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Non-invasive vagus nerve stimulation in healthy humans reduces sympathetic nerve activity

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tVNS reduces sympathetic nerve activity

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Abstract

Background: Vagus nerve stimulation (VNS) is currently used to treat refractory epilepsy and is being investigated as a potential therapy for a range of conditions, including heart failure, tinnitus, obesity and Alzheimer's disease. However, the invasive nature and expense limits the use of VNS in patient populations and hinders the exploration of the mechanisms involved.

Objective: We investigated a non-invasive method of VNS through electrical stimulation of the auricular branch of the vagus nerve distributed to the skin of the ear – transcutaneous VNS (tVNS) and measured the autonomic effects.

Methods: The effects of tVNS parameters on autonomic function in 48 healthy participants were investigated using heart rate variability (HRV) and microneurography. tVNS was performed using a transcutaneous electrical nerve stimulation (TENS) machine and modified surface electrodes. Participants visited the laboratory once and received either active (200 μ s, 30Hz; n = 34) or sham (n = 14) stimulation.

Results: Active tVNS significantly increased HRV in healthy participants ($p = 0.026$) indicating a shift in cardiac autonomic function towards parasympathetic predominance. Microneurographic recordings revealed a significant decrease in frequency ($p = 0.0001$) and incidence ($p = 0.0002$) of muscle sympathetic nerve activity during tVNS.

Conclusion: tVNS can increase HRV and reduce sympathetic nerve outflow, which is desirable in conditions characterised by enhanced sympathetic nerve activity, such as heart failure. tVNS can therefore influence human physiology and provide a simple and inexpensive alternative to invasive VNS.

Key words: vagus nerve stimulation, sympathetic nervous system, neuromodulation

Abbreviations List: ABVN – auricular branch of the vagus nerve; HF – high frequency; HRV – heart rate variability; LF – low frequency; MSNA – muscle sympathetic nerve activity; TENS – transcutaneous electrical nerve stimulation; tVNS – transcutaneous vagus nerve stimulation; VNS – vagus nerve stimulation.

Introduction

Electrical stimulation of the cervical vagus nerve has been approved for treatment resistant epilepsy in Europe and the USA for over 15 years and has been used to treat over 50,000 epilepsy patients (1). VNS is also an approved therapy for treatment resistant depression in the USA (2) and has been investigated as a potential therapy for a wide range of conditions including heart failure (3), inflammation (4), Alzheimer's disease (5), obesity (6), chronic pain (7) and tinnitus (8). However, despite positive indications from pilot studies larger scale trials are rarer. For example, even though the cognitive function of 70% of patients with Alzheimer's disease improved or did not decline during a 1 year pilot study (5), no larger scale trials have been reported.

One factor that may hinder larger trials of VNS is the invasive nature of VNS. VNS requires surgical implantation of a bipolar electrode around the cervical vagus nerve and implantation of a generator subcutaneously in the thoracic wall. This is associated with technical and surgical complications including wound infection, cardiac arrhythmia under test stimulation and electrode malfunction (9). In addition, side effects include hoarseness, dysphagia, cough and pain (10).

Given the number of conditions that VNS has the potential to benefit, a simpler, less invasive approach would enable treatment of significantly larger numbers. A potential non-invasive route for VNS is electrical stimulation of the auricular branch of the vagus nerve (ABVN), which is distributed to the external ear (Figure 1) (11). This stimulation can be performed transcutaneously by applying surface electrodes or acupuncture needles to the external ear (tVNS). Such tVNS has been trialled in patients with coronary artery disease (12, 13), epilepsy (14) and chronic pain (15), however, the outcomes investigated are varied and mostly

subjective. In addition, the stimulation parameters used differ widely, therefore, little is known about the optimal parameters for tVNS.

VNS has proven effective in pilot studies for the treatment of heart failure (16), for which a multi-centre trial is on-going (ClinicalTrials.gov Identifier: NCT01303718). Approximately 5.7 million people in the US have heart failure (17) costing the US economy \$34.4 billion every year (18). Heart failure is a leading cause of mortality and it is estimated that 50% of people die within 5 years of diagnosis. Heart failure is characterised by decreased parasympathetic and increased sympathetic nerve activity (19). Therefore, if tVNS can be shown to influence this autonomic balance towards parasympathetic predominance it could provide a method to correct imbalance in heart failure patients.

In this study we investigated the effects of tVNS on cardiovascular autonomic function in healthy participants by measuring heart rate variability. We then applied microneurography to record muscle sympathetic nerve activity directly during tVNS.

Methods

General Protocol

The study was approved by the University of Leeds Ethics Committee and conducted in accordance with the Declaration of Helsinki. Informed written consent was obtained from all participants. 48 healthy participants (24 female, 24 male; 20-62 years old) were recruited for the study. Inclusion criteria were male or female over the age of 18 years. Exclusion criteria were a history of cardiovascular disease, diabetes or hypertension. The study began between 8-10am in a dedicated study room at $21 \pm 2^{\circ}\text{C}$. All participants were asked to avoid alcohol and intense exercise 12 hours prior to attendance. They were also asked to avoid caffeine and nicotine on the morning of the study and to void their bladder before the study commenced. Participants were asked to lie on a couch in a semi-supine position while heart rate, blood pressure and respiration were monitored continuously. Data were recorded at baseline, during tVNS and during recovery and each recording period lasted 15 minutes. All participants visited the laboratory once and received either active or sham transcutaneous vagus nerve stimulation (tVNS).

Transcutaneous Vagus Nerve Stimulation (tVNS)

tVNS was performed using a Transcutaneous Electrical Nerve Stimulation (TENS) device (V-TENS Plus, Body Clock Health Care Ltd, UK) with modified surface electrodes. Electrodes were placed on the inner and outer surface of the tragus of the ear. Active tVNS ($n = 34$) was applied continuously for 15 minutes with a pulse width of $200 \mu\text{s}$ and pulse frequency of 30 Hz. Amplitude was adjusted to the level of sensory threshold (10-50 mA). Sham tVNS ($n = 14$) was performed by placing the electrodes on the tragus and increasing amplitude until the participant reported

feeling sensation. Participants were then told that the amplitude would be reduced slightly to prevent discomfort but the electrode leads were disconnected from the TENS machine without the participants' knowledge.

Heart Rate Variability (HRV)

A three lead ECG was used to monitor and record heart rate. Electrodes (Ambu, UK) were placed on left and right clavicles and costal margins. This arrangement enabled changing electrode polarities to select the lead that detected the most prominent R peak for subsequent HRV analysis (normally lead II). HRV was analysed offline using LabVIEW software (National Instruments, USA). A threshold was set to detect R peaks from an 8 minute ECG recording and R-R intervals used to produce a tachogram. The ECG was inspected to ensure all R peaks were detected and there were no abnormalities in the ECG such as ectopic beats (e.g. premature ventricular complexes). Ectopic beats could be corrected using a linear spline to average the R-R interval prior to and following the ectopic. If more than 2 ectopics were detected the recording was excluded. The resulting tachogram underwent 512 point Fast Fourier Transform with a Hanning window to calculate the power spectrum of HRV with the low frequency (LF) component at 0.04-0.15 Hz and the high frequency (HF) component at 0.15-0.40 Hz. LF and HF power were also converted to normalised units as a percentage of the total power to determine LF/HF ratio. The HF component reflects parasympathetic modulation of heart rate (20) and the LF component reflects both sympathetic and parasympathetic modulation of heart rate (21). The ratio of low frequency (LF) and high frequency (HF) oscillations of heart rate variability can be used as an index of cardiac autonomic balance such that a decrease in LF/HF ratio indicates a shift in cardiac autonomic balance towards

parasympathetic predominance and thus an improvement in HRV (20, 22). It is important to note that this may be due to either a decrease in sympathetic activity or an increase in parasympathetic activity.

Respiration

A piezo-electric transducer (Pneumotrace, UFI, USA) was placed round the thorax to monitor and record respiration rate. A respiration rate <10 breaths/min was unacceptable for HRV analysis as the HF component is respiration dependent. At slow respiration rates the HF peak of the HRV spectrum can merge with the LF peak (23). In this case the subjects were asked to use a breathing metronome set at 16 breaths/min (n = 3).

Microneurography

Muscle sympathetic nerve activity (MSNA) was recorded as previously described (24-26) in 10 volunteers receiving active tVNS (8 male, 2 female; 29-59 years). Two tungsten microelectrodes were inserted percutaneously below the knee. One electrode was inserted into the peroneal nerve (recording electrode) and the second was inserted into subcutaneous tissue 1-2cm away (reference electrode). The raw nerve signal was amplified (x50k), filtered (0.7-2 kHz; Neurolog) and digitised (16 kHz; Power 1401, CED). The data was displayed in real time and recorded on a PC (Dell laptop) using Spike2 (version 7; CED). This allowed inspection of the nerve signal during the experiment. The recording microelectrode was manipulated until a single unit could be visualised. To confirm that this was a sympathetic vasoconstrictor unit the following conditions were met; 1) the unit occurred in diastole, 2) there was no increase in activity in response to brushing the skin of the

leg, 3) activity increased in response to cold presser test or isometric handgrip test (Fig. 4a). Cold presser test comprised placing one hand in ice water (approximately 4°C) for one minute. Isometric handgrip test involved squeezing a handgrip at 50% maximal voluntary contraction for 2 minutes. Further confirmation was obtained during off-line analysis by superimposing all putative MSNA units to ensure the amplitude and shape remained constant indicating that these were recorded from the same axon (Figure. 4B,C). MSNA bursts were also inspected by rectifying and integrating (time constant 0.1s) the neurogram. MSNA single unit frequency (per min) was calculated. MSNA single unit incidence (per 100 heart beats) were also calculated to limit the effect of any changes in heart rate. Data were normalised to baseline due to a high degree of inter-individual variation.

Data Acquisition

ECG, MSNA, blood pressure and respiration data were split into two channels and fed into two data amplification systems (Coulbourn Lab Sinc V, Coulbourn Ltd, USA and Neurolog, CED, UK). Channels were independently calibrated before digitisation and storage on PCs. Data channels were then displayed on monitors using LabVIEW (National Instruments, USA) and Spike2 (CED, UK) software. The data were sampled at 12-16 kHz and stored on hard drives.

Statistical analysis

All statistical analyses were carried out using SPSS (version 18). Independent t-tests or Mann-Whitney U test were used to compare group characteristics. Repeated measures ANOVA was used to analyse time effect (baseline, stimulation, recovery) in each group alone with post hoc Bonferroni correction. To analyse the effects of

active and sham tVNS, a mixed mode ANOVA with group (active or sham stimulation) and time (baseline, stimulation, recovery) was used. . Where interactions were revealed, post hoc analyses were undertaken using repeated measures ANOVA on each group separately. The Greenhouse – Geisser correction was used where data did not meet sphericity. Linear regression was used to explore the relationship between variables. Data are presented as group mean \pm standard error of the mean (S.E.M.) unless stated otherwise. A 2-tailed probability value < 0.05 was considered statistically significant.

Results

tVNS significantly alters heart rate variability

Baseline characteristics of the active and sham tVNS groups were not significantly different (Mann-Whitney U test, $p > 0.05$; Table 1).

Repeated measure ANOVA revealed a significant decrease in LF/HF ratio during active tVNS (time effect, $p = 0.026$; Table 2; Figure 2). There was no significant change in LF/HF ratio in the sham group ($p > 0.05$). There was a modest but significant decrease in heart rate ($p < 0.005$) during active and sham tVNS. There was also a significant increase in mean BP ($p < 0.005$) during active and sham tVNS that did not recover. This finding is likely to be due to the method of measurement (Finometer) as there was no significant change in BP measurements taken using an arm sphygmomanometer (see Supplementary Figure 2) whereas the increase in BP measured using the Finometer persisted into the recovery period suggesting that the increase may be due to constriction and oedema in the finger (27).

Response to tVNS is correlated with baseline LF/HF ratio

Linear regression revealed a relationship between baseline LF/HF ratio and the change in LF/HF ratio during tVNS such that a higher LF/HF ratio predicts a greater response to tVNS ($R^2 = 0.58$; $p < 0.0005$; Figure 3A). Higher baseline LF/HF values were also observed with increasing age ($R^2 = 0.19$; $p = 0.013$; Figure 3B).

tVNS reduces muscle sympathetic nerve activity

Applying microneurography to directly record sympathetic vasoconstrictor nerve. a significant decrease in MSNA frequency (time effect, $p = 0.001$) and incidence (time effect, $p = 0.002$; Figure 4) was detected during tVNS ($n=10$). Eight of these 10 participants responded to tVNS with a decrease in MSNA, whilst the remaining 2 showed no change, which corresponded with the effects of tVNS on HRV on these individuals (Supplementary Figure 3). These participants did not differ in baseline characteristics (age, BMI, heart rate, blood pressure etc) from the rest of the tVNS group or sham groups (Kruskal-Wallis test, $p > 0.05$).

Discussion

This study shows that transcutaneous vagus nerve stimulation (tVNS) can alter cardiovascular autonomic control in healthy humans and highlights the role of the sympathetic nervous system in mediating tVNS effects. tVNS significantly decreased LF/HF ratio, indicating improved heart rate variability with a shift in cardiac autonomic balance towards parasympathetic/vagal dominance. This shift occurred alongside a decrease in MSNA, revealed by microneurography during tVNS.

tVNS effects on cardiovascular autonomic function

Increased sympathetic activity and/or reduced parasympathetic nerve activity as indicated by HRV is not only a powerful and independent predictor of poor prognosis in patients with cardiovascular disease (28, 29), but also a risk factor for mortality in healthy populations (30). Similarly, increased MSNA is associated with poor prognosis in heart failure and is also elevated in hypertension, obstructive sleep apnoea and obesity (31). The ability to favourably alter HRV and MSNA through tVNS in a healthy population is significant and could be applied to many populations where cardiovascular autonomic balance is shifted toward sympathetic predominance e.g. older or sedentary (32) populations or in conditions with sympathoexcitation such as heart failure (33). Indeed, the significant correlation between baseline LF/HF ratio and the change in LF/HF ratio during stimulation implies that tVNS may be even more effective in these populations compared to the healthy population used in this study.

Of particular interest in this study is the finding that the LF/HF ratio and MSNA remain lower than baseline levels during the recovery period after tVNS has ceased.

The stimulation and recovery period lasted 15 minutes therefore the long term effects of tVNS on cardiovascular autonomic control require further investigation, however, HRV effects of tVNS performed using acupuncture increased in HF power (indicating increased parasympathetic activity) for at least an hour after stimulation had ceased (34). The increase in HF power reported by this study is contrary to our findings. This may be due to the smaller sample size used ($n = 12$), the different tVNS technique used or the limitation of HRV analysis as an indirect measure of cardiac autonomic activity. La Marca et al. (35) demonstrated that auricular electroacupuncture increases respiratory sinus arrhythmia (RSA, mediated by the vagus nerve) in healthy participants ($n = 14$) suggesting increased vagal activity, however, this is also an indirect measure of parasympathetic activity. One of the limitations of these studies, including ours, is that they have all used healthy participants. The extent of the tVNS effects that might be observed in patient, sedentary or older populations which characteristically have reduced parasympathetic activity therefore seems likely to be underestimated.

The first clinical study of tVNS found that electroacupuncture of both ears was beneficial for patients with coronary artery disease (12, 13). Patients ($n = 10$) received tVNS for 15mins/day for 10 consecutive days. After 4 treatments, angina symptoms at rest were abolished and patients no longer required vasodilators. After 7 treatments, patients had improved exercise tolerance and were able to climb 5-7 flights of stairs without developing angina symptoms. These studies also reported that the improvement in angina symptoms persisted after the cessation of tVNS treatment for up to 3 weeks. Recently, tVNS using surface electrodes has been investigated as a possible analgesic (15, 36) and has also been trialled as an

alternative to invasive cervical VNS in patients with refractory epilepsy (14). These studies also monitored heart rate and blood pressure and reported no significant changes. Napadow et al. (15) also analysed ECG data for HRV and found tVNS had no significant effect (n = 10). These findings are contrary to the results of our study, however this seems likely to be due to the smaller sample sizes and the different stimulation parameters used.

Potential pathways of tVNS cardiovascular autonomic effects

The neurocircuitry underlying tVNS autonomic effects requires further elucidation. The auricular branch of the vagus nerve has previously received little attention and hence there is a dearth of information regarding its central projections and its peripheral distribution. Only one study in the literature investigates the distribution of the ABVN to the external ear (11). This reports that the ABVN is distributed to the tragus, concha, cymba concha and anti-helix of the ear. EEG studies have shown that tVNS of the tragus elicits vagus somatosensory evoked potentials (VSEP) (37, 38). Interestingly, stimulation at other sites in the ear not supplied by the ABVN (helix, anti-helix, scapha and lobe) did not elicit VSEPs supporting evidence that the tragus is innervated by the ABVN. Functional MRI of tVNS of the tragus in humans revealed a similar activation pattern to conventional cervical VNS further supporting the potential of tVNS at the tragus as a non-invasive alternative (39).

The central projections of the ABVN have been investigated in cats and dogs and were found to project to the nucleus tractus solitarius (NTS), which plays an integral role in relaying vagal afferent visceral information (40, 41). In addition, neuronal tracing from the junction of the concha and external auditory meatus in rats revealed

sensory afferent terminations in the NTS and dorsomedial spinal trigeminal tract (42). Indeed, the ABVN is thought to be involved in some peculiar somatovisceral reflexes. These include the ear-cough reflex (Arnold's reflex), estimated to be present in approximately 4% of the general population (43), whereby stimulation of the external auditory meatus (e.g. syringing) mimics the cough response mediated by vagal afferent innervation of the trachea. Other examples include the ear-gag reflex, ear-lacrimal reflex and auricular syncope (43, 44) although these are rare. Another interesting phenomenon involving the ABVN is pain referred to the external ear from viscera supplied by the vagus nerve in conditions such as lung cancer (45-47), gastroesophageal reflux (48) and myocardial infarction (49, 50). Furthermore, cervical vagus nerve stimulation has also been reported to cause ear pain (16, 51).

Based on the results of this study, the proposed pathway of tVNS autonomic effects could involve activation of the NTS by ABVN afferents. This could activate the caudal ventrolateral medulla to inhibit the rostral ventrolateral medulla and thus reduce sympathetic output (52). In addition, the NTS could also activate the dorsal motor nucleus of the vagus and the nucleus ambiguus to increase parasympathetic activity (53). However, the effects of tVNS on parasympathetic activity are unclear.

Scope of tVNS therapy for cardiovascular diseases

VNS is already being trialled as a potential heart failure therapy and has resulted in positive clinical outcomes (3). Our findings support the use of tVNS as a non-invasive method of VNS for cardiovascular diseases. Of particular interest is the finding that tVNS reduces sympathetic outflow. Sympathoexcitation is the hallmark of many conditions including heart failure, hypertension and obstructive sleep apnoea

(31). Further, auricular electroacupuncture has beneficial effects in coronary artery disease (12, 13). The tVNS approach described here may therefore offer a simple, non-invasive and economical alternative that could make vagus nerve stimulation a widely available therapy and potentially improve quality of life for patients with a broad range of cardiovascular diseases.

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Figure Legends

Figure 1. The distribution of the auricular branch of the vagus nerve to the external ear (shaded area, extrapolated from Peuker and Filler(11)). C, concha; T, tragus; CyC, cymba concha.

Figure 1

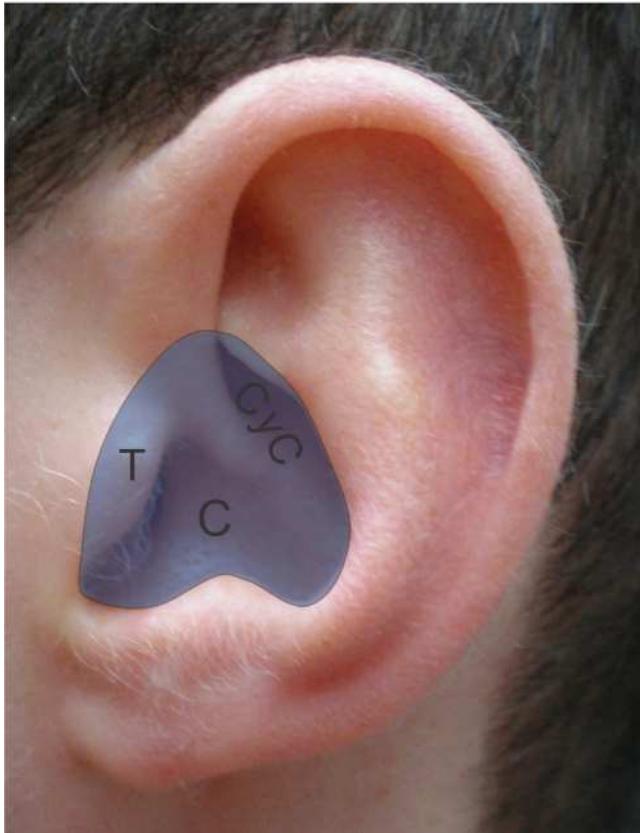


Figure 2 There is a significant decrease in LF/HF ratio during active tVNS ($p = 0.026$) whereas there is no significant change during sham tVNS.

Figure 2

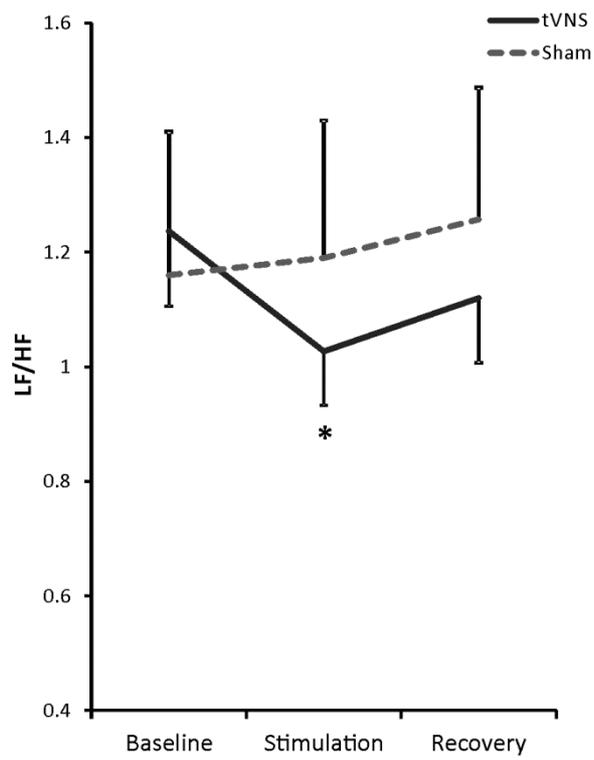


Figure 3. There is a relationship between baseline LF/HF ratio and change in LF/HF ratio during tVNS indicating that higher LF/HF ratios predict a greater decrease in LF/HF during tVNS ($R^2=0.58$; $p<0.0005$; A). There is a relationship between age and baseline LF/HF ratio ($R^2=0.19$; $p=0.013$; B).

Figure 3

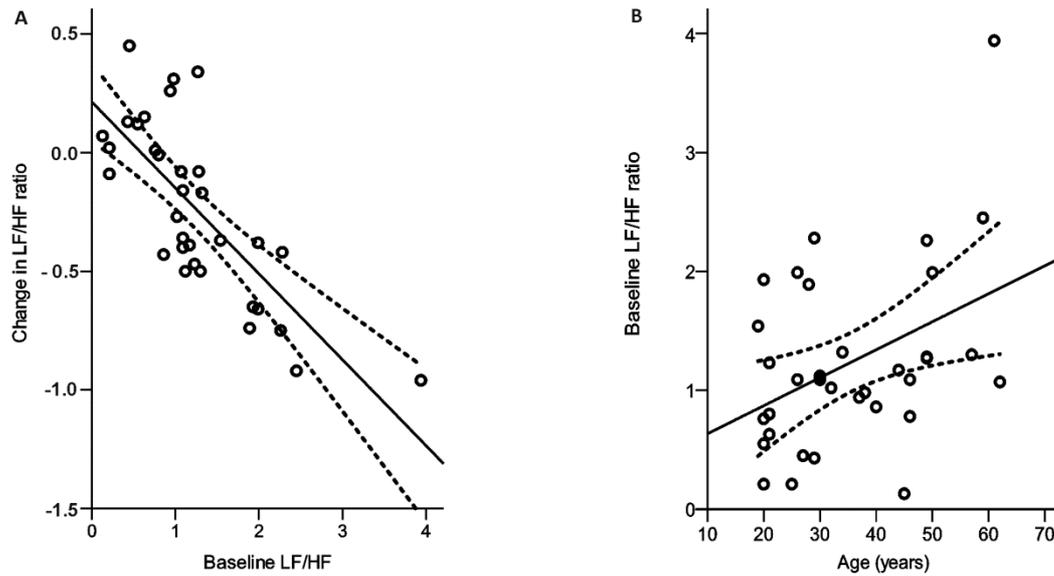
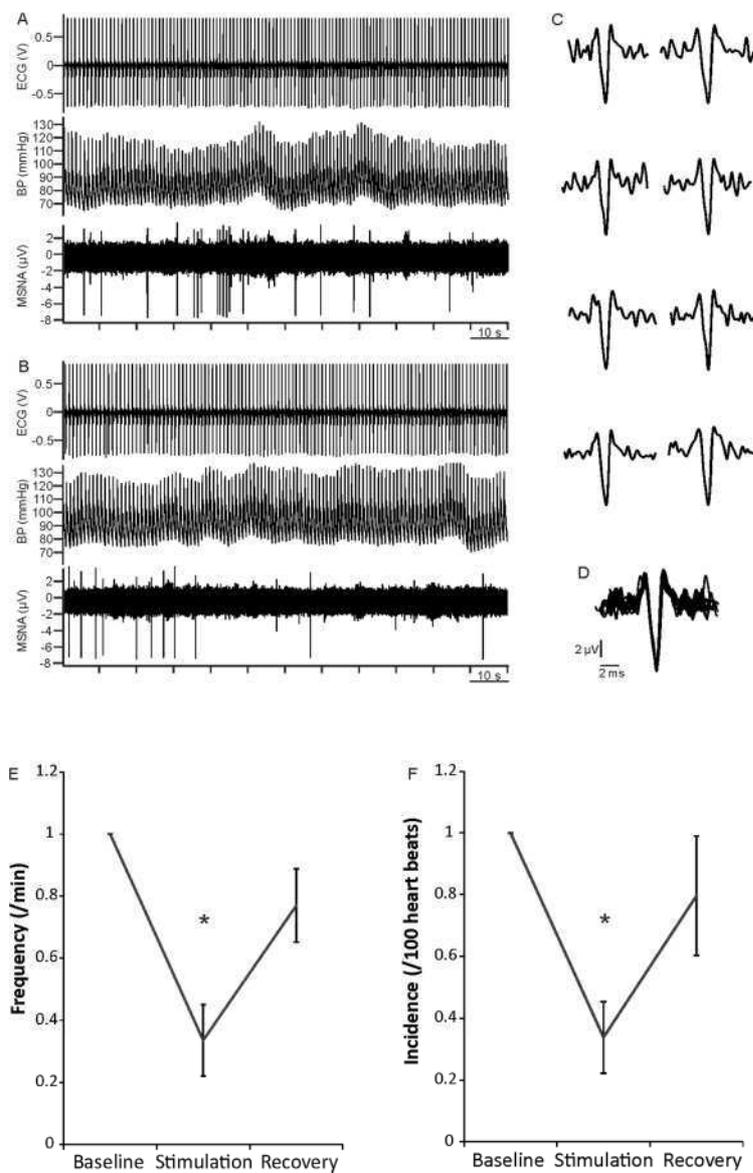


Figure 4 Example microneurography recording during baseline (A) and during tVNS (B) indicating electrocardiogram (ECG), blood pressure (BP) and MSNA. MSNA units in more detail (C) and overlaid (D). tVNS significantly reduces MSNA frequency (E; $*P = 0.0001$) and incidence (F; $*P = 0.0002$; normalized data; $n = 10$).

Figure 4



	Sham	Active
Number	14 (6 ♀; 8 ♂)	34 (18 ♀; 16 ♂)
Age (years)	38 ± 3.48	34 ± 2.3
BMI (kg/m²)	23.9 ± 0.67	24.9 ± 0.71
Heart rate (bpm)	64 ± 2.49	64 ± 1.31
Mean BP (mmHg)	79 ± 3.59	83 ± 1.99

Table 1 Baseline characteristics. There was no significant difference in baseline characteristics between active and sham tVNS groups.

	Sham tVNS			Active tVNS		
	Base	Stimulation	p	Base	Stimulation	p
Total power (ms²)	2463.75 ±732.50	2789.22 ±843.02	n.s.	2735.31 ±422.23	3212.17 ±497.45	n.s.
LF Power (ms²)	615.52 ±168.65	664.63 ±160.85	n.s.	906.39 ±133.74	821.49 ±177.62	n.s.
HF Power (ms²)	1109.41 ±395.90	1286.40 ±469.05	n.s.	972.67 ±208.32	1043.02 ±178.65	n.s.
LF/HF	1.16 ±0.30	1.19 ±0.32	n.s.	1.26 ±0.15	1.04 ±0.14	0.026

Table 2 Heart rate variability values for sham and active tVNS groups There was a significant decrease in LF/HF ratio during active tVNS ($p = 0.026$). There was no significant (n.s.) change in total power, low power or high power during active tVNS. There was no significant change in any HRV values in the sham tVNS group.

Supplementary Data

Electrode positioning for tVNS

Before undertaking the main study presented in this paper, pilot studies were performed to refine the tVNS methodology. Since the auricular branch of the vagus nerve is distributed to parts of the external ear including the tragus, concha and cymba concha¹ (Figure.1), the effect of stimulating these three areas by specific electrode placement at the ear was investigated.

Methods

To determine an effective electrode configuration three different electrode configurations were investigated in 63 healthy volunteers (34 female, 29 male; 20-66 years old); tragus, tragus + cymba or concha. The efficacy of tVNS of either the right ear only or both ears simultaneously was also investigated in the same participants (Supplementary Table 1). tVNS was performed continuously for 15 minutes with a pulse width of 20 μ s and pulse frequency of 15 Hz. Amplitude was adjusted to the level of sensory threshold (10-50 mA). Data were recorded for 15 minutes at baseline, during tVNS and recovery period.

Right side only			Left and right sides		
Tragus	Cymba + Tragus	Concha	Tragus	Cymba + tragus	Concha
10	8	3	24	11	7

Supplementary Table 1 Number of participants in each electrode configuration group

To analyse the effects of different electrode configurations, mixed mode ANOVA with group (electrode configuration) and time (baseline, stimulation, recovery) was used. Where interactions were revealed, post hoc analyses were undertaken using repeated measures ANOVA on each group separately. The Greenhouse – Geisser correction was used where data did not meet sphericity.

Results

There was no significant change in HRV during tVNS when data from all participants were analysed (repeated measures ANOVA; Supplementary Table 3). However, HRV analysis indicated that there were responders (those who had a decrease in LF/HF ratio during tVNS; $n = 31$) and non-responders ($n = 32$). Baseline LF/HF ratio was significantly higher in responders compared to non-responders (LF/HF ratio 1.32 and 1.04 respectively; $p = 0.038$; Supplementary Table 2). Comparing the two groups revealed no significant differences in other baseline characteristics such as body mass index (BMI), age, resting heart rate and blood pressure (Mann-Whitney U test, Supplementary Table 2).

To determine if there were differences between the responder and non-responder groups during tVNS a mixed mode ANOVA was performed to analyse the interaction between group and stimulation. There was a significant interaction between group and time ($p < 0.0005$). To understand the factors underlying this significance, further analysis of the responder group alone revealed a significant decrease in LF/HF ratio (time effect, $p < 0.0005$; repeated measures ANOVA; Supplementary Table 3) during tVNS indicating a shift in autonomic balance towards parasympathetic predominance. Conversely, there was a significant increase in LF/HF ratio in the non-responder group (time effect, $p < 0.0005$; Supplementary Table 3) indicating that the interaction was due to an increase in LF/HF ratio in the non-responder group compared to a decrease in the responder group. These experiments indicated that tVNS effectively decreased the LF/HF ratio in a subset of participants.

	tVNS	tVNS responder	tVNS non-responder
Number	63 (34 ♀; 29 ♂)	31 (16 ♀; 15 ♂)	32 (18 ♀; 14 ♂)
Age (years)	38±1.66	38±2.39	37±2.35
BMI (kg/m²)	24.5±0.57	24.2±0.54	24.8±1.00
Heart rate (bpm)	65±1.16	63±1.79	66±1.48
Mean BP (mmHg)	80±1.46	80±2.17	81±2.00
LF/HF	1.18±0.11	1.32±0.15*	1.04±0.15*

Supplementary Table 2: Baseline characteristics There was a significant difference in baseline LF/HF ratio between responders and non-responders (*p = 0.038). There was no significant difference in other baseline characteristics.

Using the responder subset of participants alone the effect of electrode configuration was investigated. A mixed mode ANOVA revealed no impact of electrode configuration on LF/HF ratio (main effect for electrode configuration, $p > 0.05$). This suggests that the electrode configuration did not influence the effectiveness of tVNS in reducing LF/HF in the responder group. Due to ease of application tVNS of the tragus of both left and right ears was used for all subsequent investigations.

	Total group (n = 63)			Responders (n = 31)			Non-responders (n = 32)		
Total Power (ms²)	2072.56 ±291.50	2033.08 ±343.28	ns	1953.7 ±345.9	2041.1 ±298.3	ns	2187.7 ±340.4	2025.3 ±293.6	ns
LF Power (ms²)	578.83 ±92.29	588.49 ±81.16	ns	623.0 ±94.8	567.4 ±93.4	ns	536.0± 93.3	608.9 ±91.9	ns
HF Power (ms²)	823.99 ±143.75	779.12 ±123.28	ns	596.4 ±210.3	761.2 ±163.3	ns	1044.5 ±206.9	796.5 ±160.7	ns
LF/HF	1.18 ±0.11	1.16 ±0.10	ns	1.32 ±0.15	0.95 ±0.15	<0.0005	1.04 ±0.15	1.35 ±0.14	<0.0005

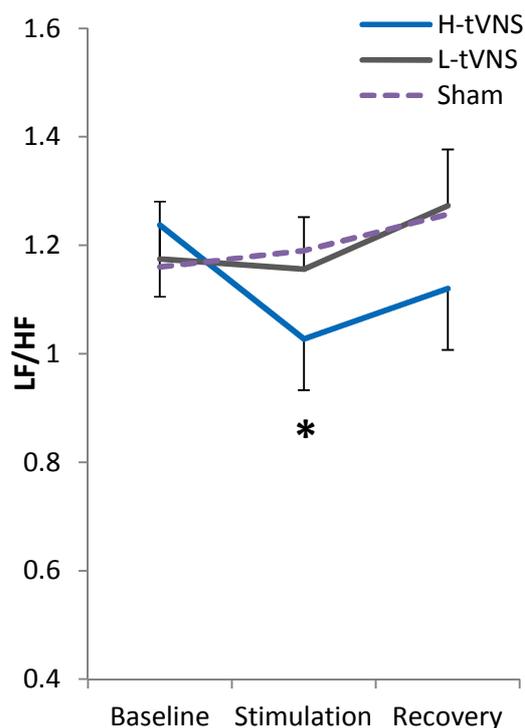
Supplementary Table 3: Heart rate variability values for L-tVNS responders and non-responder groups. There is a significant increase in LF/HF ratio during stimulation in the responder group ($p < 0.0005$) and a significant increase in LF/HF ratio in the non-responder group ($p < 0.0005$).

Stimulation Parameters for tVNS

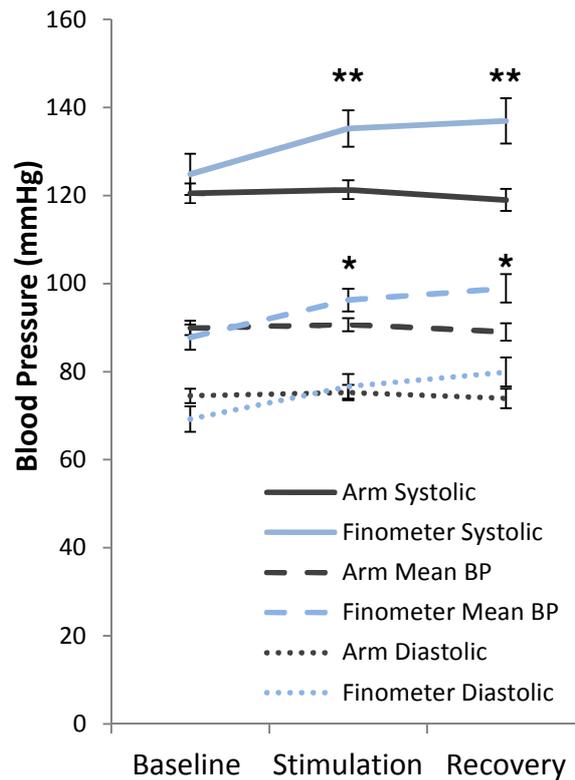
The stimulation parameters (20 μ s pulse width at 15 Hz) used in the initial study investigating electrode configuration reduced LF/HF ratio in 49% healthy participants (n = 31/63). In order to improve the efficacy of tVNS, the effects of increasing pulse width to 200 μ s and pulse frequency to 30 Hz were investigated in 34 healthy participants. The results of these experiments are presented in the main text, however, here we compare the effects of the original low pulse width and frequency (L-tVNS; 20 μ s at 30 Hz; n = 63) stimulation with the final high pulse width and frequency parameters (H-tVNS; 200 μ s at 30 Hz) used in the main study. In addition, sham stimulation (electrodes placed on the ear but no electrical stimulation used) was also investigated in 14 healthy participants. There were no significant differences in baseline characteristics between L-tVNS, H-tVNS and sham groups (including BMI, age, sex, baseline heart rate, BP or LF/HF ratio; Kruskal-Wallis test, $p > 0.05$).

Increase pulse width and pulse frequency (H-tVNS) is more effective in altering cardiovascular autonomic function

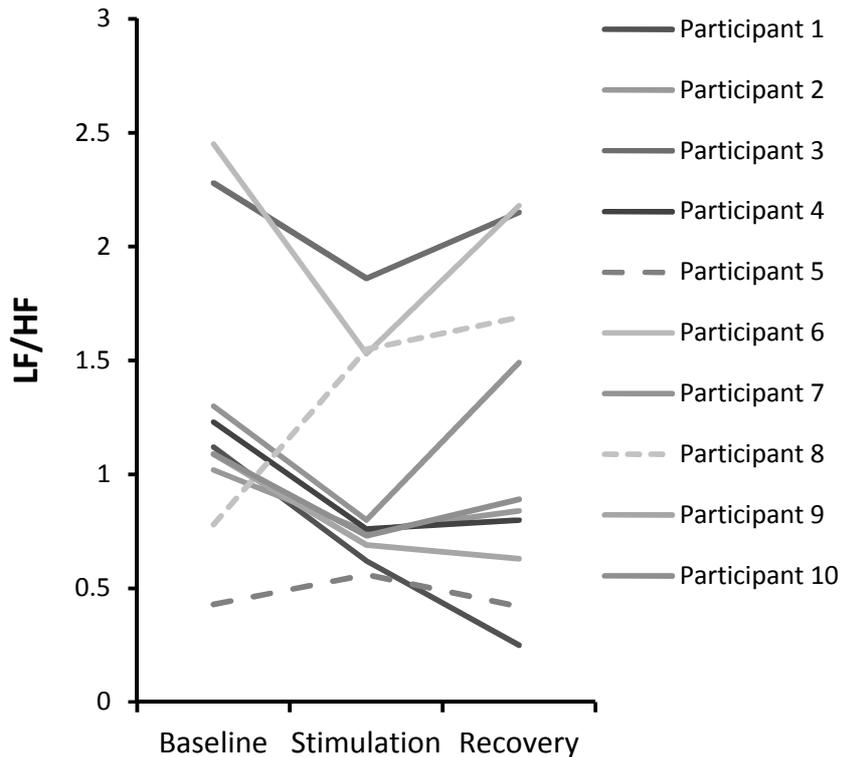
There was a significant interaction between stimulation parameters and time (mixed mode repeated measures ANOVA, $p = 0.048$), indicating that the stimulation parameters contributed to the change in LF/HF ratio. Further analysis of the H-tVNS group alone revealed a significant decrease in LF/HF ratio (repeated measures ANOVA, time effect, $p = 0.026$; Supplementary Figure 1) whereas there was no significant change in LF/H ratio in L-tVNS and sham groups. The percentage of those who responded to tVNS with a decrease in LF/HF ratio was 67 % in the H-tVNS group compared to 49 % in the L-tVNS group. These results indicate that H-tVNS is more effective in altering HRV in healthy participants.



Supplementary Figure 1 Comparison of high pulse width and frequency tVNS and low pulse width and frequency tVNS on HRV. There is a significant decrease in LF/HF ratio during H-tVNS (n=34; $p=0.026$) whereas L-tVNS (n=63) has no significant effect. There is a significant difference in LF/HF ratio response between H-tVNS and L-tVNS group ($p=0.048$).



Supplementary Figure 2: Methods of blood pressure measurement. There is a significant increase in systolic and mean BP during tVNS and recovery ($p < 0.0005$) measured using a Finometer which is in place throughout the experiment. The Finometer calibration system was used during experiment, however it was temporarily switched off during recordings for 15 minutes. It is possible that this affected accurate BP monitoring. It has also been reported that long term Finometer measurements may cause local oedema affecting BP detection². To investigate this, BP was also measured from the arm using an automatic BP machine. Three arm readings of BP were taken at baseline, during stimulation and during recovery and the average of the readings compared to the Finometer measurements. There was a significant difference between the mean and systolic BP results measured using the 2 methods during tVNS and the recovery period (mixed mode repeated measures ANOVA; * $p = 0.023$ and ** $p = 0.004$ respectively). There was no significant difference in diastolic BP measurements. Systolic and mean BP measured using the Finometer increase during tVNS and recovery compared to arm BP measurements which remain stable.



Supplementary Figure 3 Individual HRV responses in participants who received microneurography. LF/HF ratio decreased in 8 participants during tVNS and this corresponded with a decrease in MSNA. LF/HF ratio increased in 2 participants who showed no change in MSNA during tVNS.

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