated in the Figure. Statistical comparison among groups were made by two-way ANOVA repeated measures (**$p<0.01$, ***$p<0.001$; see 'Results' section for details). In the plot in (**B**), vertical box brackets with solid and dashed lines denote the comparisons between the corresponding solid symbols and between the open symbols, respectively. **C** Forced swimming test. Immobility time was measured on the first and the eighth days after CS (labeled day 1 and day 8 in the figure). ***$p<0.001$ (One-way ANOVA, $n=4$)

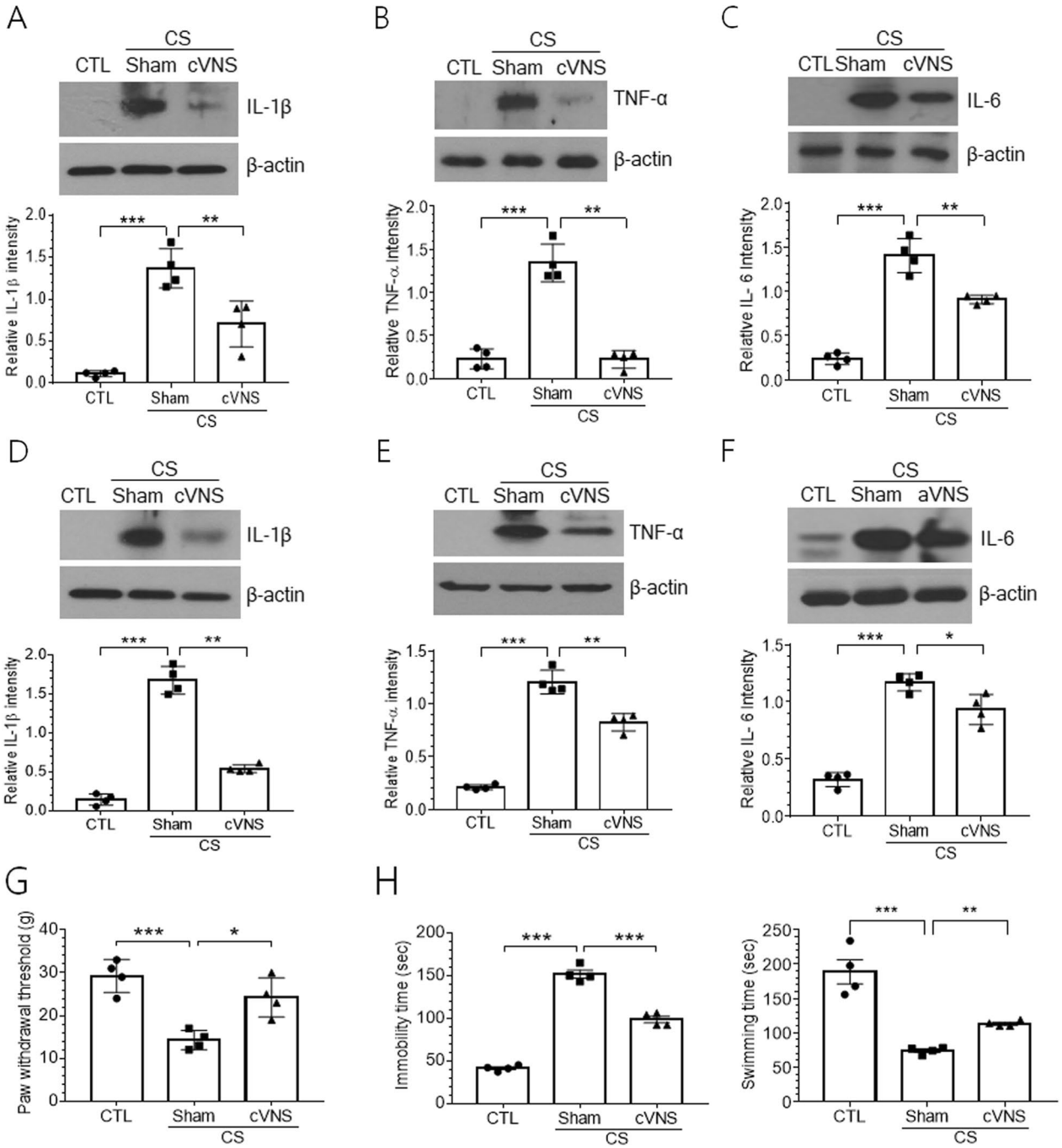

Fig. 8 Regulation of gastric and hippocampal inflammation by cVNS is related to behavioral effects. Rats were subjected to CS (5 days) and subsequently cVNS (7 days), and 24 h after the last VNS, CS+cVNS group, along with CS+Sham and CTL groups, were used for von Frey test (**G**) and forced swimming test (**H**). Animals were then sacrificed and protein was extracted from the gastric tissue (**A**–**C**) and the hippocampus (**D**–**F**) for Western blot analysis. **A**–**F** Western blot analysis. Upper images are the representatives of four independent experiments for IL-1β, TNF-α and IL-6 proteins in the gastric tissue (**A**–**C**) and in the hippocampal tissue (**D**–**F**). Quantification of protein band intensity relative to actin is plotted in lower panels. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (One-way ANOVA). **G** Von Frey hair test. Values of withdrawal threshold to stimulations on the left hind limbs were measured 40 times and averaged for individual animals. **H** Forced swimming test. Duration of immobility and swimming were analyzed for 5 min period for individual animals. In **G** and **H**, mean behavioral data collected from four different animals were compared among experimental groups. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (One-way ANOVA)

the swimming time was significantly improved by cVNS (Fig. 8H).

We further examined the production of inflammatory cytokines in the hippocampal subfields by immunofluorescence staining. Both IL-1β and TNF-α proteins were highly induced by CS in granule cells in the dentate gyrus and CA3 and CA1 pyramidal cells, and the signal intensity was decreased by cVNS (Fig. 9A, B).

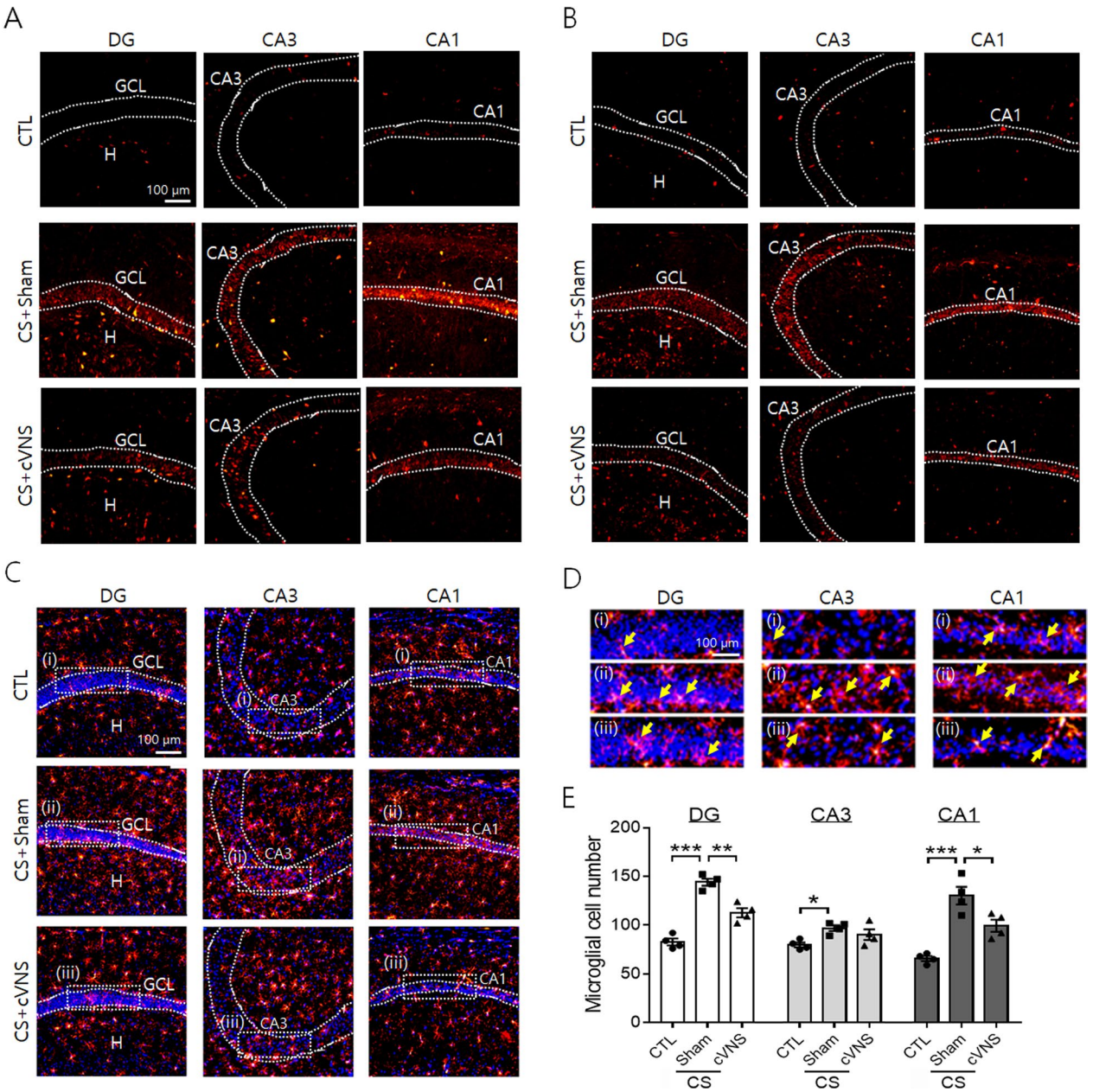

Fig. 9 Immunofluorescence localization of IL-1β, TNF-α and Iba-1 proteins in hippocampal subfields after cVNS in CS animals. In **A** and **B** images showing IL-1β and TNF-α protein signals (in red), respectively, borders of granule cell and CA3 and CA1 pyramidal cell layers were demarcated after merging with Hoechst nuclear-stained images (not shown). **C**, **D** Immunofluorescence view of Iba-1-labeled microglial cells (red) in the hippocampus. Hoechst-stained nuclei demarcated the neuronal cell layers (dotted lines). Images in **D** are the enlarged view of rectangle areas in (**C**). Yellow arrows indicate Iba-1-labeled microglial cells in the granule cell layer (GCL) in dentate gyrus (DG) and CA3 and CA1 pyramidal regions. **E** Quantification for the number of the microglial cells in the microscopic fields (4.3 × 4.3 μm^2) in the areas of dentate gyrus (DG) and CA3 and CA1 hippocampal formation. Cell numbers in the microscopic fields were averaged by counting 8 to 10 nonconsecutive sections per hippocampal tissue and the mean data from 4 hippocampal preparations were compared among experimental groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (One-way ANOVA)

Immunofluorescence-labeling of microglial cells also revealed clear increases in cell number in the hippocampal regions of dentate gyrus and CA3 and CA1 subfields after CS (Fig. 9C, E). In CS animals, Iba-1-labeled cells were frequently found within or around the border of hippocampal cell layers. For instance, microglial cells were detected in the inner part of the granule cell layer, and they were also seen within the CA3 and CA1 pyramidal cell layers (yellow arrows in Fig. 9D). The density of Iba-1-labeled microglial cells was reduced in the subfields of dentate gyrus and CA1, but not in CA3 area (Fig. 9E). Microglial cells were found less in the granule cell layer and CA1 pyramidal cell layer in CS+cVNS compared to CS+Sham group (Fig. 9D).

Discussion

Previous studies have reported that microglial cells are activated in the spinal cord of CS animals and related to chronic muscle pain [2]. Here, we provide evidence that CS increases the production of inflammatory cytokines in the hippocampus and an application of VNS either in an acute or a chronic form attenuates inflammation and improves pain response and depression-like behavior in CS animals. Pharmacological blockade of α7 nAChR abrogated the suppressive effects of VNS on the production of inflammatory cytokines, suggesting the requirement of cholinergic nerve activity for anti-inflammation.

A multiple continuous stress model was originally developed to study gastric ulcer, ischemia, and dysfunction in gastric motility [29, 30]. In addition, CS animals show alterations in endocrine function and chronic pain [4, 31]. Having noted that abnormal regulation of gastrointestinal function in CS animals can affect brain function and the vagus nerve acts as a bridge connecting between gut and brain (brain–gut axis) [7], we explored the potential regulatory function of vagus nerve activity in CS-related neuroinflammation and neurological abnormalities. We found that CS consistently increased levels of TNF-α, IL-1β, and IL-6 in the gastric and hippocampal tissues and induced morphological changes of microglial cells. Also, the number of microglial cells were increased in the hippocampal subfields including granule cell layer and CA3 and CA1 pyramidal cell layers, suggesting CS-mediated induction of the proliferation and migration activities of microglial cells. In several neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), and prion disease, neuroinflammation in brain tissues is noted with dystrophic morphology of microglial cells [32, 33], and here the microglial cells produce reactive oxygen species (ROS), phagocytic receptors, proinflammatory cytokines and others causing neurotoxic effects [34]. It is thus presumed that microglial cell may interact with neurons and contribute to neuroinflammation in CS animals.

The vagus nerve is composed of afferent and efferent components of which 80% account afferent fibers [35], implicating the significance of afferent vagus signaling in mediating brain–gut axis. Afferent vagus nerve activity is transmitted to raphe nucleus, brachial nucleus, locus coeruleus and ventral tegmental nucleus and further propagated into the forebrain areas [7, 16, 36]. Stimulation of visceral sensory fibers of the vagus nerve activates brain's reward system [16]. Moreover, preclinical and animal studies provide evidence that VNS can improve the symptoms of epilepsy, depression, and tinnitus [13, 14, 21, 37, 38], and also regulate neuroinflammation in the brain of animals injected with 6-OHDA and in LPS-injected animals for endotoxemia [39–41].

Here, we found that both acute and chronic forms of VNS downregulated the production of IL-1β, TNF-α and IL-6 in the hippocampus of CS animals and the cholinergic nerve activity was involved in VNS-modulated neuroinflammation. Cholinergic nerve fibers are projected into the hippocampus mainly through the medial septum-diagonal band of Broca and activate cholinergic receptors in target cells in the hippocampus [26–28]. Our data show that α7 nAChR proteins are expressed at moderate levels in the hippocampus of both control and CS animals and significantly increased by VNS. While our data showing the increased production of inflammatory cytokines by α7 nAChR antagonist MLA in CS+VNS animals indicate the requirement of α7 nAChR activity for VNS-mediated anti-inflammation, it is unknown at this moment how VNS-mediated activation of α7 nAChR is linked to the signaling pathway leading to decreased expression of inflammatory cytokines in hippocampal neurons. According to our data, inflammatory cytokine IL-1β was mostly induced from hippocampal neurons of CS animals and downregulated by VNS. It was reported that the activation of α7 nAChR in microglial cells was related to LPS-induced transition of microglial cell M1 and M2 [42]. Augmented cholinergic inputs could affect directly on microglial cells or indirectly through the postsynaptic neurons. Neuron–microglial communication is made through chemical mediators such as inflammatory cytokines, metalloproteases, and chemokine signaling and plays a determining role in neuronal survival [33, 43, 44]. It will be of great interest to determine whether VNS modulates the effects of fractalkine–CX3CR1 signaling for the regulation of microglial neurotoxicity in the hippocampus of CS animals [45]. Finally, VNS increased levels of ChAT enzymes in the hippocampus, implying the increased production of acetylcholine in the presynaptic terminal and possibly contributing to presynaptic facilitation. Coincidental activation for the synthesis

of presynaptic neurotransmitters and the postsynaptic receptors may facilitate the anti-inflammatory activity of VNS in target neurons in the hippocampus.

ME/CFS, representing the diverse abnormal pathophysiological processes in gastrointestinal, immune, and psychobiological functions, with high commodities, does not reveal clear etiology so far. One of the pathophysiological features of ME/CFS is a decrease in parasympathetic activity, showing reduced activation of α7 AChR [46]. It was also reported that the activation of α7 nAChR in microglial cells increases mitochondrial activity which is related to neuroprotection [47, 48]. This notion is consistent with our current data showing the role of VNS-induced α7 nAChR activity in regulating neuroinflammation.

Conclusion

This study demonstrates that VNS attenuates hippocampal neuroinflammation caused by CS in rats and improves pain sensitivity and depressive-like behavior. Given that CS animals partially show the symptoms associated with CFS [2], VNS may be considered as the possible therapeutic strategy for CFS known to involve complex neuroimmune activities, as neuroimmune communication is a key principle explaining VNS efficacy in the brain (e.g., neuron–microglial interaction) as well as in the visceral organs [49].

Abbreviations

CFS: Chronic fatigue syndrome; VNS: Vagus nerve stimulation; aVNS: Acute vagus nerve stimulation; cVNS: Chronic vagus nerve stimulation; CS: Continuous stress; α7nAChR: α7 nicotinic acetylcholine receptor; 5-HT1AR: 5-HT1A receptor; ChAT: Cholinergic acetyltransferase; DG: Dentate gyrus; GCL: Granule cell layer.

Acknowledgements

We thank Seung A. Jung for technical support. We are grateful to all members in the Neurophysiology Laboratory at Daejeon University for helpful discussion.

Authors' contributions

UN conceptualized, supervised, analyzed data, and wrote the manuscript. KK and BJ performed the experiments. JP supervised CS experiment and gastric tissue analysis. All authors read and approved the final manuscript.

Funding

This work was supported by the Basic Science Research Programs through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2018R1A6A1A03025221 and NRF-2016R1D1A1B01014190).

Availability of data and materials

All data generated and analyzed during this study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

All protocols involving live and postoperative animal care were approved by the Daejeon University Institutional Animal Use and Care Committee and were in accordance with the Animal-Use Statement and Ethics Committee Approval Statement for Animal Experiments provided by Daejeon University (Protocol Number: DJUARB2019-029, Daejeon, Korea).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Received: 25 July 2021 Accepted: 20 January 2022
Published: 2 February 2022

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Different Transcutaneous Auricular Vagus Nerve Stimulation Parameters Modulate the Anti-Inflammatory Effects on Lipopolysaccharide-Induced Acute Inflammation in Mice

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Abstract: Vagus nerve stimulation (VNS) is considered a potential method for anti-inflammation due to the involvement of the VN in the cholinergic anti-inflammatory pathway (CAP) formation of a connection between the central nervous system and peripheral immune cells that help relieve inflammation. However, whether a non-invasive transcutaneous auricular VNS (taVNS) modulates the inflammation levels via altering the parameter of taVNS is poorly understood. This study aimed to determine the differential inhibitory effects of taVNS on lipopolysaccharide (LPS)-induced systemic inflammation using electrical stimulation parameters such as pulse frequency and time. The taVNS-promoted CAP activity significantly recovered LPS-induced tissue injuries (lung, spleen, and intestine) and decreased inflammatory cytokine levels and tissue-infiltrated immune cells. Interestingly, the anti-inflammatory capacity of taVNS with 15 Hz was much higher than that of taVNS with 25 Hz. When a cytokine array was used to investigate the changes of inflammation and immune response-related cytokines/chemokines expression in taVNS with 15 Hz or 25 Hz treatment in LPS-induced endotoxemia in mice, most of the expression of cytokines/chemokines associated with pro-inflammation was severely decreased in taVNS with 15 Hz compared to 25 Hz. This study demonstrated that the taVNS parameter could differentially modulate the inflammation levels of animals, suggesting the importance of taVNS parameter selection for use in feasible interventions for acute inflammation treatment.

Keywords: transcutaneous auricular vagus nerve stimulation (taVNS); anti-inflammation; cytokines; electrical stimulation parameters; coronavirus disease 2019 (COVID-19)

Citation: Go, Y.-Y.; Ju, W.-M.; Lee, C.-M.; Chae, S.-W.; Song, J.-J. Different Transcutaneous Auricular Vagus Nerve Stimulation Parameters Modulate the Anti-Inflammatory Effects on Lipopolysaccharide-Induced Acute Inflammation in Mice. *Biomedicines* **2022**, *10*, 247. https://doi.org/10.3390/biomedicines10020247

Academic Editor: Carmela Matrone

Received: 6 December 2021
Accepted: 21 January 2022
Published: 24 January 2022

1. Introduction

The vagus nerve (VN) controls the parasympathetic nervous system as one of the cranial nerves [1]. Over 80% of afferent nerves in the VN mostly convey the body's sensory information to the central nervous system [2]. For example, once the VN detects any inflammatory process in the body, the efferent fibers of the VN activate postsynaptic excitatory potentials to modulate immune response via the α-7 nicotinic acetylcholine receptors (α7nAChR)-mediated pathway [3,4]. Acetylcholine from vagal efferent fibers interacts with α7nAChR in the immune cells like macrophages and dendritic cells of tissues, blocking the release of pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), IL-1β, and IL-8 [5,6]. These inflammatory reflex reactions reduce cytokine production and inhibit the body's systemic inflammatory response [5]. Considering the anti-inflammatory modulation of the VN, VN stimulation (VNS) is a medical tool for treating inflammatory-related diseases. Many researchers have evaluated

the effect of electrical stimulation of the VN on inflammatory disorders such as lung injury, sepsis, and rheumatoid arthritis [5,7–9].

An electrical stimulator of the VN was approved by the United States Food and Drug Administration to treat epilepsy in 1997 [10]. Early devices of VNS delivered the electrical impulses to the VN invasively. An electrode was surgically implanted into the left chest under the collarbone and then connected to the left VN of the patient [11,12]. More recent VNS devices are non-invasive and easily modulate VNS via the transcutaneous region such as the auricular region [13,14]. VNS devices were classically generated to treat drug-resistant diseases such as epilepsy or depressive disorder [10,13,15]. VNS provides a therapeutic intervention to avoid the side effects of chemical agents as a non-drug therapy.

Currently, the world is witnessing the coronavirus disease 2019 (COVID-19) pandemic [16,17]. COVID-19 often causes extreme immune reactions in the human body, which may lead to severe organ damage via an inflammatory cytokine storm [18,19]. Thus, preventing or modulating cytokine release is an important strategy to impede multi-tissue damage-related mortality in COVID-19. To reduce the excessive inflammatory cytokine levels in COVID-19, targeting α7nAChR activity via VNS can effectively control the further aggravation induced by the activation of the immune system. Several researchers also suggested that VNS could be a potential adjunct therapy for inflammatory disorders originating from COVID-19 [20–22]. In this study, we used a lipopolysaccharide (LPS)-induced endotoxin mice model to upregulate the expression levels of inflammatory factors. The anti-inflammatory effects of transcutaneous auricular VNS (taVNS) were observed in the lung, spleen, and intestines, which are innervated by the α7nAChR-mediated cholinergic anti-inflammatory pathway (CAP) of the VN [5,6,23]. We also determined that different combinations of electrical parameters such as the frequency and time of taVNS affected the expression levels of pro- and/or anti-inflammatory cytokines in the lung, spleen, intestine, and serum. We found that specific stimulation parameters of taVNS can be used to modulate the rate of inflammation in vivo.

The present study reveals the effectiveness of taVNS in reducing systemic inflammation and demonstrates the therapeutic potential of taVNS in the treatment of acute inflammation, to be considered for application in clinical trials for COVID-19 as an adjuvant therapy.

2. Materials and Methods

2.1. LPS-Induced Endotoxemia Mice Model

This study used 6–8-week-old male C57BL/6 mice that weighed 25~27 g (purchased from Orientbio Inc., Seongnam, Korea). All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Korea University and followed the animal ethics and welfare standards according to the IACUC guidelines. To prepare the LPS-induced endotoxemia model, mice were anesthetized by isoflurane. A single intraperitoneal injection of LPS (2 mg/kg, *Escherichia coli* 0111: B4; Sigma Aldrich, St. Louis, MO, USA) was conducted between stimulation with taVNS. Animals were sacrificed 2 h after LPS administration, based on previous studies [24,25] and our own preliminary analysis, and then serum and tissue samples were collected. All samples were stored at −80 °C until use.

2.2. Transcutaneous Auricular VNS

Two electrodes coated with a gold-plated platinum hook were placed on the auricular concha of the left ear (Figure 1A). Electrodes were connected to a stimulator (Neurive Inc., Seoul, Korea). Both the cymba and cavum conchae of the auricular were biphasically stimulated with the same parameter. The LPS was applied between two taVNS treatments with the indicated stimulation parameter in each group (Figure 1B,C). The charged-balanced biphasic waveforms during stimulation were shown in Figure 1D.

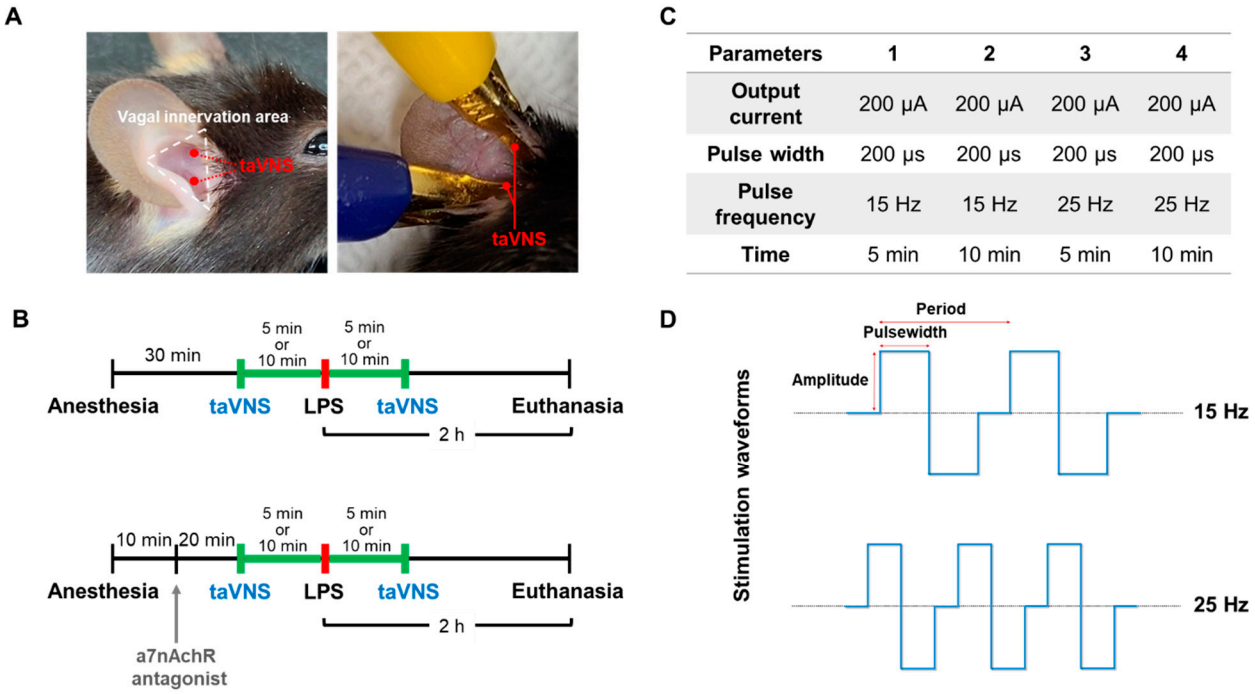

Figure 1. Experimental design and procedure of taVNS in the LPS-induced endotoxemia in mice. (**A**) Mice were bilaterally treated with taVNS, followed by the cymba and cavum concha of the vagus innervation area in the ear. (**B**) Under anesthesia, mice were stimulated with taVNS treatments for 5 or 10 min before and after LPS injection. The animals were euthanized after 2 h of LPS administration, and the whole blood and tissues were collected. (**C**) Four different taVNS parameters were used during stimulation in this study. (**D**) Schematic image showing the charge-balanced stimulation waveform of taVNS with low pulse frequency (**top**) and high pulse frequency (**bottom**).

2.3. Administration of α7nAChR Antagonist

Methyllycaconitine citrate (MLA) (Tocris Bioscience, Bristol, Avon, UK) was used for the α7nAChR intervention as a specific α7nAChR antagonist according to a previous method [26]. MLA was dissolved in phosphate-buffered saline (PBS) and then administered to mice intraperitoneally at a dose of 5 mg/kg before LPS injection (Figure 1B).

2.4. Enzyme-Linked Immunosorbent Assay (ELISA)

Serum and intestine tissues from mice were used to determine the levels of pro-inflammatory cytokines. The concentrations of TNF-α, IL-6, and IL-1β were analyzed by mouse-specific ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

2.5. Western Blotting

Total proteins of spleen tissues were lysed. An equal amount of protein was subjected to immunoblotting using antibodies against myeloperoxidase (MPO) (1:1000; Invitrogen, Carlsbad, CA, USA) and β-actin (1:2000; Santa Cruz Biotechnology, Dallas, TX, USA) for primary antibodies. The same amounts of proteins were electrophoresed on SDS-PAGE and then transferred onto PVDF membranes (Millipore, Darmstadt, Germany). Blocked membranes with 5% skim milk were incubated with primary antibodies overnight at 4 °C. The next day, the membranes were incubated with a secondary antibody (HRP-goat anti-rabbit IgG antibody, 1:3000, Invitrogen) for 1 h at room temperature. Images were subsequently captured using a Fusion Solo Imaging System (Vilber Lourmat, Marne-la-Vallée, France). Immunoreactive protein bands were quantified using ImageJ software.

2.6. Hematoxylin and Eosin Staining

At sacrifice, lung and intestine tissues were removed from the mice, fixed in 4% paraformaldehyde, embedded in paraffin, and sliced into 5-μm sections using a rotary microtome (Leica RM2255, Weztlar, Germany). For hematoxylin and eosin (H&E) staining, the deparaffinized tissue section was incubated in hematoxylin solution (Sigma) for 5 min and Eosin-Y solution (Sigma) for 1 min with tap water washing. Images were taken using Olympus BX43 microscope (Olympus Co., Tokyo, Japan). Inflammatory cell accumulation in the alveolar space, interalveolar septum thickening, alveolar congestion, alveolar hemorrhage, and cellular hyperplasia were considered for lung injury scoring: nil, 0; mild, 1; moderate, 2; severe, 3 [27]. Morphological examination with these five pathological features was performed in blind analysis by two examiners. The villus height was determined by the vertical distance from the crypt opening to the tip of the villus. The crypt depth was defined from the base of the level of the crypt opening.

2.7. Myeloperoxidase Staining

The deparaffinized 5-μm sections were incubated in MPO antibody (1:1000, Invitrogen) for 30 min at room temperature and washed with PBS. They were then incubated with a secondary antibody (1:1000, peroxidase-labeled goat anti-rabbit IgG) for 30 min. After the final wash with PBS, diaminobenzidine (DAB; DAKO, Santa Clara, CA, USA) was applied on the slide to detect the bound antibody. Hematoxylin was then used to evaluate the presence of neutrophils, as described previously [28].

2.8. RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from lung and spleen tissues using TRIzol™ reagent (Invitrogen) to analyze the relative gene expression. Then, PrimeSript™ first strand cDNA Synthesis Kit (Takara Bio, Tokyo, Japan) was used for the reverse transcription of 1 μg of RNA to 20 μL of cDNA, according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed using the obtained cDNA as a template with the Power® SYBR Green PCR Master Mix kit (Life Technologies Co. Ltd., Woolston, UK). The relative expression levels of *TNF-α*, *IL-6*, *IL-1β*, *IL-8*, *TGF-β*, and *IL-10*, were calculated by the $2^{(-\Delta\Delta Ct)}$ method with normalization to *β-actin*. The specific primer sequences used in this study were: *TNF-α*, 5′-CCC CAA AGG GAT GAG AAG TT-3′ (forward) and 5′-CAC TTG GTG GTT TGC TAC GA-3′ (reverse); *IL-6*, 5′-CCG GAG AGG AGA CTT CAC AG-3′ (forward) and 5′-CAG AAT TGC CAT TGC ACA AC-3′ (reverse); *IL-1β*, 5′-TCG CAG CAG CAC ATC AAC AAG-3′ (forward) and 5′-CAT GTC CTC ATC CTG GAA G-3′ (reverse); *IL-8*, 5′-CCC GCG TTA GTC TGG TGT AT-3′ (forward) and 5′-AAC AGC CCA TAG TGG AGT GG-3′ (reverse); *TGF-β*, 5′-TTG CTT CAG CTC CAC AGA GA-3′ (forward) and 5′-TGG TTG TAG AGG GCA AGG AC-3′ (reverse); *IL-10*, 5′-ATG CAG GAC TTT AAG GGT TAC TTG-3′ (forward) and 5′-AGA CAC CTT GGT CTT GGA GCT TA-3′ (reverse); *β-actin*, 5′-AGC CAT GTA CGT AGC CAT CC (forward) and 5′-CTC TCA GCT GTG GTG GTG AA-3′ (reverse).

2.9. Cytokine Array

To analyze the inflammatory-related cytokines, chemokines, growth factors, and angiogenic markers in serum samples, the concentration and purity of the isolated proteins were determined using the BCA protein assay kit (Pierce, Rockford, IL, USA) and UV spectrum. The antibody array slide (RayBiotech, Norcross, GA, USA, #L308) consisted of 308 nitrocellulose membrane kits to detect 308 mouse proteins in duplicated capture antibodies with positive and negative control antibodies. Briefly, the array slide was blocked with 400 μL of blocking solution for 30 min and incubated with samples for 2 h at room temperature. After being washed with the manufacturer-supplied buffers, the membranes were immersed in biotin-conjugated anti-cytokine antibodies and then incubated for 2 h with gentle shaking. Subsequently, Cy3-conjugated streptavidin solution was added to generate the chemiluminescent signals at each spot in the membrane. The fluorescence signal intensity was measured using GenePix 4100A microarray scanner (Axon

Instrument, San Jose, CA, USA) within 24–48 h at 10-μm resolution, optimal laser power, and photomultiplier (PMT). The quantified scan images with GenePix software (Axon Instrument, San Jose, CA, USA) calculated the average signal of the duplicate spots and then normalized it to the control spot signals. The protein information for data mining was annotated using UniProt DB. Graphic visualization was used in ExDEGA software (Ebiogen Inc., Seoul, Korea).

2.10. Statistical Analysis

All data were obtained from triplicate experiments and have been expressed as the mean ± standard deviation. A student's two-tailed *t*-test and one-way analysis of variance with Prism 5 software (GraphPad, San Diego, CA, USA) were used. The *p*-values are shown in the figures, and the differences were considered statistically significant at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3. Results

3.1. taVNS Reduced the Expression Levels of Pro-Inflammatory Cytokines in the Serum of the LPS-Induced Inflammation

The pro-inflammatory cytokines were rapidly evoked in the LPS-induced endotoxemia group. After systemic inflammation via LPS administration, TNF-α and IL-1β highly increased in serum. Electrical stimulation with taVNS significantly inhibited the expression levels of TNF-α and IL-1β, indicating excellent anti-inflammatory efficacy of taVNS (Figure 2A). We also analyzed the CAP-mediated inhibitory effect of taVNS on systemic inflammation using LPS application pre-treatment with the α7nAChR antagonist, MLA. A reverse increase in TNF-α and IL-1β was detected in MLA-treated group with taVNS on LPS-induced inflammation compared to the MLA-untreated group (LPS + taVNS) (Figure 2A). These results indicate that the decrease in pro-inflammatory cytokines levels by taVNS was weakened by inhibiting α7nAChR using MLA. MLA treatment slightly promoted the levels of pro-inflammatory cytokines compared with LPS injection alone, but not significantly. These ELISA results demonstrated that taVNS reduced the expression levels of pro-inflammatory cytokines in LPS-induced systemic inflammation via activation of α7nAChR.

We found two studies that have reported that specific VNS parameters can affect the cytokine levels in serum [24,29]. It is unclear whether the parameters of taVNS, specifically pulse frequency and time, differentially affect the inflammatory cytokine expression in the serum of the endotoxin model. To address this, we delivered taVNS with 15 and 25 Hz for 5 and 10 min to LPS-induced endotoxemia individually (n = 5–10). Other parameters, including the pulse width and amplitude, were not changed. The electrical stimulation videos of taVNS with 15 Hz and 25 Hz were represented in Supplementary video, respectively. All experimental groups with taVNS in LPS-induced inflammation significantly decreased the serum TNF-α and IL-1β levels compared to the non-taVNS group in LPS induction (Figure 2B). In particular, taVNS stimulation with low pulse frequency (15 Hz) produced a significant inhibitory effect on serum TNF-α and IL-1β compared to the high pulse frequency (25 Hz) regardless of the time parameter. These results demonstrate that taVNS had anti-inflammatory effects via activation of α7nAChR on LPS endotoxemia, and pulse frequency of taVNS can be an important parameter for the regulation of pro-inflammatory cytokines levels in serum.

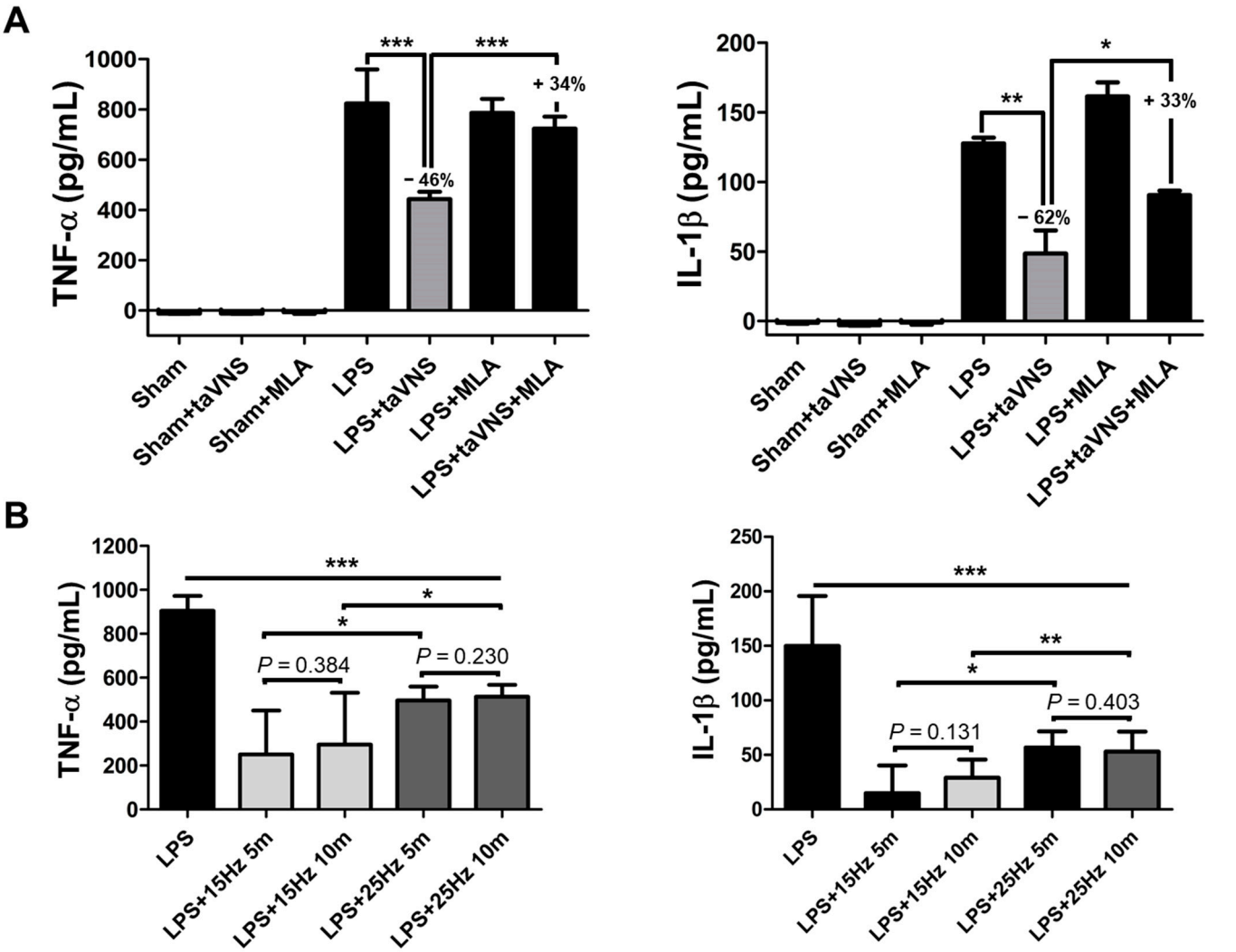

Figure 2. A decrease in pro-inflammatory cytokine releases by taVNS in LPS-induced endotoxemia. (**A**) Mice were treated with taVNS and/or MLA treatment for acute inflammation from LPS. The expression levels of serum TNF-α and IL-1β were measured by ELISA. The decrease and recovery percentages of each cytokine were represented as a number. (**B**) The expression levels of serum TNF-α and IL-1β were determined using ELISA pre- and post-treatment of taVNS with different pulse frequency and time parameters. Data have been presented as the means and SD; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, compared to the corresponding control.

3.2. Anti-Inflammatory Effect of taVNS on Spleen Tissue of LPS-Induced Inflammation

Since the concept of CAP from the Tracey group in 2000, this theory has been confirmed in multilevel organs such as the spleen, lung, and gut [5]. These organs are regulated by efferent fibers of the VN when inflammation is evoked, following which the vago-parasympathetic reflex activates and then targets these multi-organs of α7nAChRs for anti-inflammation [6,23,30]. Therefore, we investigated whether taVNS stimulated the vagal anti-inflammatory effect of the spleen, lung, and intestine. The swelling of LPS-induced spleens was relieved by treatment of taVNS as the LPS-untreated groups (Sham) (Figure 3A). The rate of MPO expression of the spleen in LPS-induced endotoxemia increased (four to five-fold) compared to the sham groups. Intensive expression with MPO on the spleens of the LPS-induced inflammation indicated an increase in leukocyte extravasation to the spleen during the inflammatory condition. However, it significantly reduced the expression levels of MPO on the spleen of LPS + taVNS treated mice in a pulse frequency-dependent manner (Figure 3B). The qRT-PCR analysis showed that taVNS decreased the relative

expression of genes such as *TNF-a*, *IL-1β*, *IL-6*, and *IL-8*, which have a stimulatory role in inflammation (Figure 3C), compared to the only LPS-treated group. The expression levels of these pro-inflammatory cytokine genes were significantly decreased when taVNS treatment was applied to LPS-induced endotoxemia regardless of the taVNS parameter. Compared to the taVNS-treated groups with different parameters, taVNS treatment with 15 Hz displayed a more significant decrease in the mRNA levels of *TNF-a*, *IL-1β*, *IL-6*, and *IL-8* than taVNS treatment with 25 Hz. The taVNS with 15 Hz_5 min treatment group showed lower expression levels of *TNF-a* and *IL-6* than the taVNS with 15 Hz_10 min treatment group, but not the taVNS with 25 Hz treatment group. The relative expression of anti-inflammatory cytokine mRNA, including *IL-10* and *TGF-β* encoding genes, was also determined using different pulse frequencies of taVNS. The significantly decreased *IL-10* and *TGF-β* gene expressions were evaluated in the taVNS-treated groups, except for taVNS with 25 Hz_10 min in *TGF-β* gene expression. These results demonstrated that taVNS significantly reduced the inflammatory reaction of the spleen in LPS-induced endotoxemia, and the pulse frequency of taVNS is capable of regulating the expression levels of inflammatory cytokine genes in the spleen.

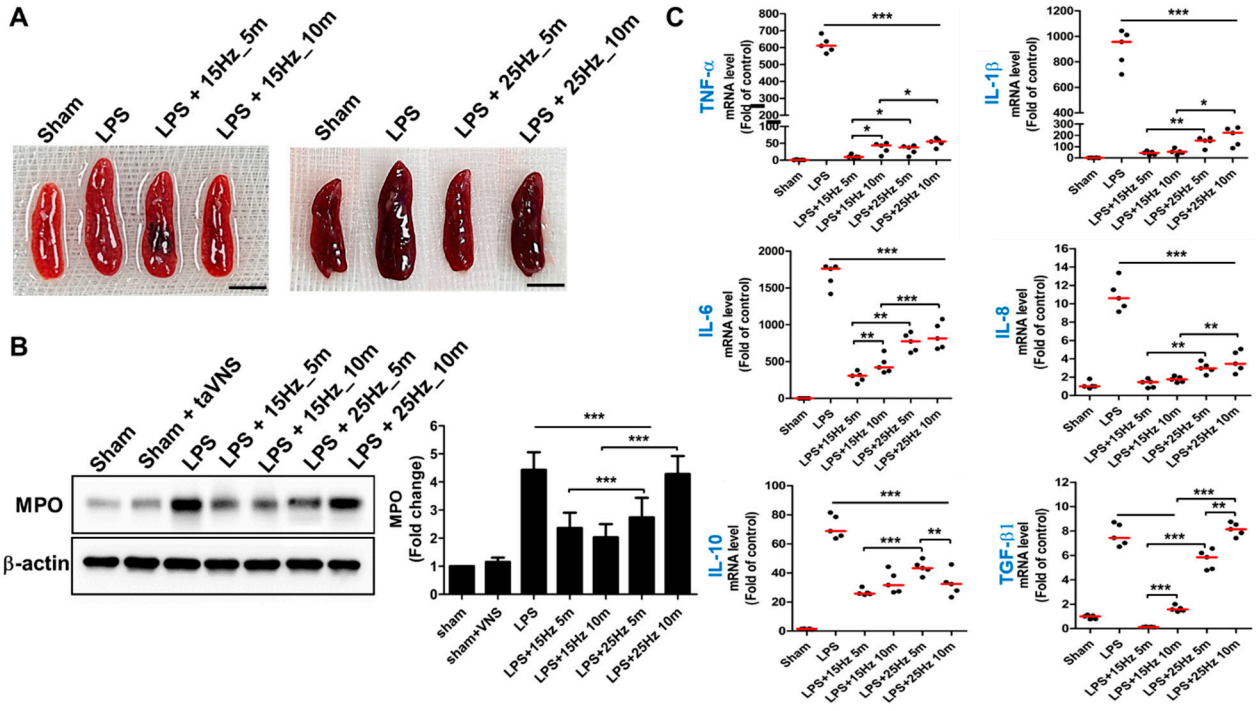

Figure 3. taVNS reduced inflammation of the spleen in LPS-induced endotoxemia. (**A**) Representative images of the spleen after treatment of taVNS with different pulse frequencies and time parameters. Scale bar: 5 mm. (**B**) Western blot analysis using an antibody against MPO was used to analyze the relative expression levels of neutrophils in the indicated stimulation conditions of taVNS on LPS-induced inflammation. The intensity ratios for MPO were presented as a graph using ImageJ. (**C**) The mRNA levels of pro- and anti-inflammatory cytokines were determined by qPCR. All results have been presented as the means and SD; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, compared to the control.

3.3. Anti-Inflammatory Effect of taVNS on Lung Injury of LPS-Induced Endotoxemia

Next, we validated whether taVNS reversed the inflammatory response of the lung in LPS-induced endotoxemia. The representative images of the lung after taVNS treatment on the LPS endotoxemia model show the morphological difference between the LPS and LPS with taVNS groups (upper panel of Figure 4A). H&E staining also determined a significant anti-inflammatory effect of taVNS on LPS-induced damage in mice lungs, compared to only LPS-exposed mice (lower panel of Figure 4A, Supplementary Figures S1 and S2).

The congested alveolar wall was distinguishable, and the edema phenomenon of the interalveolar septum was decreased in the LPS + taVNS group, compared to the Sham and LPS-treated group. Next, MPO stain was used in this experiment to evaluate inflammatory cell infiltration. As shown in Figure 4B, positive staining of MPO was highly observed in the lung of LPS-induced endotoxemia, but upon treatment with taVNS, there was a remarkable reduction in the strong expression of MPO of lung injury by LPS. The proportion of MPO-positive stained area significantly decreased and disappeared in taVNS-treated groups compared to only LPS-induced group (Figure 3B). The lung injury score and coverage rate of MPO stain in the lung are presented in Figure 4C. The relative expression levels of pro- and anti-inflammatory cytokines were also analyzed in treatment with or without taVNS on LPS-induced lung injury. Consistent with the qRT-PCR spleen results, all groups with taVNS showed significant alleviation of pro-inflammatory cytokine expression in lungs after taVNS treatment. Above all, treatment of taVNS with 15 Hz caused a significant decrease in the expression of *IL-1β* and *IL-6* encoding genes compared with 25 Hz, but the fold change increase or decrease in the taVNS-treated group with different time parameters (5 min and 10 min) was not observed in the same parameter of taVNS (Figure 4D). These results showed the protection and recovery effects of taVNS on LPS-induced lung injury.

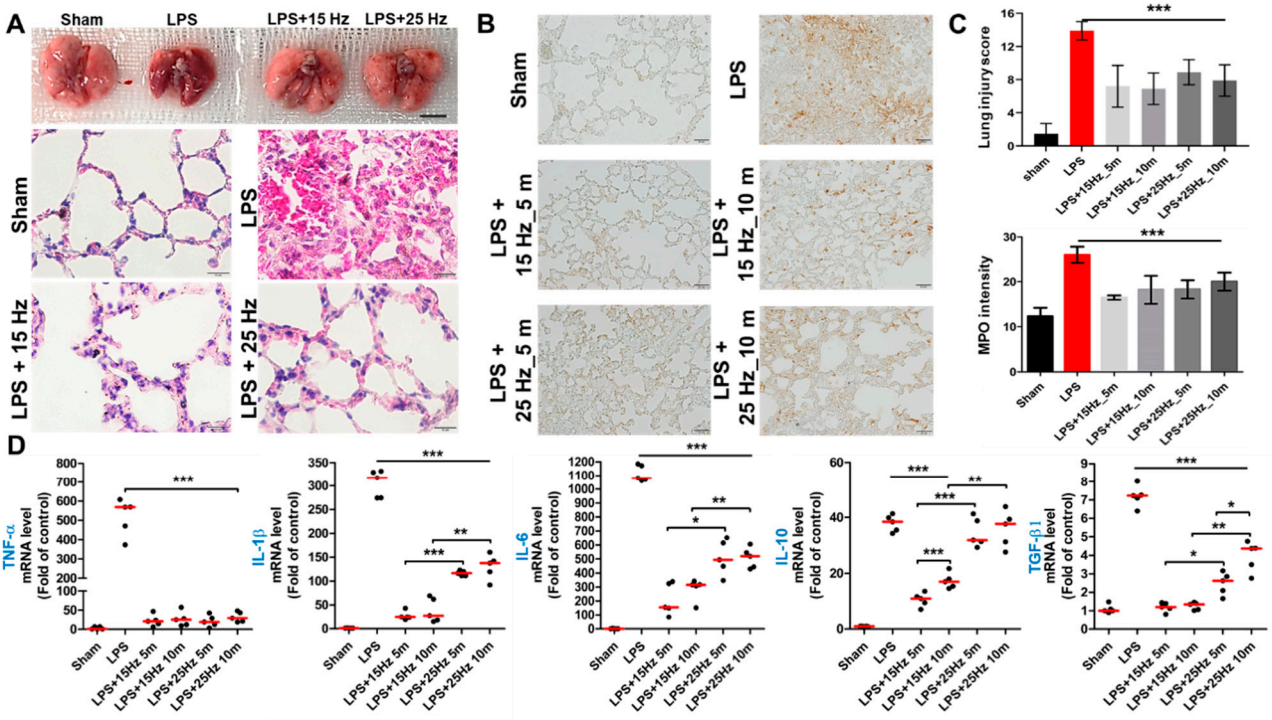

Figure 4. Anti-inflammatory effect of taVNS on the lung of LPS-induced endotoxemia. (**A**) The representative images of the lung after treatment of taVNS with 15 Hz and 25 Hz. Scale bar: 5 mm. The results of H&E staining were determined using the lung injury score upon observation under a light microscope. Scale bar: 10 μm. (**B**) MPO staining was determined by the infiltrated immune cells on the lung. Scale bar: 20 μm. (**C**) The relative scoring of lung injuries was compared as a graph in three independent images. Positively stained area was measured in three different images in each group and then represented on a graph. (**D**) The expression levels of pro- and anti-inflammatory cytokines genes were determined by qPCR, and significance was compared among groups. Data have been presented as the means and standard deviation (n = 5–10); * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, as compared to the corresponding control.

3.4. Anti-Inflammatory Effect of taVNS on Intestinal Inflammation Induced by LPS

Regarding the anti-inflammatory effect of taVNS on the intestine, the pro-inflammatory cytokine levels of intestines were determined by ELISA and indicated a significant down-

regulation of TNF-α, IL-6, and IL-1β compared with the only LPS-treated group (Figure 5A). Different expression levels of these pro-inflammatory cytokines were observed in the taVNS with 15 Hz and 25 Hz groups (decrease rate in the 15 Hz_10 min treated group, −65% (TNF-α), −85% (IL-6), −38% (IL-1β); 25 Hz_10 min group, 22% (TNF-α), −10% (IL-6), −15% (IL-1β). Particularly, taVNS with 15 Hz showed some variation compared to 25 Hz. This may imply that the stimulation condition of the taVNS with 15 Hz group was unstable.

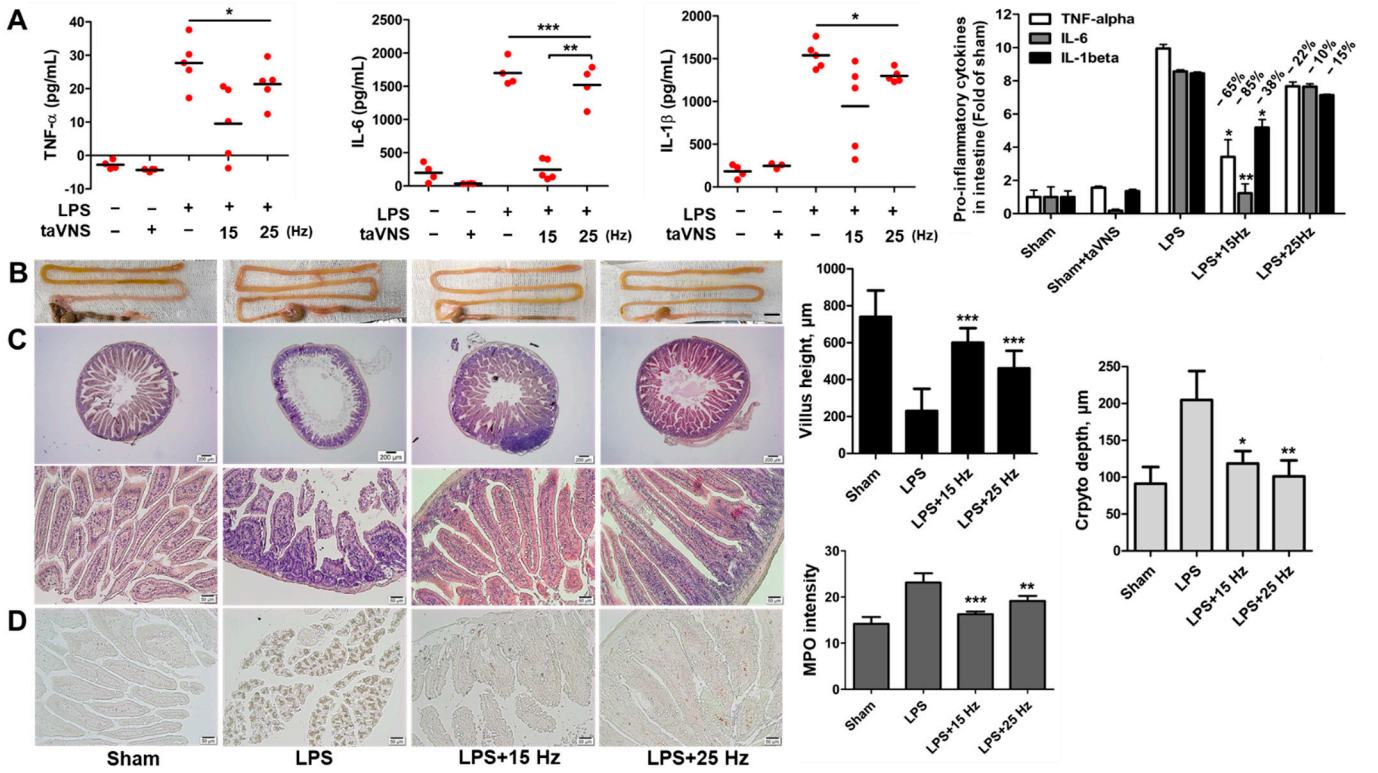

Figure 5. taVNS induced anti-inflammatory effect on the intestine of LPS-induced endotoxemia. (**A**) Determination of the pro-inflammatory cytokines in the intestine of LPS-induced inflammation with or without taVNS treatment. The relative expression levels of pro-inflammatory cytokines in response to taVNS treatment of LPS-induced endotoxemia were indicated. Fold change of groups was represented as a graph and calculated decreases rate in taVNS treated groups. (**B**) The morphological changes of the intestine were represented. Scale bar: 10 mm. (**C**) H&E staining showed the histological change of the intestine upon observation under a light microscope. Scale bar: 200 μm (upper panels) and 50 μm (lower panels). The villus height and crypto depth of the intestine were determined and represented by a graph. (**D**) Immunohistochemical staining with anti-MPO antibodies showed a significant decrease in neutrophils in LPS-induced inflammation by interventions as of taVNS. Scale bar: 50 μm. All taVNS groups were treated with electrical stimulation for 10 min. Data have been presented as the means and SD; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, compared to the corresponding control.

The intestine in LPS-induced endotoxemia was swollen and longer compared to the untreated group. The treatment of taVNS on LPS-induced inflammation reduced these morphological changes in the LPS-treated group (Figure 5B). To assess the protective capacity of taVNS on intestine injury via LPS, histological evaluation was performed using H&E and MPO stain in LPS-induced intestine with taVNS 15 Hz and/or 25 Hz (Figure 5C). Histological scores and MPO intensity-graph of the intestine showed the effective anti-inflammatory capacity of taVNS. Moreover, a difference in the recovery efficacy of taVNS between 15 Hz and 25 Hz on acute inflammation was also observed in mice gut.

3.5. Electrical Frequency of the taVNS Parameter Regulated Inflammatory Cytokines in the Serum of LPS-Induced Endotoxemia

We next investigated the whole inflammatory molecule level changes triggered at two different pulse frequency parameters upon taVNS treatment of the LPS-induced endotoxemia to analyze the different anti-inflammatory effects of taVNS on systemic inflammation. The heatmap image shows the differentially expressed 50 cytokines/chemokines related to inflammation and immune response between LPS-induced inflammation treated with or without taVNS. taVNS downregulated most of the cytokines/chemokines activated by LPS induction (Figure 6A). taVNS with 15 Hz suppressed the cytokines/chemokines evaluated values more severely than taVNS with 25 Hz. The scatter plot analysis also presents the differential downregulation of the upregulated cytokines/chemokines by LPS between taVNS with 15 Hz and 25 Hz, as shown by the distribution of cytokines/chemokines dots (Figure 6B). Of these 50 cytokines, the pixel intensity of TNF-α, IL-6, IL-1β, TGF-β1, TLR4, and IL-10 and fold change of chemokines (CCL/CXCL) and interleukins (IL) presented in Figure 6C and Supplementary Figure S3 also indicate differential modulation of inflammatory cytokine levels through different taVNS pulse frequencies. The pixel intensity of 34 significantly downregulated chemokine/cytokines at 15 Hz compared to 25 Hz is presented in Supplementary Figure S4. These results imply that taVNS differentially improved systemic inflammation via modulation of the expression levels of cytokines/chemokines using changes in the pulse frequency parameter of taVNS.

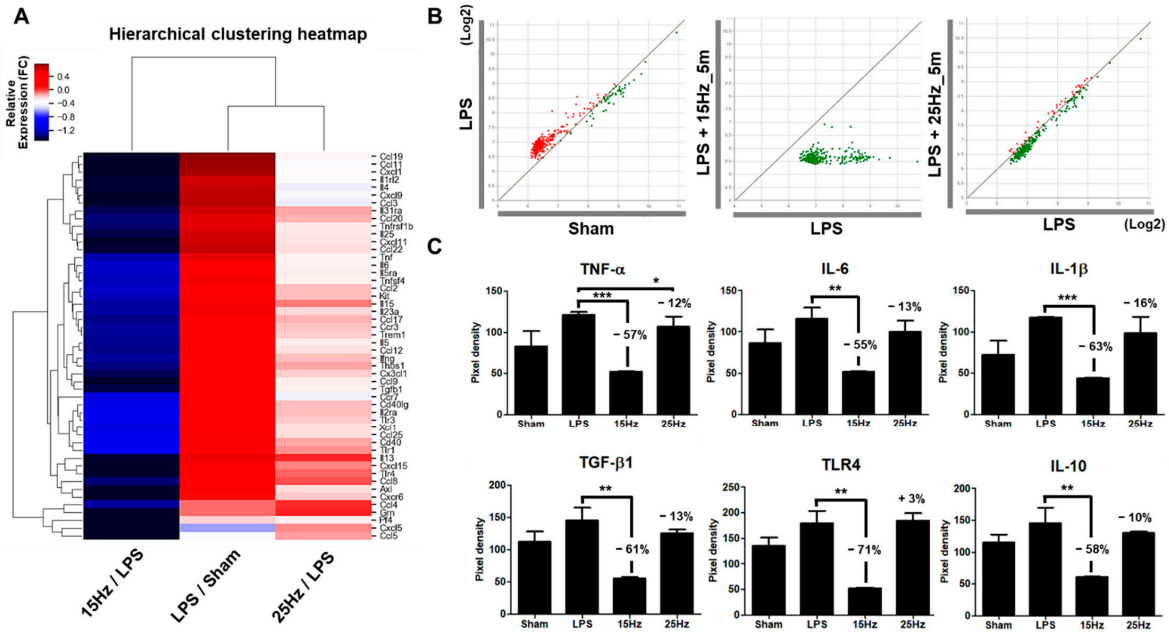

Figure 6. Changes in inflammatory cytokines and chemokines in the plasma of LPS-induced endotoxemia with taVNS. (**A**) Most cytokines and chemokines were upregulated by LPS injection (LPS/Sham). Both taVNS groups (15 Hz and 25 Hz) attenuated the expression levels of cytokines and chemokines in serum. taVNS with 15 Hz was more significantly reduced than with 25 Hz. Clustering software generated a heatmap. The color depicts the levels of fold change. (**B**) Scatter plot analysis showed the expression changes of cytokines and chemokines for the LPS-induced inflammation verse sham (**left**), taVNS with 15 Hz on the LPS-induced inflammation versus the LPS-induced inflammation (**middle**), and taVNS with 25 Hz on the LPS-induced inflammation versus the LPS-induced inflammation (**right**). Upregulation is presented as red dots and downregulation as green dots. (**C**) The representative pixel density of pro- and anti-inflammatory-related cytokines, namely TNF-α, IL-6, IL-1β, TGF-β1, TLR4, and IL-10 in the serum of the sham, LPS applied groups and taVNS with 15 Hz or 25 Hz on LPS-induced groups. Data have been presented as the means and SD; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ to determine the significance levels compared to the LPS group.

4. Discussion

VNS has three main fibers: A-, B-, and C-fibers, which can be delivered by sensory afferent and motor efferent signals to regulate vital functions in the body's autonomic nervous system [31]. These nerve fibers of the VN innervate different physiological changes via combined or individual fiber form [32]. For example, the activation of A- and B-fibers of the VN is associated with anti-inflammatory effects, while C-fibers activation is involved in triggering cardioinhibitory effects [24,33]. Each fiber of the VN has distinct stimulation thresholds for activation because of its different axon diameters, conduction velocity, and myelination [34,35]. Typically, the higher the stimulation current levels, the smaller nerve fiber activation in the peripheral nervous system occurs [36]. The increasing pulse width and amplitude also selectively activate small diameter nerve fibers while inhibiting the activation of large diameter fibers [37]. This means that the bioelectric stimulation parameter can differentially modulate the activation of VN fibers and consequently exhibit desired physiological effects [32]. The most important goal of taVNS research is the selective and efficient nerve activation using taVNS parameters, such as pulse frequency, duration, amplitude, time, and electrical current rate, to apply as a therapeutic tool. However, it has been poorly defined, and the stimulation parameter of taVNS for neuromodulation therapy needs to be optimized. We first tested how different pulse frequencies and times of taVNS affect the anti-inflammatory effects on VN innervated tissues and serum using an LPS-induced endotoxemia model. taVNS effectively reduced LPS-induced inflammation, as indicated by a decrease in pro-inflammatory cytokines expression, histopathological scores, and leukocyte infiltration. These anti-inflammatory effects of taVNS were changed by the stimulation parameter of pulse frequency and time. Among them, the result of the cytokine array showed the most obvious difference between 15 Hz and 25 Hz of taVNS during inhibition of the LPS-induced inflammation. taVNS with 15 Hz severely downregulated the levels of cytokines and chemokines in serum, whereas taVNS with 25 Hz did not. These findings indicate that the rate of inflammatory cytokine production can be modulated by regulating the pulse frequency of taVNS in various inflammatory conditions.

In 2020, Piruzyan et al. showed that electrical stimulation with a high-frequency pulse current more effectively suppressed the excessive production of inflammatory cytokines than a low-frequency pulse current [38]. However, this report included different electrical stimulation systems and anti-inflammatory mechanisms without stimulation of the VN. Another recent study supports our results that low pulsing frequency selectively provided the optimal intensity range to activate the A- and B-fibers of the VN [39]. The low stimulation threshold of A- and B-VN fibers need to achieve the activating vagal anti-inflammatory pathway that distinguishes the C-fiber of the VN to regulate the heart rate [24]. The electrical stimulation condition of taVNS with 15 Hz may be more effective in the activation of CAP through A- and B-fibers of vagal signaling than a higher pulse frequency of taVNS. This does not mean that taVNS with 15 Hz is optimal for regulating inflammation because proper inflammation response is necessary to defend the body against infection. Tsaava et al. suggested that different pulse widths, duration, and amplitude may play an important role in the modulation of inflammatory-related cytokines via the VN [29]. However, our taVNS equipment could not regulate other parameters except Hz and time, which was a limitation of this study. The taVNS parameters need to be fine-tuned in the future.

The fibers in the cervical VN consist of a mixed formation, which co-activates side effect-inducing fibers [40]. It has been known that the inhibitory effect of intestinal inflammation was supposed to be related to vagal C-fibers with a high stimulation threshold but typically also activated other fibers in the cervical VN [41,42]. The selective activation of fibers in the VN is essential to the treatment of distinct diseases if VNS is used as a treatment method [43]. Compared with cervical VNS, taVNS is easy to apply for therapy on ears. In addition to convenience, taVNS is a safe method because it indirectly regulates the VN without directly connecting with the vagal fibers. Thus, taVNS has not been reported to cause cardiac dysfunction [44].

Inflammation is a protective reaction of the host against exogenous pathogens, stress, and injury that must control and balance the body's immune system [43]. Excessive inflammatory response due to autoimmunity or uncontrolled inflammatory pathway of host cells leads to several inflammatory diseases such as rheumatoid arthritis, atopic dermatitis, and chronic inflammation in humans [45,46]. COVID-19 also causes serious inflammatory responses and many chemical drug therapies have been trialed such as corticosteroids, tocilizumab, IL-6 inhibitor, and intravenous immunoglobulin. However, specific approaches for COVID-19 are currently lacking [16,47–51]. The anti-inflammatory role of VNS can specifically reduce overproduced inflammatory cytokine levels via CAP activation. Two case studies of using VNS treatment for COVID-19 have highlighted its potential clinical benefit in treating patients with COVID-19 [21,52]. taVNS is a non-invasive and safe therapy. Therefore, VNS may be considered as a supplement treatment if large clinical trials prove its efficacy in treating patients with COVID-19.

5. Conclusions

We developed a stimulating electrode system of transcutaneous auricular VN and determined the effective anti-inflammation capacity of taVNS. Different taVNS pulse frequency parameters differentially modulated the rate of inflammation injuries on the spleen, lung, and gut, regulation of inflammatory-related cytokines expression, and chemokines levels. taVNS appears to be an effective therapeutic tool against inflammation disorders in humans following optimization of the taVNS parameters.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biomedicines10020247/s1, Video S1: taVNS 15 Hz and 25 Hz treated mice. Figure S1: Histological evaluation of the lung in taVNS 15 Hz for 5 min and 25 Hz for 5 min on LPS-induced endotoxemia. Figure S2: Histological evaluation of the lung in taVNS 15 Hz for 10 min and 25 Hz for 10 min on LPS-induced endotoxemia. Figure S3: Fold change of CCL/CXCL/IL in 15 Hz and 25 Hz taVNS on endotoxemia mice. Figure S4: The pixel intensity of chemokines in 15 Hz and 25 Hz taVNS on endotoxemia mice.

Author Contributions: Y.-Y.G. and J.-J.S. designed the experiments; Y.-Y.G., W.-M.J. and C.-M.L. performed the experiments; Y.-Y.G. and J.-J.S. analyzed and interpreted the data; S.-W.C. and J.-J.S. provided the facilities; Y.-Y.G. and J.-J.S. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Korea Medical Device Development Fund grant funded by the Korea government (the Ministry of Science and ICT, the Ministry of Trade, Industry and Energy, the Ministry of Health & Welfare, the Ministry of Food and Drug safety) (Project Number: 1711138343, KMDF_PR_20200901_0168).

Institutional Review Board Statement: This study was conducted in accordance with Korea University guidelines for animal experiments after approval by the institutional Animal Care and Use Committee (IACUC) of Korea University (approval number, Korea-2021-0015 approved on 27 January 2021).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are all contained within the article.

Acknowledgments: We thank Chol Hyuk from the School of Medical Science at Korea University for assisting in the taVNS stimulation waveforms analysis. We also thank and research director Guk-han Kim from Neurive Institute for providing taVNS equipment.

Conflicts of Interest: The authors declare no conflict of interest.

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Review

Manipulation of the inflammatory reflex as a therapeutic strategy

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https://doi.org/10.1016/j.xcrm.2022.100696

SUMMARY

The cholinergic anti-inflammatory pathway is the efferent arm of the inflammatory reflex, a neural circuit through which the CNS can modulate peripheral immune responses. Signals communicated via the vagus and splenic nerves use acetylcholine, produced by Choline acetyltransferase (ChAT)+ T cells, to downregulate the inflammatory actions of macrophages expressing α7 nicotinic receptors. Pre-clinical studies using transgenic animals, cholinergic agonists, vagotomy, and vagus nerve stimulation have demonstrated this pathway's role and therapeutic potential in numerous inflammatory diseases. In this review, we summarize what is understood about the inflammatory reflex. We also demonstrate how pre-clinical findings are being translated into promising clinical trials, and we draw particular attention to innovative bioelectronic methods of harnessing the cholinergic anti-inflammatory pathway for clinical use.

INTRODUCTION

By the end of the 20th century, the key influence of the central nervous system (CNS) on modulating our systemic inflammatory response was well recognized. Cytokines released by immune cells in response to pathogens have the capacity to transmit signals across the blood-brain barrier (BBB) through a variety of mechanisms, including stimulation of the afferent (sensory) vagus nerve.[1,2] This stimulates a reciprocal response via the hypothalamic-pituitary-adrenal (HPA) axis.[1]

Building on these initial observations, researchers found that the CNS transmits efferent signals more directly via neural circuits, specifically the efferent vagus nerve of the parasympathetic nervous system, to exert a systemic anti-inflammatory effect.[3] They termed this the cholinergic anti-inflammatory pathway (CAP) after the acetylcholine-mediated effects of the vagus nerve. The combination of the afferent and efferent arms of this vagal-immune interaction is termed the "inflammatory reflex."[4] These seminal observations led to the proposed concept of harnessing the systemic anti-inflammatory activity of the efferent arm of the vagus nerve as a therapeutic platform targeting chronic inflammatory diseases.[5,6]

The aim of this review is to highlight the historical evidence that supports the concept of harnessing the potential of the parasympathetic nervous system as a complementary anti-inflammatory therapy. In addition, we will describe more recent work translating these observations into the clinical trials arena. In particular, we will highlight the exciting advances in the realm of bioelectronics as potential non-pharmacological therapies.

THE INFLAMMATORY REFLEX

Kevin Tracey and colleagues made the seminal observation that acetylcholine (ACh) and nicotine attenuated pro-inflammatory actions of macrophages. ACh is the key parasympathetic system neurotransmitter. This led to the hypothesis that these ACh-mediated anti-inflammatory effects were mediated via peripheral nicotinic (rather than muscarinic) receptors (nAChR).[3] The cholinergic vagus nerve is the mediator of the parasympathetic nervous system. *In vivo,* transection of this nerve (vagotomy) in rats subjected to LPS-induced endotoxemia led to a more aggressive systemic inflammatory response, characterized by earlier onset of shock and higher serum and liver levels of the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α), typically released from macrophages.[3] Electrical stimulation of the distal arm of the transected vagus attenuated this response.[3,7] These findings revealed that the vagus nerve, previously thought only to be activated in response to peripheral inflammation,[1] was also capable of modulating the inflammatory response through its efferent projections, the now-called cholinergic anti-inflammatory pathway (CAP).[4]

While these initial observations were important, they did not explain the full story. Further animal studies found that splenectomy and transection of the splenic nerve abolished the effects of vagus nerve stimulation (VNS) on systemic TNF-α released in response to endotoxemia and polymicrobial sepsis[8–10] In other words, the vagus nerve modulated the TNF-α response of nAChR-positive splenic macrophages through signals transmitted via the splenic nerve. Specifically, the α7nAChR subtype was responsible for the anti-inflammatory effects of ACh, as

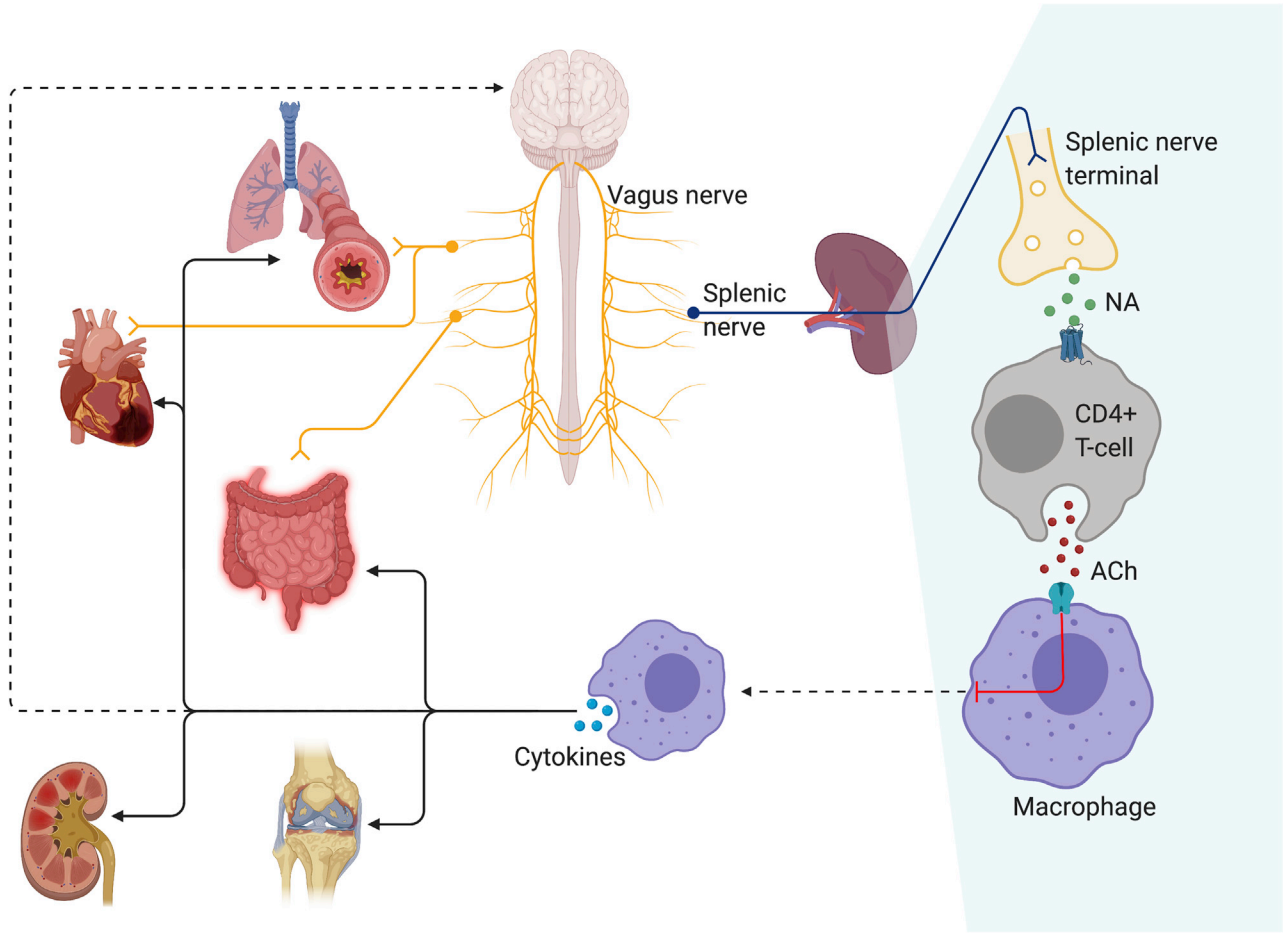

Figure 1. The cholinergic anti-inflammatory pathway

Through the inhibition of splenic macrophages, the vagus nerve attenuates inflammatory responses in multiple bodily systems, including the lungs, GIT, myocardium, synovia, and kidneys. The vagus nerve may also mediate some of its effects directly through innervation of viscera (e.g., lungs, heart, GIT). Suppression of the systemic inflammatory response can likewise influence neuroinflammation.

ACH, acetylcholine; NA, noradrenaline.

demonstrated in α7nAChR knockout (KO) mice.[8,9,11] The splenic nerve is believed to synapse not with macrophages directly (splenic neurons are catecholaminergic, not cholinergic[12]), but instead with choline acetyltransferase-positive (ChAT+), β2-adrenergic-receptor-positive (β2AR+) T cells, which release non-neuronal ACh in response to noradrenaline signaling. Nude mice, devoid of functional lymphocytes, are insensitive to the anti-inflammatory effects of VNS. ChAT+ T cells have been identified at synapses with splenic nerve terminals and are necessary for VNS inhibition of endotoxin-induced TNF-α release.[13] Furthermore, a series of experiments by Vida and colleagues confirmed β2AR-expressing lymphocytes to be crucial for VNS-induced anti-inflammatory activity.[14] Nude mice and β2AR KO mice were insensitive to VNS, but the effect was restored by the transferring of β2AR+ T cells into these animal models. The transfer of β2AR KO lymphocytes into nude mice did not restore the effect of VNS. The findings of Rosas-Ballina[13] and Vida[14] in combination identify β2AR+, ChAT+ lymphocytes as an essential mediating step between the splenic nerve and macrophages, completing Tracey's model of the CAP as it is understood today (see Figure 1).[15]

It should be noted that alternative theories to the CAP have been proposed. The concept of a di-synaptic connection between the vagus and splenic nerve has been questioned.[16] This was based on the observation that VNS did not induce detectable action potentials in the splenic nerves of rats, and an anatomical connection could not be identified at a synaptic level.[17] This led to an alternative concept that the efferent arm of the inflammatory reflex is not the CAP, but rather the sympathetic nervous system.[17] Subsequent research demonstrated that action potentials were transmitted via the sympathetic chain and splanchnic nerves, in turn innervating the splenic nerve and ultimately inhibiting cytokine release.[16–18] Another group found that the anti-inflammatory effects of stress and activation of autonomic C1 neurons in the brainstem were not attenuated by subdiaphragmatic vagotomy.[19] It was similarly found that vagotomy did not exacerbate the effects of endotoxemia, whereas splanchnic neurotomy did.[18] However, a substantive body of

work including Borovikova and colleagues[3,20–22] has supported the importance of the CAP mediated via the splenic nerve and β2AR-expressing lymphocytes. Martelli and colleagues attributed this discrepancy to an interruption of the HPA axis. However, this failed to explain why stimulation of the distal vagus nerve (which was transected proximally and therefore incapable of transmitting an afferent signal to the hypothalamus) would result in suppression of splenic TNF release without any alteration of corticosterone levels.[3] Furthermore, the corticosterone antagonist mifepristone did not block the effects of the CAP.[19] Abe and colleagues showed that VNS could activate the CAP regardless of whether it was applied to the distal or proximal limb of the vagus,[23] while the contralateral vagus nerve was blocked with lignocaine, potentially suggesting the existence of a second efferent arm to the CAP that could be stimulated via the afferent vagus nerve.

Vida and colleagues[9] reported that suppression of serum TNF levels in a mouse model of systemic sepsis could also be achieved by splenic nerve stimulation (SNS), and while VNS was dependent on the α7nAChR, SNS was effective in α7nAChR KO mice. Their findings suggested that, although the α7nAChR is an essential component of the CAP at the level of vagus-to-splenic nerve signaling, splenic nerve-to-macrophage signaling may be possible through alternate, α7nAChR-independent mechanisms, though such a mechanism has yet to be identified. Further supporting these findings is the recent finding that while T cells certainly appear capable of forming synapse-like structures with splenic neurons,[24] and ChAT+ lymphocytes in the spleen are primarily concentrated in the white pulp where sympathetic are situated,[13] a synaptic connection with ChAT+ lymphocytes could not be identified on confocal microscopy.[25]

These findings suggest that the CAP model may be one of the additional pathways by which our nervous system modulated the systemic inflammatory response. For example, Murray and colleagues have proposed that splenic neurons may communicate with ChAT+ lymphocytes via neurotransmitter diffusion or chemotaxis through the CXCL13 chemokine,[25] which is upregulated by sympathetic activity, rather than through synaptic transmission. Detailed characterization of alternative or additional pathways within the inflammatory reflex will be essential to developing the therapeutic potential of this field, particularly if these pathways present additional therapeutic targets or help to explain treatment failure in a sub-group of patients. Nevertheless, it is our opinion that there is a convincing and growing wealth of evidence for targeting the vagus nerve and the α7nACh receptor as anti-inflammatory therapies.

As discussed above, the α7nAChR has been identified as the extracellular target of the CAP through KO studies.[8,9,11] The intracellular effects of α7nAChR activation, however, are numerous and not mutually exclusive. In non-neuronal cells, intracellular-signaling cascades are activated via ligand binding of intracellular molecules and tyrosine kinase-mediated increases in intracellular calcium, rather than by ion-channel opening, as seen in neurons.[26] In macrophages and monocytes, anti-inflammatory effects of VNS are mediated by the recruitment of the tyrosine kinase JAK2 to the α7nAChR and subsequent phosphorylation of STAT3, which blocks cytokine transcription by NF-κB.[27]

The JAK2/STAT3 pathway is not the only one implicated in intracellular signaling of the α7nAChR (see Figure 2). Other molecules have been implicated and may interact with STAT3 or act independently (see Figure 2). These include inhibition of mitogen-activated protein kinase (MAPK) pathways such as ERK1/2,[28] the activation of adenylyl cyclase (AC) 6, which in turn activates the cAMP-CREB-cFOS pathway,[29] signaling via heme-oxygenase 1[30,31] and heat shock protein (HSP)-70,[32] and the suppressed phosphorylation of IκB.[33] Through other pathways, α7nAChR may downregulate cell surface expression of NF-κB-inducing receptors CD14 and Toll-like receptor (TLR)-4[34,35] and enhance autophagic activity,[36–38] further contributing to the anti-inflammatory phenotype. ACh, which enters the cytoplasm during states of inflammation, can also act on mitochondrial α7nAChR, preventing the release of mtDNA and the activation of the NLRP3 inflammasome complex responsible for release of cytokines IL-1β and HMGB1.[39] α7nAChR activation promotes the expression of microRNA-124, which inhibits IL-6 and TNF-α release.[40] The proposed mechanisms of action of microRNA-124 include targeting of IκB and inhibition of TNF-α-converting enzyme (TACE), but also the *suppression* of STAT3, which was paradoxically found to be an essential mediator of IL-6 production.[40] Conflicting results of this type demonstrate that elements of this signaling cascade are still poorly understood and that there may be multiple intracellular pathways through which the α7nAChR can act. While the majority of "CAP-targeted" therapies to date are directed toward extracellular components such as the vagus nerve and α7nAChR, further investigation of downstream pathways will help to clarify the mechanism underlying the CAP and may present new therapeutic targets.

THE INFLAMMATORY REFLEX IN DISEASE

Inflammation is implicated in the pathogenesis of a broad range of human diseases. The demonstration of the significant systemic anti-inflammatory properties of the inflammatory neural reflex makes the CAP an attractive therapeutic target. This could potentially be harnessed pharmaceutically by targeting the α7nAChR or bioelectronically via both VNS and other methods such as splenic ultrasound (see Figure 3). Here, we review evidence on the role of the CAP and its therapeutic potential in inflammatory diseases.

Rheumatological disease

Rheumatoid arthritis (RA), a chronic inflammatory condition of synovial membranes, and related connective tissue disorders are the most studied with regard to the potential of exploiting the inflammatory reflex therapeutically. Extensive pre-clinical research over the past twenty years has led to more recent exploratory early clinical trials, resulting in an expanding body of work supporting this therapeutic strategy in connective tissue disorders.

Administration of nicotine,[41] selective α7nAChR-agonist AR-R17779,[42] and partial agonist GTS-21[43,44] mitigate joint swelling[42–44] and reduce both radiological[42,44] and histological[41] measures of bony erosion in animal models of collagen-induced arthritis (CIA). α7nAChR-selective antagonist methyllycaconitine

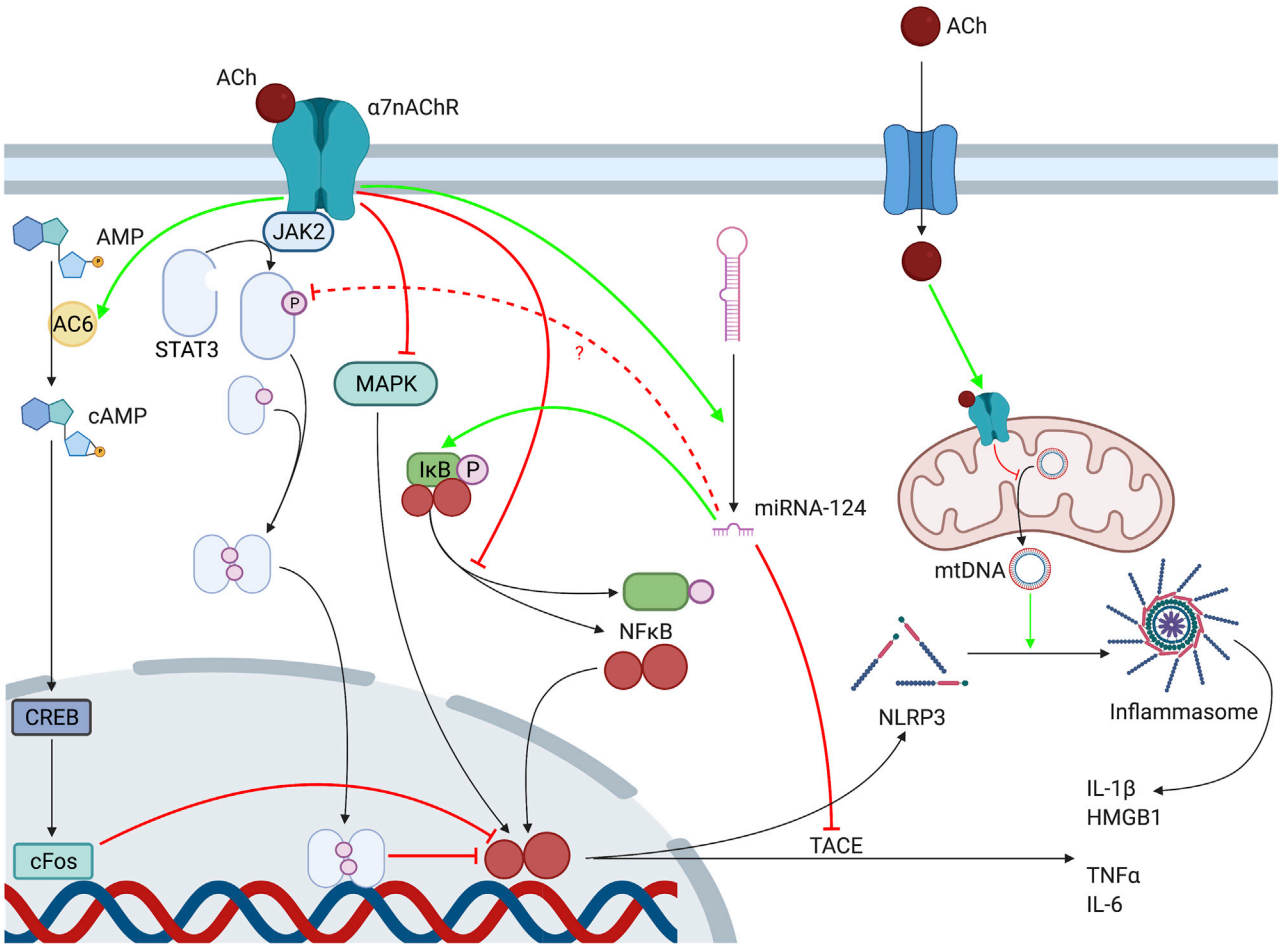

Figure 2. Proposed intracellular mechanisms of the α7nAChR

AC6, adenylyl cyclase 6; Ach, acetylcholine; AMP, adenosine monophosphate; cAMP, cyclic adenosine monophosphate; CREB, cAMP response element-binding protein; JAK2, Janus kinase 2; MAPK, mitogen-activated protein kinase; miRNA, microRNA; NF-κB, nuclear factor κ-B; NLRP3, NOD-, LRR-, and pyrin domain-containing protein; STAT3, signal transducer and activator of transcription 3.

(MLA) attenuated the effect of GTS-21.[43] These clinical and radiological findings are associated with a significant fall in systemic pro-inflammatory cytokines findings (e.g., TNF-α,[41–44] IL-6,[41–43] and IL-1β[44]). Analysis of synovial fluid reveals a parallel fall in pro-inflammatory cytokines with a consequent reduction in joint inflammatory cells[42] and osteoclasts.[44]

The synovia of CIA mice treated with GTS-21 has significantly reduced expression of CD11c, a relatively specific marker of dendritic cells (DCs), known to be pathogenic in RA.[43] One potential mechanism for this is that GTS-21 inhibited the differentiation of bone marrow-derived DCs from progenitor cells *in vitro*, and this differentiation was inhibited by MLA.[43]

Nicotine treatment results in lower levels of synovial Th17 cells, a subset of $CD4^+$ lymphocytes believed to promote inflammation in RA through secretion of IL-17a.[45] α7nAChR KO exacerbates CIA with greater levels of joint destruction, higher serum levels of TNF-α and chemokine MCP-1, and a shift to a higher ratio of inflammatory cytokine-producing Th1 cells to IL-10-producing Th2 cells.[46]

Overall, these studies demonstrate that the α7nAChR has anti-inflammatory and potentially disease-modifying effects on RA. A range of cells including macrophages, fibroblasts, T cells, and B cells expressing these receptors can be found in the synovia of patients with RA.[47] Though it remains to be determined whether infiltration of these cells is actually stimulated directly by ACh or by α7nAChR-independent mechanisms downstream of macrophage activation and cytokine release, or whether α7nAChR expression is purely a secondary marker of inflammatory cell activation.

The vagus nerve has been implicated in RA. Vagotomy was associated with greater levels of synovial neutrophil infiltration and hyperalgesia in a mouse model of antigen-induced arthritis.[21] Two studies demonstrated that the disease-exacerbating effects of vagotomy were attenuated by α7nAChR stimulation.[42,48] While these effects only trended toward statistical significance, the studies used unilateral vagotomy solely, which may, because of compensation from the contralateral nerve, have more modest effects than that of bilateral vagotomy seen in other disease models.[20] Furthermore, VNS has demonstrated

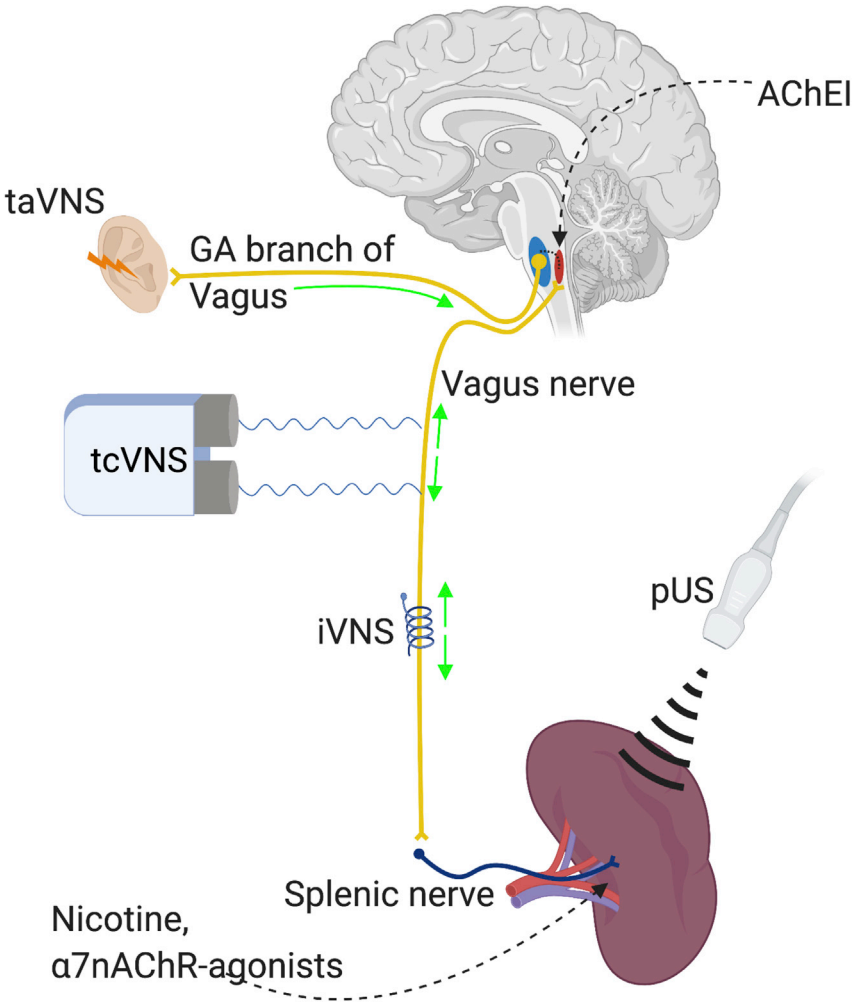

Figure 3. Stimulation of the CAP

The CAP can be stimulated pharmacologically through centrally acting acetylcholinesterase inhibitors (AChEI) and peripherally acting nicotine or α7nAChR agnoists. Non-pharmacological stimulation is achieved through invasive and non-invasive VNS or pUS. iVNS, invasive vagus nerve stimulation; taVNS, transauricular vagus nerve stimulation; tcVNS, transcervical Vagus nerve stimulation.

efficacy in the treatment of CIA in at least two studies, one using electrical stimulation[49] and the other mechanical.[50] Together, these studies reveal that VNS inhibits joint inflammation and destruction[49,50] and release of inflammatory cytokines.[50] These studies led to a clinical trial of VNS in RA patients.[51] Eighteen RA patients were implanted with VNS and experienced a rapid improvement in disease severity as measured by the internationally validated disease activity score-28 (DAS28). This DAS is derived from the assessment of 28 specific joints by a healthcare professional combined with both a patient self-reported score for health and well-being and input of blood systemic inflammatory markers (e.g., ESR or CRP). In addition, a corresponding reduction in TNF-α release on lipopolysaccharide (LPS)-whole blood culture was observed, a measure of the systemic pro-inflammatory state, in which blood samples from participants are exposed to an inflammatory trigger *ex vivo*. When VNS was suspended for two weeks, symptoms relapsed but were once again attenuated on reactivation of the stimulator. Recently a sham-controlled pilot study assessed the safety of an implantable VNS device in RA patients but was underpowered to assess clinical efficacy. However, it is worth noting that 5 of 10 patients (seven blinded, three unblinded) in the treatment arm demonstrated a clinically significant change in disease severity score, versus zero out of four sham controls.[52] Neither of these trials reported serious adverse events, though a high proportion (89% in the former,[51] 57% in the latter;[52] 60% of in the treatment arm and 50% of controls) reported mild to moderate side effects. Many adverse events are attributable to procedural complications (i.e., secondary to the surgery itself), but cough and hoarseness are well recognized adverse effects of device activation. In epilepsy, these effects are generally well tolerated by patients and do not limit treatment compliance.[53] Large-scale placebo-controlled trials are now required to definitively address the therapeutic efficacy of this treatment strategy.

Gastrointestinal disease

Inflammatory bowel disease (IBD) encompasses ulcerative colitis (UC) and Crohn's disease (CD), disorders characterized by inflammation of the gastrointestinal tract (GIT). This inflammation is largely mediated by the activation of macrophages and cytokine pathways including TNF-α. The emergence of anti-TNF biologic therapies has transformed the treatment of IBD, particularly CD,[54] but neither are without risk nor universally effective. Heart-rate variability (HRV) is reduced in IBD,[55,56] implying a disruption of vagal tone, which, as discussed in the introduction, can be associated with greater inflammatory activity and represents an attractive target for new therapies.

Evidence for the importance of the inflammatory reflex in IBD comes from *in vivo* studies. α7nAChR KO mice demonstrate more severe responses to dextran sulfate sodium (DSS)-induced colitis, with more severe symptoms, higher disease activity scores, higher tissue and serum levels of cytokines (IL-1β, IL-6, IL-18, and TNF-α), and higher mortality rates,[38,57] though not all studies confirm this finding.[58] α7nAChR-selective agonist PNU282987[59] and partial agonist encenicline[60] (the effects of which are significantly attenuated by MLA) inhibit the development of DSS-induced colitis, reflected by less histological damage,[59,60] less macrophage infiltration,[59,60] and lower tissue levels of cytokines.[59] Of note, other studies have found α7nAChR activation to be ineffective once DSS-induced colitis is established (i.e., if agonists are administered three days after

DSS).[60,61] Similar benefits to α7nAChR agonism have been demonstrated in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis, a model considered more representative of CD than UC.[60,62] However, an earlier study found two agonists (AR-R17779 and GSK1345038A) to be ineffective in TNBS- and DSS-colitis; low doses were paradoxically harmful whereas high doses were protective.[63] The authors theorized that higher agonist doses might have α7nAChR-independent effects. This hypothesis requires replication and further investigation.

Vagotomy was found to be associated with a 50% greater risk of IBD in a recent epidemiological study including over 15,000 vagotomized patients and more than 600,000 age-matched controls.[64] A series of comprehensive experiments by one research group further demonstrated this phenomenon. Galantamine, an acetylcholinesterase inhibitor (AChEI), has demonstrated promising results in preventing the induction of TNBS-[65] and DSS-induced colitis,[66] an effect mediated by central muscarinic (mAChR) activation of the CAP[65] and dependent on the vagus nerve and α7nAChR. Vagotomy leads to an increase in colitis severity and tissue cytokine levels in mouse models of DSS- and dinitrobenzene sulfonic acid (DNBS)-induced colitis.[22] Similar effects were seen following splenic denervation and splenectomy.[66] However, VNS, which has demonstrated efficacy in models of IBD,[67] has also been shown to reduce bowel inflammation independently of splenic innervation, and instead via cholinergic stimulation of α7nAChR-expressing resident macrophages in the gut.[68] This pathway has recently been termed the "enteric-CAP."[69] Thus, the vagus nerve once again appears key to the α7nAChR-mediated effects in IBD. Further research has suggested that the vagus nerve is capable of inducing α7nAChR-independent anti-colitic effects, such as by the recruitment of regulatory T cells (Tregs).[58]

To date, there have been few published clinical trials of cholinergic agonists in IBD. Studies have shown that transdermal nicotine provides clinically meaningful benefits as an adjuvant therapy in active UC.[1] These trials were based on the hypothesis that nicotine is the causative agent behind the inverse relationship between smoking and UC risk and predate discovery of the CAP. The effect of nicotine on UC remains unexplained, and while it is tempting to imagine a mechanistic role for the α7nAChR between nicotine and UC, such a hypothesis would require further and more specific investigation. It is also important to note that smoking has the opposite relationship with CD, and some have even proposed that nicotine may be responsible for this effect via immunomodulatory mechanisms, though this lacks convincing evidence.[70] Semapimod (a.k.a. CNI-1493), a small molecule that centrally activates the CAP,[7] failed to reduce severity in CD patients over placebo after three doses. It did demonstrate intra-patient improvements after repeated dosing in an open-label continuation trial, but a high rate of infusion site reactions limited its tolerability.[71] Large-scale trials are required to more accurately define responders while limiting systemic side effects in this patient population.

Because such off-site effects may prevent the delivery of α7nAChR agonists at the doses required for clinical benefit, efforts have therefore been made to target the CAP more directly using VNS. Two small open-label trials,[72] one published in abstract form only,[73] have trialed VNS in a total of 23 CD patients, reporting clinically meaningful improvements in clinical and endoscopic disease scores. However, a relatively high number (9–11) of cases experienced worsening of disease. Again, more substantive clinical trial data is required to evaluate the efficacy and safety of VNS in IBD.

Lung disease

In a mouse model of acid-induced acute lung injury,[74] markers of inflammation, including excess lung water, lung vascular permeability, and bronchoalveolar lavage levels of leukocytes, were significantly reduced by the administration of nicotine, choline, and α7nAChR-selective agonist PNU-282987. These acid-induced effects were enhanced in α7nAChR KO mice. Leukocytes expressed higher cytoplasmic levels of NF-κB, and this effect was abrogated by treatment with nicotine. Vagotomy exacerbates the inflammatory response in animal models of ventilator-induced lung injury (VILI).[20] Stimulation of the inflammatory reflex using α7nAChR agonists[20,74,75] or VNS[20,75] mitigated these inflammatory responses. α7nAChR agonist GTS-21 proved effective in reducing both organ injury and inflammatory markers in radiation-induced lung injury.[76] Recently, neostigmine, an AChEI, demonstrated efficacy in a model of allergic asthma. This effect was associated with an increased expression of α7nAChR in the lungs.[77]

These studies present a strong case for the role of the CAP in lung inflammation and justify further study of the vagus nerve and its effects in the lungs. However, the relationship between the vagus nerve and the lungs may be more complex than that seen in inflammatory diseases of other organs. Activation of mAChR has bronchoconstrictive and possibly pro-inflammatory effects in the lungs.[78] The use of beta-adrenergic agonism and muscarinic antagonism in the symptomatic treatment of COPD and asthma is based on this very concept. On the other hand, activation of other α7nAChR+ airway mucosa cells (e.g., type 2 innate lymphoid cells [ILC2s] and pulmonary neuroendocrine cells [PNECs]) downregulates the production of pro-inflammatory cytokines.[79] It is possible that vagal release of ACh, which is capable of activating not only the α7nAChR but also other ACh receptors, in the airway mucosa could activate both pro- and anti-inflammatory processes. There are therefore two important clinical questions: how do these two pathways behave together *in vivo*, and does the balance tip toward a pro- or anti-inflammatory endpoint when there is an increase in vagal tone?

One study found that VNS inhibited LPS-induced TNF production in cardiac and hepatic tissues, but not in the lungs.[80] However, other studies[20,75] have found VNS to be protective against VILI and to impair anti-inflammatory processes in the lungs including IL-6 release.[20] So, while evidence is variable, there is an increasing body of work supporting the hypothesis that vagus nerve activity mitigates inflammation in the lungs as it does in other organs, though whether this effect results solely from the spleen-mediated CAP or is also influenced by direct parasympathetic innervation of the airways warrants further investigation.

α7nAChR are expressed by various cells within lung mucosa.[81] The downstream effects of α7nAChR activation in lung tissue are heterogeneous, complex, and remain to be fully elucidated but are important to consider when targeting the α7nAChR for therapeutic purposes. It has been postulated on the basis of

in vitro studies and some animal models that α7nAChR activation may increase the metastatic potential of lung cancers[81] and fibrogenesis in pulmonary fibrosis.[80,82] However, these hypotheses lack clinical evidence to date. In fact, vagotomy has associated with a higher risk of lung and other cancers in pre-clinical and human epidemiological studies.[79] Therefore, the CAP may actually have anti-cancer properties.

While there are some conflicting studies and unanswered questions, the trend of available evidence indicates that the CAP has therapeutic potential in lung disease, warranting further pre-clinical and clinical study. Other inflammatory lung diseases such as sarcoidosis, in which TNF-α-release from macrophages is central to the pathogenesis,[83] also present tempting targets for future interventions.

CNS disease

Underlying inflammation forms the basis for the pathogenesis of many CNS diseases. These include not only the classic "inflammatory" disorders such as encephalitis and multiple sclerosis (MS), but also degenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) and psychiatric disorders including depression and schizophrenia. The CNS immune system, which was once considered immune-privileged, operates somewhat independently, but not totally isolated, from the peripheral system. The BBB, under normal physiological conditions, limits the influence of peripheral immune cells on the CNS. A specialized subset of macrophages known as microglia govern innate immunity.[84] The α7nAChR appears to play an important role in modulating the neuroinflammatory response in CNS diseases; the clinical use of VNS in epilepsy and depression are therapeutic examples of this. However, as we will demonstrate, the intricacies of the CNS immune system add an extra layer of complexity to its relationship with the CAP.

ACh is a ubiquitous and multifunctional neurotransmitter of the CNS, and α7nAChR are expressed abundantly on neuronal and non-neuronal cells, including microglia, astrocytes (CNS glial cells), BBB endothelial cells, and oligodendrocyte precursors (responsible for myelin production).[85] Microglia mediate inflammatory processes through the release of TNF-α, amongst other mechanisms, and are sensitive to the anti-inflammatory effects of α7nAChR activation,[85] presenting a suitable target for the CAP.

The risk of AD correlates with genetic variation in the α7nAChR.[86] α7nAChR KO mice exhibit enhanced depression-type behaviors,[87] and in a transgenic model of AD, experience fewer deficits and less neurodegeneration.[88] In an ischemic stroke model, α7nAChR KO confers smaller infarct size with corresponding preservation of neurological function.[89] One study found that α7nAChR KO was protective against experimental autoimmune encephalomyelitis (EAE),[90] the gold-standard rodent model of MS. Two other studies found that KO did not alter the EAE phenotype.[91,92] Though all three found that nicotine was protective against EAE, α7nAChR KO attenuated this protection.[90–92] The reason proposed for this discrepancy was that α7nAChR activation not only mediates the migration and activation of pathogenic Th1 and Th17 cells in EAE, but also the actions of antigen-presenting cells (APCs) within the CNS, which are necessary to trigger EAE.[90] Other research suggests that nicotine confers some of its anti-inflammatory effects through other nicotinic-receptor subtypes,[91] including α9nAChR.[90,93] Nevertheless, PNU-282987 has demonstrated clinical efficacy in EAE, improving clinical severity scores, reducing leukocyte infiltration into the CNS, and reducing mRNA expression of IL-6, IL-1β, IL-18, and TNF-α while also inducing autophagy by microglia and splenic macrophages. Thus, while α7nAChR may have a number of heterogeneous and possibly opposing functions, therapeutic strategies targeting the receptor are likely to be neuroprotective in EAE based on current evidence.

Pharmacological α7nAChR activation has also demonstrated therapeutic potential in models of other CNS disorders including AD,[94] PD,[95] schizophrenia,[96] ischemic stroke,[97] intracerebral haemorrhage,[98] LPS-induced anxiety and depression,[99] traumatic brain injury (TBI),[100] and cardiopulmonary bypass-induced brain injury.[101] AChEIs are licensed for use in improving cognition in AD. Historically, clinical efficacy of AChEIs was thought to be mediated via increasing synaptic concentrations of ACh and thus compensating for the loss of cholinergic neurons. However, it now appears likely that at least some of the clinical benefit is conferred through its anti-inflammatory effect.[102] That being said, at least three α7nAChR-selective agonists have been studied in clinical trials of human AD patients, but none have progressed past stage two, either because of adverse effects, insufficient clinical benefit, or without explanation.[103]

The only diseases in which VNS is currently licensed for clinical use are epilepsy, depression, and headache, fundamentally CNS disorders. There is emerging clinical evidence for efficacy in other CNS disorders including AD[53] and stroke.[104] VNS has proven to be very efficacious in reducing seizure frequency in treatment-refractory epilepsy,[105] though its mechanism of action is not understood. It was first used in epilepsy over 30 years ago, long predating characterization of the CAP, and was thought to directly inhibit the electrical activity of partial seizures. Soon thereafter, research showed that chronic intermittent stimulation induced long-term neural network changes, reducing seizure frequency.[106]

VNS demonstrates disease-modifying effects in pre-clinical models of schizophrenia by restoring normal neuronal activity and by reversing the hypersensitive amphetamine psychomotor response.[107] It has been shown to enhance the rate of recovery after established ischaemic[108] and haemorrhagic[109] stroke. These effects might be seen as neuroplastic rather than neuroprotective. Cholinergic circuits and microglia are believed to have important neuroplastic properties.[110] It is not clear from these studies that VNS exerts its effect through anti-inflammatory mechanisms or via the α7nAChR. One might expect that CNS inflammation would activate intrinsic cholinergic circuits and directly modulate inflammation without the unnecessary steps of involving the vagus nerve and spleen. Certainly, microglia appear susceptible to intrinsic cholinergic circuits with anti-inflammatory and neuroprotective effects.[109] However, there is evidence to support a role for VNS in neuroinflammation and that CAP suppression of systemic inflammation could mediate this.

VNS reduces CNS levels of inflammatory cytokines and other biomarkers in models of systemic endotoxaemia.[110,111] In the

Table 1. Clinical trials using electrical stimulation of the CAP

Treatment	Population	Reference	Findings
iVNS	rheumatoid arthritis	Koopman et al., 2016[52]	n = 18 RA patients were implanted with VNS and stimulated up to four times daily. Stimulation was associated with a reduction in disease activity (DAS28) and impaired TNF release on LPS-whole blood culture. These measures relapsed when stimulation was suspended for 14 days but improved again after reactivation. Clinical improvement was maintained at 84 days post-implantation.
	Crohn's disease	Sinniger et al.,[74] 2020	n = 9 patients with active CD receiving azathioprine or no treatment were implanted with VNS. Over twelve months, five experienced improvement in symptomatic (CDAI) and six in endoscopic (CDEIS) measures of severity. Two experienced worsening of disease and were removed from the study.
	rheumatoid arthritis	Genovese et al., 2020[53]	n = 14 treatment-refractory RA patients. 3 received treatment in an open label pilot study. The remaining 10 were randomized to receive 1 min of stimulation daily (n = 3), four times daily (n = 4), or sham procedure (n = 4) using a novel design of VNS device. 5 of 10 actively treated subjects demonstrated clinical improvement versus no controls. There was a significant reduction in cytokine (IL-1β, IL-6, and TNF) response to LPS-whole blood culture in the treatment group. MRI features of RA did not improve. One case of transient Horner's syndrome and another of transient vocal cord paralysis, amongst other adverse effects, were reported.
TcVNS	healthy participants	Lerman et al., 2016[117]	n = 20 (10 tcVNS and 10 sham controls, randomized). 3 courses of tcVNS over one day (2 min to each Vagus nerve per course) significantly reduced cytokine (TNF-α, IL-1β) and chemokine (MIP-1α, MCP-1, IL-8) response to LPS-whole blood culture compared to baseline and to controls.
	healthy participants	Brock et al., 2016[118]	n = 20 (internal controls). A single course of 120 s of tcVNS to each vagus nerve was sufficient to induce a small but significant reduction in circulating TNF-α levels, but no other cytokines, after 24 h. Blood samples were not challenged with LPS.
	Sjögren's syndrome	Tarn et al., 2018[119]	n = 15 female participants (internal controls). 3 weeks of twice daily tcVNS was associated with (1) improvement in fatigue score (n = 12/15), (2) reduced cytokines (TNF-α, IL-6, IL-1β, IP-10) and chemokine (MIP1α) response to LPS-whole blood culture, and (3) a transient rise in circulating T cells, NK cells, and NKT cells after first administration only.
	rheumatoid arthritis	Drewes et al., 2020[120]	n = 36, 16 with active RA and 20 with low activity RA. 120 s of tVNS three times daily for four days was associated with a significant reduction in DAS28-CRP and IFN-γ in non-stimulated blood (but not other cytokines) in the active group only. There was also a statistically significant reduction in blood pressure in this group, possibly indicative of vagus nerve activity. Surprisingly, the low activity group actually experienced a reduction in cardiac vagal tone and in serum levels of IL-10.
TaVNS	impaired glucose tolerance	Huang et al., 2014[121]	n = 35 who received 12 weeks of taVNS experienced a reduction in fasting plasma glucose, 2-h plasma glucose, and Hba1c compared with n = 30 receiving no treatment in a parallel non-randomized observational study. However, a sham-placebo group (n = 35) experienced a similar reduction in 2-h plasma glucose and Hba1c.
	acute STEMI and MIRI post-PCI	Yu et al., 2016[122]	n = 95 (47 taVNS, 48 sham-controls, randomized). TaVNS was applied before and throughout percutaneous coronary intervention (PCI). Intervention group demonstrated (1) fewer arrhythmias, (2) more favorable echocardiographic features, and (3) lower levels of serum cardiac enzymes and cytokines (TNF-α, IL-6, IL-1β, HMGB-1).
	rheumatoid arthritis and healthy participants	Addorisio et al., 2019[123]	Two days of twice-daily taVNS was associated with lower cytokine levels on LPS-whole blood assay in two separate studies (n = 9, TNF-α assay. n = 19 TNF, IL-1β, and IL-6 assays). n = 9 patients

(*Continued on next page*)

Table 1. *Continued*

Treatment	Population	Reference	Findings
			with RA experienced a significant reduction in disease activity (DAS28), sustained for at least one week after treatment.
	systemic lupus erythematosus	Aranow et al., 2021[124]	n = 18 (12 taVNS and 6 controls) received 5 min of taVNS or sham procedure daily for four days in a double-blinded RCT. One subject was excluded and replaced due to a respiratory tract infection. 83.3% of taVNS participants experienced a meaningful reduction in subjective measurements of pain and fatigue at 12 days versus 16.7 and 0% of controls, respectively. However, improvements in objective measures by blinded physicians of disease activity were not statistically significant, nor were inflammatory markers or cytokine levels.

iVNS, invasive VNS; taVNS, transauricular VNS; tcVNS, transcervical VNS; SLE, systemic lupus erythematosus.

context of addressing was that this observed VNS anti-inflammatory activity a peripheral response, investigators have shown that this anti-inflammatory CNS effect is inhibited in vagotomized mice, supporting the role of the efferent vagus mediating these effects on neuroinflammation.[112] Supporting this hypothesis, splenectomy impaired the anti-inflammatory effects of α7nAChR-agonism in TBI.[100] Again, this may not be the most suitable model for organ-specific neuroinflammation, as TBI is accompanied by a systemic inflammatory response and translocation of peripheral immune cells across the disrupted BBB.[100] Systemic inflammation is associated with deleterious effects in most neurodegenerative disorders.[112] So, it is plausible that VNS would have neuroprotective effects mediated via the efferent nerve.

OTHER SYSTEMS

The cholinergic system, which is found in both neuronal and non-neuronal cells, mediates complex functions in all organs of the body. Therefore, it is plausible that manipulation of the CAP would have therapeutic potential in chronic inflammatory end-organ injury. In a comprehensive set of experiments, Inoue and colleagues[23] illustrated the capabilities of the CAP in protecting the kidney from renal ischemia-reperfusion injury (IRI). VNS, when applied 24 or 48 h before ischemia, attenuated cytokine response and acute kidney injury. This effect was not seen in α7nAChR KO or splenectomized mice. The efferent vagus nerve does not innervate the kidney,[113] and blocking of the sympathetic renal nerve actually prevented renal IRI,[23] so VNS appears to exert its reno-protective effect indirectly through the spleen.

Deletion of the α7nAChR results in larger infarct size and greater inflammatory response in mice subjected to myocardial infarction.[36] α7nAChR agonists[114] and VNS[115] have the opposite effect. A non-invasive form of transcutaneous VNS, discussed below and in Table 1, has demonstrated cardioprotective effects during acute myocardial infarction in a human randomized control trial (RCT).[116] Unlike the kidney, the heart receives extensive parasympathetic input from the vagus nerve, though whether the cardioprotective effects of VNS are mediated via direct myocardial innervation, the splenic CAP, or both has not been investigated.

Vagotomy has an exacerbating effect on models of pancreatitis.[125] Stimulation of the CAP with α7nAChR agonists[125] or centrally acting agents[122] attenuates the disease process.

CLINICAL APPLICATIONS AND FUTURE DIRECTIONS

As the therapeutic potential of the CAP becomes increasingly realized in pre-clinical studies, efforts have begun to translate this work into new clinical therapies (see Figure 3).

Attempts at pharmacological stimulation of the CAP have had mixed success. As discussed above, an RCT of Semapimod in CD failed to meet its primary endpoint, and the medication was poorly tolerated.[73] A pilot study of GTS-21 in 14 healthy volunteers failed to attenuate the inflammatory response in LPS-induced endotoxemia *in vivo*. The pharmacokinetics of GTS-21 were found to vary greatly between participants in this study. It is possible that insufficient doses were used and it is likely that the study was underpowered.[126] On the other hand, an RCT of galantamine successfully reduced serum levels of TNF-α, increased levels of IL-10, and improved insulin resistance in patients with metabolic syndrome.[127] α7nAChR and other AChR have extensive non-immune actions on other cell types. These actions could be responsible for some of these agents' adverse effects, limiting their clinical use.

Bioelectronic therapies, which target the CAP more specifically, such as VNS, offer an attractive alternative to drug-based therapies modulating cellular α7nAChR expression. Pilot studies of invasive VNS (iVNS), which have shown promise in RA[51,52] and IBD,[72] are described above and in Table 1. As already discussed, VNS implantation is generally well tolerated but is not without adverse effects. Patients may be reluctant, or even physically unsuitable, to undergo implantation. Implantation may be impractical and unjustified in acute or monophasic illnesses which require urgent, but not long-term, intervention and therefore would be more suited to a temporary form of immunomodulation. In recognition of these limitations, but staying cognizant of the significant burden of chronic inflammatory diseases, the industry has expressed significant interest in developing non-invasive bioelectronic devices for these diseases.

The vagus nerve can be stimulated transcutaneously (tVNS) by placing an electrode over the cervical vagus nerve in the neck (transcervical VNS, tcVNS) or the auricular branch in the cymba

concha of the external ear (transauricular VNS, taVNS). A device for the former is FDA approved for use in migraine, though efficacy was only demonstrated in a sub-group analysis of patients adherent to treatment,[128] so patient concordance may limit the efficacy of such a treatment in practice. More recently, the FDA fast-tracked approval for tVNS use in treating respiratory symptoms of COVID-19.[129] Stimulation of the afferent vagus nerve with taVNS activates vagal brainstem nuclei (see Figure 3).[130,131] It is proposed that signals in these nuclei are, in turn, relayed to the efferent vagus nerve based on the observation that taVNS can have systemic autonomic effects. Certainly, both tcVNS and taVNS have demonstrated anti-inflammatory effects in an animal model of endotoxaemia.[132] These effects were eliminated by both vagotomy and an α7nAChR antagonist, suggesting involvement of the CAP. TaVNS has also demonstrated anti-inflammatory and disease-modifying effects in models of post-operative ileus.[130] Neuroprotective effects have been demonstrated in PD,[133] post-operative cognitive dysfunction,[134] and ischemic stroke.[131] In more recent studies, such effects were associated with a reduction in intracerebral cytokine release and an upregulation of α7nAChR expression.[133,134] Intracerebral upregulation of the α7nAChr has also been observed in response to PNU-282987 with apparent anxiolytic and antidepressant effects.[135]

Table 1 summarizes the findings of human clinical trials aimed at harnessing the CAP using iVNS and tVNS. Several other tVNS trials not listed in Table 1 have been conducted,[136] for example in conditions for which iVNS has already been approved (epilepsy and migraine) and in patients with other neurological conditions (e.g., depression and PD) where symptomatic improvement has been demonstrated. However, unlike the conditions listed in Table 1, the anti-inflammatory role of VNS in these neurological disorders remains less well defined and is not typically measured in trial outcomes, so the effects of tVNS may or may not be anti-inflammatory. One short, double-blinded pilot study in systemic lupus erythematosus (SLE) showed improvement in only subjective markers of disease but not in objective measures or inflammatory markers.[137] However, the overall evidence from these studies, though small in size and number, supports a potential therapeutic benefit for both iVNS and tVNS across a range of inflammatory disorders, warranting larger RCTs.

Recently, an innovative method of stimulating the CAP using non-invasive pulsed ultrasound (pUS) was proposed.[138] Administration of abdominal ultrasound was found to attenuate subsequent renal IRI in rats. This effect was dependent on functional $CD4^+$ T cells[139] and was not observed following splenectomy, KO or inhibition of the α7nAChR, or splenic denervation.[124] Prevention of acute kidney injury (AKI) was associated with impaired inflammatory potential of splenic leukocytes and reduced levels of serum and renal cytokines including TNF-α and IL-6.[124] It is believed that the pUS stimulates the CAP downstream of the vagus nerve in the spleen. Focusing pUS waves at the site of splenic nerve terminals stimulates the release of noradrenaline and ACh in a process dependent on ChAT+ $CD4^+$ T cells and the α7nAChR.[138] Splenic[138,140,141] or abdominal[142] pUS has proven effective in preventing the effects of endotoxaemia,[138] prolonging survival in sepsis[141] and reversing the effects of established inflammatory arthritis[140] and DSS-colitis.[142] pUS applied to the spleen or neck reduces infarct size 3-fold in myocardial ischemia-reperfusion injury exacerbated by hyperglycaemia.[143] Focused pUS may also have anti-inflammatory uses at other sites. The afferent fibers of the vagus nerve be stimulated at the porta hepatis in the liver, modulating hypothalamic insulin sensitivity resulting in attenuation of hyperglycaemia[138] with associated reduction in weight gain and an overall down-regulation of hepatic inflammation.[69] Together, these results present a potential therapeutic role in modulating the CAP across a range of organ systems.

In addition to being non-invasive and apparently safe, a theoretical advantage to splenic pUS is that it avoids the non-CAP effects on other organs in which the vagus has been implicated, such as in the lungs.[79,82] By the same token, however, limiting the CAP effects to those mediated by splenic macrophages could be less efficacious in conditions such as IBD, where vagal innervation of the GIT might play a role.[68] The effects of pUS on DSS-colitis, administered non-specifically throughout the abdomen, were almost absent in splenectomized mice, aside from a mild improvement in bloody stool frequency and evidence of AChR+ cell recruitment in the mesenteric lymph node.[142] Recently, however, pUS was shown to be effective in DSS-colitis by stimulating the CAP at the level of the celiac ganglion.[144] Human clinical trials of pUS in inflammatory disease are now warranted to assess its therapeutic potential and answer these questions.

CONCLUSION

The inflammatory reflex is a pervasive homeostatic mechanism that can influence inflammatory diseases across all bodily systems. The nature of certain interactions between the CAP and individual organs remains unclear, as with the CAP's downstream cellular mechanisms. Nevertheless, evidence to date presents the CAP as an enticing therapeutic target for a wide range of diseases. Findings of pre-clinical experiments are now being translated into small but promising clinical trials. Pharmacological manipulation of the CAP remains somewhat elusive but warrants further study. Bioelectronic techniques capable of harnessing the CAP through invasive and non-invasive methods such as VNS and splenic pUS have shown promising results and may present an innovative form of anti-inflammatory therapy.

ACKNOWLEDGMENTS

S.C.D., C.B., and M.J.K. gratefully acknowledge the support of Science Foundation Ireland (SFI) and the Health Research Board (HRB) of Ireland.

AUTHOR CONTRIBUTIONS

M.J.K. and S.C.D. searched the literature and wrote the first draft of the manuscript. C.B. and K.T. critically revised each version of the manuscript.

DECLARATION OF INTERESTS

All authors declare no competing interests.

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frontiers | Frontiers in Physiology

ORIGINAL RESEARCH
published: 04 July 2022
doi: 10.3389/fphys.2022.897257

Percutaneous Auricular Vagus Nerve Stimulation Reduces Inflammation in Critical Covid-19 Patients

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Covid-19 is an infectious disease associated with cytokine storms and derailed sympatho-vagal balance leading to respiratory distress, hypoxemia and cardiovascular damage. We applied the auricular vagus nerve stimulation to modulate the parasympathetic nervous system, activate the associated anti-inflammatory pathways, and reestablish the abnormal sympatho-vagal balance. aVNS is performed percutaneously using miniature needle electrodes in ear regions innervated by the auricular vagus nerve. In terms of a randomized prospective study, chronic aVNS is started in critical, but not yet ventilated Covid-19 patients during their stay at the intensive care unit. The results show decreased pro-inflammatory parameters, e.g. a reduction of CRP levels by 32% after 1 day of aVNS and 80% over 7 days (from the mean 151.9 mg/dl to 31.5 mg/dl) or similarly a reduction of TNFalpha levels by 58.1% over 7 days (from a mean 19.3 pg/ml to 8.1 pg/ml) and coagulation parameters, e.g. reduction of DDIMER levels by 66% over 7 days (from a mean 4.5 µg/ml to 1.5 µg/ml) and increased anti-inflammatory parameters, e.g. an increase of IL-10 levels by 66% over 7 days (from the mean 2.7 pg/ml to 7 pg/ml) over the aVNS duration without collateral effects. aVNS proved to be a safe clinical procedure and could effectively supplement treatment of critical Covid-19 patients while preventing devastating over-inflammation.

Keywords: COVID-19, SARS-CoV-2, hyperinflammation, cytokine storm, aVNS, nervus vagus stimulation

OPEN ACCESS

Edited by:
Vaughan G. Macefield, Baker Heart and Diabetes Institute, Australia

Reviewed by:
Milan Stengl, Charles University, Czechia
Christopher G. Wilson, Loma Linda University, United States

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Specialty section:
This article was submitted to Autonomic Neuroscience, a section of the journal Frontiers in Physiology

Received: *15 March 2022*
Accepted: *14 June 2022*
Published: *04 July 2022*

Citation:
Seitz T, Szeles JC, Kitzberger R, Holbik J, Grieb A, Wolf H, Akyaman H, Lucny F, Tychera A, Neuhold S, Zoufaly A, Wenisch C and Kaniusas E (2022) Percutaneous Auricular Vagus Nerve Stimulation Reduces Inflammation in Critical Covid-19 Patients. Front. Physiol. 13:897257. doi: 10.3389/fphys.2022.897257

INTRODUCTION

Covid-19

Covid-19 is an infectious disease caused by SARS-CoV-2 invading epithelial cells via angiotensin 2-converting enzyme (ACE2) receptors abundantly expressed in alveolar epithelial cells as well as in the heart (Hoffmann et al., 2020). Covid-19 may lead to severe pro-inflammatory cytokine storms and hemophagocytosis, derailed sympatho-vagal balance, culminating in severe hypoxemia, major respiratory distress, cardiovascular damage, and increased thrombotic and/or thromboembolic events. In particular, elevated levels of interleukin-1β, IL-2, IL-6, IL-10, TNF, IFNalpha, IFNbeta, interferon-γ, macrophage inflammatory proteins 1α and 1β, and VEGF appear in patients with Covid-19–associated cytokine storm (Huang et al., 2020; Yi et al., 2020; Zhu et al., 2020). Several clinical and laboratory abnormalities, such as elevated CRP, ferritin and DDIMER levels are

described during cytokine storm in addition to the elevated systemic cytokine levels (Fajgenbaum and June 2020). Further, on the one hand a repression of anti-inflammatory cytokines, such as IL-10, on the other hand an excessive secretion was described in critical COVID-19 (Rabaan et al., 2021).

The Vagally-Controlled Immune System

The immune system is mediated and modulated by afferent and efferent fibers of the vagus nerve, the major nerve of the parasympathetic nervous system (Pavlov and Tracey, 2012). The nerve acts as a major component of the neuroendocrine-immune axis (Bonaz, Sinniger and Pellissier, 2016) and is a key factor in response to infection. While the parasympathetic vagus nerve exerts only anti-inflammatory effects, the sympathetic nervous system may have both pro-inflammatory and anti-inflammatory effects. The vagus nerve is involved in multiple pathways within immune reflexes: the anti-inflammatory hypothalamic-pituitary-adrenal axis (HPAA) and the cholinergic anti-inflammatory pathway (ChAIP) (Borovikova et al., 2000; Tracey, 2007; Bonaz, Sinniger and Pellissier, 2016).

In both reflexes, inflammatory mediators (e.g., pro-inflammatory cytokines and/or endotoxins) activate the afferent vagal fibers projecting this inflammatory information to the nucleus of the solitary tract (NST) which, in turn, project to the dorsal motor nucleus of the vagus nerve. In the HPAA, activated efferents to hypothalamus stimulate the release of corticotrophin-releasing hormone which stimulates the secretion of adrenocorticotropic hormone from the pituitary gland. The adrenocorticotropic hormone reaches adrenal glands and stimulates the release of glucocorticoids, acting on the spleen so that pro-inflammatory cytokines and the resulting peripheral inflammation are reduced. In the ChAIP, activated cholinergic efferents to the spleen release acetylcholine at their preganglionic synaptic endings, with acetylcholine binding to surface receptors of macrophages and suppressing the release of pro-inflammatory cytokines by these macrophages.

In view of cytokine storms in Covid-19, it is instructive to observe that over-inflammation with the associated unrestrained cytokine release results when the tonic neural activity of ChAIP is impaired (Mercante, Deriu and Rangon, 2018), which highlights the importance of ChAIP, the involved vagus nerve and its stimulation.

The Vagus Stimulation

Vagus nerve stimulation can be performed through invasive, non-invasive, and minimally-invasive methods (Kaniusas et al., 2019a). For invasive stimulation, a cuff electrode is implanted at the cervical level typically wrapped around the left cervical branch of the vagus nerve, showing high implantation risks and costs (Mertens et al., 2018). For non-invasive stimulation, surface skin electrodes are used on the outer ear (Ellrich, 2011), yielding easy-to-use, but diffuse transcutaneous stimulation of both vagally and non-vagally innervated regions. The associated minor side effects include headache, pain and skin irritation at the stimulation site. The afferent branches of the cervical vagus nerve can also be non-invasively stimulated via surface skin electrodes of a hand-held device applied at the neck (Barbanti et al., 2015), with potential side effects such as prickling at stimulation site, neck pain, dizziness, headache.

We focus on the percutaneous minimally invasive auricular vagus nerve stimulation (aVNS) where miniature needle electrodes are positioned in the outer ear regions innervated mainly by the vagus nerve (Kaniusas and Samoudi, 2020). The percutaneous aVNS shows minor side effects (Kaniusas et al., 2019a; Kaniusas, et al., 2019b): local skin irritation of the ear (with the incidence <10%) and inadvertent bleeding (<1%) can occur, which can be reduced down to <0.05% when erroneous placement of needles directly into auricular vessels is avoided using transillumination of the outer ear to visualize auricular vascularization (Kampusch et al., 2016; Roberts et al., 2016).

The percutaneous aVNS targets Aβ-fibers in the ear responsible for cutaneous mechanoreception and touch sensation, which project to NST in the brainstem and activate visceral and somatic projections (Frangos, Ellrich and Komisaruk, 2015). The NTS is involved in the aforementioned control of autonomic immune systems, as well as cardiorespiratory and cardiovascular regulation (Thayer, Mather and Koenig, 2021).

The Auricular Vagus Stimulation in Immune System

aVNS reduced pro-inflammatory cytokines in atrial fibrillation (Stavrakis et al., 2015) and increased norepinephrine levels (Beekwilder and Beems, 2010) which supports anti-inflammatory aVNS effects. aVNS reduced systemic tumor necrosis factor in mice with lethal endotoxemia or polymicrobial sepsis (Huston et al., 2007). aVNS reduced pro-inflammatory cytokines and suppressed lipopolysaccharide-induced inflammatory responses in endotoxemic rats (Zhao et al., 2012). In humans, anti-inflammatory effects were shown in rheumatoid arthritis (Bernateck et al., 2008; Koopman et al., 2016), inflammatory bowel disease (Crohn's disease, ulcerative colitis), and postoperative ileus (Tracey, 2007; Marshall et al., 2015; Bonaz, Sinniger and Pellissier, 2016), and lobectomy (Salama, Akan and Mueller, 2017).

Hypothesis

In line with our theoretical hypothesis (Kaniusas and Szeles, 2020a) suggesting aVNS as a potential treatment of Covid19-originated acute respiratory distress syndrome and the associated co-morbidities through activation of anti-inflammatory pathways, we present here a randomised prospective study on clinical effects of aVNS on inflammation related markers in critical but not yet ventilated patients.

METHODS

Patient Recruitment

Patients admitted to the intensive care unit (ICU) of the Department of Infectious Diseases and Tropical Medicine, Klinik Favoriten, Vienna, Austria due to Covid-19 were

screened by the attending physician regarding the following inclusion and exclusion criteria:

Inclusion criteria (all criterion are needed for inclusion):

- Positive for SARS-CoV-2 by RT-PCR test (defined as a C_T value less than 30)
- Acute respiratory failure requiring non-invasive respiratory support
- PaO_2/FiO_2 <200

Exclusion criteria (one criteria is sufficient for exclusion):

- Age <18 years
- Pregnancy (to be excluded using serum beta HCG in women of childbearing age)
- Signs of infection, eczema, or psoriasis at the application site
- Active malignancy
- Implanted cardiac pacemaker, defibrillator, or other active implanted electronic devices
- Patient unable to consent
- Heart rate <60 beats/min
- Known vagal hypersensitivity
- History of haemophilia

If all inclusion criteria and none of the exclusion criteria were met, the attending physician informed the patient about the study. After written consent was obtained, randomisation of the study group ($n = 10$) into the VNS group or the standard of care (SOC) group was performed with a computer-based randomisation tool. The time of inclusion was considered as the time point 0 (T0). Following the COVID-19 guidelines of Open Critical Care (COVID-19 Guidelines Dashboard - Open Critical Care, 2021), SOC implicates that patients with respiratory insufficiency receive 10 mg of dexamethasone for 10 days and prophylactic anticoagulation therapy.

Procedure

aVNS was started immediately for patients selected in the VNS group. This procedure was performed using an AuriStim device (Multisana GmbH, Austria). AuriStim is a single-use, miniaturized, and battery-powered electrical stimulator. The stimulator delivers monophasic varying polarity pulses (pulse width 1 ms) with a fixed amplitude (3.8 V) every second (stimulation frequency is 1 Hz), and a duty cycle (3 h ON/3 h OFF). The resistance of the needle and needle to tissue interface is about 4–7kOhm so that the resulting peak current amount to about 0.5–0.9 mA, residing at or below the suggested limits of about 1.5 mA.

Multi-punctual percutaneous aVNS was mediated via three miniature needle electrodes inserted into vagally (solely or partly) innervated regions of the auricle. These regions were the cymba concha (vagal nerve is found in 100% of cases) (Peuker and Filler, 2002), cavity of concha (45%), and the crura of antihelix (9%). Needles were located close to local blood vessels—as identified by transillumination of the auricle (Kaniusas et al., 2011)—with the auricular nerves nearby (Dabiri et al., 2020).

Following insertion of the needles, an intermittent stimulation cycle of 3 hours of activity and 3 hours of rest was initiated, equating to four cycles of 3 hours of stimulation in 24 h. During the treatment period the device was swapped to avoid a decrease of function due to low battery. The procedure was performed until either the patient was discharged from the ICU, transferred to another ward, or died.

If patients were responsive, the visual analogue scale (VAS) was documented four times per day and at time of device placement to document pain at the insertion site. The VAS is a Likert scale that ranges from 0 ("no pain") to 10 ("pain as bad as it could possibly be"). If the VAS was over five during aVNS, the stimulation was stopped. Patients had the option of terminating the procedure immediately if they wished due to discomfort. Furthermore, patients were clinically examined and interviewed for potential side effects of aVNS.

To evaluate inflammation, macrophage activation, anti-inflammatory, and coagulation biomarkers, blood samples (1 serum tube per time point) were drawn in both groups at the following time points:

- Day 1 (every 4 h): T0 (Time of inclusion in the study), T4 (4 h after T0), T8, T12, T16
- Day 2 (every 8 h): T24, T32, T40
- Day 3 (every 12 h): T48, T60
- Day 4 (every 12 h): T72, T84
- Day 5 (every 12 h): T96, T108
- Day 6 (every 12 h): T120, T132
- Day 7 (every 12 h): T144, T156
- The blood samples were immediately centrifuged for 15 min at 3,000 rpm and then serum was frozen at −20°C. The analysis was performed within a 2-week period at a certified immunology laboratory.
- Supplementary blood samples were drawn every day between 6:00 and 6:30 a.m. for additional inflammation (CRP, ferritin) and coagulation (DDIMER, fibrinogen) biomarkers that were tested immediately afterwards.

Blood samples were collected for 7 days after study inclusion or until patients were discharged from this ICU, transferred to another ward, or died.

Statistical Analysis

Basic characteristics of the participants were collected including gender, virus variant, time since symptom onset, comorbidities, and respiratory situation. The study presents a Proof of Concept-Study and financial resources were limited, therefore no power analysis was performed prior to the study.

The mean, median, standard deviation, minimum, and maximum were calculated for categorical variables.

Inflammation parameters were analysed on the log-scale using linear mixed models. A random intercept was introduced for each patient and a time-dependent correlation structure was modelled for the residual variance-covariance matrix, taking into account the unequally spaced intervals. A heteroscedasticity correction was applied when residuals were

TABLE 1 | Basis parameters of the study participants.

	VNS (*n* = 5)	SOC (*n* = 5)
Age (in years)		
Mean	55.6	53.2
Min-Max	42–68	43–64
Standard deviation	8.69	7.83
Gender		
	40% female 60% male	60% female 40% male
Comorbidities		
Hypertension	60%	40%
Obesity	80%	80%
Mean BMI	35.32	33.22
Diabetes	40%	60%
Chronic artery disease	20%	0%
Chronic renal failure	20%	0%
Chronic lung disease	0%	0%
Thyroid disease	0%	20%
Active cancer	0%	0%
Hematological Disease	0%	0%
Rheumatological disease	0%	20%
Current Smoking	20%	0%
SARS-CoV-2 Vaccination	0%	0%
Virus variant	80% B.1.617.2 (Delta) 20% unknown	100% B.1.617.2 (Delta)
Time between symptom onset and ICU admission (in days)		
Mean	9.60	7.80
Min-Max	7–16	5–12
Standard deviation	3.26	2.64
Time between symptom onset and study Inclusion (in days)		
Mean	10.4	8.4
Min-Max	8–17	6–12
Standard deviation	3.32	2.06
Need of non-invasive ventilation at time of study inclusion	80%	80%
Horowitz Index at time of study inclusion		
Mean	124.7	103.8
Min-Max	69.8–190.7	65.6–180
Standard deviation	46.95	40.81
Length of aVNS (in days)		
Mean	12	—
Min-Max	3–18	—
Standard deviation	6.23	—
Therapy		
Corticosteroids	100%	100%
Other immunosuppressive agents	0%	0%
Antimicrobial therapy	80%	80%

not homogeneously distributed over time. Restricted maximum likelihood was used to estimate the model parameters. A type I error rate of 5% was used for inferential statistics. In each model, we checked that the residuals were homoskedastic. The analyses were performed with R Statistics 4.1.1.

Ethical Considerations

The study was approved by the local ethics committee (EK 21-079-0521) and Austrian Federal Office for Safety in Health Care BASG. The study was registered at ClinicalTrials.gov (NCT05058742).

RESULTS

The study was conducted from June to December 2021 and included 10 patients (5 patients randomised each to the VNS and SOC groups). Basic characteristics of the study participants are

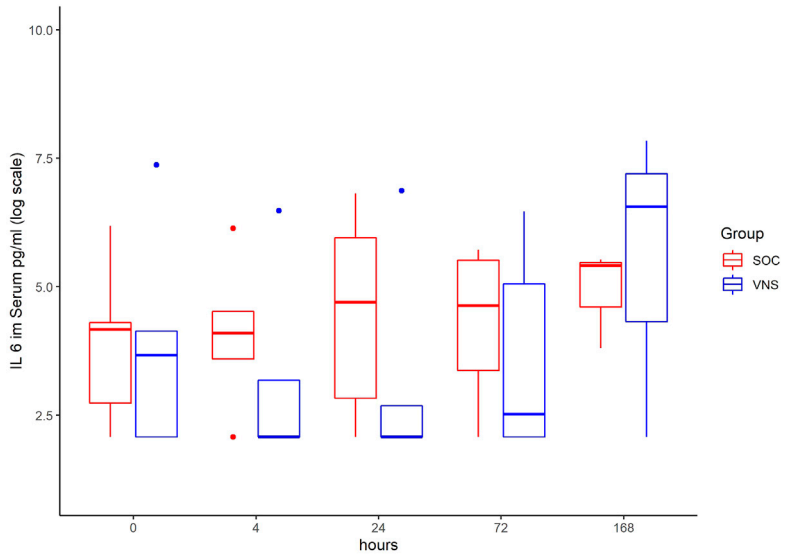

FIGURE 1 | Median IL-6 level with Interquartile Range of patients receiving auricular Vagus Stimulation (VNS = blue) and patients only receiving Standard of Care (SOC = red) after 0, 4, 24, 72, and 168 h of study inclusion.

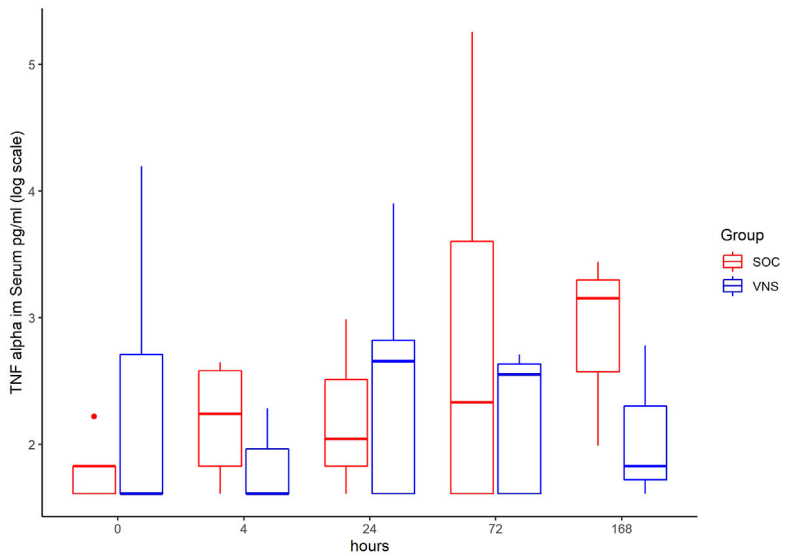

FIGURE 2 | Median TNF alpha level with Interquartile Range of patients receiving auricular Vagus Stimulation (VNS = blue) and patients only receiving Standard of Care (SOC = red) after 0, 4, 24, 72, and 168 h of study inclusion.

listed in **Table 1**. The mean age of the participants was 53–56 years, in the majority the virus variant B.1.617.2 was detected.

Tolerance and Safety

No adverse event was documented while using aVNS. No patient opted to terminate aVNS early. Mean VAS at the time of device placement was 3.4 (range 2–5). Otherwise, VAS was documented four times a day. The mean VAS was 1.9 (range 0–3). Eighty percent required non-invasive ventilation at the time of study inclusion with a Horrowitz Index between 65.6—190.7.

Inflammation Parameters

Serum levels of IL-6, IFNγ, TNFα, Calprotectin, IL-18, S100A12, sIL2-receptor and IL-10 were analysed at an average of 16.1 time points

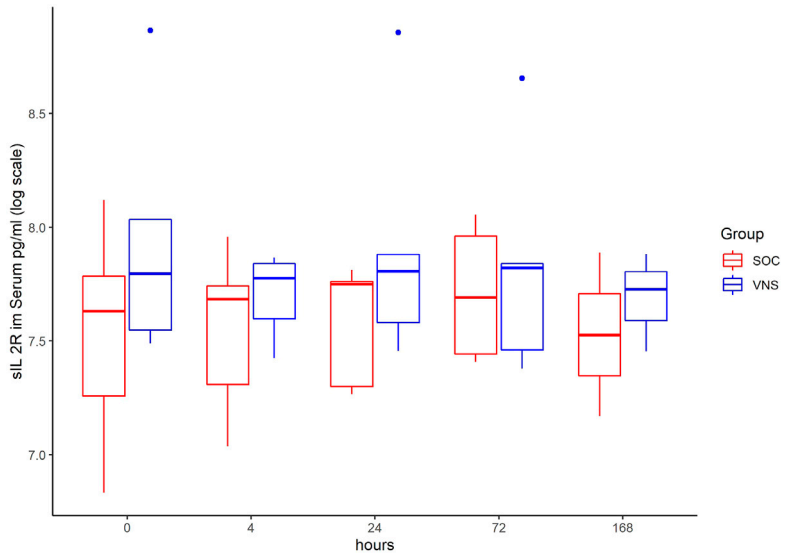

FIGURE 3 | Median sIL-2R level with Interquartile Range of patients receiving auricular Vagus Stimulation (VNS = blue) and patients only receiving Standard of Care (SOC = red) after 0, 4, 24, 72, and 168 h of study inclusion.

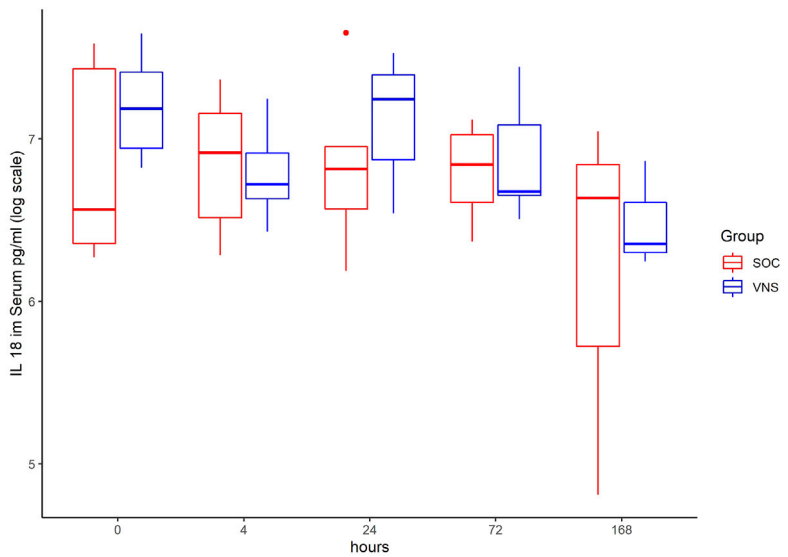

FIGURE 4 | Median IL-18 level with Interquartile Range of patients receiving auricular Vagus Stimulation (VNS = blue) and patients only receiving Standard of Care (SOC = red) after 0, 4, 24, 72, and 168 h of study inclusion.

(range 9–20) and a mean time of 6 days. CRP, ferritin, Fibrinogen and DDIMER levels at an average of six time points (range 2–8) and a mean time of 6 days per patient.

The inflammatory parameter levels at the time points 4, 24, 72, and 168 h in patients of the VNS group compared to the SOC group are shown in **Figures 1**–**11**. IFNγ did not show an elevated value at any timepoint.

The temporal courses and dynamic ranges of all parameters at each time point and patients are demonstrated in **Supplementary Material**.

Difference Inflammation and Clinical Parameters Between VNS and SOC Group

The decrease of IL-6 ($p = 0.048$) and TNFalpha ($p = 0.048$) from T0 to T4 was statistically significantly stronger in VNS than SOC group, as well as the decrease of DDIMER from T0 to T24 ($p = 0.025$). Further, the decrease of CRP from T0 to T72 ($p = 0.003$) was statistically significantly stronger in VNS group, as well as the decrease of CRP ($p = 0.018$) and fibrinogen ($p = 0.002$) from T0 to T168 was statistically significantly stronger in VNS group. On the contrary, the increase of IL-10 from T0 to T72 and T168 was

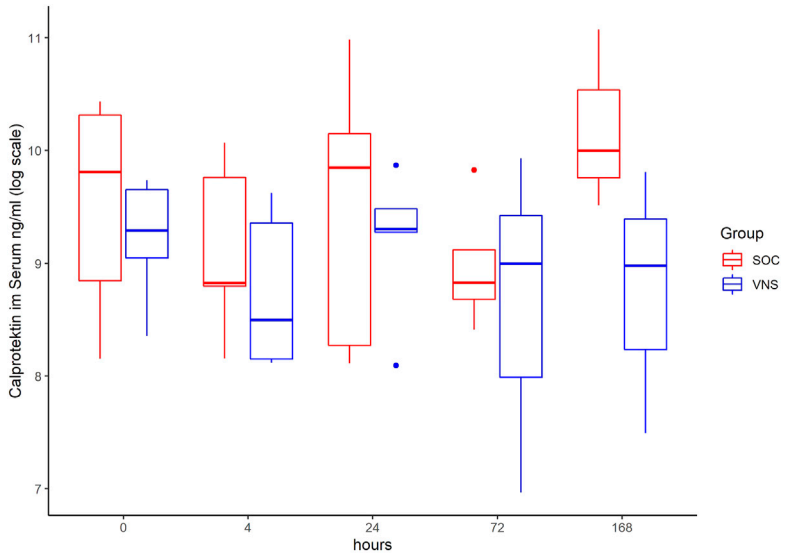

FIGURE 5 | Median Calprotektin level with Interquartile Range of patients receiving auricular Vagus Stimulation (VNS = blue) and patients only receiving Standard of Care (SOC = red) after 0, 4, 24, 72, and 168 h of study inclusion.

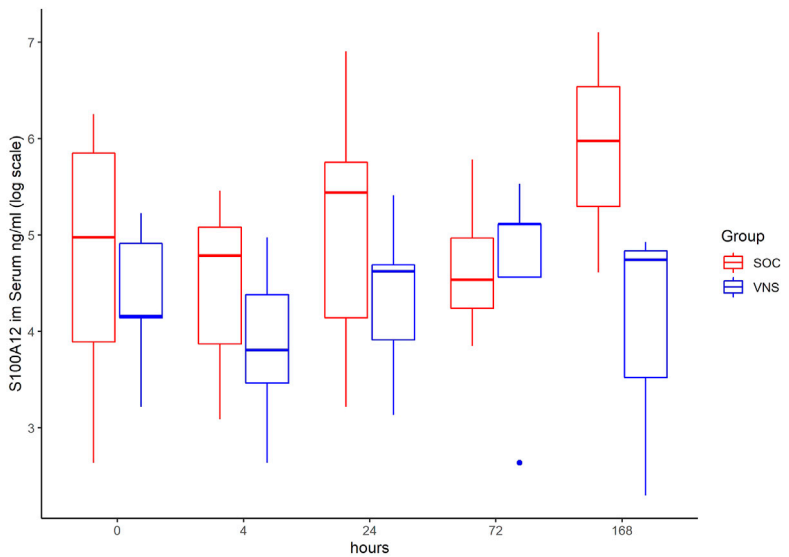

FIGURE 6 | Median S100A12 level with Interquartile Range of patients receiving auricular Vagus Stimulation (VNS = blue) and patients only receiving Standard of Care (SOC = red) after 0, 4, 24, 72, and 168 h of study inclusion.

significantly higher in the VNS group ($p = 0.041$ and $p = 0.048$). The exact calculation is shown in **Supplementary Material**.

An increase of Horowitz index (Pao2/FiO2-Ratio) was seen in the VNS group, as seen in **Figure 12**.

DISCUSSION

In this study we evaluated aVNS as a novel procedure to reduce COVID-19 associated cytokine storm. The procedure has been shown to be well tolerated and safe.

Decrease of serum levels of IL-6, TNF Alpha, DDimer between T0 and T4 was significantly more pronounced in the aVNS versus SOC group. Here the mean decrease of TNFα level was 19.3 pg/ml at T0 to 6.4 pg/ml at T4. In contrast, within the control group including patients without aVNS mean TNFα increased from 6.3 pg/ml at T0 to 8.7 pg/ml. The mean decrease of IL-6 level in aVNS group was from 341.4 pg/ml at T0 to 140.2 at T4. In the control group the mean IL-6 rose from 129 pg/ml 131.8 pg/ml. Therefore, aVNS treatment may have a significant impact on patient outcome given that a correlation with worse outcome in

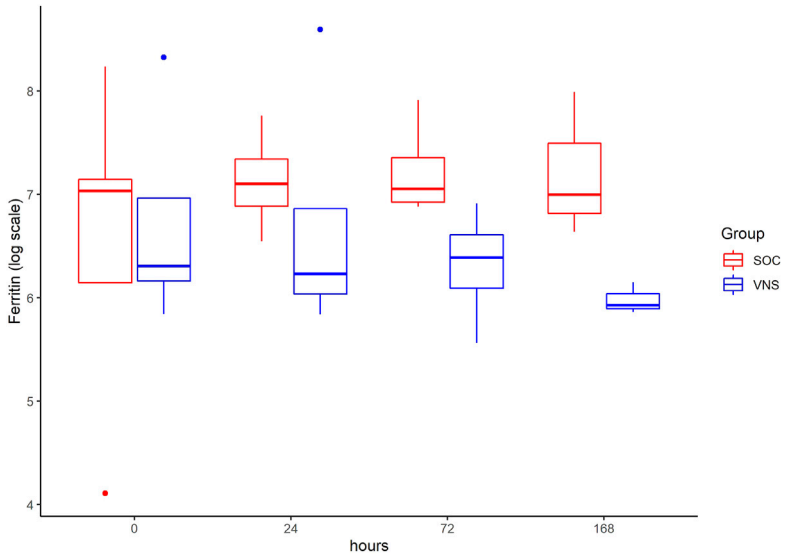

FIGURE 7 | Median ferritin level with Interquartile Range of patients receiving auricular Vagus Stimulation (VNS = blue) and patients only receiving Standard of Care (SOC = red) after 0, 24, 72, and 168 h of study inclusion.

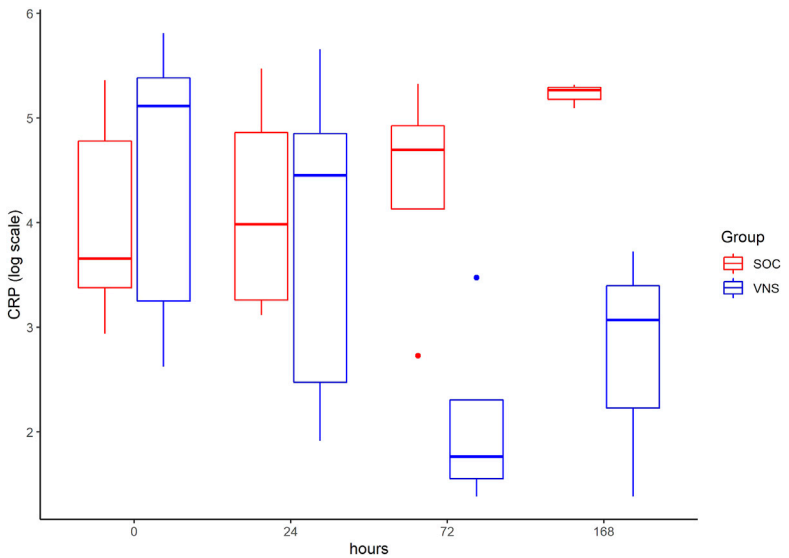

FIGURE 8 | Median CRP level with Interquartile Range of patients receiving auricular Vagus Stimulation (VNS = blue) and patients only receiving Standard of Care (SOC = red) after 0, 24, 72, and 168 h of study inclusion.

Covid-19 has been reported along with high pro-inflammatory levels of IL-6 and TNFα (del Valle et al., 2020). Among others, extensive IL-6 secretion can trigger coagulation and vascular leak syndrome leading to cardiomyopathy, coronary artery disease and myocardial dysfunction (Rabaan et al., 2021).

Conversely, the anti-inflammatory cytokine IL-10 increased in all patients 4 h after the start of aVNS (from 2.7 pg/ml as the mean at T0 to 7 pg/ml at T4) and was significantly higher over time compared to the control group (from 8.6 pg/ml to 8.6 pg/ml). However, in all patients the levels were only slightly elevated. Extremely high and low levels of IL-10 are considered to play a role in the cytokine storm of COVID-19 (Rabaan et al., 2021). The clinical impact of the slight elevation of IL-10 after receiving aVNS is not clear so far.

In all patients the nonspecific inflammation biomarker CRP decreased (from a mean level of 151.9 mg/dl at T0 to 31.5 mg/dl at T7) while receiving aVNS. In comparison to patients who were not treated with aVNS the mean CRP levels increased (from

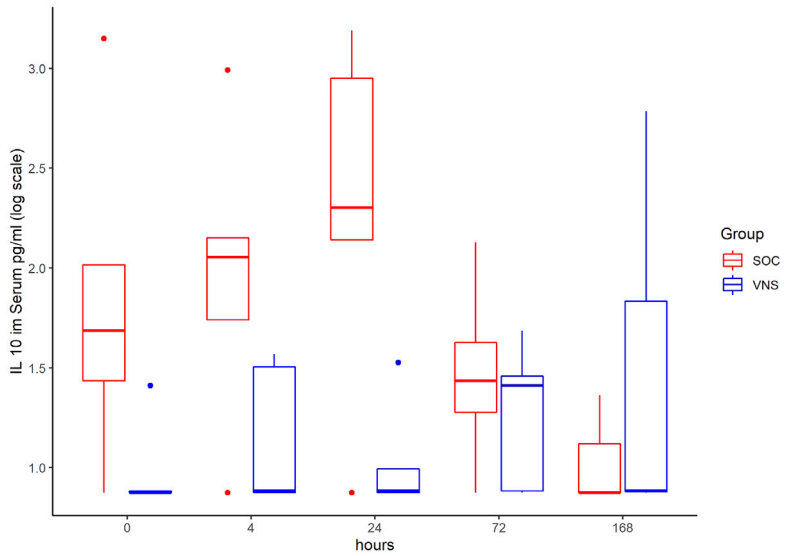

FIGURE 9 | Median IL-10 level with Interquartile Range of patients receiving auricular Vagus Stimulation (VNS = blue) and patients only receiving Standard of Care (SOC = red) after 0, 4, 24, 72, and 168 h of study inclusion.

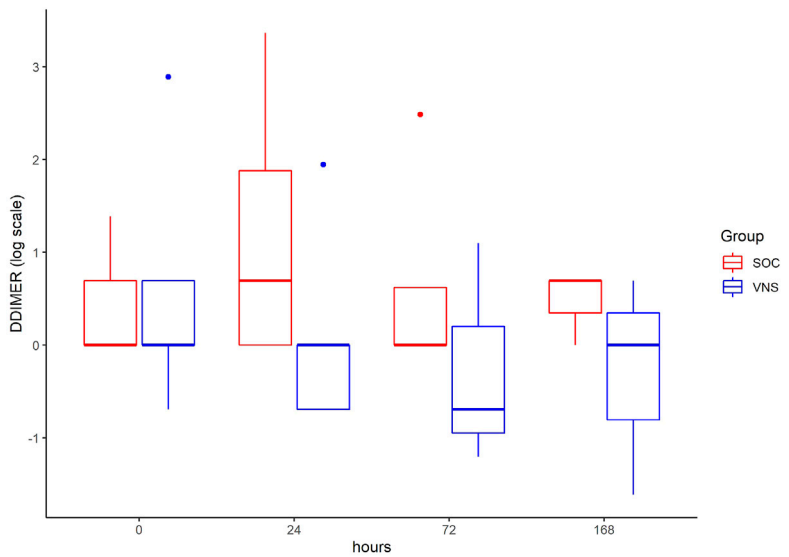

FIGURE 10 | Median DDIMER level with Interquartile Range of patients receiving auricular Vagus Stimulation (VNS = blue) and patients only receiving Standard of Care (SOC = red) after 0, 24, 72, and 168 h of study inclusion.

83.9 mg/dl to 187.1 mg/dl). Ferritin decreased as well initially, but there was no clear difference in comparison to the control group. In all patients, the inflammation and coagulation biomarkers DDIMER (from 4.5 µg/ml at T0 to 1.5 µg/ml at T168) and fibrinogen (from 4.6 g/L at T0 to 3.7 g/lat T168) decreased initially after the start of aVNS, while in the control group the mean fibrinogen levels increased from 4.4 g/L to 7.5 g/L and mean DDIMER levels decreased from significantly less from 1.8 µg/ml to 1.7 µg/ml. It is not known that the patients receiving aVNS have a better clinical outcome due to the lower inflammation parameters; however, in another study elevated CRP, ferritin, and DDIMER were found to predict worsening outcomes in Covid-19 (Caricchio et al., 2021), which may indicate a favourable effect of aVNS. Other studies have shown that a decrease of inflammation parameters throughout the clinical course improved patient outcomes in Covid-19 (Zhou et al., 2020) and reduced risk and severity of ARDS (Wu et al., 2020). Furthermore, in this study a decrease of macrophage activation parameters such as

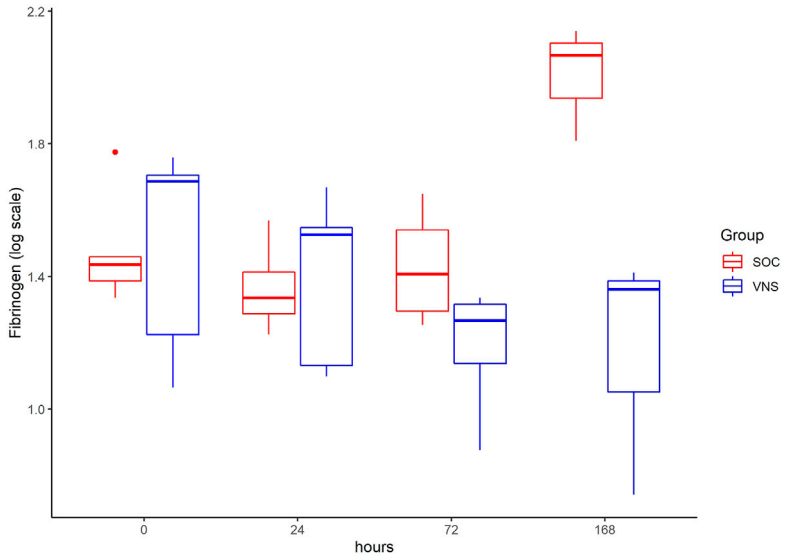

FIGURE 11 | Median Fibrinogen level with Interquartile Range of patients receiving auricular Vagus Stimulation (VNS = blue) and patients only receiving Standard of Care (SOC = red) after 0, 24, 72, and 168 h of study inclusion.

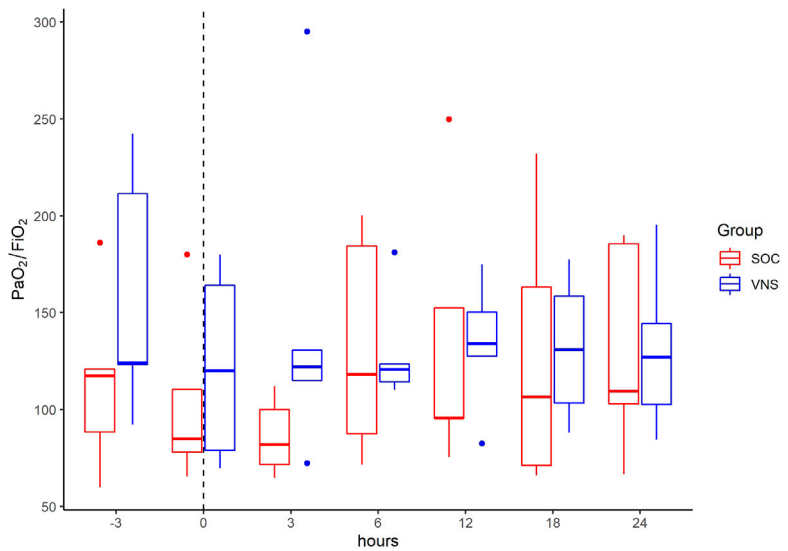

FIGURE 12 | Median Horowitz index (PaO_2/FiO_2) with Interquartile Range of patients receiving auricular Vagus Stimulation (VNS = blue) and patients only receiving Standard of Care (SOC = red) prior to, at time of study inclusion (dashed line) and up to 24h afterwards.

sIL-R, calprotectin, and IL-18 in all patients 4 h after initiation of aVNS was observed, in contrast to the SOC group, in which an increase by 40–60% was registered.

It is noteworthy that the early aVNS-induced decline of proinflammatory mediators such as IL-6 was not sustained during the observation period. aVNS most likely has a complex effect on the regulation of the inflammatory cascade during COVID-19. Along these lines it is interesting to note that aVNS did not have a down-regulatory effect on all proinflammatory parameters alike. While IL-6 and TNF-alpha decreased early following aVNS treatment, other mediators of phagocyte activation such as calprotectin or IL-18 seemed to be less affected. Calprotectin has crucial activities in the regulation of immune homeostasis and inflammation and triggers inflammation through interaction—as an endogenous agonist - with the proinflammatory phagocyte receptor TLR4. Binding to TLR4 initiates a signaling cascade including IL-6 expression in an NF-κB-dependent manner (Vogl et al., 2007). Through induction

of IFN-gamma IL-18 primes mononuclear phagocytes for TNF-alpha-induced IL-6 expression, which might be particularly relevant during the later stages of viral infection. Independently of IFNγ or other cytokines, IL-18 exhibits proinflammatory characteristics such as increases in cell adhesion molecules, nitric oxide synthesis, and chemokine production via NF-kB activation (Kohka et al., 1998). It is thus possible that aVNS has a direct effect on the early phase of IL-6 induction while the effect on a later phase of IL-6 release that is predominantly secondary to other proinflammatory mediators is less pronounced. Alternatively, desensitization of the aVNS effect on IL-6 production might develop, as has been described for vagus nerve stimulation-induced weight loss (Khan et al., 2017), probably involving desensitisation of adrenergic receptors involved in the therapeutic effect, a process that leads to reduced receptor responsiveness after prolonged stimulation (Carrara et al., 2021).

To the best of our knowledge, this is the first prospective randomised study using aVNS in patients with critical Covid-19. Although the potential positive impact of vagus nerve stimulation on cytokine storm in patients with severe Covid-19 was often discussed (Bonaz et al., 2020a, 2020b; Bara, de Ridder and Maciaczyk, 2020; Kaniusas and Szeles, 2020b; Guo et al., 2021; Wang et al., 2022), clinical data is scarce. Boezaart et al. reported two cases of patients with severe Covid-19 receiving transcutaneous aVNS in addition to standard of care in which case a rapid decrease of IL-6 was observed and aVNS was well tolerated (Boezaart and Botha, 2021). However, in contrast to our study, there was no comparison to a control group. Further, in our study percutaneous stimulation with miniature needle electrodes was preferred over the transcutaneous stimulation with surface electrodes in order to avoid diffuse stimulation of vagal and non-vagal nerves in the auricle as well as to avoid large stimulation voltages (to overcome skin barrier). In addition, an uncomfortable tragus clip was also avoided for the comfort of the patient.

At this point in time the Covid-19 pandemic has been ongoing for more than 2 years and the clinical presentation of the disease is known to vary widely. Although most cases of Covid-19 are mild or asymptomatic, in some cases the initial stage of viral replication can be followed by a stage of hyperinflammatory response to SARS-CoV-2 infection resulting in severe disease with acute respiratory distress syndrome (ARDS) or even multi-organ failure (Fajgenbaum and June 2020). The pathomechanism of the 'cytokine storm' is not fully explained. Tay et al. (Tay et al., 2020) postulated that SARS-CoV-2 is a cytopathic virus inducing death of infected cells during viral replication due to pyroptosis: an inflammatory form of programmed cell death caused by secretion of proinflammatory cytokines such as IL-6 and IFNγ. The excessive secretion of cytokines recruits activated macrophages and T cells to the site of infection inducing local inflammation that could lead to tissue damage. This local inflammation again stimulates systemic cytokine production further escalating the cytokine storm. This excessive inflammatory response can cause further local tissue damage, like destruction of lung parenchyma resulting in ARDS and multi-organ failure. Furthermore, the excessive recruitment of activated macrophages can lead to hemophagocytosis which can promote organ failure at several sites. Postmortem analysis has demonstrated the presence of hemophagocytosis in lung, heart, liver, bone marrow, and the reticuloendothelial organs (Fox et al., 2020; Bryce et al., 2021) in patients with severe Covid-19.

Several medications have been developed to reduce cytokine expression or systemic inflammation, such as antibodies against different interleukin receptors or cortisone. However, interference with the immune system can cause an array of different problems such as increased risk of secondary infection (Fajgenbaum and June 2020).

The non-invasive vagus nerve stimulation on the neck has shown a decrease in inflammatory markers such as CRP and procalcitonin, as stated in a non peer-reviewed preprint (Tornero et al., 2021a; 2021b), whereas this type of stimulation was successfully applied in managing respiratory symptoms in two case studies (Staats et al., 2020; Tornero et al., 2021a). In general, neuromodulatory applications have a strong rationale for their use in acute and chronic Covid-19 symptoms (Pilloni et al., 2020) along various pathways, such as modulation of anti-inflammatory responses, amelioration of musculoskeletal pain and fatigue, augmenting rehabilitation, and reducing mental distress (Baptista et al., 2020). Antinociceptive effects of aVNS were also demonstrated, in line with the use of the same stimulation device in chronic cervical pain and chronic low-back pain (Sator-Katzenschlager et al., 2003, 2004).

Limitations of the study are the lack of interpretation of clinical outcome and the small number of participants. Further we did not include many clinical parameters. We showed an increase of Horowitz Index after start of VNS. However, interpretation must be cautious, because it can be influenced by many factors, like breathing index or ventilation method. These limitations should be addressed in future studies.

In summary, this study shows that aVNS has the potential to reduce expression of pro-inflammatory proteins and increase expression of anti-inflammatory proteins in patients with severe Covid-19. Given the good tolerance and low risk of side effects, non-invasive aurical vagus stimulation might present a good option for additional treatment of patients with hyperinflammatory Covid-19.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethikkommission Stadt Wien. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TS, JS, RK, HW, AZ, SN, CW, and EK conceived and planned the study and contributed to the design and implementation of the research. TS, AG, JH, HA, and RK screened the patients for inclusion and exclusion criteria and collected samples and prepared them for analysis. FL and AT carried out the simulations. TS, RK, JH, JS, and EK contributed to the interpretation of the results. TS and EK took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

FUNDING

A funding was applied for the study at the Austrian Institute for Economic Research and was granted (GED—Wi-2021/155). The funding was only used to pay for the devices and analysis of the inflammation parameters.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.897257/full#supplementary-material

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Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin

Borovikova V. Lyudmila, et al. (2000)
Nature 405:458-462
DOI: 10.1038/35013070

ABSTRACT

Vertebrates achieve internal homeostasis during infection or injury by balancing the activities of proinflammatory and anti-inflammatory pathways. Endotoxin (lipopolysaccharide), produced by all gram-negative bacteria, activates macrophages to release cytokines that are potentially lethal1,2,3,4. The central nervous system regulates systemic inflammatory responses to endotoxin through humoral mechanisms5,6,7,8. Activation of afferent vagus nerve fibres by endotoxin or cytokines stimulates hypothalamic–pituitary–adrenal anti-inflammatory responses9,10,11. However, comparatively little is known about the role of efferent vagus nerve signalling in modulating inflammation. Here, we describe a previously unrecognized, parasympathetic anti-inflammatory pathway by which the brain modulates systemic inflammatory responses to endotoxin. Acetylcholine, the principle vagal neurotransmitter, significantly attenuated the release of cytokines (tumour necrosis factor (TNF), interleukin (IL)-1β, IL-6 and IL-18), but not the anti-inflammatory cytokine IL-10, in lipopolysaccharide-stimulated human macrophage cultures. Direct electrical stimulation of the peripheral vagus nerve in vivo during lethal endotoxaemia in rats inhibited TNF synthesis in liver, attenuated peak serum TNF amounts, and prevented the development of shock.

VNS for the treatment of inflammatory disorders of the gastrointestinal tract

Bonaz Bruno, et al. (2017)
In: Majid, A. (eds) Electroceuticals. Springer, Cham.
DOI: 10.1007/978-3-319-28612-9_9

ABSTRACT

The brain and the gut communicate bi-directionally through the autonomic nervous system of which the vagus nerve is a major component. The vagus nerve has a well-documented anti-inflammatory activity through its afferents and the hypothalamic-pituitary-adrenal axis. More recently, an anti-inflammatory role of vagal efferents has also been discovered through the cholinergic anti-inflammatory pathway. Vagus nerve stimulation, used in the treatment of drug resistant epilepsy and depression, could be an effective tool to treat inflammatory disorders of the gastro-intestinal tract, such as inflammatory bowel disease, irritable bowel syndrome, as well as postoperative ileus which are characterized by an autonomic imbalance with a low vagal tone.

https://link.springer.com/chapter/10.1007/978-3-319-28612-9_9