THE EFFECTS OF NONINVASIVE ULTRASOUND STIMULATION ON VAGUS NERVE ACTIVITY AND HEART RATE VARIABILITY

By

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A Thesis Submitted to The W.A. Franke Honors College

In Partial Fulfillment of the Bachelors degree
With Honors in

Neuroscience and Cognitive Science

THE UNIVERSITY OF ARIZONA

M A Y 2 0 2 2

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I. Abstract

Anxiety rates have skyrocketed in recent years with nearly one third of adolescents reporting that anxiety has negatively impacted their well-being. Additionally, nearly 50% of patients using standard treatment options are left with unwanted side effects or minimal symptom relief (Kessler et al., 2009). One alternative to current anxiety treatments is Vagus Nerve Ultrasound Stimulation (VNS), a fast-acting and less expensive approach to treat anxiety. Prior research has demonstrated that VNS effectively decreased inflammation markers and temporarily modulated heart rate in animal models (Coiado et al., 2016, Wasilczuk, 2019). In order to further investigate VNS as a potential treatment option, we analyzed fluctuations in heart rate variability (HRV). HRV is a measure of variation between individual heart beats and is an indicator of physiologic control via an interplay between the parasympathetic and sympathetic nervous systems. We investigated the effects of VNS on heart rate, HRV, and examined whether increased HRV predicts lower anxiety in healthy individuals. The results demonstrated a significant difference in baseline normalized RMSSD (a measure of heart rate variability) between sham and interval stimulation groups ($F_{2,44}$ = 4.252, p= 0.020). There were significant differences in RMSSD and HR across timepoints within all groups, which may be indicative of effects due to pressure or temperature rather than the VNS itself. Changes in HRV did not predict differences in anxiety or mood scores. Future research is required to further examine the effects of ultrasound at different pulse parameters and carrier frequencies in enhancing or inhibiting cardiac vagal control.

I. Introduction

Considered one of the most prevalent psychiatric disorders, anxiety is characterized by excessive worrying, hyperarousal or maladaptive behaviors to cope with or avoid stress (Niles, Lebeau, Liao, Glenn, & Craske, 2012). While almost everyone experiences occasional uneasiness or worrying, anxiety disorders are psychiatric disorders that cause significant burden on the individuals plagued by them. Some common disorders included in the DSM-V (Diagnostic and Statistical Manual of Mental Disorders Fifth Addition) are Generalized Anxiety Disorder, Panic Disorder, Social Anxiety Disorder, Specific Phobias, along with numerous others. Unfortunately, despite the pharmacological and psychological treatments available today, the prevalence of anxiety continues to climb to reach roughly 25% of the population (Santabarbara et al., 2021). Nearly one third of adolescents endorse experiencing the ravaging effects of anxiety disorders in their own lives, and 8.3% of adolescents suffer from severe impairment due to anxiety disorders (Kessler et al., 2009). These statistics illustrate the severity of this disorder. Anxiety has a sizable burden on both the individual and family unit, impacting everything from work performance to social functioning. According to Erickson et al. (2009), individuals with increased anxiety demonstrated lower overall work performance and were less likely to advance in their career as compared with their colleagues without anxiety. Additionally, researchers investigating the effects of anxiety on the family unit found that family members of individuals with anxiety disorders experience a sizable burden, which worsens depending on the severity of the anxiety disorder (Senaratne et al., 2010). Anxiety disorders have been associated with degraded employment trajectories, reduced performance compared to those without anxiety diagnoses, and elevated unemployment rates (Waghorn et al. 2005). From direct healthcare costs to more indirect costs associated with disability or inhibited work performance, mental disorders account for a greater economic burden than many somatic diseases (Trautmann, Rehm, & Wittchen, 2016).The total global economic costs associated with mental disorders have been estimated at approximately $2.5 trillion (Trautmann et al., 2016). Due to the significant burden of anxiety, as well as its increasingly pervasive effects in our world today, it is evident that the need for novel anxiety treatments is imperative in order to engender growth and improvement in quality of life of individuals suffering from this psychiatric disorder.

Historically, anxiety treatments have ranged from pharmacotherapy to psychological therapy and oftentimes a combination of both. While not all anxiety disorders require intervention, those that significantly interfere with the individual's ability to function warrant treatment. Pharmacological treatments typically include drugs such as selective serotonin reuptake inhibitors, selective norepinephrine reuptake inhibitors, tricyclic antidepressants, calcium modulators, anxiolytics such as benzodiazepines and reversible monoamine oxidase A inhibitors. Unfortunately, individuals often experience side effects to medications including dizziness, diarrhea, nausea, jitters, somnolence as well as other symptoms. Benzodiazepines do not lead to the jitteriness associated with SSRIs and SNRIs; however, there is a risk of drug dependency as a result of long-term use in patients with predisposition to drug use, which often eliminates Benzodiazepines from a first line of treatment (Bandelow et al., 2014). Not only are there often adverse effects associated with many of the prevalent drugs used to target symptoms of anxiety, but often, patients are resistant to treatment and hesitant to start taking psychotropic drugs. Additionally, medications often require a significant amount of time to have an effect, and many individuals will discontinue treatment before seeing symptom relief. An alternative to psychotropic drugs is Cognitive Behavioral Therapy (CBT). However, research has demonstrated poor response rates with approximately 48% of patients still experiencing anxiety symptoms after CBT (Durham et al., 2005). CBT trains individuals to alter thinking and behavior in order to better cope with stressors, and exposure therapy, one form of CBT, helps individuals to confront their fears and decrease anxiety associated with a particular trigger. Unfortunately, these approaches are often expensive and lack efficacy, making them less than ideal treatment options for most of patients. While the standard therapeutic approaches might be effective for some, nearly 50% of patients are left with unwanted side effects or minimal relief from their symptoms of anxiety (Kessler et al., 2009). Not only do current anxiety treatments lack efficacy for many individuals, but many anxiety disorders are significantly undertreated, which may be, in part, a result of a lack of promising treatment options. Thus, novel interventions ought to be further examined. To overcome some of the limitations associated with the standard treatment options, one alternative is Vagus Nerve Stimulation (VNS), a fast-acting and potentially less expensive approach to treat anxiety compared to drugs and psychotherapy.

II. Vagus Nerve Stimulation (VNS)

The vagus nerve extends from the brain to the abdomen and functions as a major component of the parasympathetic nervous system, which impacts numerous functions including mood regulation and immunity. The vagus nerve plays a role in regulating the function of internal organs, and impacts respiratory rate, digestion, heart rate, and certain reflexes. The primary role of the vagus nerve is to carry information from organs including the gut, heart or lungs back to the brain through afferent fibers (Breit et al., 2018). Due to the various functions of the vagus nerve, it has become an attractive target as a potential treatment for disorders ranging from depression to inflammatory bowel disease. Treatments impacting this nerve aim to elevate vagal efferent activity while simultaneously decreasing the production of cytokines (Breit et al.; 2018). Because vagal control is associated with stress regulation and vagal afferent fibers impact monoaminergic brain structures, stimulating the vagus nerve may be an effective means to treat individuals with psychiatric disorders, including anxiety.

Stimulation of the vagus nerve has been investigated in the context of certain animal models. For example, research by Noble and colleagues (2019) demonstrated that electrical stimulation to the vagus nerve leads to conditioned fear extinction in rat models, leading researchers to presume that it may have promising effects in human stress responses as well.

However, VNS was first used as a means to treat epilepsy as early as the 1880s when seizures were thought to be caused by changes in blood flow to the brain (Lanska, 2002). Approximately a century later, the first implantable device was utilized in treating seizures resistant to pharmacological control (Bonaz et al, 2013). The mechanism by which VNS functions in reducing seizures associated with epilepsy is still unclear, although some stipulate that affecting vagal activity may decrease the likelihood of a seizure occurring in an area of the brain associated with increased excitability, such as the limbic system and the thalamus (Bonaz et al., 2013).

While it was not originally designed to treat depression, VNS used to treat epilepsy resulted in an improvement in mood in many individuals, leading researchers to further examine the effect of VNS on mood. Vagus Nerve Stimulation was approved by the FDA as a treatment for depression in 2005 for adult patients with depression unresponsive to traditional pharmaceutical methods. In addition to ECT (electroconvulsive therapy) and rTMS (transcranial magnetic stimulation), VNS is now considered a neuromodulation technique for patients resistant to traditional treatments for depression, and nearly two thirds of patients noted a promising response to VNS (Muller et al 2018). According to a study by Bajbouj et al., more than half of the patients who received VNS saw at least a 50% reduction in severity of depression based on scores on the Hamilton Rating Scale for Depression and nearly 40% of patients met remission criteria (Bajbouj et al., 2010).

While it has been shown to be an effective treatment for epilepsy as well as depression, a primary issue with traditional VNS is that electrical stimulation to the vagus nerve often requires surgical implantation. Not only is this method invasive, but it also often results in numerous side effects from dysphonia to cough. However, ultrasound stimulation appears to be a safe alternative to electrical stimulation as it is noninvasive and has a demonstrated safety record. Studies have indicated that there were no adverse events in over 150 subjects when ultrasound devices with intensity below FDA limits were used to image the vagus nerve (Giovagnorio and Martinoli, 2000). A study by Coiado et al demonstrated that ultrasound stimulation to the vagus nerve reduces heart rate for a short period of time without any extended adverse effects (2016).

Additionally, several animal models have indicated that low-intensity ultrasound stimulation may be effective in modulating vagal activity. Wasilczuk and colleagues (2019), for instance, found that 5 minutes of ultrasound stimulation could decrease an indicator of inflammation, TNF-alpha, which is suspected to be mediated by vagal activity. Thus, this research seems to demonstrate that low-intensity ultrasound stimulation to the cervical branch of the vagus nerve appears to be a safe and noninvasive means to modulate vagal activity.

III. Heart Rate Variability

Heart rate variability (HRV) is defined as variability in the duration of intervals between consecutive heartbeats called interbeat intervals (IBI). A healthy heart has significant variation in the timing of the individual heartbeats; these oscillations of the heart are constantly changing and allow for rapid adaptation to changes in homeostasis. Blood pressure, autonomic balance, vascular tone, regulation of the heart and gut are all measures related to HRV (Shaffer, 2017). Heart rate variability is generated by heart-brain communication and is an indicator of physiologic control via the relationship between the autonomic nervous system. HRV reflects the interplay of inhibition or activation in one or both branches of the autonomic nervous system (Bernston et al., 1991). The heart is innervated by the parasympathetic nervous system via the right branch of the vagus nerve which enters at the sinoatrial node. Blocking vagal synapses via pharmacological methods at the heart’s sinoatrial node destroys this relationship between respiration and heart rate, indicating that the vagus nerve is specifically responsible for variability in heart rate within the respiratory frequency band (Japundzic et al, 1990; McCabe et al., 1985). Thus, there is evidence that measures used to examine respiratory-linked heart rate variability could be used to assess vagal control.

While it is important for a heart to have the flexibility to cope with a changing environment, a higher HRV is not always better due to the possibility of pathological conditions producing a higher HRV. One example of this is the association between elevated HRV due to cardiac abnormalities and an increased risk of mortality. However, ideal ranges for HRV are associated with improved health, resilience, and capacity for self-regulation. Functions such as emotional processing and attention are related to elevated vagally-mediated HRV (Shaffer, 2017).

Heart rate variability can be examined in the short-term as well as over a 24-hour period. Two processes generate short-term HRV: the dynamic relationship between the two branches of the autonomic nervous system and the mechanisms that regulate heart rate by respiratory sinus arrhythmia (RSA), fluctuations in vascular tone and baroreceptor reflex. RSA particularly refers to the acceleration and deceleration of heart rate mediated by the vagus nerve (Karemaker, 2009). Twenty-four-hour HRV on the other hand is influenced by core body temperature, metabolism, circadian rhythm and the renin-angiotensin aldosterone system. In comparison with short-term HRV, 24-hour heart rate variability is the recommended metric for clinical HRV and has greater predictive power (Shaffer, 2017).

There are numerous metrics used to analyze HRV based on time-domain, frequency- domain, and other non-linear measurements. In this paper, I will primarily be focusing on RMSSD, root mean square of successive respiratory rate intervals (differing more than 50ms). RMSSD is determined by calculating the time between heartbeats (in ms), then the subsequent values are squared and averaged prior to obtaining the square root of the total. The RMSSD is reflective of variance between beats in heart rate and is one of the primary time-domain measures used to estimate vagally-mediated HRV changes. Additionally, RMSSD has been demonstrated to be less affected by respiration than RSA (Shaffer, 2017). However, the RMSSD is more influenced by parasympathetic nervous system activity than the SDNN (the mean standard deviations of all the inter-beat intervals. Because RMSSD is influenced by fluctuations in the autonomic nervous system, but less impacted by changes in respiration, it appears to be an appropriate measure to utilize when examining the effects of vagal ultrasound stimulation on HRV.

IV. Specific Aims

The purpose of this thesis is to investigate the effects of low-intensity ultrasound as a means to alter vagal activity, inferred by heart rate variability, in order to determine whether vagal ultrasound stimulation can reduce anxiety. Due to the fact that lower HRV is associated with increased levels of anxiety and depression, we hypothesized that stimulation to the vagus nerve would increase HRV across participants and subsequently lower self-reported anxiety scores on the Spielberger State Anxiety Scale. The specific aims for this project included: 1) to examine whether delivery of ultrasound to the vagus nerve affects heart rate; 2) to demonstrate that delivery of ultrasound to the cervical branch of the vagus nerve temporarily modulates HRV; 3) to determine whether Vagal ultrasound stimulation specifically enhances HRV; and, 4) to test whether increased HRV in turn predicts lower anxiety in healthy individuals. In order to investigate these three aims, participants received one of three protocols with the ultrasound transducer placed over the cervical branch of the vagus nerve: sham stimulation, two minutes of uninterrupted ultrasound stimulation administered in short pulses, or two minutes of stimulation divided into 30-second intervals rest periods between intervals of stimulation. Finding that either of the latter two conditions enhanced HRV and reduced anxiety would provoke future research examining vagus nerve stimulation as a potential treatment for anxiety and anxiety disorders.

VI. Methods

The participant sample included 60 undergraduate students, ranging from ages 18-25, recruited from the Psychology Department SONA's subject pool. Exclusion criteria were defined as diagnosis of a medical or psychological condition, use of psychotropic medications, history of cardiac condition(s), history of seizure(s), pregnancy, use of nonprescription drugs or less than 4 hours of sleep within the 24 hours prior to ultrasound stimulation.

In this experiment, the ultrasound device used was Telemed MicroUS which has a 5MHz transducer. The device was used in the B-mode, which is standard for medical imaging and has been used to image the vagus nerve in clinical settings. All stimulation was delivered below the FDA intensity safety limits of 720mW/cm^2. According to multiple studies (O'Brian 2007, Nyborg, 2002; Dalecki, 2004), there have been no associated adverse side effects to ultrasound administered at this level.

Two investigators were present throughout the extent of the experiment. Prior to their participation in the study, participants completed a consent form, medical questionnaire, as well as exclusion criteria to screen participants eligible for the study. In order to examine the changes in participants' experiences before and after stimulation, a series of questionnaires were administered including: the Spielberger State Anxiety Scale (Julian 2011), the Visual Analogue Mood Scale (Stern et al. 1997), and the Positive and Negative Affect Schedule (Watson, Clark & Tellegen, 1988). The Spielberger State Anxiety Scale (STAI) is considered one of the most widely used scales to examine self-reported anxiety. It is straightforward to administer and score. Researchers found that the STAI was considered to demonstrate satisfactory validity when compared with other anxiety scale scores (Julian 2011). Both the Visual Analogue Mood Scale (VAMS) and the Positive and Negative Affect Schedule were also determined to be effective methods to reflect changes in mood across participants (Stern et al. 1997; Watson, Clark & Tellegen, 1988). To account for fluctuations in mood or anxiety due to the effect of removing participants' cellphones during their one hour of participation in the study, participants were given the Nomophobia scale (Lin, Griffiths & Pakpour, 2018). Because increases in heart rate variability are hypothesized to also have an effect on mindfulness, participants were given the Toronto Mindfulness Scale to assess changes in mindfulness scores (Lau et al., 2006). Each of the questionnaires were administered before and after the treatment with placebo or ultrasound stimulation.

Participants were randomly assigned to one of three conditions: two minutes of sham stimulation, two minutes of continuous stimulation, or two minutes of interval stimulation (with intervals consisting of 30 seconds of stimulation followed by a 30 second rest period, totaling a four-minute period). Participants were blind as to which condition that they were assigned to. Across all three conditions, the participant's electrocardiogram (ECG) was recorded using a J&J psychophysiological system. Electrodes with conducting gel were placed on subjects' wrists and stabilized using medical tape. Participants were also given a consumer heart rate monitor, Polar H10, to wear around their chest; this monitor was also utilized to actively record heart rate and heart rate variability. Participants' heart rate (Polar) and ECG (J&J) were recorded at five time points: baseline, during stimulation, one minute after stimulation, 10 minutes after stimulation, and 20 minutes after stimulation.

The ECG recordings were exported for processing using QRSTool and custom matlab scripts based on CMetX software, a tool developed by Allen, Chambers and Towers (2007) to compute heart rate variability (HRV) metrics. According to Hibbert et al. (2012), HRV indices processed using QRSTool and CMetX have been shown to be consistent with predicted indices of parasympathetic and sympathetic activity, supporting the field validity of QRSTool and CMetX as a reliable method of measuring HRV. We utilized QRSTool to identify and remove artifacts such as missed beats via interpolation based on the QRS intervals surrounding the segment containing a potential artifact. All contaminated segments were cut out using data markers and not included when calculating HR or HRV metrics. For the interval condition, the 30 second intervals were segmented for analysis of the interval on and off stimulation periods. We computed the RMSSD values by taking 30-second windows of ECG data and averaging across all artifact-free windows. For windows with artifacts, no data were included, and the next window began following the artifact. All data were reviewed by the author to verify the identified peaks of the QRS intervals. Before analyzing the RMSSD and heart rate data for potential interactions, we calculated Z-scores of baseline RMSSD to eliminate outliers with scores greater than 2s.d.

For the analysis of changes in heart rate variability and heart rate over time, we ran a two-way repeated measures ANOVA with group (3 levels: interval stimulation, continuous stimulation, and sham) by time (5 levels: pre, during, post1, post10, and post20) interactions. We then normalized the ECG data by dividing all values by the baseline heart rate and heart rate variability, and then ran a two-way rmANOVA with group (3 levels: interval stimulation, continuous stimulation, and sham) by time (4 levels: normalized during, normalized post1, normalized post 10, normalized post20) interactions. We utilized the Mauchly's Test of Sphericity to determine whether the assumption of sphericity was met and used the Greenhouse-Geisser epsilon and Huynh-Feldt epsilon corrections when appropriate. All mean differences are presented with a standard error of the mean.

VII. Results

Prior to examining changes in physiologic and survey data over time, we ran a 1-way ANOVA to analyze baseline differences in heart rate, RMSSD, anxiety (STAI) scores, and mood (PANAS) scores across the three stimulation groups. We found a significant difference across groups in RMSSD ($F_{2,47} = 4.56$, $p = 0.016$), but not in heart rate, STAI, or PANAS scores ($F_{2,55} < 0.22$, $p > 0.80$). The post hoc analysis revealed a significant effect between baseline RMSSD in the sham and interval groups ($p = 0.004$). Due to the inconsistencies in baseline RMSSD scores across groups, HR and RMSSD data was baseline-normalized for further analysis. The 2-way rmANOVA [3 stimulation groups (sham, continuous, interval) × 4 time points (during, post-1, post-10, post-20)] on normalized RMSSD values showed a significant main effect of time ($F_{3, 132}$ = 5.46 and p = 0.006) and group ($F_{2,44}$ = 4.25, p = 0.020), but no significant group × time interaction effect. Because the sphericity assumption was violated, we utilized the Greenhouse-Geisser correction. The post hoc analysis revealed a significant decrease between timepoints 'during' and 'post1' (p < 0.001) and a significant decrease between timepoints 'during' and 'post10' (p < 0.001; Figure 1). Additionally, the post hoc analysis revealed differences between the sham and interval stimulation groups (p = 0.01; Figure 1) and between continuous and interval stimulation conditions (p=.027; Figure 1).

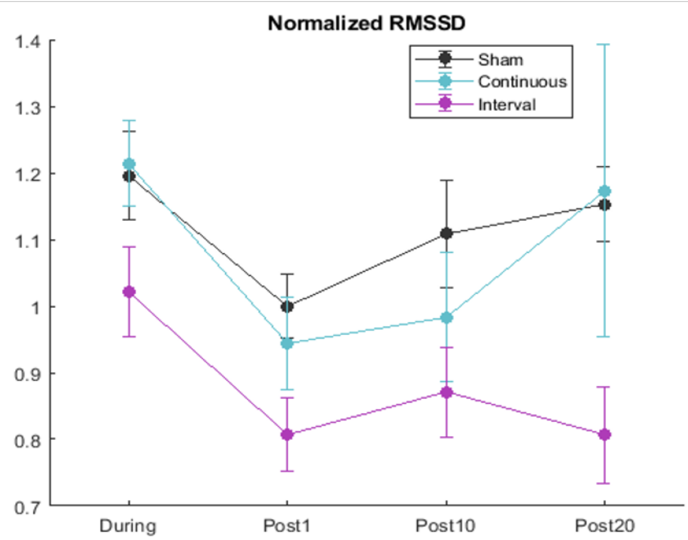

Figure 1: Changes in normalized RMSSD across the time points during, one minute post stimulation, 10 minutes post stimulation and 20 minutes post stimulation. Scores were normalized to pre-stimulation baseline levels so that scores above 1 reflect an increase, while scores below 1 reflect a decrease, from pre-stimulus baseline

A second 2-way rmANOVA [3 stimulation groups (sham, continuous, interval) × 4 time points (during, post-1, post-10, post-20)] on baseline-normalized heart rate data revealed a significant main effect of time ($F_{3,132}$ =21.56, p < 0.001, Figure 2). Post hoc analysis revealed a significant increase from timepoints ‘during’ to ‘post 1’ ($p < 0.001$), ‘post10’ ($p < 0.001$), and ‘post20’ ($p < 0.001$), as well as a significant decrease between timepoints ‘post1’ and ‘post10’ ($p < 0.036$, Figure 2). There were no significant group, or group × time effects. As shown in Figure 2, heart rate decreased briefly during stimulation in all three groups and then increased one minute after stimulation.

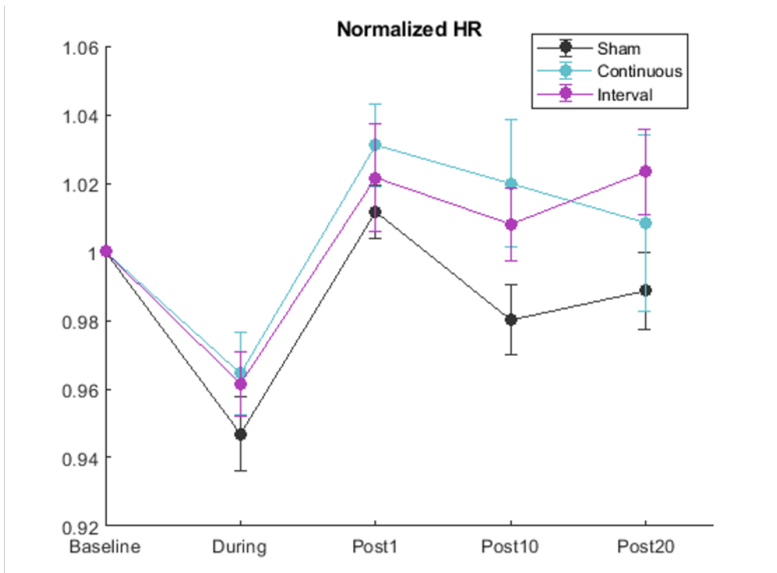

Figure 2: Normalized heart rate during stimulation and after stimulation. Scores were normalized to pre-stimulation baseline levels so that scores above 1 reflect an increase, while scores below 1 reflect a decrease, from pre-stimulus baseline

Heart rate was recorded during each 30-second timeframe of interval stimulation and analyzed for any changes as a result of the ultrasound stimulation. Figure 3 reflects changes in heart rate during interval stimulation where the first, third, fifth and seventh intervals reflect a decrease in heart rate when the stimulator was on, and the second, fourth, and sixth intervals demonstrate brief increases in heart rate during rest periods. In contrast to the pattern across intervals 1-7, heart rate decreased further during the eighth interval before spiking sharply during the first "post" recording obtained one minute later. This pattern in heart rate was not observed in RMSSD for the interval stimulation group.

Figure 3: Heart rate during each of the 8 intervals where the ultrasound device was placed on and off of the neck over the vagus nerve and the first recording immediately following stimulation. Odd numbered intervals represent placement on the neck, whereas even-numbered intervals represent when the transducer was off the neck.

The 2-way rmANOVA for STAI (anxiety scores) at two timepoints (before and after stimulation) across the three conditions showed significant effects for time ($F_{54,1}$=6.75, p = 0.012), and the post hoc demonstrated a mean decrease of 2.56 +/- .986 in anxiety scores after stimulation across all groups. A 2-way rmANOVA for mood (PANAS) scores revealed a significant time effect ($F_{1,55}$=12.29, p < 0.001), but no group or group ´ × time effects. The post hoc demonstrated a mean difference of 2.24 +/- .639 between the two timepoints, where there was an increase in positive mood scores after stimulation. As shown in Figure 4, the continuous group appeared to show a less marked change compared to the sham and interval stimulation groups, though these group differences were not statistically significant. There were no significant differences in VAMS mood scores before and after stimulation.

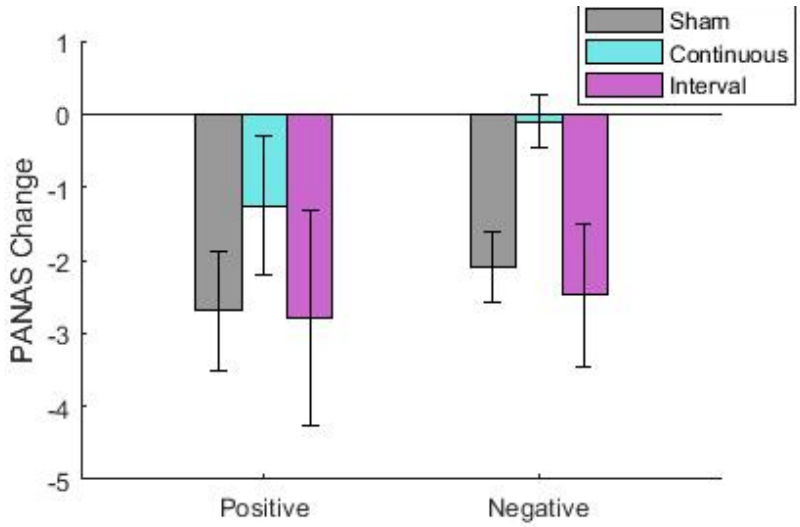

Figure 4: Differences in positive and negative mood scores before and after stimulation across the three groups.

Additionally, Nomophobia scores were examined alongside changes in anxiety scores as shown in Figure 5 below. Dividing Nomophobia scores into two groups: those above 60 and those below 60, demonstrated an increase in standard deviation in anxiety scores in the group scoring above 60 (15.96) compared with those below 60 (9.24).

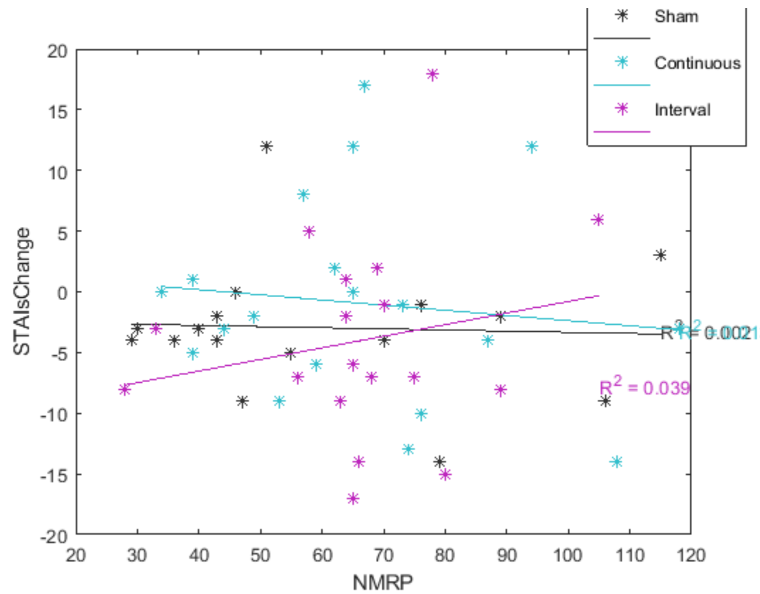

Figure 5: Higher Nomophobia scores (above 60) associated with greater standard deviation in anxiety scores

VIII. Discussion

One potential concern regarding the comparison of RMSSD across conditions is the baseline difference observed across groups as shown in Figure 6 below.

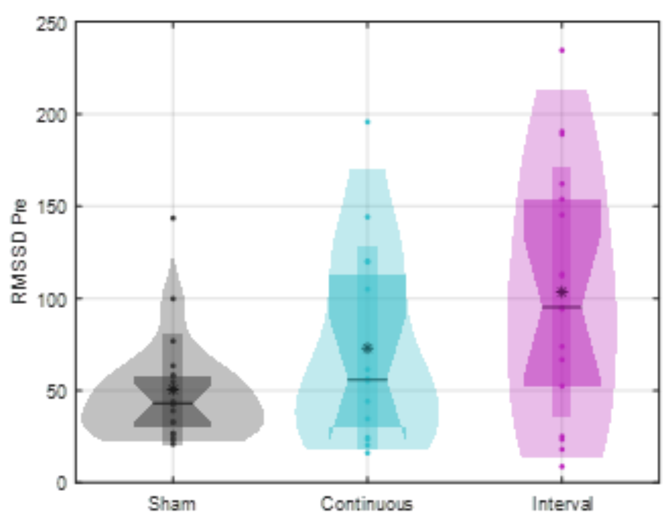

Figure 6: Baseline differences in RMSSD across sham, continuous, and interval stimulation groups, where the interval condition appeared to have a larger distribution of baseline RMSSD values

While outliers were removed, the presence of a greater number of participants with elevated baseline RMSSD in the interval stimulation group may have contributed to group differences. Because of these differences in baseline RMSSD, we chose to use normalized RMSSD values for the comparison across groups during the analysis.

The presence of differences in RMSSD over the five time points across all groups (interval stimulation, continuous stimulation and sham stimulation) is perhaps indicative of an effect due to experimental procedure as opposed to the ultrasound stimulation itself. While the decrease in heart rate during stimulation appears to be indicative of a physiological effect, this change in heart rate was observed in the sham condition as well. Because of this, the decrease in HR may be a result of tactile stimulation or a thermal effect due to the presence of cool ultrasound gel rather than the ultrasound. While there is a significant difference in normalized RMSSD between the sham and interval stimulation group, because there was no significant difference in RMSSD or heart rate between the continuous and sham conditions, it is likely that the difference between the interval stimulation and sham stimulation group is due to the alternating “on and off” nature of the interval stimulation rather than ultrasound.

While we initially hypothesized that an increase in heart rate variability (RMSSD) would be observed in both the continuous and interval stimulation conditions, instead, it appears that the interval stimulation condition contributed to possible dysregulation or lack of any effect rather than enhancement of cardiac vagal control. However, this may be due to experimental protocol, ultrasound carrier frequency, or pulse parameters. In one animal study by Wasilczuk et al. (2019), the experimenters utilized a protocol in which they surgically exposed the vagus nerve to ensure focus of the ultrasound transducer throughout all experiments, rather than stimulating noninvasively. Wasilczuk's group created an incision at the midline of each of the animals' jaws and dissected the carotid sheath in order to expose the left vagus nerve completely. Additionally, they applied ultrasound for 5 minutes with a pulse repetition frequency of 10Hz and utilized a transducer frequency of 250kHz. In contrast, our protocol involved only 2 minutes of noninvasive stimulation to the vagus nerve, with a transducer frequency of 5MHz. Finally, Wasilczuk's group only examined changes in levels of the inflammation marker TNF- α, rather than tracking fluctuations in RMSSD or heart rate over the course of stimulation. Another study, by Coiado et al. (2015), demonstrated that ultrasound significantly decreased heart rate ($p= 0.02$) and cardiac output ($p= 0.005$); however, they further examined the effects of ultrasound on the vagus nerve by comparing vagotomy (in which the vagus nerve was cut) vs non-vagotomy groups (in which the vagus nerve was fully intact). They concluded that the vagotomy group showed similar ultrasound-induced cardiac effects as the non-vagotomy group, suggesting that the vagus nerve itself may be less involved in the effects of ultrasound modulation (Coiado, 2015). However, in this study by Coiado et al., the animals received 5% isoflurane anesthesia throughout the procedure which may have impacted results as anesthesia has been shown to affect autonomic nervous system activity (Skovsted, 1977). Coiado's group utilized a 1MHz ultrasound transducer and delivered bursts of 2.0 MPa PRPA consecutively in 10 second intervals for a total of 30 seconds followed by bursts of 2.5MPa PRPA in three 10-second intervals, and finally bursts of 3.0MPa again in three 10-second intervals. Because the transducer that they used was non-FDA approved and differed from the settings used in our protocol, these differences may have significantly impacted our findings. Moreover, the research by Coiado et al. regarding the effectiveness of ultrasound in the vagotomy group necessitates further research on the involvement of the vagus nerve in inducing changes in heart rate due to ultrasound.

Additionally, in a study by Juan and colleagues (2014), the researchers utilized ultrasound to noninvasively stimulate the left cervical branch of the vagus nerve using 1.1 MHz frequency and intensities ranging from 13.6 to 93.4W/cm^2. The researchers observed vagus nerve inhibition in most cases by recording amplitudes of compound action potentials. They found that the peak amplitude decreased, and that peak latency increased during the ultrasound exposure (Juan, 2014). This finding is more consistent with the difference in RMSSD observed in the interval stimulation group compared with the sham stimulation group, in which the recording 20 minutes after stimulation revealed an overall decrease in RMSSD in the interval stimulation group.

While we anticipated anxiety scores would mirror fluctuations in RMSSD, neither anxiety scores nor mood scores significantly differed across groups. However, this may be explained by the lack of clinical levels of anxiety or the nature of self-reported anxiety and mood scales. Further research on physiological markers for anxiety such as cortisol may be interesting to examine in the context of vagal ultrasound stimulation. Additionally, the increase in standard deviation in anxiety scores in individuals scoring 60+ on the Nomophobia scale may be indicative of an increase in variability of responses to the stimulation with higher baseline anxiety due to lack of access to electronics.

While our results are indicative of possible effects due to ultrasound in modulating vagal tone, further research is required to investigate ultrasound in vagal disinhibition or enhancement. It remains unclear whether our findings result from the ultrasound stimulation itself or whether they are due to tactile or thermal effects from the ultrasound transducer. In the future, we plan to re-run the interval stimulation condition by administering ultrasound without removing the transducer from the subject's neck during on or off periods of stimulation to assess the presence of tactile effects. Additionally, further research is needed to determine the ideal pulse parameters and carrier frequencies for vagal ultrasound stimulation. We plan to further examine different protocols in administering ultrasound to determine possible pressure or temperature effects. Ultimately, while our findings regarding VNS, heart rate variability and perceived changes in anxiety scores are inconclusive, ultrasound stimulation to the vagus nerve ought to be further investigated to better assess its effects on cardiac vagal control and its efficacy as a potential treatment for anxiety disorders.

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