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Effects of short and prolonged transcutaneous vagus nerve stimulation on heart rate variability in healthy subjects

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Abstract

The vagus nerve is strategically located in the body, and has multiple homeostatic and health-promoting effects. Low vagal activity predicts onset and progression of diseases. These are the reasons to activate this nerve. This study examined the effects of transcutaneous vagus nerve stimulation (t-VNS) on a main index of vagal activity, namely heart rate variability (HRV). In Study 1, we compared short (10 min) left versus right ear t-VNS versus sham (no stimulation) in a within-subjects experimental design. Results revealed significant increases in only one HRV parameter (standard deviation of the RR intervals (SDNN)) following right-ear t-VNS. Study 2 examined the prolonged effects of t-VNS (1 hour) in the right ear. Compared to baseline, right-t-VNS significantly increased the LF and LF/HF components of HRV, and SDNN in women, but not in men. These results show limited effects of t-VNS on HRV, and are discussed in light of neuroanatomical and statistical considerations and future directions are proposed.

Introduction

The vagus nerve, the tenth cranial nerve, has important physiological and homeostatic roles because of its strategic location and multiple functions in the body. This nerve contains both afferent (sensory) (80%) and efferent (motor) (20%) pathways. The sensory vagal pathways terminating in the nucleus of the solitary tract transmit a wide range of signals to the brain, including cardiac, digestive and immunological signals \cite{1}. The motor pathways descend from the nucleus ambiguus and nucleus dorsalis nervi vagi in the brainstem to many visceral organs including the lungs, heart, pancreas and gastrointestinal tract, bridging these organs with the central nervous system (CNS) \cite{1}.
Efferent vagus nerve activity is measured non-invasively via Heart Rate Variability (HRV). Classical physiology dictates that increased efferent vagus nerve activity leads to a slowing of the heart rate, via inhibition of the sinoatrial node [2] by release of acetylcholine, the main vagal nerve neurotransmitter. Measuring the time between individual heartbeats, done with softwares that determine the distance between the R waves on the electrocardiogram (ECG), provides information about the instantaneous heart rate. HRV represents the time differences between successive heartbeats (also known as the beat-to-beat intervals), and is synonymous with RR variability [2]. Analysis of the time differences between successive heartbeats can be accomplished with reference to time (time domain analysis) or frequency (frequency domain analysis). Examples of the former include the standard deviation of the RR intervals (SDNN) and the square root of the mean squared differences of successive RR intervals (RMSSD). Frequency domain analysis, which may more closely reflect HRV components, provides information of how power (variability) distributes as a function of frequency bands. The different spectral (frequency) components include very low frequency (VLF), low frequency (LF) and high frequency (HF) power components [3]. Extensive physiological and pharmacological studies have examined the neural contributions to the frequency components of HRV. For example, administration of acetylcholine antagonists or vagotomy down modulates the HF power component and electrical vagus nerve stimulation (VNS) increases HF power [2]. These results indicate that the HF power component reflects efferent vagus nerve activity to the sinoatrial node. The LF component is a measure of sympathetic and parasympathetic activity [2]. The ratio of low- to high-frequency spectral power (LF/HF) has been proposed as an index of sympathetic to parasympathetic balance of heart rate fluctuation. Furthermore, there is evidence that HF is strongly correlated with total vagal neuronal activity [4]. Experimentally, pharmacologic activation of the efferent vagus nerve pathway is associated with increases in HRV [5].

Measures of HRV have been strongly correlated with morbidity and mortality in diverse diseases [6,7]. Furthermore, low HRV has been shown to predict onset and/or worse prognosis in cardiovascular diseases, cancer, Metabolic Syndrome and Alzheimer’s disease [8]. In all these diseases, three pathophysiologial mechanisms often contribute to their occurrence and progression, namely inflammatory responses, sympathetic over-activity and oxidative stress [8,9]. Vagal nerve activity on the other hand, has been found to be significantly inversely correlated with oxidative stress [10], with inflammatory markers in healthy individuals as well as in those with cardiovascular diseases [11] and with norepinephrine [12, 8].

Stimulating the vagus nerve can be done via an implantable vagus nerve stimulator (VNS). Within the last decade, more than 60,000 epileptic and depressed patients have been treated by VNS. VNS has been shown to be effective in preclinical and clinical trials in depression and epilepsy [13,14]. However, due to surgery and electrical stimulation of efferent nerve fibers, frequent side effects including hoarseness, cough, and pain are reported [15]. Furthermore, it may not be feasible to implant such a vagus nerve stimulator in all patients. Therefore, given the potential positive impact of
VNS on health and the possible negative side effects of the implantable VNS, it is crucial to test the effects of non-invasive VNS. Transcutaneous VNS (t-VNS) applies electrical pulses to afferent nerve fibers of the auricular branch of the vagus nerve in the outer ear [16]. t-VNS has been shown to be a safe and well-tolerated method and an overall reduction of seizure frequency was observed in five patients with epilepsy after 9 months usage of t-VNS [16b]. Furthermore, Kraus and colleagues [17] showed that the brain activation patterns as a result of t-VNS clearly shared beneficial or positive features with changes observed during implantable VNS. Therefore, the t-VNS medical device received CE mark for clinical application in epilepsies.

A few animal and human studies have been conducted until now to confirm the beneficial effect of t-VNS. An animal study showed that t-VNS dose-dependently reduced systemic tumor necrosis factor levels during lethal endotoxemia and inhibited HMGB1 levels (a key inflammatory mediator) in mice with polymicrobial sepsis [18]. A human study carried out on healthy participants found that 1 hour of a continuous t-VNS on the left ear reduced significantly the sensitivity of mechanical evoked pain compared to sham condition [19]. Furthermore, a recent pilot study testing the combined effects of t-VNS and sound therapy (ST) on tinnitus patients found that short term t-VNS in the left ear improved mood, decreased tinnitus handicap scores and auditory cortical activation when it was combined with ST, compared to ST alone [20]. Thus, these studies showed the positive effect of short and long term t-VNS on subjective well-being and perception. However, none of them tested whether t-VNS influenced HRV, which could be the key factor behind these improvements. We believe that it is crucial to determine whether t-VNS affects HRV, given the predictive possibilities of HRV in multiple healthy functions and illnesses. Furthermore, both human studies used t-VNS only in the left vagal nerve tract (left ear), as such the effect of the right vagal nerve tract is remained uncovered. Therefore, our first aim was to investigate the short term effect (10 min) and long term effect (1 hour) of t-VNS on HRV in healthy volunteers. Secondly we wanted to examine whether stimulating the left or right ear would have stronger effects on HRV compared to baseline HRV and sham stimulations. Thus, we conducted two studies: study 1 was designed to test the short term (10 min) and study 2 to test prolonged effect (1 hour) of t-VNS on the HRV.

The present studies examined the effects of short (10 min – Study 1) and prolonged (1 hour – Study 2) t-VNS on the activity of the vagus nerve, indexed by HRV, in healthy men and women in an experimental within-subjects design. Furthermore, in study 1, we wanted to examine whether stimulating the left or right ear would have stronger effects on HRV. Finally, the roles of possible moderators e.g. gender and age, were explored.

Methods
We present together the methods of study 1 and 2 due to the methodological overlap between them.

Participants
This research was approved by the Institutional Review Board of the Faculty of Medicine and Pharmacy, The Free University of Brussels (VUB), Belgium. The participants were recruited from the Flemish Ninove region and in the Free University of Brussels, through personal contacts and advertisements. Participants were eligible if they did not have cardiovascular illnesses (arrhythmia, ischemic heart disease), major mental conditions, severe inflammation (e.g. rheumatoid arthritis), outer ear problems and were not taking any medication possibly influencing the autonomic nervous system. Participants were asked not to smoke, consume alcohol or caffeine 3 hours before participation. Thirty participants (aged between 23 and 58 years) took part in study 1, balanced for age and sex. In study 2, 30 participants (aged between 30 and 65 years) participated, balanced for sex. The participants received a movie ticket as a reward for their cooperation.

Instrumentation and Software

The electrocardiogram (ECG) was measured using the Nexus-4 Biofeedback System BioTrace+ (Mind Media B.V., the Netherlands) connected to a laptop in order to assess HRV. ECG data were recorded using ECG disposable electrodes, on the right and left side of the upper chest and one left electrode under the heart, digitized at the rate of 1024 Hz. This sample rate is in line with the requirements of the Task Force, which states that the ideal range for sampling rate is between 250-500 Hz or higher [2].

Abdominal respiration was measured using a flexible belt, by a sample rate of 32 samples per second. During the entire session, the participants watched an emotionally neutral movie in sitting position.

ECG analysis

After the Biotrace software recorded the ECG signal, it was exported to MATLAB R2010a (The MathWorks Inc., MA, USA). These procedures met the instrumentation requirements for recording of HRV and HRV analysis outlined by the Task Force [2]. A tachogram, containing the interbeat-intervals (IBIs) that are needed to analyze HRV, was derived from the ECG signal. Firstly, the R peaks of the ECG signal were detected via the Pan-Tompkins algorithm [21]. The R peak detections were afterwards automatically edited using the preprocessing algorithm described in [22]. Finally, the detections were visually inspected and manually corrected in accordance with published guidelines [2,23]. Based on the correct R peak locations, the IBIs were determined. Berntson and Stowell [24] defined “a prolonged IBI as being either longer than 1400 ms or longer than 150% of the mean value of the preceding 2 IBIs. A short IBI was defined as being either shorter than 400 ms or shorter than 50% of the mean value of the preceding 2 IBIs.” These were the criteria we also used for removing outliers. Thereafter, the remaining IBIs were written in a text file and analyzed using Kubios HRV analysis package 2.0, allowing for the calculation of time- and frequency-domain indices of HRV.

The Task Force [2] recommends that short term measurements of HRV should be 5 min and long term measures should be 24 h for standardization purposes. In study 1, HRV measurements
were taken during 10 min using similar conditions for each participant and using a resting baseline with spontaneous breathing in sitting position. In study 2, we used 5-min ECG recording segments for HRV analysis. Statistical analysis was focused only on the time-domain parameters SDNN (the standard deviation of RR intervals) and RMSSD (square root of the mean squared difference of successive RRs) and the frequency domain parameters Low-Frequency (LF), High-Frequency (HF) and Low/High-Frequency (LF/HF) parameters.

Stimulation by t-VNS
In study 1, transcutaneous vagus nerve stimulation (t-VNS; Cerbomed, Germany) was performed by means of electrical stimulation of the auricular branch of the vagus nerve (ABVN) in the left or right ear, in sitting position. The t-VNS consists of a handheld, battery driven electrical stimulator, connected to an ear electrode placed in contact with the skin of the concha. Impedance is measured automatically and insufficient electrode contact with the skin evokes an alarm. t-VNS applies rectangular pulses of 250 µs duration to the cymba conchae area of the outer ear, using the double ball point electrodes. This anatomical region has been shown to be supplied by the ABVN in man [16]. Stimulus intensity was adjusted according to the method of limits in order to apply electrical pulses that evoke clear tingling or pulsating sensations without any pricking pain or unpleasant perception (see below). With this stimulus intensity, primarily thick-myelinated Aδ fiber afferents were excited. t-VNS consisted of alternating pulse series of 30 s duration followed by 30 s stimulation pause. The stimulus frequency was adjusted to 25 Hz in the active stimulation session and no stimulation in the sham session. In study 1, t-VNS was applied to the left ear with 25 Hz or to the right ear with 25 Hz for 10 min duration, while the electrode on the other side remained in the ear of the participants as well. The sham session included a stimulus frequency of no stimulation with the electrodes attached (both sides). We will explain the sham condition more into detail below. In study 2, t-VNS with 25 Hz was applied only to the right ear for 1 hour.

Defining the threshold of the t-VNS
The threshold level of t-VNS stimulation included the mean of the individually detectable stimulation level and the uncomfortable stimulation level. To define the individually detectable threshold, participants were asked “Tell me when you feel anything unusual in your ear?”, and this starting from an intensity of 0.1mA and increasing 0.1mA at a time. When the self-detectable threshold was defined, the intensity was increased 0.1mA at a time while participants were asked “Tell me when the stimulation feels uncomfortable, not painful”. This set the upper threshold level. Then we repeated defining the self-detectable threshold and the uncomfortable threshold again decreasing and increasing 0.1mA at a time. The personalized threshold stimulation level was the mean of the 4 values (see figure 1).
Measures
After explaining the protocol and signing the consent form, participants were asked to complete several measures.

- **General background information**: Participants completed a short questionnaire asking for their background data including age, gender, height, weight, medication, alcohol consumption, smoking and coffee consumption. As mentioned before, participants were asked not to smoke, drink alcohol or caffeine 3 hours before starting the experiment.

- **Physical activity**: Physical activity was assessed by the short International Physical Activity Questionnaire (IPAQ) [25]. The 7-item IPAQ asks about three specific types of activity: walking, moderate-intensity activities and vigorous-intensity activities. The items in the short IPAQ form were structured to provide separate scores on walking, moderate-intensity and vigorous-intensity activities. Computation of the total score for the short form requires summation of the duration (in minutes) and frequency (days) of walking, moderate-intensity and vigorous-intensity activities, then multiplied by 3.3, 4 and 8, respectively [25]. The continuous score is expressed in MET-minutes/week, MET-minute scores being equivalent to kcal for a 60 kg person.

- **Relaxation level**: Participants were asked to fill in a one-item self-report question: ‘How relaxed are you feeling right now?’ on a visual analogue scale from 0 till 10, respectively ‘not at all’ till ‘very much’. In study 1, this was assessed at baseline, after the two stimulation conditions and after the sham condition. In study 2, this was assessed after baseline and after one hour of stimulation.

- **Perceived strength of stimulation**: Participants were asked how strong they felt the stimulation on a scale from 0 till 10, respectively ‘not at all’ till ‘very much’. In study 1, this was assessed after the left, right and sham interventions, the latter measure serving as a manipulation check of the sham condition. In study 2, this was assessed after one hour of stimulation.

Design - Study 1
We used a within-subjects experimental design, with randomized order. All participants first underwent baseline measurement. For the three other conditions, each participant was randomly assigned to one of the 6 different orders (in which the 3 intervention conditions could be ordered: e.g., Sham, Right Stimulation and Left Stimulation). During the first minutes of contact with the participant, the study rationale and methods were explained and the informed consent was obtained. Subsequently, the t-VNS electrodes, the ECG electrodes and the respiration belt were attached, after which the threshold of the participant in both ears was measured. During the entire study, the left and right electrodes stayed in the participants’ ears. Thereafter, participants completed the measurements explained above. Following this, a period of 10 minutes of baseline took place, during which participants did not receive stimulation. Thereafter, the participants underwent right stimulation, left stimulation or sham stimulation, according to their randomly allocated order. These periods lasted 10 minutes each, spaced by 5 minutes of rest and adjustment of the stimulation level. During the entire study, participants were sitting. The sham intervention included no stimulation but the electrodes were
placed in both ears. Participants did have expectations for being stimulated. They were told they would receive a lower stimulation level. Furthermore, during the sham, they were also asked whether the stimulation felt comfortable, to increase their expectations and credibility of the sham condition. Figure 2 depicts the entire study design.
Figure 2: Design of t-VNS study 1: left and right stimulation
Design - Study 2
As in study 1, we used a within-subjects experimental design. To reduce boredom, the participants were allowed to read. All preparatory stages preceding the baseline condition were identical to Study 1. The 5-min baseline did not include stimulation. Thereafter, the stimulation level was adapted to participants to avoid pain as in Study 1, and the period of one hour stimulation was started. HRV was measured during the first 5 minutes, between minutes 30 – 35 and between minutes 55-60 of the one hour stimulation. The study design is shown in figure 3.

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PLEASE INSERT FIGURE 3 HERE
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Figure 3: Design of Study 2 – Prolonged right t-VNS

Statistical Analysis - Study 1
A repeated measures analysis of variance was performed. The model included four conditions (baseline, right stimulation, left stimulation, and sham) in which HRV was repeatedly measured. Two between-subjects variables - age groups (< 40 years and > 40 years) and gender – were considered as well. A Bonferroni adjustment was used to reduce the chance of a type 1 error, and considered 4 contrasts (Sham-Baseline, Left-Baseline, Right-Baseline, Left-Right). However, since the five HRV parameters were all related to each other, we did not multiply this number of contrasts by the number of HRV indices. We thus set the statistical significance level at $\alpha = 0.05/4 = 0.0125$. We then also tested the moderators age and gender. Since the moderation tests were exploratory analyses, we did not take them into account in the Bonferroni correction. Effect size (Cohen’s $d$) was used to quantify the standardized mean difference between two within-subjects conditions and was computed as $d = \frac{M_D}{\sigma_D}$ [26], where $M_D$ is the estimated mean difference between two conditions and $\sigma_D$ is the standard deviation of the difference.

Statistical Analysis - Study 2
As in Study 1, a repeated measures analysis of variance was conducted. The model included four conditions or time points (baseline, 0-5min, 30-35min and 55-60min), during which HRV was measured. Two between-subjects variables - age groups (< 40 years and > 40 years) and gender – were considered. A Bonferroni adjustment was used to reduce the chance of a type 1 error, and considered 3 contrasts. However, since the five HRV parameters were all related to each other, we did not multiply this number of contrasts by the number of HRV indices. We thus set $\alpha = 0.05/3 = 0.0166$ as the level of statistical significance. We then also tested the moderators age and gender. Again, since the moderation tests were exploratory analyses, we did not take them into account in the Bonferroni correction. Effect size analyses was performed as in study 1.
Results - Study 1

In order to normalize the data, log transformations were used for all physiological variables (HRV parameters, respiration rate, physical activity, body mass index - BMI). The mean age of the participants was 37 years and their BMI 23.7. The participants were stimulated at a mean intensity of 0.7mA, in the left and right ear. There was no significant difference in the stimulation levels in the right ear between men and women (p>0.05), while men had significantly higher stimulation levels in the left ear compared to women (p<0.05) (data not shown). When we compared baseline HRV parameters of the participants below and above 40 years old, SDNN, RMSSD, LF and HF were significantly higher in the young group (p<0.05) while LF/HF was significantly lower in the young group (p<0.05). Concerning gender, no significant differences were found between men and women on baseline HRV measures.

Main results:
To perform the main analyses, two criteria were used in considering the covariates. First, the covariates gender, age, BMI and physical activity were considered, as these have been shown to correlate with HRV in past studies [27]. Furthermore, since participants were told not to smoke, drink alcohol or consume caffeine before participation (and since they followed these instructions), we did not consider caffeine and nicotine as extra confounders. Secondly, after testing the correlations between the remaining possible confounders and HRV at various time points, we added the stimulation level of the right ear. Since respiration rate was not correlated with HRV and was not significantly changing over the four conditions, we did not statistically control for it. Finally, order of randomization was not significantly correlated with HRV at various time points, and was thus not statistically controlled for in the following analyses.

Table 1 shows the mean ± SD of the HRV parameters in the four conditions in all participants, using their non-log-transformed results.

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PLEASE INSERT TABLE 1 HERE
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We analyzed the data without controlling for confounders, whereas table 2 shows the results controlling for all confounders. The results are very similar.

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PLEASE INSERT TABLE 2 HERE
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As shown in table 2, right stimulation significantly increased SDNN compared to baseline (p=.001; d=.726). However, this could not be replicated for the other HRV indices. Furthermore, left stimulation tended to significantly increase SDNN compared to baseline. Similar to the right stimulation, this could not be replicated in the other HRV indices. The sham versus baseline contrast was consistently not significant, considering the Bonferroni corrected p-value. Finally, no significant differences were found between the two experimental conditions (left vs. right ear t-VNS) on any HRV parameters, considering the corrected p-value.

When we investigated the subjective relaxation levels over the four conditions, participants felt significantly less relaxed in the baseline condition versus the three others (F(1, 29) = 20.34; p<0.001; F(1, 29) = 21.27; p<0.001; F(1, 29) = 12.55; p<0.001; respectively Right vs Bas; Left vs Bas and Sham vs Bas). Left stimulation versus right did not significantly differ on relaxation levels (F(1, 0.04) = 0.066; p=0.8).

The perceived strength of stimulation during left and right stimulation was significantly higher than in the sham condition (p<0.001 for both). Nevertheless, the mean perceived stimulation in the sham condition was above zero, namely 1.3 on a scale of 0-10, supporting the validity of the sham condition. Furthermore, we also asked the participants about their levels of pain and 75.9% reported low pain levels (1 or 2 on a Likert-scale). Pain levels were also not significantly related to any HRV levels in each of the 4 conditions. These results support the attempt to prevent pain in the procedure and suggest that pain was not a confounder.

**Moderators**

To test the condition x age and the condition x gender interactions, an omnibus test was performed. The likelihood ratio test was not found to be significant, in relation to several HRV indices (p> 0.05 for all). Thus, no further analyses were performed to examine age and gender as moderators.

**Conclusion**

This study tested the effects of left versus right short-term t-VNS and sham stimulation, on HRV. We could conclude that right ear t-VNS led to significant increases in HRV compared to baseline, however, only in one parameter, namely SDNN. Using our corrected significance level, left ear t-VNS did not increase HRV.
**Study 2**

Study 1 revealed minimal effects of t-VNS in the right ear which were a possible result of administering a brief stimulation only, namely of 10 minutes. Furthermore, there could be a ‘contamination’ due to a carry-over effect (Left, Right and Sham), though no relation was found between order of condition and HRV levels. It was thus important to test in a subsequent study if a prolonged stimulation in the right ear could have more substantial effects on HRV. This was the aim of the following experiment.

**Results - Study 2**

In order to normalize the data, log transformations were used for all physiological and crucial variables which were not normally distributed (HRV parameters, physical activity, body mass index - BMI). Due to skewness, analyses were performed and presented on log-transformed data. After transforming all data, outliers were detected. We had to exclude the data of one woman and one man due to technical problems and extreme outlier data.

The mean age of the sample was 44 years and their BMI 24.8. The mean stimulation level for the 1 hour stimulation period was 1 mA, and was not significantly different between men and women (p > 0.05). Baseline log SDNN, log RMSSD and log LF were not significantly different between men and women (p >0.05), while baseline HF was significantly higher in women than in men (p < 0.05) and LF/HF was significantly higher in men than in women (p < .05). The relaxation levels before and after t-VNS were not significantly different in the total sample (p >.05). However, men had a significantly higher relaxation level after stimulation than before (p < 0.05). In women, no difference was found over time (See Table 3).

Table 3 shows the mean ± SD of HRV parameters in the four time conditions in all participants, using their non-log-transformed results.

PLEASE INSERT TABLE 3 HERE

**Main results**

To perform the main analyses, two criteria were used for considering the covariates. First, the covariates gender, age, body mass index (BMI) and physical activity were considered, as these have been shown in past studies to correlate with HRV [27]. Furthermore, since participants were told not to smoke, drink alcohol or consume caffeine before participation (and since they followed the instructions), we did not consider these variables as extra confounders, as in Study 1. Secondly, after testing the correlations between the remaining possible confounders and HRV at various time points, no other variable was found to be significantly correlated with HRV, and hence, we did not need to statistically control for confounders.
Considering the Bonferroni correction, SDNN tended to significantly increase after 35 minutes and after one hour, versus baseline. LF and LF/HF significantly increased after 35 minutes of stimulation (p < 0.0166). No other HRV parameters or time points showed significant results.

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PLEASE INSERT TABLE 4 HERE
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However, when controlling for confounders, as shown in table 4, there was no significant contrast for any HRV parameter, during one hour of right ear stimulation. In other words, HRV was not changing as a function of duration of right ear stimulation compared to baseline levels, once confounders were statistically controlled for.

**Moderators**
A significant time x gender interaction for SDNN was observed (F(3, 63) = 3.239; p<.05) with an effect size of \(\eta^2 = .134\). The effects of time were more consistent in women than in men during the three time points (respectively p=0.019, p=0.005 and p=0.018 for the three periods in women, while p=0.705, p=0.012 and p=0.064 in men). This could not be replicated for the other HRV indices (p>.05) (data not shown).

**Conclusions**
Study 2 revealed no overall effects of right ear t-VNS on HRV in the total sample of participants, beyond effects of confounders. However, when we did not control for confounders, LF and LF/HF significantly increased after 35 minutes of stimulation. Furthermore, in women, t-VNS appeared to increase significantly the SDNN over time more consistently than in men (see Figure 4).

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PLEASE INSERT FIGURE 4 HERE
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**General discussion**
In this research, we wished to test the effects of brief t-VNS (10 min – Study 1) and prolonged t-VNS (one hour – Study 2) on Heart Rate Variability (HRV), an index of vagal nerve activity [4]. In study 1, we compared the effects of left versus right ear stimulation by brief t-VNS stimulation on HRV. We found that only right ear stimulation significantly increased SDNN compared to baseline. Furthermore, left stimulation tended to significantly increase SDNN compared to baseline. However, in both sides, this could not be observed in the other HRV indices. Finally, no significant differences could be found
between left and right ear stimulation on any HRV parameter and no moderation was found by gender or age.

Following the results of this first study, we focused on right ear stimulation and extended the stimulation duration in study 2 from 10 min to 1 hour, where we measured HRV in three time periods. We found that HRV did not change as a function of stimulation duration in the entire sample. However, a significant time x gender interaction for SDNN was observed - The effects of time were more consistent in women than in men at 0-5 min, 30-35 min and 55-60 min. Thus, our results demonstrate modest effects of longer, 1 hour t-VNS on HRV, mainly in women. In both studies, SDNN was the more consistent parameter to change. This parameter could be more sensitive to stimulation, or it could reflect a chance finding.

Considering these results, one could question why this vagal nerve stimulation does not increase HRV more substantially? Furthermore, the most consistent effects of t-VNS observed in the present study was with SDNN. Could this mean that t-VNS is not activating efferent vagus nerve fibers projecting down to the heart? Alternatively, is HRV indeed a reliable indicator of vagal nerve activity? To answer the first question, we will first briefly review studies examining the effects of invasive vagal nerve stimulators on HRV. In a study in dogs, VNS treatment enhanced HRV [28]. Similarly, a study in rabbits founds that intermittent VNS, but not constant VNS, increased the HF (mainly vagal) component of HRV [29]. Furthermore, some human experiments have been done and found contradictory results. In patients treated for seizures by VNS, one study found a significant increase in the HF component [30], while another study suggested that VNS does not affect HRV [31]. Indeed, several studies in epileptic patients found no significant changes in HRV as a result of acute VNS [32,33]. On the other hand, one study in patients with major depression showed that HRV increased significantly in switched on conditions during VNS (30sec) in six patients compared to stimulation-free intervals and baseline [34], showing reversible changes in HRV as a function of VNS. When considering long-term VNS, one study, in which refractory epileptic patients received VNS during 3 months, failed to show a significant effect of left VNS on cardiac autonomic functioning [35]. Thus, also in invasive VNS studies, HRV was not consistently increased.

Several observations could explain the lack of effects of VNS on HRV. During VNS treatment, the left vagus nerve is stimulated below the cardiac branches of the vagus nerve, which may explain why the cardiac function is unaffected by routine VNS treatment. The device is stimulating the vagus nerve because one study found it to activate brain regions (e.g. anterior cingulate cortex) [17], which are known to be associated with HRV [50]. t-VNS, similar to VNS, primarily excites thick-myelinated Aβ fibers, which do not innervate the heart [36], with no effects on thinner A or C fibers [37]. The fibers of the auricular branch of the vagus nerve, which were most directly stimulated in the present study, project to the NTS as shown by tracer studies in cat and rat [38]. Electrophysiological recordings from the NTS in rats demonstrated activation of secondary sensory neurons by auricular stimulation [39]. Recently, activation of the NTS and deactivation of the hippocampus by t-VNS has been documented...
in an fMRI trial in people [40]. Beyond activation of the main target NTS by t-VNS, the complete cerebral activation and deactivation pattern under t-VNS resembles that of invasive VNS [28]. Thus, it is possible that since t-VNS activates non-cardiac fibers and since it operates via a complex indirect system of brain regions and nuclei, the effect of t-VNS on HRV may be minimal. Finally, it is possible that some or one of the confounders mediated the effects of t-VNS on SDNN since after controlling for their effects, t-VNS no longer affected SDNN. Alternatively, effects of invasive VNS and t-VNS might be only evident in patients with low HRV.

In our studies, t-VNS had little effect on HRV in the full sample. However, we found gender to moderate the effects of prolonged t-VNS. In women, t-VNS increased SDNN more consistently than in men. Past studies have found inconsistent gender differences in HRV [41,42]. Gender differences have been proposed in the neurohormonal control of the cardiovascular system during various contexts e.g. standing, physical activity [43]. In contrast, considering anatomical issues, one study in rats found no gender differences in vagal innervation of the heart [44]. However, it is possible that gender differences may exist in the neuronal pathways between the site of t-VNS at the concha of the ear and the cardiac vagal fibers. Furthermore, gender differences may exist in neuronal sensitivity or in levels of reactivity of certain nuclei along the pathway. Finally, neurohormonal differences between men and women may also affect gender differences in responses to t-VNS. These issues require further investigation in future studies.

Concerning the stronger effect in the right ear, there are various explanations. The right vagus nerve primarily innervates the sinoatrial node which affects the heart rate, whereas the left vagus innervates the atrioventricular node which has less influence on the frequency [45]. Furthermore, the left vagus has proportionally fewer cardiac efferent fibers [46] and is thus preferentially stimulated in clinical practice rather than the right vagus, to reduce negative cardiac effects [47].

We now examine some evidence from the literature regarding our second question – Is HRV a valid measure of efferent vagal nerve activity? Several lines of evidence suggest that HRV is a marker, albeit nonspecific, of autonomic tone, and that heart rate turbulence may be a marker of baroreceptor sensitivity [48]. The high-frequency components of HRV are associated with vagus nerve/parasympathetic effects, whereas the low-frequency components are due to sympathetic and parasympathetic activation [49]. There is direct evidence that actual vagal nerve activity, measured in anaesthetized rats, is strongly correlated with the HF component of HRV (r=0.88) [4]. Though compelling, this evidence needs to be further investigated in people.

This research had three main limitations. First, the studies included small samples, particularly for moderation analyses. Despite this limitation, referring to the methodological criteria of Wheat and Larkin [27] concerning HRV-Biofeedback studies, the present study met all the applicable criteria for stringent methodology (e.g. sham control condition in Study 1, correcting for skewness, consideration of confounders, reducing type 1 error). The second limitation is the duration of stimulation. Future
studies should consider testing long-term t-VNS (e.g. 1 month) with larger samples, including men and women of different ages, and possibly with measuring other outcomes associated with vagal activity, e.g. activity in brain regions [50], pro-inflammatory cytokines [1] or other physiological changes associated with parasympathetic activity such as pupil diameter. A third limitation was the request from participants to abstain from consuming caffeine, alcohol and smoking only 3 hours before participation.

Conclusions
No consistent changes in HRV could be found as a result of acute (10 min) or prolonged (1 hour) transcutaneous vagal nerve stimulation. However, it seems that right t-VNS stimulation has more effects on HRV, and changes could be found more consistently in women than in men. Future studies may wish to examine these issues further, while addressing our studies’ limitations.

Acknowledgement
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References
3 Huston JM, Tracey KJ. The pulse of inflammation: Heart rate variability, the cholinergic anti-inflammatory pathway and implications for therapy. J Intern Med 2011;269:45–53.


40 Frangos, Ellrich, Dell’Italia, Wise, Komisaruk, Society for Neuroscience Abstracts 891-09, 2012


Uncomfortable (t2)  

Detectable (t1)  

Detectable (t3) 

Uncomfortable (t4)  

---  

Is increased 0.1mA at a time until uncomfortable  

Is decreased 0.1mA at a time until detectable  

t: Threshold  

Personalized threshold level of stimulation = \((t1+t2+t3+t4)/4\)  

Figure 1
<table>
<thead>
<tr>
<th>Activity</th>
<th>Adjust</th>
<th>Rest</th>
<th>Baseline or sham</th>
<th>R or L VNS</th>
<th>Rest or R or L VNS</th>
<th>Rest or sham</th>
<th>L or R VNS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Questionnaires</td>
<td>General background</td>
<td>Relaxation level</td>
<td>Perceived strength of stimulation</td>
<td>Relaxation level</td>
<td>Perceived strength of stimulation</td>
<td>Relaxation level</td>
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<tr>
<td>ECG</td>
<td>ECG</td>
<td>ECG</td>
<td>ECG</td>
<td>ECG</td>
<td>ECG</td>
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</tr>
<tr>
<td>Time</td>
<td>10</td>
<td>5</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td>10</td>
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</tbody>
</table>

Figure 2
<table>
<thead>
<tr>
<th></th>
<th>20 min</th>
<th>5 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Adjustment</strong></td>
<td>baseline ECG</td>
<td>adjustment t-VNS</td>
<td>0-5 min ECG</td>
</tr>
<tr>
<td><strong>Questionnaires</strong></td>
<td>Relaxation level</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>t-VNS threshold</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3
Figure 4
Table 1: Mean and standard deviation of the different HRV parameters in the 4 conditions

<table>
<thead>
<tr>
<th></th>
<th>SDNN (ms)</th>
<th>RMSSD (ms)</th>
<th>LF (ms²)</th>
<th>HF (ms²)</th>
<th>LF/HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>56.6 ± 24.1</td>
<td>42.1 ± 24.1</td>
<td>1017.7 ± 852.8</td>
<td>839.3 ± 1001.4</td>
<td>2.2 ± 2.5</td>
</tr>
<tr>
<td>Right</td>
<td>65.9 ± 35.6</td>
<td>45.1 ± 26.2</td>
<td>1286.2 ± 1224.4</td>
<td>1008.4 ± 1468.6</td>
<td>2.2 ± 2.0</td>
</tr>
<tr>
<td>Left</td>
<td>61.1 ± 24.2</td>
<td>43.1 ± 26.1</td>
<td>955.9 ± 768.1</td>
<td>867.2 ± 1149.4</td>
<td>2.1 ± 2.1</td>
</tr>
<tr>
<td>Sham</td>
<td>60.9 ± 27.2</td>
<td>45.3 ± 26.2</td>
<td>1203.4 ± 1188.2</td>
<td>931.9 ± 1175.5</td>
<td>2.3 ± 2.7</td>
</tr>
</tbody>
</table>

Values are presented as means ± SD. HRV: Heart Rate Variability; SDNN: Standard deviation of the RR intervals; RMSSD: Root Mean Square of the Successive Differences; LF: Low-Frequency; HF: High-Frequency
Table 2. Statistical significance and effect sizes of contrasts between various experimental conditions, with statistically controlling for confounders

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Log (SDNN) p-value</th>
<th>Log (SDNN) Effect size (d)</th>
<th>Log (RMSSD) p-value</th>
<th>Log (RMSSD) Effect size (d)</th>
<th>Log (LF) p-value</th>
<th>Log (LF) Effect size (d)</th>
<th>Log (HF) p-value</th>
<th>Log (HF) Effect size (d)</th>
<th>Log (LF/HF) p-value</th>
<th>Log (LF/HF) Effect size (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right vs B</td>
<td>0.001</td>
<td>0.726</td>
<td>0.207</td>
<td>0.264</td>
<td>0.038</td>
<td>0.431</td>
<td>0.143</td>
<td>0.308</td>
<td>0.912</td>
<td>0.023</td>
</tr>
<tr>
<td>Left vs B</td>
<td>0.015</td>
<td>0.514</td>
<td>0.729</td>
<td>0.066</td>
<td>0.797</td>
<td>0.052</td>
<td>0.697</td>
<td>0.084</td>
<td>0.347</td>
<td>0.195</td>
</tr>
<tr>
<td>Sham vs B</td>
<td>0.025</td>
<td>0.471</td>
<td>0.269</td>
<td>0.214</td>
<td>0.357</td>
<td>0.178</td>
<td>0.969</td>
<td>0.007</td>
<td>0.582</td>
<td>0.110</td>
</tr>
<tr>
<td>Left vs Right</td>
<td>0.427</td>
<td>0.156</td>
<td>0.209</td>
<td>0.240</td>
<td>0.022</td>
<td>0.450</td>
<td>0.107</td>
<td>0.307</td>
<td>0.233</td>
<td>0.226</td>
</tr>
</tbody>
</table>

B: Baseline; S: Sham; SDNN: Standard deviation of the RR intervals; RMSSD: Root Mean Square of the Successive Differences; LF: Low-Frequency; HF: High-Frequency; Bonferroni corrected p-value= 0.0125.
Table 3: Means and standard deviations of the different HRV parameters in the 4 time conditions

<table>
<thead>
<tr>
<th></th>
<th>SDNN</th>
<th>RMSSD</th>
<th>LF</th>
<th>HF</th>
<th>LF/HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>45.03 ± 16.19</td>
<td>32.2 ± 19.5</td>
<td>671.1 ± 679.7</td>
<td>415.4 ± 523.1</td>
<td>2.5 ± 2.0</td>
</tr>
<tr>
<td>0-5 min</td>
<td>44.4 ± 16.3</td>
<td>32.1 ± 19.4</td>
<td>615.8 ± 503.1</td>
<td>519.6 ± 766.0</td>
<td>2.7 ± 2.8</td>
</tr>
<tr>
<td>30-35 min</td>
<td>55 ± 19.4</td>
<td>32.5 ± 15.0</td>
<td>1021.6 ± 824.5</td>
<td>445.3 ± 456.1</td>
<td>3.4 ± 2.2</td>
</tr>
<tr>
<td>55-60 min</td>
<td>51.7 ± 19.2</td>
<td>34.1 ± 17.3</td>
<td>1026.5 ± 948.5</td>
<td>516 ± 655.5</td>
<td>3.7 ± 3.7</td>
</tr>
</tbody>
</table>

Values are presented as means ±SD. SDNN: Standard deviation of the RR intervals; RMSSD: Root Mean Square of the Successive Differences; LF: Low-Frequency; HF: High-Frequency.
Table 4. Statistical significance and effect sizes of contrasts between various experimental conditions, with statistical control over confounders

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Log (SDNN) p-value</th>
<th>Effect size (d)</th>
<th>Log (RMSSD) p-value</th>
<th>Effect size (d)</th>
<th>Log (LF) p-value</th>
<th>Effect size (d)</th>
<th>Log (HF) p-value</th>
<th>Effect size (d)</th>
<th>Log (LF/HF) p-value</th>
<th>Effect size (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-5 min vs. Bas</td>
<td>0.777</td>
<td>0.004</td>
<td>0.564</td>
<td>0.016</td>
<td>0.355</td>
<td>0.041</td>
<td>0.087</td>
<td>0.133</td>
<td>0.051</td>
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<tr>
<td>30-35 min vs. Bas</td>
<td>0.992</td>
<td>0.000</td>
<td>0.766</td>
<td>0.004</td>
<td>0.870</td>
<td>0.001</td>
<td>0.354</td>
<td>0.041</td>
<td>0.391</td>
<td>0.035</td>
</tr>
<tr>
<td>55-60 min vs. Bas</td>
<td>0.778</td>
<td>0.004</td>
<td>0.792</td>
<td>0.003</td>
<td>0.510</td>
<td>0.021</td>
<td>0.884</td>
<td>0.001</td>
<td>0.863</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Bas: baseline; SDNN: Standard deviation of the RR intervals; RMSSD: Root Mean Square of the Successive Differences; LF: Low-Frequency; HF: High-Frequency; Bonferroni corrected p-value= 0.0166