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Transcutaneous Auricular Vagus Nerve Stimulation (taVNS): Development, Safety, Parametric Optimization, and Neurophysiological Effects

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A dissertation submitted to the faulty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Graduate Studies.

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To my loving parents – Huda and Wadih

To my brothers – Karam and Alan

To my best friends – Chris, Greg, Joe, Josh, Thomas

To my mentor – Dr. George

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KEY TO SYMBOLS OR ABBREVIATION

ABVN	Auricular Branch of the Vagus Nerve
ACC	Anterior Cingulate Cortex
ACh	Acetylcholine
Amg	Amygdala
ANOVA	Analysis of Variance
ANS	Autonomic Nervous System
BG	Basal Ganglia
BOLD	Blood Oxygen Level Dependent
BPM	Beats Per Minute
CB	Cerebellum
CCK	Cholecystokinin
Cing	Cingulate
CN	Cranial Nerve
CNS	Central Nervous System
DBS	Deep Brain Stimulation
DLPFC	Dorsal Lateral Prefrontal Cortex
DRN	Dorsal Raphe Nucleus
ECG	Electrocardiogram
ECT	Electroconvulsive Therapy
EEG	Electroencephalogram
FA	Flip Angle (MRI)

FDA	Food and Drug Administration
fMRI	Functional Magnetic Resonance Imaging
HR	Heart Rate
Hyp	Hypothalamus
IDE	Investigational Device Exemption
imOCC	Inferomedial Occipital Gyrus
IPG	Implantable Pulse Generator
IRB	Institutional Review Board
LC	Locus Coeruleus
MDD	Major Depressive Disorder
MUSC	Medical University of South Carolina
NBM	Nucleus Basalis Meynert
NRS	Numerical Rating Scale
NT	Neurotransmitter
NTS	Nucleus Tractus Solitarius
nVNS	Noninvasive Vagus Nerve Stimulation
OFC	Orbitofrontal Cortex
PB	Parabrachial Nucleus
PET	Positron Emission Tomography
PFC	Prefrontal Cortex
PNS	Parasympathetic Nervous System
PT	Perceptual Threshold

PVN	Periventricular Nucleus
RCT	Randomized Clinical Trial
RF	Radio Frequency
rTMS	Repetitive Transcranial Magnetic Stimulation
SD	Standard Deviation
SEM	Standard Error of the Mean
SNS	Sympathetic Nervous System
STG	Superior Temporal Gyrus
TA	Time of Acquisition
TAU	Treatment as Usual
taVNS	Transcutaneous Auricular Vagus Nerve Stimulation
tcVNS	Transcutaneous Cervical Vagus nerve Stimulation
TE	Echo Time (MRI)
TENS	Transcutaneous Electrical Nerve Stimulation
Thal	Thalamus
TMS	Transcranial Magnetic Stimulation
TNF	Tumor Necrosis Factor
TR	Repetition Time (MRI)
TRD	Treatment Resistant Depression
TTL	Transistor-Transistor Logic
vmPFC	Ventromedial Prefrontal Cortex
VNS	Vagus Nerve Stimulation

ABSTRACT

BASHAR WADIH BADRAN. Transcutaneous Auricular Vagus Nerve Stimulation (taVNS): Development, Safety, Parametric Optimization, and Neurophysiological Effects. (Under the direction of Mark S. George)

Cervically implanted vagus nerve stimulation (VNS) is a FDA-approved treatment for epilepsy and major depressive disorder (MDD). Additionally, VNS is a reemerging area of interest, showing promise in numerous animal studies with significant translatable applications. The cost, surgical risk, and human translation difficulty makes noninvasive VNS a highly-desired alternative.

We have developed a transcutaneous auricular vagus nerve stimulation (taVNS) system that electrically stimulates the auricular branch of the vagus nerve (ABVN). We aimed to answer the following questions in this body of work: 1) whether taVNS is safe and feasible 2) if taVNS stimulates the vagus system similarly to implanted VNS 3) if the neurobiological effect of taVNS is similar to implanted VNS.

We measured physiological recordings in healthy adults during taVNS to determine whether taVNS has vagus-mediated effects. In our first trial (n=15), we explored the physiological effects of 9 various stimulation parameter combinations (various pulse widths and frequencies) as a broad search of the physiological effect. A second, follow-

up trial was conducted (n=20) to determine the best candidate parameter that optimally activates the parasympathetic nervous system. Lastly, we developed and conducted a novel concurrent taVNS/fMRI trial (n=17) to determine the neurobiological effect of taVNS and its afferent targets. All three trials consisted of 2 visits each, in a randomized, controlled, crossover design in which taVNS was delivered to either the left tragus (active) or earlobe (control).

The first physiological trial revealed relevant, immediate heart rate decreases during taVNS followed by a sympathetic rebound upon termination of stimulation. Of the nine parameters tested, two had the largest effect on heart rate (500μs, 10Hz; 500μs, 25Hz). These two parameters were tested in the follow-up trial, which demonstrated that both parameters decrease heart rate, with 500μs 10Hz having the largest physiologic effect. Lastly, findings from the taVNS/fMRI trial demonstrate the neurobiological effect of taVNS mimics that of cervically implanted VNS and targets several cortical and subcortical vagus afferent pathway targets.

taVNS in our paradigms was feasible, safe, and demonstrated neurobiological effects that are similar to implantable VNS. Future trials should conduct parametric optimization using the taVNS/fMRI protocol as it reliably targets vagus nerve afferents as well as further explore optimizing taVNS as a possible therapeutic and research tool.

CHAPTER 1

OVERVIEW OF VAGUS NERVE STIMULATION (VNS)

Human Anatomy of the Vagus Nerve

Cranial Nerve X

Cranial nerves (CN) serve as a pathway for which information is exchanged between the central nervous system (CNS) and the periphery. It would not be possible to integrate outside sensory information with the CNS without CNs. There are 12 cranial nerves (1), all of which play an important role in human sensation and perception. Important senses relied on daily, such as smell (CN I), vision (CN II), hearing (CN VIII), and taste (CN IX) are all relayed to the brain from the periphery via cranial nerves. 10 of the 12 CN's first point of entry into the CNS is through the brainstem and have widespread afferent cortical and subcortical targets and effects.

CN X, otherwise known as the vagus nerve, is a mixed sensory and motor nerve that originates from the medulla in a region known as the nucleus tractus solitarius (NTS). Latin for "wandering nerve," the vagus nerve's efferent projections travel throughout the thorax and abdomen, targeting nearly every major organ in the body. The vagus nerve's primary role serves to monitor and regulate the organs depicted in f**igure 1-1a** (2)

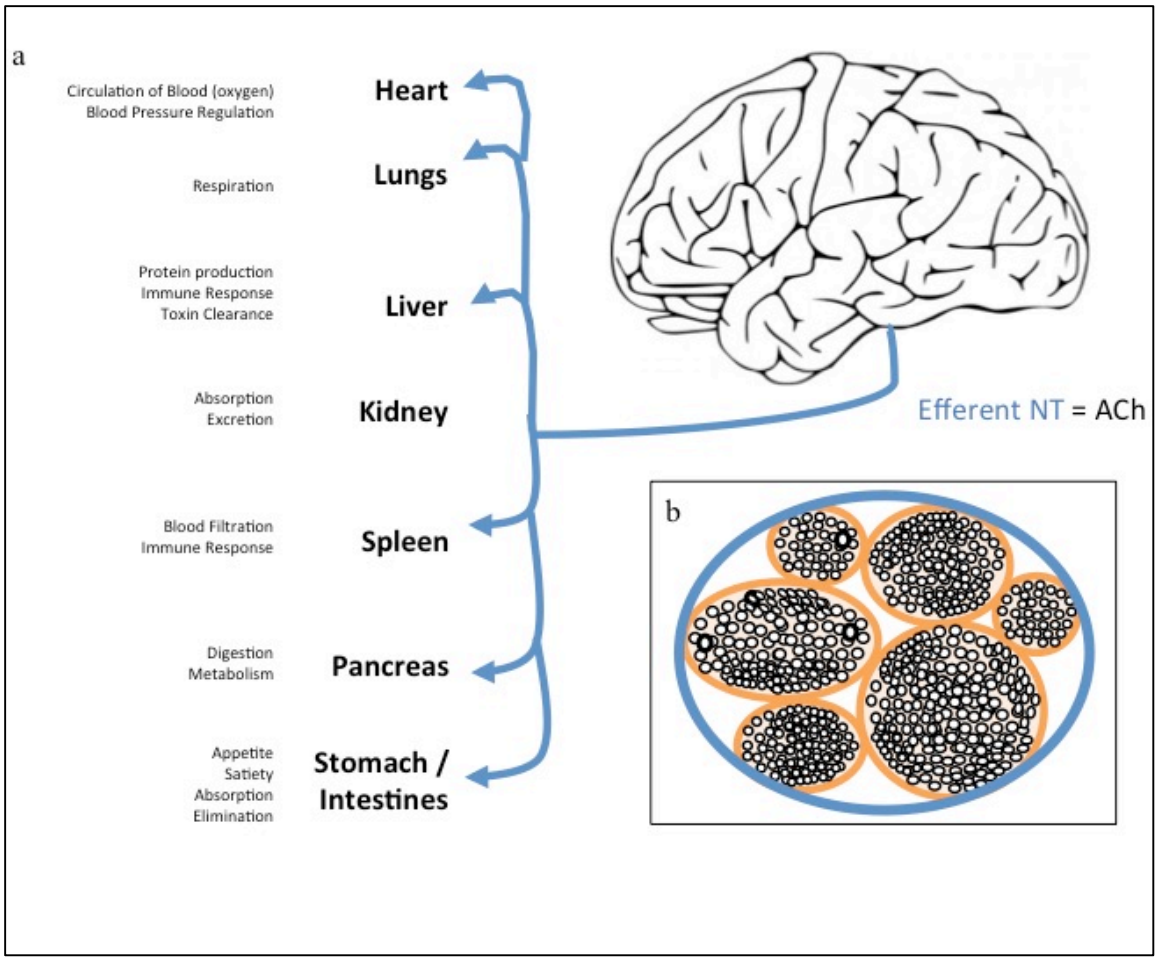


Figure 1-1 Schema of the Vagus Nerve. a) Visualization of the vagus nerve path through the human torso. The vagus nerve exits the brain and wanders vertically down through the entire human torso targeting nearly every major organ in the body. Listed are some of the major organs and their functions modulated by the vagus nerve. **b) A cross section of the vagus nerve.** This cross-sectional diagram demonstrates how the vagus nerve is composed of multiple bundles of nerves. The vagus nerve houses over 100,000 individual nerves, each compartmentalized into bundles and surrounded by gristle. These numerous bundles form one large nerve known as the vagus.

and convey information to the CNS (afferent projections) as well as from the CNS to the organs (efferent projections). It's efferent effect is primarily parasympathetic (3), as the vagus releases acetylcholine (ACh) onto its targets, which binds to muscarinic Ach receptors inducing their behavioral effects.

The vagus nerve is not one large nerve, but rather a large track of nerve bundles surrounded by gristle (**Figure 1-1b)** housing over 100,000 individual nerves. These nerves are about 80% afferent projecting nerves and 20% efferent nerves (4), although it is nearly impossible to determine exactly which nerves serve what purpose given its complexity. It is also extremely difficult to isolate behavioral or physiological effects from an individual nerve within the bundle, as intricate in-vivo microsurgery is required.

Peripheral Targets

Nearly every major organ in the human body has a connection to the vagus nerve, which enables bidirectional communication of information regarding relevant bodily functions performed by the organ. Summarized in (**Figure 1-2**) are functional domains that these target organs can be classified into: cardiovascular (heart), ingestion (esophagus, tongue), metabolism (stomach, intestine), inflammation (spleen), glucose regulation and toxin filtration (pancreas, liver, kidney). These domains are integral to daily life activities and are constantly monitored by the CNS via the vagus nerve.

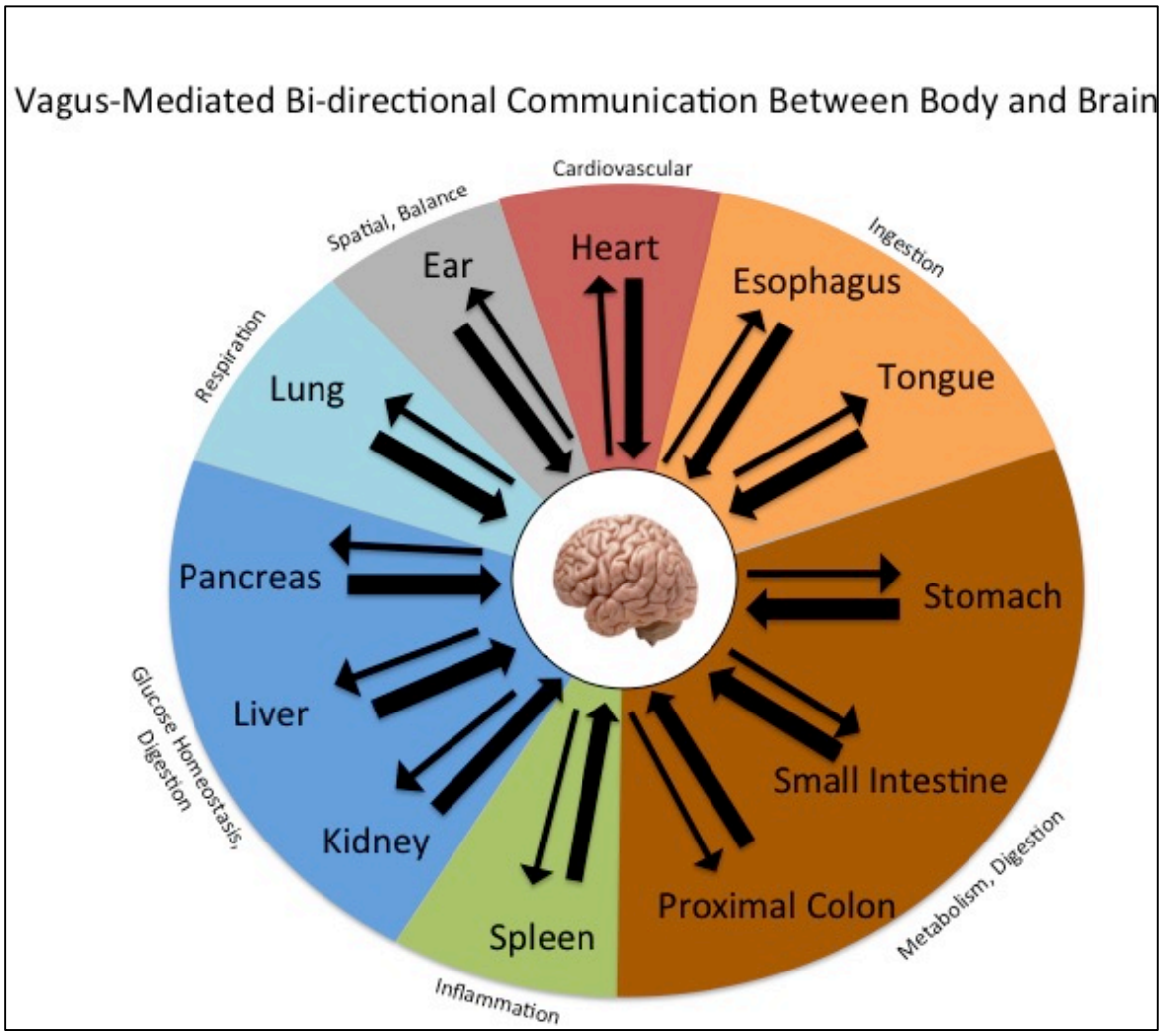


Figure 1-2 Behavioral domains that the vagus nerve regulates. The vagus nerve has bi-directional communication with the periphery. It receives input from these organs as well as sends centrally driven information to them to regulate their action. This vast two-way communication tract can be utilized to treat peripheral diseases of target organs and central neuropsychiatric diseases.

Afferent (periphery to central) vagal communication can be easily exemplified by the feeding satiety signal. Cholecystokinin (CKK) and leptin are produced in the gut when an individual eats and stomach fills with food. CCK and leptin generate a satiety "signal" transmitted from the stomach to the CNS via the vagus nerve. This signal alerts the brain and elicits a termination of feeding behavior (5, 6).

An example of efferent vagal communication is best demonstrated by the parasympathetic relaxation of heart rate (7, 8). This parasympathetic response is initiated in the periventricular nucleus (PVN) of the hypothalamus, sending efferent projections down through the NTS and to the heart and lungs, releasing ACh and slowing heart rate (9). This bidirectional communication and direct regulation of function of vital bodily organs makes for an extremely large and intricate nerve system.

Afferent Brain Targets

The first entry point of the vagus nerve into the CNS (**Figure 1-3**) is the NTS (10, 11). From the NTS there are direct projections to the locus coeruleus (LC) and parabrachial nucleus (PB) (12). These two brain regions are responsible for many of the behavioral effects of vagus nerve stimulation (VNS), which will be discussed in the later part of this chapter. Krahl et al demonstrated lesions of the LC cause the anti-epileptic effect of VNS to disappear (13) which confirm the LC main role as a vagus central hub.

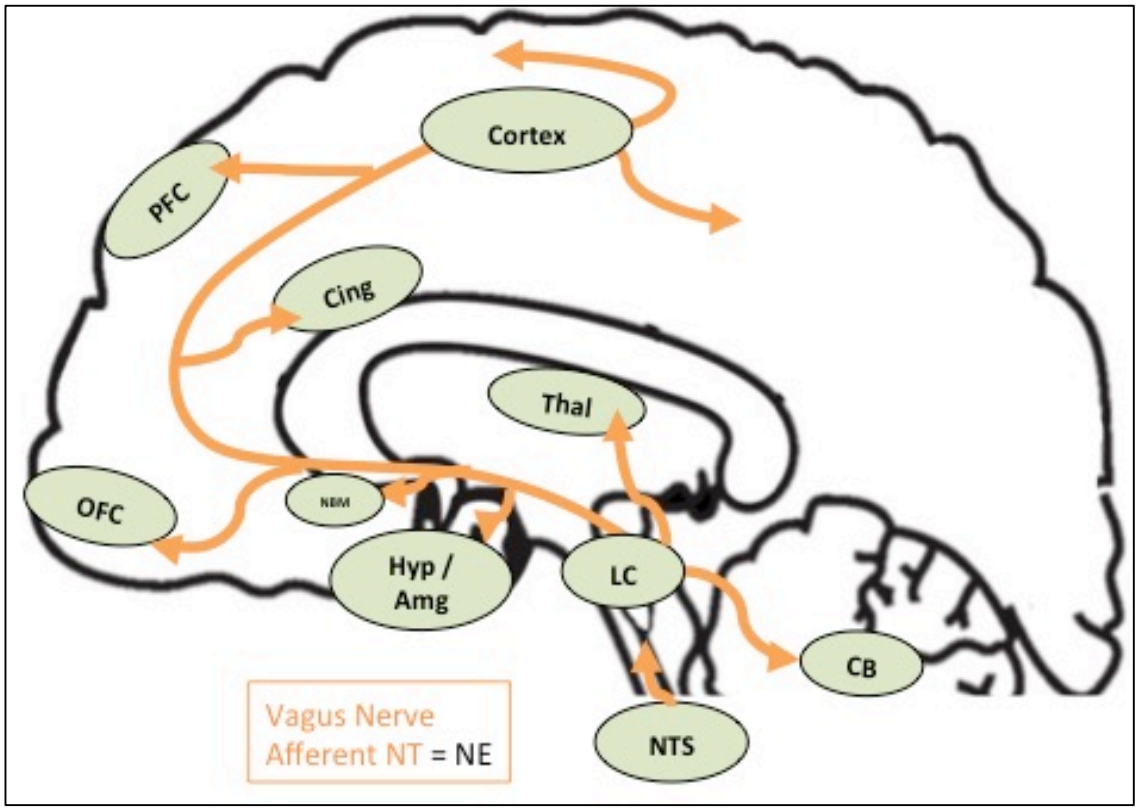


Figure 1-3 Afferent pathway of the vagus nerve. The first point of entry of the vagus nerve is in the nucleus tractus solitarius (NTS). From there the signal is immediately projected up to the locus coeruleus (LC), the primary producer of norepinephrine for the brain. From there the signal propagates in three directions 1) directly to the cerebellum (CB) 2) up to the thalamus (THAL), and 3) frontally to the hypothalamus (Hyp), amygdala (Amg), and nucleus basalis (NBM). Passing these deeper brain structures, the afferent path leads to important mood and cognitive processing networks like the orbital frontal cortex (OFC), cingulate cortex (Cing), and prefrontal cortex (PFC). Effects are not limited to the named structures, as there are unlisted widespread, diffuse cortical effects.

The LC is the primary producer of norepinephrine (NE) in the CNS, a key neurotransmitter and alert signal of the brain. From the LC there are ascending projections branching directly to the thalamus (Thal), hypothalamus (Hyp), cerebellum (CB), orbitofrontal cortex (OFC), prefrontal cortex (PFC), cingulate gyrus (Cing), amygdala (Amg) nucleus basalis of meynert (NBM) and the rest of the cerebrum (14).

Although NE is the primary afferent neurotransmitter involved in the afferent vagal pathway, the LC also influences serotonin release through direct projections to the dorsal raphe nucleus (DRN) (15) which is the brain's primary producer of serotonin and independently has a wide range of ascending brain targets, many of which overlap the ascending LC pathway.

Early Vagus Nerve Stimulation

Initial Animal Trials Exploring VNS

In the early 20th century, Otto Loewi conducted a famous experiment (16) that is credited with discovering neurotransmitter communication in nerves. Loewi stimulated the intact nerve of a frog heart maintained in a solitary perfusion chamber. He observed the slowing of heart rate and collected the chamber fluid, transferring the fluid to a second chamber, which contained a denervated frog heart. When the second, untethered heart was bathed in the new fluid, it beat rate also slowed. This study concluded that the fluid must contain a chemical released upon electrical stimulation of the nerve which Loewi called "vagusstoff." Eventually, this chemical was validated and now known as acetylcholine

(ACh). This discovery earned Loewi the Nobel Prize in Physiology or Medicine in 1936, shared with Sir Henry Hallett Dale for "their discoveries relating to chemical transmission of nerve impulses."

Following Loewi's experiment which demonstrated th peripheral effects of direct electrical VNS, there were several important animal studies (**Table 1-1**) exploring the central effects of VNS leading up to the inception of VNS as a human therapeutic tool to eventually be used for intractable epilepsy and major depressive disorder (MDD). These trials span a series of half a decade and are not assumed to be the only VNS trials conducted during this time, but rather pivotal positive trials that served as integral findings in the development of VNS as a modality. They all involve direct, in-vivo electrical stimulation of the vagus nerve, as Loewi did to demonstrate neuronal and behavioral changes.

Bailey and Bremer conducted the first of these studies in 1938 (17). This study demonstrated that VNS in cats increased synchronized electrical potentials of the orbital frontal cortex as measured by electrogram. VNS was then conducted in monkeys under anesthesia by MacLean and Pribram in 1949 and reported in MacLean's book in 1990 (18). Their study suggested changes in the lateral frontal cortex associated with stimulation. Dell and Olson conducted their own VNS study in awake cats in 1951 (19) which demonstrated relevant slow-wave changes in the amygdala and thalamus. Radna and MacLean followed up with a second VNS trial with monkeys in which they

Table 1-1. Early VNS studies leading to human trials

Year	Author	Model	Brain region	Findings
1938	Bailey & Bremer	Cats	OFC	↑ synchronized electrical signals on EEG
1949	MacLean & Pribram	Monkeys	LFC	Inconsistent, slow waves on EEG
1951	Dell & Olsen	Awake cats	Amg, Thal, ARS	Slow wave response on EEG
1981	Radna & MacLean	Monkeys	Limbic	↑ single unit activity
1981	Radna & MacLean	Monkeys	Striopallidum	↑ single unit activity
1987	Zabara et al.	Dogs	Cortical	Medication-induced seizure termination. Protection 4x stimulation period. Parameters established.
1992	Zabara et al.	Dogs	Cortical	

OFC-Orbital Frontal Cortex; LFC-Lateral Frontal Cortex; Amg-Amygdala, Thal-Thalamus, ARS-Anterior Rhinal Sulcus

demonstrated single unit activity effects as a result of stimulation in the thalamus, cingulate, and limbic structures (20, 21).

The invention of therapeutic VNS is credited to Dr. Jacob Zabara, who was the first individual to consider VNS as a treatment for neurological disorder. In the late 1980's, Zabara conducted a VNS trial in dogs that had pharmacologically induced seizure disorder (strychnine) (22, 23). VNS in these dogs elicited cortical changes as measured by EEG and halted motor seizures and tremors. He then conducted a follow-up trial, again in canines, optimizing VNS as a seizure suppressor and also demonstrated that VNS had a behaviorally positive long-term effect that persisted beyond the stimulation period (24). These studies are cited as the pivotal animal trials that justified VNS as an implantable therapeutic device for humans.

VNS for Epilepsy in Humans

Although Zabara is credited with the innovated application of therapeutic VNS in 1985, it is forgotten that a century prior, an American neurologist by the name of James Leonard Corning suggested that seizures could be attenuated using transcutaneous vagal nerve stimulation through the neck. In 1883 (25, 26) Corning built a device (**Figure 1-4, US National Library of Medicine Public Domain**) which he hypothesized would stimulate the vagus nerve, decrease cerebral blood flow, and reduce epileptic seizure frequency and duration. He also suggested it be used as a prophylactic therapy. Unfortunately for

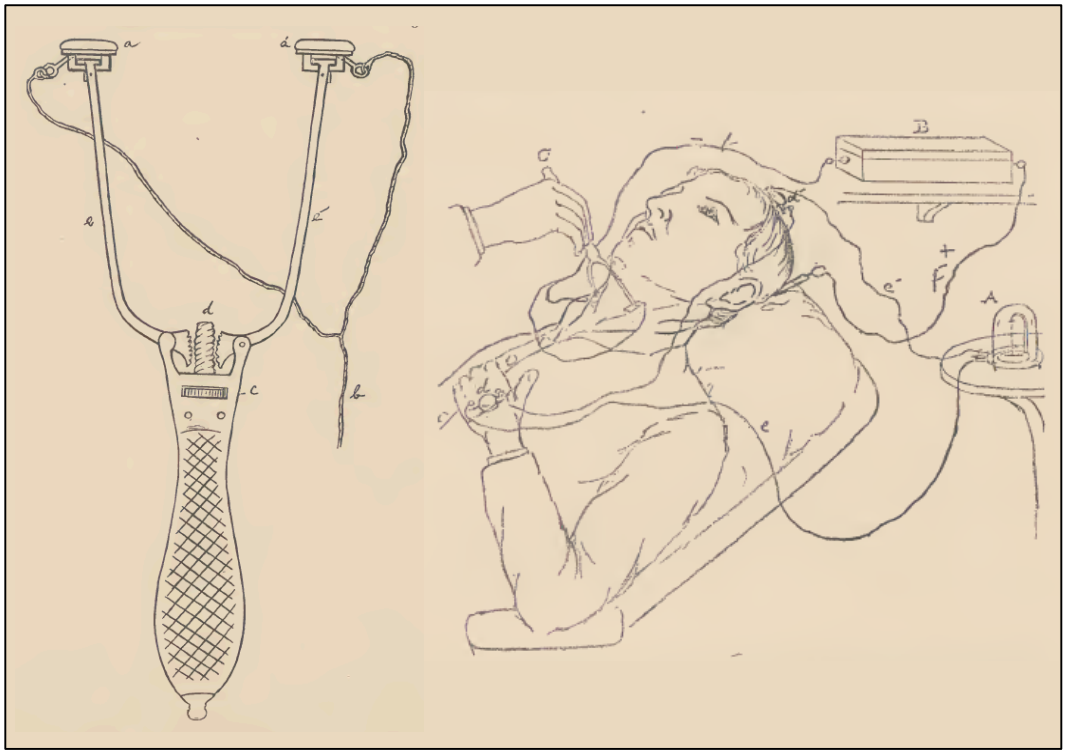


Figure 1-4. The Corning fork. Developed in 1883 by James L. Corning, this device served two purposes 1) bilateral carotid compression, which was believed to treat epilepsy, and 2) direct electrical stimulation to the carotid sheath, stimulating the bilateral pneumogastric nerves as a prophylactic epilepsy therapy. This figure is from the US National Library of Medicine where the original manuscript may be found and falls under public domain use (26).

Corning, his colleagues did not adopt his technology and it disappeared by the early 1900's.

In 1987, Zabara co-founded Cyberonics, Inc. (now LivaNova), along with Reese Terry, and began developing a human VNS device based on his promising animal trials. The first human implanted with a VNS device was in 1988 at Wake Forest Gray Medical School in North Carolina by Dr. J. Kiffen Penry and neurosurgeon William Bell (27). Eventually, four patients were implanted in this inconclusive safety and feasibility trial. Side effects were described (hoarseness, stimulation sensation, hiccups). Several more clinical trials (28, 29) were conducted in the early 1990's leading up to European approval of the Cyberonics VNS device to treat epilepsy in 1994 (30, 31), and subsequent United States FDA approval in 1997. Degorgio et al (32) demonstrated nearly 20% of individuals had a >75% reduction in seizure frequency at 12-months post implantation, and a median reduction on seizure frequency of nearly 50%. Sackeim et al (33) demonstrated the acute response rate in refractory epilepsy as being approximately 30%. As of 2017, according to Cyberonics, over 100,000 patients worldwide have been implanted with a VNS device as a treatment for intractable epilepsy.

Modern Vagus Nerve Stimulation

Implantation and Programming

A VNS system can be implanted by any surgeon trained in the head and neck. It is an outpatient surgical procedure with few serious complications (32, 33). The implantation

site is the left branch of the mid-cervical vagus nerve, which is accessed through the neck. A helical bipolar electrode (three-helix cuff) is wrapped around the nerve (**Figure 1-5**). Wires are run from the electrode cuff to a subcutaneously implanted pulse generator in the left chest. This pulse generator, or "can" contains a lithium battery and constant current pulse generator with a lifespan of approximately 5 years although second generation devices are being developed to have lifespans of over 10 years.

Following implantation, there is a two-week period in which the patient can recover from the minor procedure. The patient then returns after this two-week period to have their pulse generator programmed by their providing physician in an outpatient setting. Programming of the device is completely wireless, using a proprietary wand that connects to the device using radio frequency (RF). Through a portable computer, the wand can program specific parameters (current (mA), duty cycle (on/off time) and frequency (Hz)). The pulse generator also contains a reed switch, which enables the patient to turn off the device by swiping a strong static magnet over it. This enables the patient to test if the device is still functioning, but more importantly allows for user control of the device in the case of side effects.

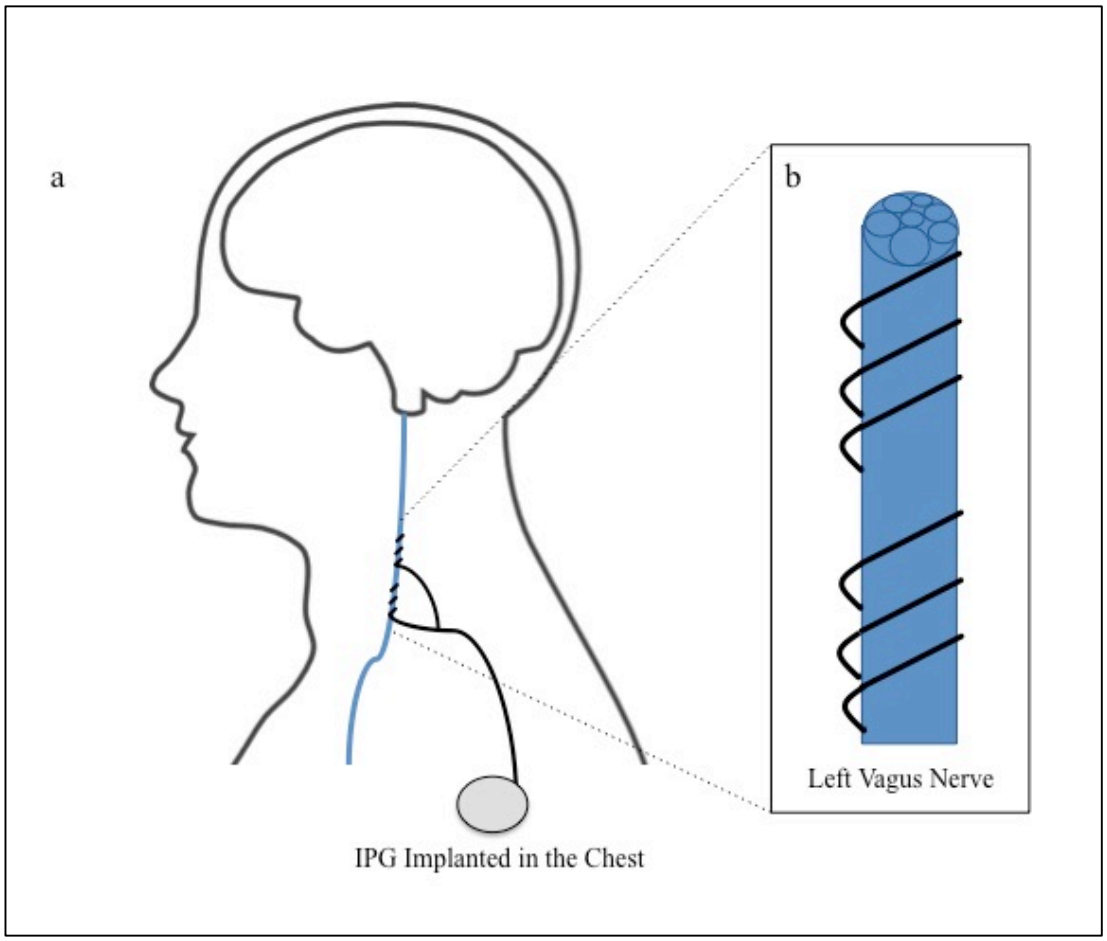


Figure 1-5. Modern cervically implanted VNS. a) VNS systems have two key components 1) an implantable pulse generator (IPG) which contains a battery and microcontroller delivering current, and 2) bipolar helical electrodes that wrap around the left cervical vagus nerve. **b)** This zoom view of the vagus nerve demonstrates how the electrodes are wrapped around the vagus nerve bundle.

Parameters

The question of optimal parameters arises with every form of neuromodulatory techniques such as transcranial magnetic stimulation (TMS), electroconvulsive therapy (ECT) and deep brain stimulation (DBS). The parameter space for these modalities is extremely vast. As presented in **Figure 1-6**, pulse width (μs), current (mA), frequency (Hz), duty cycle (on/off time), and dose titration (% threshold) are all parameters that can be manipulated to optimize desired behavioral response. Although the parameter space is large, many of our current therapies are based on the effects seen in preclinical animal studies, which are then translated to human studies.

Initial trials of the anti-seizure effect in a canine model demonstrated in Zabara's trials determined an optimal stimulation frequency of 20-30Hz, at a constant 20V with a pulse width of 200μs (24). Those findings guided initial multi-site clinical trials that eventually could determine optimal stimulation parameters to reduce seizure frequency in epilepsy patients.

One notable difference is the pulse width increased by a magnitude of more than 2 (from 200μs to 500μs) from animal trials to human trials. This is a result of neuronal chronaxie, which is the minimum pulse width size required to fire a nerve fiber using an electric current. Imach and Ranck discuss that a pulse width of 200-700μs is the optimal pulse width to fire nerve fibers as it maximizes the peak firing percentage rate while minimizing inefficient excess and side effects (34, 35). Short pulse width would

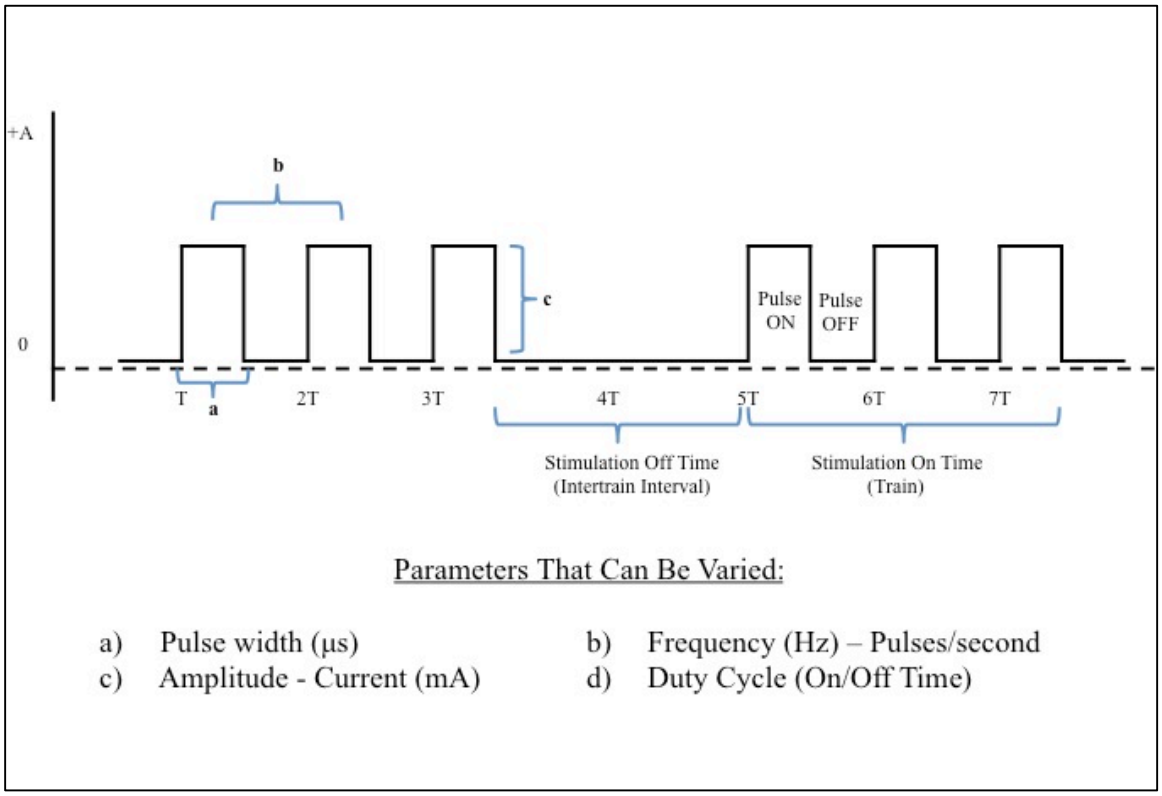


Figure 1-6 Direct electrical current waveform. Direct square wave electrical current can be delivered at various parameters. This figure demonstrates key properties of the waveform that can be changed to achieve desired biologic effects.

require significantly higher voltages to consistently and reliably depolarize nerves, whereas larger pulse widths, although they maximally depolarize nerves, demonstrate higher side effects and are using electrical energy from the device inefficiently.

Agnew and McCreery explored the question of optimal stimulation frequency the initial 1987 anti-epileptic findings by (36, 37). They demonstrated that higher frequency ranges (>50Hz) in fact cause damage to the vagus nerve. This damage is not seen in lower operating frequencies. Low frequencies (1Hz) were explored in human clinical trials as a control (32) (38)and showed minimal behavioral effects, effectively constraining the frequency space in humans to between 1Hz and 50Hz.

The current accepted parameters for VNS are as follows: current– 0.25-0.75mA; pulse width– 250-500μs; frequency– 20-30Hz; duty cycle – On 30s, Off 5min. These parameters are set as ranges and increased to a maximally tolerable level dependent on immediate side effects listed as hoarseness of voice, throat pain, coughing and headache (32, 33).

VNS for Resistant Depression

During the late 1990's, as the VNS for epilepsy pivotal FDA-trials were coming to an end, a clerk at the Florida hotel all follow-up patients stayed at noticed their moods were improving. Anecdotally, and lacking objective depression measures, this was relayed to the study team and followed-up by a prospective study in 14 individual (39) showing a

trend of mood-enhancing effects of VNS. In retrospect, given the wreath of depression neuroimaging research accessible today, VNS for depression is accepted to potentially have an anti-depressant effect with its afferent brain targets.

Several multi-site trials were conducted in the early 2000's to determine whether VNS was an effective antidepressant in patients with extremely resistant major depressive disorder (MDD). The first trial (33, 40, 41) was a four-site, open-label trial in 59 patients. Acutely, 8 weeks of VNS produced a 31% response rate in these patients with a 15% remission rate. Over time, these individuals improved, with the two-year response and remission rates increasing to 44% and 22% respectively. European open-label trial findings were similar to their US counterparts (42). These findings posed Cyberonics to conduct a pivotal FDA-approval seeking randomized control trial in 222 extremely resistant MDD patient. The findings of this pivotal trial were disappointing, with a large sham response rate (10%) and reduced overall effect by condition, demonstrating non-significant acute benefits compared to sham (43). These patients were followed for two years after implantation and the response rate more than doubled at two year follow up compared to the acute treatment phase. There seems to be a cumulative, long-lasting effect of VNS that is not being accounted for and is still unknown mechanistically.

VNS was FDA approved for chronic or recurrent depression in 2005 based on the findings by George et al. (44). This study demonstrated that when VNS therapy was compared against treatment as usual in a multi-site comparison trial with a followed, non-

implanted matched cohort receiving treatment as usual (TAU), VNS produced significant, long-term, durable benefits. It still lacks class 1 evidence as a treatment for depression and as of 2017 plans are being developed by LivaNova (acquired Cyberonics in 2016) to conduct a pivotal, randomized controlled FDA trial for class 1 evidence of VNS as a therapy for chronic recurrent depression.

Key VNS Functional Neuroimaging Trials

Several neuroimaging trials have been conducted using positron emission tomography (PET) and functional magnetic resonance imaging (fMRI). Initial VNS imaging trials were conducted in 1992 by Garnett et al using positron emission tomography (PET) scanning (45). PET detects pairs of gamma rays, which are emitted by a radiotracer that is injected into the patient and used as a measure of metabolism. Oxygen 15 ($H_2{}^{15}O$ PET) is an excellent tracer for neuronal activity, as the cells require oxygen for metabolism and neuronal firing. In this early trial in patients implanted with a stimulator for epilepsy, VNS-increased blood flow was discovered in the thalamus and cingulate. This study was followed-up by Henry and colleagues (46) who demonstrated areas of increased blood flow in the brainstem, thalamus, and hypothalamus and decreased blood flow in the medial temporal cortex and hippocampus. These two studies, although not inclusive of all PET studies, provided the groundwork for functional neuroimaging that followed using fMRI.

fMRI uses strong magnetic fields (>1.5T) to measure blood oxygen level dependent (BOLD) signals in the brain (47) that serve as a surrogate marker of neuronal activity. Oxygen binds to hemoglobin and is carried to neurons in the brain for metabolism. The differences in magnetic properties of oxygenated vs. deoxygenated hemoglobin make this imaging modality possible.

Faraday's law of induction dictates that electricity is induced when there is a changing magnetic field around a coil of wire which poses concern for conducting fMRI combined with VNS as the electrodes may heat up causing harm to the patient and the pulse generator may function improperly due to the magnetic field of the scanner. Using a specific orientation of implantation for the pulse generator as well as a specific head coil to decrease the magnetic field delivered to the VNS system, Bohning et al (48) developed the first VNS/fMRI method at the Medical University of South Carolina (MUSC). This landmark study was followed by an important VNS/fMRI study in depressed adults showing the frequency and dose effects of VNS in a pathologic group (49) and subsequently by Mu et al (50).

Nahas et al (51) followed by conducting several longitudinal VNS/fMRI scans on patients to explore the brain effects of VNS as a function of time. His findings reveal that BOLD signals increased as a function of how depressed the patient was and how strong the stimulation parameters were. Serially over time, these overall BOLD activations decreased. Since then, there have been several VNS/fMRI studies from groups all over

the world (48-54) demonstrating relevant BOLD signal intensity increases in vagal afferent brain regions which are summarized in **Table 1-2**.

Recent Strides in VNS

Promising Animal Trials

VNS is seeing a resurgence in the scientific community over the past 15 years. In the late 1980's and early 1990's, much of the literature was attempting to determine the basic feasibility and method of VNS. After that was established, the mid-1990's to early 2000s were dominated primarily by human clinical trials, translating the early animal studies into two FDA-approved treatments for intractable epilepsy and chronic resistant depression. Since the mid-2000's to today, the field of basic VNS research has boomed, with hundreds of papers being published a year on this interesting form of neuromodulation. VNS has been explored in dozens of different disease animal models, but the most promising are in the following central and peripheral disorders: obesity, inflammation/sepsis, tinnitus and stroke. This section will highlight some of the hallmark studies that are of high impact in the field and will most likely (if not already) be translated into human studies.

The first promising animal trial exploring the effects of VNS (implanted in the thorax near the stomach rather than cervical for epilepsy and depression) dates to 2001 in which 4 weeks of VNS in 27 rabbits demonstrated decreased food intake and weight loss

Table 1-2. VNS/fMRI studies

Year	Author	n	Subjects	Parameters	Control	Findings
2001	Bohning et al.	9	TRD	20 Hz; 500 μs; 0.5-1.25 mA; 13 sec on, 103 sec off	n/a	Feasibility of fMRI to measure VNS effects on TRD patients
2002	Lomarev et al.	6	TRD	5 Hz or 20 Hz; 500 μs; 0.25-1.25 mA; 13 sec on, 103 sec off	Yes, 5 Hz Tone	20 Hz > 5 Hz BOLD response. 20 Hz > 5 Hz to tone (arbitrary stimulus)
2004	Mu et al.	12	TRD	20 Hz; 0.25-1.25 mA (max tolerated at 500 μs); 13.6 sec on, 41 sec off	n/a	130 μs - insufficient global activation 250 μs - sufficient activation & deactivation 500 μs - insufficient global deactivation
2007	Nahas et al.	9	TRMD	20 Hz; 500 μs; variable mA; 13.6 sec on, 41 sec off	Yes, 0 mA	VNS acutely activates R insula, deactivates vmPFC.
2002	Sucholeiki et al.	4	Epilepsy	varied	n/a	Feasibility of fMRI to measure VNS effects on epilepsy patients
2002	Narayanan et al.	5	Epilepsy	30 Hz; 250 μs; 0.5-2.0 mA; 30 sec on, 30 sec off (48 x1)	none	VNS induced ↑ activity in: b/l thalami, b/l insula >> L BG, L Postcentral g, R post. STG, L>R imOcc g.
2003	Liu et al.	5	Epilepsy	30 Hz; 250 μs; 1.25-1.75 mA; 30 sec on, 66 sec off		2 patients with thalamic activation has greatest seizure response. All patients had frontal and occipital activation.

TRD-Treatment resistant depression; TRMD-Treatment resistant mood disorders; vmPFC- ventromedial Prefrontal Cortex; b/l- bilateral, R-right, L-left; BG-basal ganglia; STG- superior temporal gyrus; imOcc-inferomedial occipital gyrus

*Refer to Dietrich et al. for review of areas of anatomic activation

(55). There have been several follow-up trials since, demonstrating VNS decreases food intake and weight gain (56-58). The most notable of these trials was the 2010 trial in which obese Gottingen minipigs (58) were implanted with VNS devices and not only lost weight but opted for a healthier diet. These findings translated to human clinical trials in the 2012 EMPOWER randomized control trial (59) testing blockade of the vagus nerve for weight loss. Unfortunately, the EMPOWER trial did not find significant behavioral effects compared to sham. This trial was followed by a 2014 large scale, multi-site RCT called ReCharge (60) which addressed limitations from the EMPOWER trial and demonstrated significant, long-term weight loss benefits that eventually became FDA-approved in 2015.

The human inflammatory system is a body's natural response to pathogens and trauma. These responses can be triggered by infectious and non-infections conditions (61) and when left unchecked, the inflammatory cascade becomes a systemic response that can become deadly. In 2000 a study published in Nature (62) described a landmark study in which rats were given a lethal endotoxic event that was intended to develop into septic shock. After administration of the endotoxin, electrical stimulation of the vagus nerve decreased the release of pro-inflammatory cytokines (tumor necrosis factor (TNF), interleukins 1B, 6, and 18). Ultimately this suppression of inflammatory cytokines prevented the rats from entering septic shock from the endotoxin. Since then there have been dozens of trials exploring the anti-inflammatory effect of VNS, most notably attenuating heart failure progression in canines (63).

Tinnitus is an auditory phantom perceptual disorder in which individuals hear a sound in the absence of an external stimulus. The leading cause of tinnitus is acoustic trauma (64) in which hair cells are damaged and no longer provide cochlear input of specific environmental auditory frequencies, causing a spontaneous overexpression of that frequency in the auditory cortex that presents as internally generated sound. To date there are no effective treatments for tinnitus; often patients are sent home with this debilitating condition and told to manage it on their own.

Michael Kilgard's group at the University of Texas, Dallas have demonstrated that pairing cervically implanted VNS in a tinnitus rat model paired with auditory stimulus is able to reorganize the auditory cortex and reverse the pathological changes that induce tinnitus(65-68). When VNS was delivered to the rats in a tinnitus model, there was no decrease in tinnitus symptoms, neither were any symptom reductions seen with the tone therapy alone. This suggests there is a synergistic effect of VNS combine with a paired stimulus that is directing plastic changes to occur in the cortex. Kilgard calls this concept "targeted plasticity"(66) in which various cortical targets can be selectively changed dependent on the paired stimulus. This group is exploring VNS induced targeted plasticity as a treatment for other neurological disorders in animal models involving cortical reorganization, including stroke (69, 70) and have successfully moved into human clinical trials for both these promising treatments (71-73).

Lastly, VNS has been shown to rescue the brain if stimulation begins immediately after trauma. In 2009 a study by Ay et al (74) demonstrated a rescuing effect of VNS in a rat model of ischemic stroke. Rats that received vagal stimulation immediately after the focal cerebral ischemia had not only significantly better neurological scores compared to the control (non-VNS rats), but these rats also had infarcts that were nearly 50% smaller in area than the control rats. This was replicated in 2011 by the same group (75) and is very promising as a future potential immediate therapy for ischemic stroke. VNS seems to be neuroprotective and keeps ischemia from spreading.

Noninvasive Vagus Nerve Stimulation (nVNS)

Although cervical VNS is relatively safe and effective in seizure prevention (30, 76), the risks involved in surgical implantation as well as its high procedural cost (about $30-50,000) makes it less appealing and accessible as a treatment modality. Additionally, only about 30% of implanted patients have a clinical response, despite undergoing surgery and spending large amounts of money. Having a non-invasive method as an alternative or to determine ultimate responders would greatly improve VNS acceptance as a treatment modality.

Noninvasive VNS (nVNS) can potentially be administered at two locations. The first and most obvious method is via transcutaneous electrical stimulation of the cervical vagus accessed through neck near the carotid artery (carotid sheath). This method, known as transcutaneous cervical VNS (tcVNS) was first described 125 years ago by Corning (26).

It can be delivered experimentally in a research setting by attaching transcutaneous electrical nerve stimulation (TENS) electrodes to the neck and stimulating the underlying tissue and nerve with either alternating or direct current high-frequency electrical stimulation. A commercially available device, marketed as gammaCore, safely delivers tcVNS (25Hz, constant current <60mA) to the cervical vagus nerve. This device has been explored to treat various neurological disorders, including headache, migraine (77-80). The optimal parameters and duty cycle is still unknown and needs to be developed further, although as of early 2017 the gammaCore device gained US FDA-approval.

nVNS may also be administered through the auricular branch of the vagus nerve (ABVN) that innervates the ear, more specifically the conchae and the external auditory canal (81). This noninvasive method is called transcutaneous auricular vagus nerve stimulation (taVNS) was first developed in 2000 (82). Since then, there have been several groups that have conducted studies on this novel form of neuromodulation (83-88) which uses low electrical current stimulation (<10mA) to stimulate the auricular vagus nerve. Many laboratories conduct this novel method experimentally by building their own, miniature electrodes that target this nerve and there is also a commercially available device (European only, not for purchase in the US) called Nemos® claiming to treat epilepsy using their proprietary device.

Whether nVNS enters the brain via the brainstem and targets vagal afferent brain regions has been explored in a handful of studies combining nVNS with fMRI. There have only

been five taVNS/fMRI trials in which stimulation was conducted concurrently with imaging (89-93) and one tcVNS/fMRI trial (94). These studies are summarized in detail in Chapter 4 of this dissertation. In general, these studies demonstrate similar findings to the cervically implanted VNS/fMRI, with relevant BOLD signal activations and deactivation in the afferent vagal brain regions (brainstem, thalamus, insula, amygdala). Brainstem activation is inconsistently viewed in these functional imaging trials, as the sample sizes are often small and breathing artifact often washes out this small region of interest.

There are two major problems with nVNS: 1) the parameter space is unknown, and 2) surrogate markers of vagal activation are difficult to determine. With cervically implanted VNS, the vagus nerve is directly stimulated, whereas nVNS jumps through several hoops to get to this point. Firstly, the electricity is delivered through the skin targeting underlying nerves that are not visible. This requires more electricity to be delivered in the case of tcVNS, potentially recruiting surrounding nerves (glossopharyngeal nerve, laryngeal nerve) in the area and losing its focal effect, or in the case of taVNS, recruitment of off target nerves in the area (auriculotemporal nerve, lesser occipital nerve). taVNS still does not have a consensus target location to stimulate and there is a debate that has arisen as to whether one targets the tragus or the conchae of the ear. Aside from off-target questions, current stimulation parameters arise. Stimulation current intensity (mA), frequency (Hz), and pulse width (μs) all vary throughout the various early nVNS trials in the literature.

Before the field begins large-scale use of nVNS, optimal stimulation targets and parameters must be determined. It is common to rush to clinical trials with novel methodology, but often these trials risk failure due to the lack of pre-clinical optimization. This lack of optimization is the impetus for this dissertation body of work which is consists of the systematic, parametric testing and optimization of taVNS which is integral to guiding future trials. This dissertation describes three sequential studies conducted at the Medical University of South Carolina. The first two experiments determined feasibility, safety, and optimal taVNS parameters that modulate the parasympathetic nervous system in healthy adults. We suspected that heart rate can be used as a surrogate marker of vagal activation. The third experiment is a neuroimaging trial which taVNS was administered in the fMRI scanner to explore the direct brain effects. The combination of these three trials were planned to give this new field a benchmark as to parametric-specific afferent and efferent effects of taVNS.

CHAPTER 2

STUDY 1: A CONTROLLED TRIAL EXPLORING THE SAFETY, FEASIBILITY and HEART RATE EFFECTS of taVNS

Study Summary

Background: Transcutaneous auricular vagus nerve stimulation (taVNS) is hypothesized to stimulate the vagal system via the auricular branch of the vagus nerve (ABVN). The optimal parameters of stimulation are still unknown and given the parasympathetic role of the vagus nerve, it is important to establish a safety profile of this novel form of stimulation, as well as the effect of various parameters on heart rate (HR).

Objective: The objective of this study is to investigate the safety profile and HR effects of 1-minute sessions of nine various taVNS parameters (pulse width: 100μs, 200μs, 500μs; frequency: 1Hz, 10Hz, 25Hz).

Methods: We enrolled 15 healthy individuals in this 2-visit, controlled, crossover trial. Each experiential visit was identical, in which participants received either active (tragus) or control (earlobe) stimulation. 9 stimulation parameters were administered, each for 1 minute flanked by a baseline and recovery period. Participants were monitored for adverse events while their HR was recorded the entirety of their visit. Statistical analysis

was conducted on overall effect of condition (all 9 parameters combined; active vs control) for the entire time course (120s) as well as a focused analysis on the independent stimulation period (60s) and recovery period (60s). Multivariate analysis exploring the individual parameter effects (active vs control) was also conducted.

Results: An overall effect of condition was revealed comparing active taVNS to control for all parameters. No overall effect of condition was found on HR during the stimulation period although active taVNS was found to significantly suppress the sympathetic HR rebound in the post-stimulation period ($p<0.001$) compared to control. Upon multivariate analysis, several parameters of higher pulse width and frequency (500μs 10Hz and 500μs 25Hz) significantly induced bradycardia during stimulation and attenuated the sympathetic recovery spike (100μs, 10Hz and 500μs 10Hz).

Conclusion: taVNS is feasible and safe for 1-minute stimulation periods in healthy adults with no adverse events observed. Two specific parameters (500μs 10Hz and 500μs 25Hz) are revealed to be further studied as likely optimal parameters at modulating parasympathetic response via vagal activation.

Introduction

Autonomic nervous system

The vagus nerve, as described in Chapter 1, is a large bundle of nerves that spans the entire length of the body and targets every major bodily organ. The autonomic effect it

has on these organs is primarily parasympathetic, with acetylcholine (ACh) being the key neurotransmitter released on these organs and responsible for this effect (16).

The autonomic nervous system regulates vital organ behavior and is unconsciously activated in response to certain sensory triggers, most notably affecting heart rate (HR), respiration, and vasoconstriction/dilation (10). There are two independent and opposite autonomic systems (95). The first, known as the sympathetic nervous system (SNS), is generally accepted to be excitatory and accelerates HR, respiration, and vasoconstriction of vessels. This is known as the "fight or flight" response which allows for heightened arousal. The splanchnic nerve carries these excitatory signals to the viscera and release norepinephrine onto its target organs. The second, independent system is the parasympathetic nervous system (PNS), which slows HR and respiration. In general, it elicits a slowing and relaxation of organs that are targeted. All parasympathetic nervous system signals are sent via the vagus nerve, which releases Ach onto the organs to induce the inhibitory effect. Both the SNS and PNS are tonically and reciprocally active to maintain body homeostasis. Both the SNS and PNS activity arise from signals sent from the hypothalamus, known as the central hub of the autonomic nervous system.

The Effect of Cervically Implanted VNS on HR

Otto Loewi demonstrated the slowing effects of the heart via the release of a ACh onto the heart in the late 1900's(16). Since then, as the autonomic nervous system was studied, hundreds of studies have demonstrated the slowing of HR as a major response of

activating the parasympathetic nervous system. This is easily demonstrated by the carotid massage (96, 97), a procedure in which gently rubbing on the vagus nerve via the carotid sinus stimulates the vagus and induces a parasympathetic slowing of the heart.

A major concern during the inception and development of vagus nerve stimulation (VNS) is that direct electrical stimulation of the vagus nerve will elicit a powerful parasympathetic response. During clinical trials, this was heavily monitored for and tracked throughout implantation and treatment course (30). Anecdotally, there are theories suggesting implanting a VNS electrode on the right cervical vagus could induce more cardiac side effects than the left, although this was investigated in a VNS trial exploring both left and right VNS for chronic heart failure which demonstrated equal safety profiles (98).

There also have been several trials suggesting VNS has no effect on HR. Early VNS trials by Holder (99) and Uthman (28) determined no change in HR in VNS implanted humans. Ramsay (100) retrospectively determined there was no HR effect in acute monitoring of epilepsy patients implanted with VNS. Setty A.B. and colleagues conducted a prospective trial explored the effect of VNS on ten individuals implanted with VNS for epilepsy and showed no change in cardiac rhythm during the 30s stimulation period (101). Contrary to these three early studies, a recent study in 2001 by Frei and Osorio showed a decrease in HR (bradycardia) immediately upon starting stimulation, followed by increased HR (tachycardia)(102). Although this was the first description of immediate HR effects of

VNS, their findings were highly variable between patients (no one consistent physiological signature), although a consistent pattern within patients.

Rationale Behind Study 1

Stimulating the vagus nerve in humans elicits a decrease in HR. Since the therapeutic VNS duty cycle is 30s and intensity relatively low, it is supposed that may explain the lack of major HR adverse effects. To date, there have been no prospective studies conducted directly exploring the effects electrical stimulation of the ABVN via taVNS delivered to the ear on HR.

If stimulating the ABVN enters the vagus pathway, safety should be highly considered as the parasympathetic effects of the vagus nerve may cause inadvertent adverse events. To demonstrate the feasibility and safety profile of this novel form of stimulation that is suspected to stimulate the vagus system, it is important to conduct a systematic, parametric study exploring the HR effects of various taVNS parameters. Additionally, given the large parameter space of taVNS, this trial was conducted to determine whether some parameters modulate the vagal system and HR more effectively than others, using HR modulation as a potential surrogate measure of optimal parameters. This is not intended to be an exhaustive exploration of parameters but rather a reasonable combination of high and low settings loosely based on prior cervically implanted trials (24, 27). We hypothesize parameters of higher energy density (larger pulse width, faster frequency) would be more effective at modulating HR in a similar manner as prior

cervically implanted VNS trials (32) and these parameters could pose the highest safety risk.

Methods

Overview

We conducted a 2-visit, controlled, crossover trial in healthy individuals exploring the HR effects of taVNS. Individuals came to the Medical University of South Carolina (MUSC) brain stimulation laboratory for two separate 1-hour experimental visits (active/control visits, counterbalanced design). Each visit was identical except for stimulation condition (**Figure 2-1a**). The trial was approved by MUSC Institutional Review Board (IRB) and is registered on ClinicalTrials.org (NCT02835885).

Participants and Inclusion Criteria

15 healthy adults (7 female) were enrolled after meeting the following inclusion criteria: Age 18-45, no personal or family history of seizure, mood, or cardiovascular disorders, no facial or ear pain, no recent ear trauma, no metal implants including pacemakers, not pregnant, no dependence on alcohol or recent illicit drug use, not on any pharmacological agents known to increase seizure risk (Bupropion, neuroleptics, albuterol, theophylline, antidepressants, thyroid medications, or stimulants).

taVNS Stimulation System and Parameters

A custom developed stimulation system was developed at the MUSC Brain Stimulation Laboratory and used in this trial. It consists of a commercially available, FDA-cleared Digitimer DS7A constant current stimulator (Digitimer Ltd., USA) used with custom-built electrodes (built by BWB and AWB) (**Figure 2-2a**). Electrodes had a stimulation surface diameter of 1cm and Ten20 conductive paste (Weaver and Company, USA) was used to deliver stimulation to ear targets. Participants lay supine with their neck and head propped up in a comfortable position with a pillow. They were instructed to stay awake and maintain a still, comfortable position. Stimulation targets were prepped with alcohol swabs (70% isopropyl alcohol) to clean surface oils and decrease skin resistance.

Stimulation parameters varied by pulse width (100μs, 200μs, 500μs) and frequency (1Hz, 10Hz, 25Hz) creating 9 different combinations of stimulation parameters. These parameters were chosen to cover a wide range (low to high) of pulse width and frequencies. The current (mA) of electrical stimulation was delivered at 200% of each participant's individual perceptual threshold (PT). A PT was conducted for each of the three pulse widths investigated in this trial (100μs, 200μs, 500μs) in which the lowest electrical current perceived was recorded and repeated for each stimulation condition (tragus and earlobe). This is due to the large impact pulse width has on PT as well as

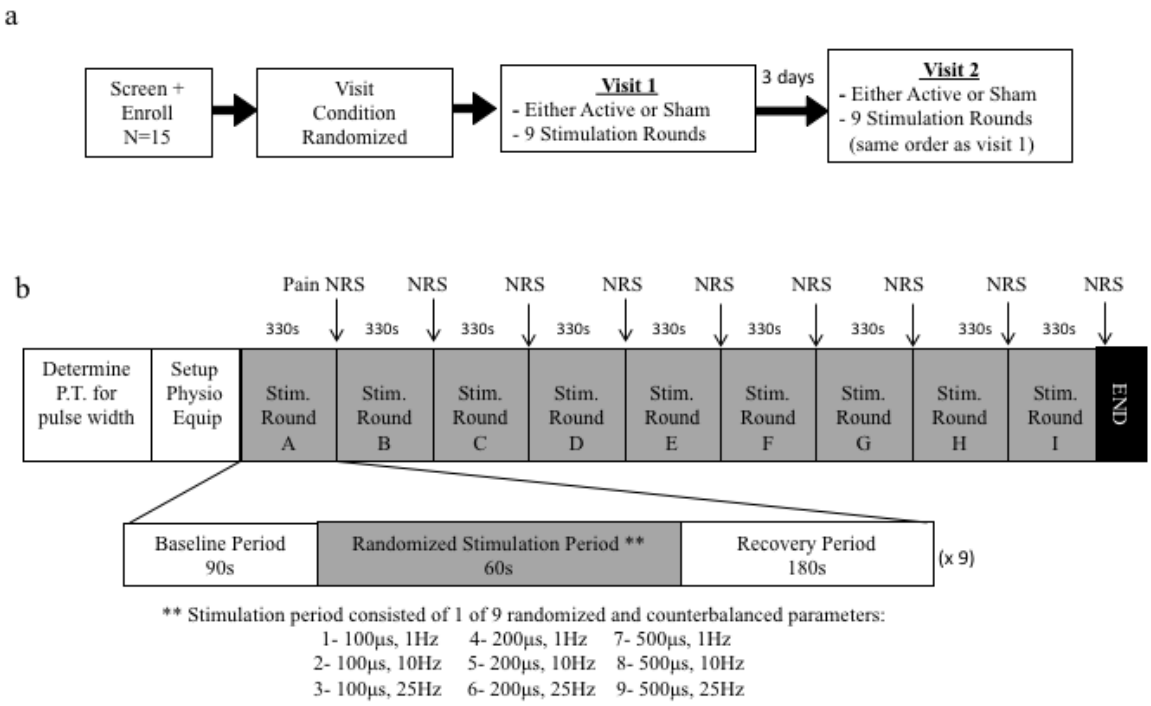


Figure 2-1 Overview of Study a) timeline demonstrating flow of participants through trial. **b)** Experimental visit timeline. Each participant attended two identical visits structured as presented in the figure.

sensitivity variance between target sites. Each stimulation period lasted 1minute, flanked by a 90s baseline and 180s recovery period. This is repeated 9 times, once for each stimulation parameter in a randomized order between subjects (orders were kept identical within both subject visits). See **Figure 2-1b** for an overview of experimental design.

Ear Stimulation Targets

This controlled study employed two different stimulation conditions (**See Figure 2-2b**). The active condition was direct electrical stimulation delivered to the inner side of the left tragus (anode on electrode in the outer ear canal, cathode on the surface of the tragus). Currently there is a debate as to optimal active stimulation position. Some groups have chosen to stimulate the conchae/pinna of the ear, whereas our group decided to stimulate the inner part of the tragus. The tragus location was chosen based on the review of several prior studies exploring the tragus nerve anatomy (81, 103), tragus-evoked potentials (104-106), auricular acupuncture trials (107, 108), and an early taVNS/fMRI trial (91). More generally, the hypothesis is derived from the idea that the tragus stimulation point is closest to the root of the ABVN and stimulation would be most efficient delivered there.

The control condition used was the left earlobe, thought to have little auricular vagus nerve innervation (81). Aside from the placement, the control stimulation condition received identical stimulation as the active condition. This condition was included order to explore the hypothesized non-vagal effects of ear targets. The proximity of the earlobe

region serves as a very stringent control and used to model the physiological response of the other nerves in the ear. Subjects were not informed which condition they were getting or which position was thought to have greater vagal effects.

Safety and Tolerability Reporting

Participants were constantly monitored for major and minor adverse events during each stimulation session regardless of condition. Major adverse events were categorized as: extreme decreases in HR (HR) to levels less than 35BPM, respiration difficulty, and cardiac arrest. Minor adverse events were categorized as: skin discomfort, irritation, headache, facial pain, and dizziness. Procedurally, stimulation was to be aborted if the observing personnel noticed any adverse events (this did not occur).

The participant reported pain ratings of each stimulation parameter after each stimulation block using a numerical rating scale (NRS) ranging from 0-10 after each of the nine stimulation parameters. “0” was used as the lowest rating for no sensation perceived, “10” was the highest pain rating for extreme intolerable pain. Participants could use 0.5 increments with a rating of 1 representing the lowest rating where stimulation is felt with no pain.

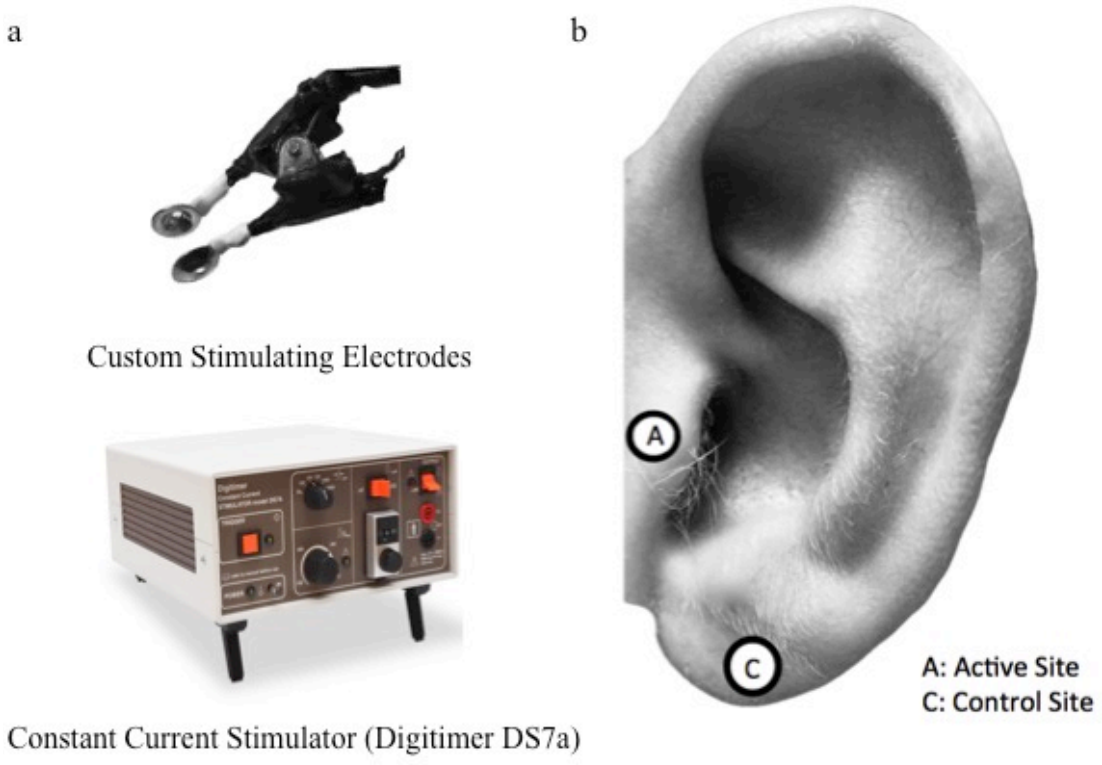


Figure 2-2 Stimulation system and ear targets. a) taVNS was delivered using a FDA-cleared constant current stimulator and custom stimulation electrodes. **b)** Schematic of left ear demonstrating targets. Active stimulation was delivered to the tragus, control to the earlobe.

HR Data Acquisition, Preprocessing, Statistics

Physiological measures were recorded using a 3-channel Thought Technology system (Thought Technology Ltd), which measured HR using a blood volume pulse (BVP) sensor worn on the right index finger. BioGraph Infinity Software was used for both online safety monitoring as well as offline analysis. All HR data was down-sampled to 8Hz and exported to be analyzed in IBM SPSS Statistics version 23 (IBM Corp, USA).

HR was analyzed in 5-second bins. There were 12, 5s bins for stimulation period (totaling 60s); 12, 5s bins for recovery (totaling 60s) and the baseline used was the final 5-seconds before stimulation started. Change scores for the stimulation period were calculated as the difference in HR in beats per minute (BPM) during stimulation bin and baseline. Change scores for the recovery period were calculated from the final stimulation 5-second bin (bin 12). All subjects HR was blindly scanned for artifact and all subject data was included in the analysis (no data removed).

A repeated measures ANOVA was conducted to determine the overall effect of stimulation condition (active vs. control) on change in HR over time throughout the entire time course (120s), as well as focused analysis on the stimulation period (60s) and recovery period (60s) independently. Secondly, individual parametric effects of HR by a multivariate analysis was conducted. Similar analyses were conducted for the stimulation and recovery period, and determined partial eta-squared was used to determine effect sizes.

Results

Participants, Perceptual Thresholds (PT), and Stimulation Current

15 healthy, right-handed individuals (7 female, 8 male, mean age 26.5 SD 4.99) were included in this study. All participants completed both visits without any dropouts. Perceptual thresholds (PT) varied by stimulation site and pulse width. Mean PTs (n=15) were as follow (mean ± SD mA): 100μs (tragus- 4.64 ± 1.28; earlobe 3.29 ± 0.92) 200μs (tragus- 2.66 ± 0.80; earlobe 1.82 ± 0.63) 500μs (tragus- 1.5 ± 0.46; earlobe 0.98 ± 0.35). Using a paired 2-tailed t-test, it was determined that tragus perceptual thresholds were higher than earlobe perceptual thresholds for each pulse width ($p<0.01$).

The current at which taVNS was delivered was a scale multiplier of the PT (200%). Mean stimulation currents were as follow (mean ± SD mA): 100**μs** (tragus- 9.28 ± 2.56; earlobe 6.57 ± 1.83) 200**μs (**tragus- 5.32 ± 1.60; earlobe 3.64 ± 1.26**)** 500**μs** (tragus- 3.0 ± 0.93; earlobe 1.97 ± 0.70). Using a paired 2-tailed t-test, it was also determined that tragus stimulation currents were higher than earlobe perceptual thresholds for each pulse width ($p<0.01$). **Table 2-1** outlines PTs and stimulation data.

Adverse Events and Pain Ratings

There were no minor or major adverse events during the experimental sessions or spontaneously reported following exit of the trial. No rapidly accelerated or sustained

drops in HR were seen during the 1-minute stimulation periods. Minor, temporary light redness was seen at the sight of stimulation that disappeared within 5 minutes of stimulation completion.

Parametric NRS scores for pain are described in **table 2-2.** The lowest rating a participant could make when they felt stimulation was a 1. For the nine various parameters, as the pulse width and frequency increased, as did the NRS, although the highest mean tragus NRS pain rating was 2.133, SD 1.34 (500μs, 25Hz) and the highest mean earlobe NRS pain rating was 1.23, SD .42 (100μs, 10Hz). Although some of these NRS scale ratings show statistical significance between stimulation conditions, the behavioral differences of such low pain ratings are not accurately reflected.

Table 2-1: Perceptual Threshold and Stimulation Currents for Each Pulse Width Setting.

	Perceptual Threshold ± SD (mA)		**Stim. Current ± SD (mA)**		
Pulse Width	Tragus (Active)	Earlobe (Control)	Tragus (Active)	Earlobe (Control)	**Significant (p value)**
100μs	4.64 ± 1.28	3.28 ± 0.91	9.28 ± 2.56	6.57 ± 1.83	Y (p=0.002)
200μs	2.66 ± 0.80	1.82 ± 0.63	5.32 ± 1.60	3.64 ± 1.26	Y (p=0.003)
500μs	1.5 ± 0.47	0.99 ± 0.35	3 ± 0.93	1.97 ± 0.71	Y (p=0.002)

Table 2-2: Mean NRS Pain Ratings Reported for Each Parameter Tested.

Parameter		Mean NRS Pain Rating ± SD		Significant? (p value)
		Tragus (Active)	Earlobe (Control)	
100μs	1 Hz	1.27 ± 0.59	1 ± 0	N
	10 Hz	1.67 ± 0.82	1.23 ± 0.42	N
	25 Hz	1.57 ± 0.82	1.13 ± 0.35	N
200μs	1 Hz	1.27 ± 0.46	1.0 ± 0.0	N
	10 Hz	1.43 ± 0.82	1.03 ± 0.13	N
	25 Hz	2.1 ± 1.36	1.33 ± 1.05	N
500μs	1 Hz	1.2 ± 0.56	1.07 ± 0.26	N
	10 Hz	1.77 ± 0.86	1 ± 0	Y (0.004)
	25 Hz	2.13 ± 1.34	1.17 ± 0.36	Y (0.006)

Overall Effect of Stimulation Condition on HR (HR)

The overall pattern of effect on HR (HR) over time is illustrated in **Figure 2-3**. taVNS has very recognizable physiologic signature – when stimulation begins (stimulation period), HR decreases immediately and is sustained at this lower level. Upon termination of stimulation (recovery period), there is an immediate reorientation spike in HR that elevates past baseline for nearly 30 seconds which then regresses back to the mean resting HR. The nine different stimulation parameters each have a varied effect on HR, with some inducing large decreases while other parameters are less effective.

To determine the overall effect of taVNS on HR, all active and all control changes in HR during stimulation were grouped, in 5s bins for a total of 12 consecutive bins. As demonstrated in **figure 2-4**, when all parameters are grouped together, both active (tragus) and control (earlobe) stimulation have a bradycardia effect, with an active mean HR decrease from baseline of 1.43 beats per minute (BPM), SEM 0.20, and control mean HR decrease of 1.02, SEM 0.20. In a repeated measures ANOVA statistical comparison, this effect was not significant.

A repeated measures ANOVA was conducted on the one-minute post-stimulation period change in HR (from the final 5 second bin of stimulation). The sympathetic rebound that occurs upon termination of taVNS was blunted by active stimulation compared to control demonstrating a condition effect of rebound spike ($p<0.001$)**.**

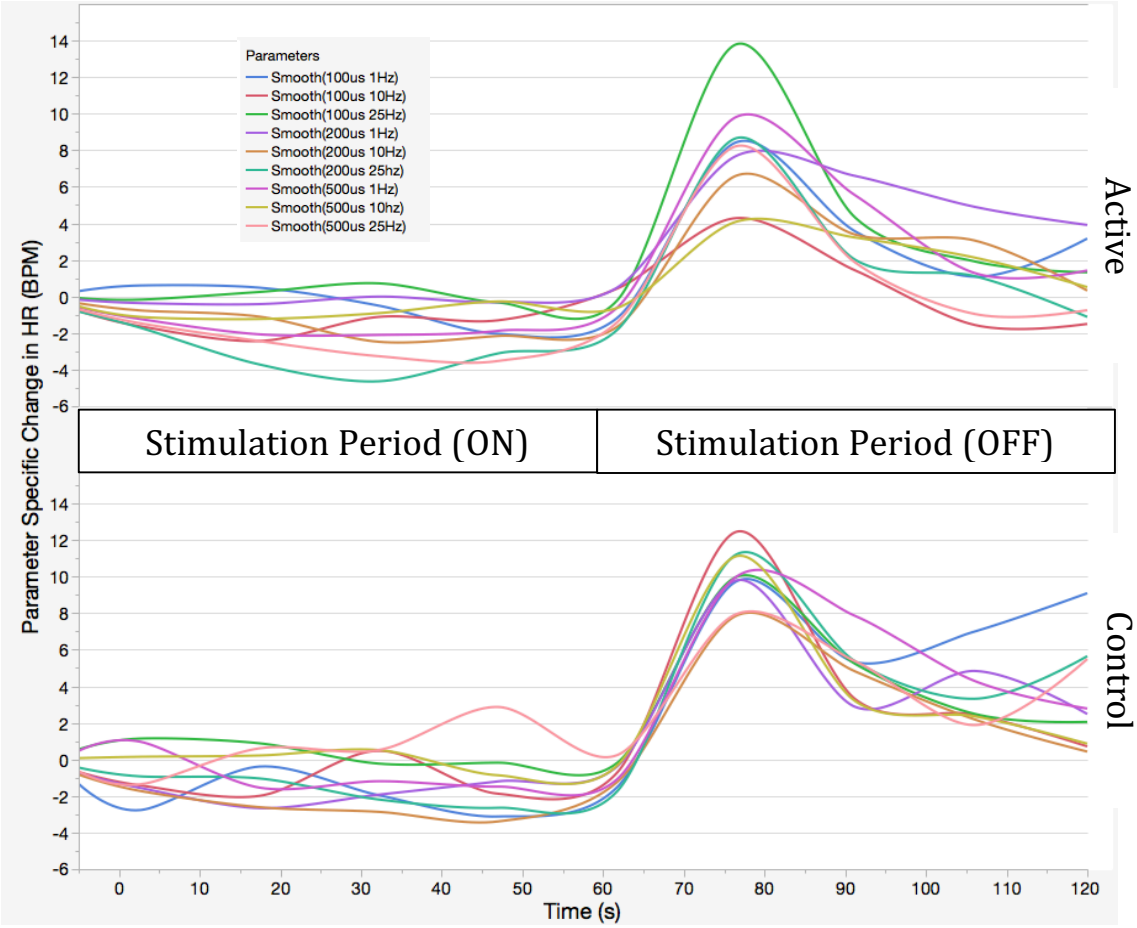


Figure 2-3 Overview of mean HR changes over time for all 9 parameters. This figure presents the effect of both active and control stimulation on heart rate. Stimulation seems to have an immediate bradycardia effect during stimulation, followed by a tachycardia rebound when stimulation is turned off.

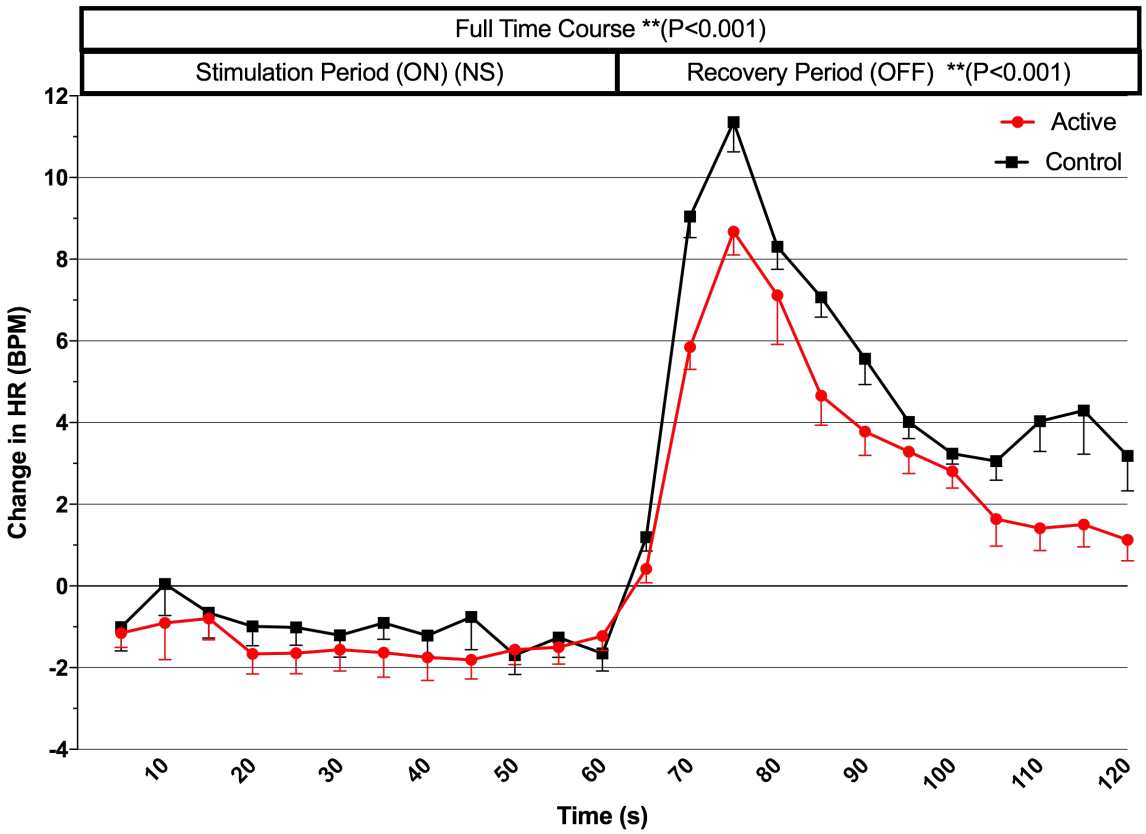


Figure 2-4 Mean change in HR over time (active v control). This figure presents the mean change of all 9 parameters tested in this trial, revealing a decrease in HR during stimulation and a recovery heart rate spike upon termination. Overall time course analysis reveals an overall effect of condition (P<0.001). There was non-significant effects of condition on the stimulation-induced bradycardia. Active stimulation had a significantly lower recovery heart rate spike (P<0.001).

The peak HR rebound was achieved during the third 5-second bin (15 seconds post-taVNS) with a max rebound in HR for active taVNS of 8.153BPM and control stimulation of 11.361 BPM. The sympathetic spike time-course analysis revealed a significant condition*time interaction ($p<0.001$), as active stimulation returns to baseline much quicker than control stimulation.

Multivariate Analysis of HR to Determine Parametric Effects

The grouped data can subsequently be split based on specific parameters in a multivariate analysis. Some parameters were hypothesized to have a biologic effect whereas others were not. **Table 2-3** shows the mean effects on HR during the stimulation period, separated by individual parameters. Several parameters were determined to have an effect of condition on the decrease of HR in the stimulation period. There were two parameters that had large, significant effects by condition in which active stimulation decreased HR more than control stimulation, and in which there was no decrease in HR in the control condition. These were 500μs, 25Hz (active HR -3.13BPM, control 0.799, $p<0.001$) and 500μs, 10Hz (Active HR -.929 BPM, control .290, $p=0.01$). The HR trace during stimulation for these two parameters is presented in **Figure 2-5**.

A multivariate analysis was also performed on the sympathetic reorientation spike in HR. Several parameters were demonstrated to suppress this sympathetic spike during the recovery period. These data are demonstrated in **Table 2-4.** The optimal parameter for

Table 2-3: Multivariate Analysis of Mean Change in HR from Baseline

Parameter		Mean Change in HR from Baseline ± SEM (Average over 60s Stimulation Period)			Condition Effect? (p value)
		Tragus (Active)	Earlobe (Control)	Partial Eta Sq.	
100μs	1 Hz	-0.744 ± .781	-2.40 ± .781	0.007	N
	10 Hz	-1.17 ± .0.461	-0.891 ± 0.461	0.001	N
	25 Hz	0.24 ± 0.362	0.524 ± 0.362	0.001	N
200μs	1 Hz	0.07 ± 0.631	-2.01 ± 0.631	0.016	Y (0.008)
	10 Hz	-1.54 ± 0.353	-2.86 ± 0.353	0.021	Y (0.005)
	25 Hz	-3.57 ± 0.436	-1.81 ± 0.436	0.024	Y (0.006)
500μs	1 Hz	-2.17 ± 0.337	-0.857 ± 0.337	0.022	Y (0.006)
	10 Hz	-0.93 ± 0.335	0.290 ± 0.335	0.019	Y (0.01)
	25 Hz	-3.13 ± 0.545	0.799 ± 0.545	0.072	Y (<0.001)

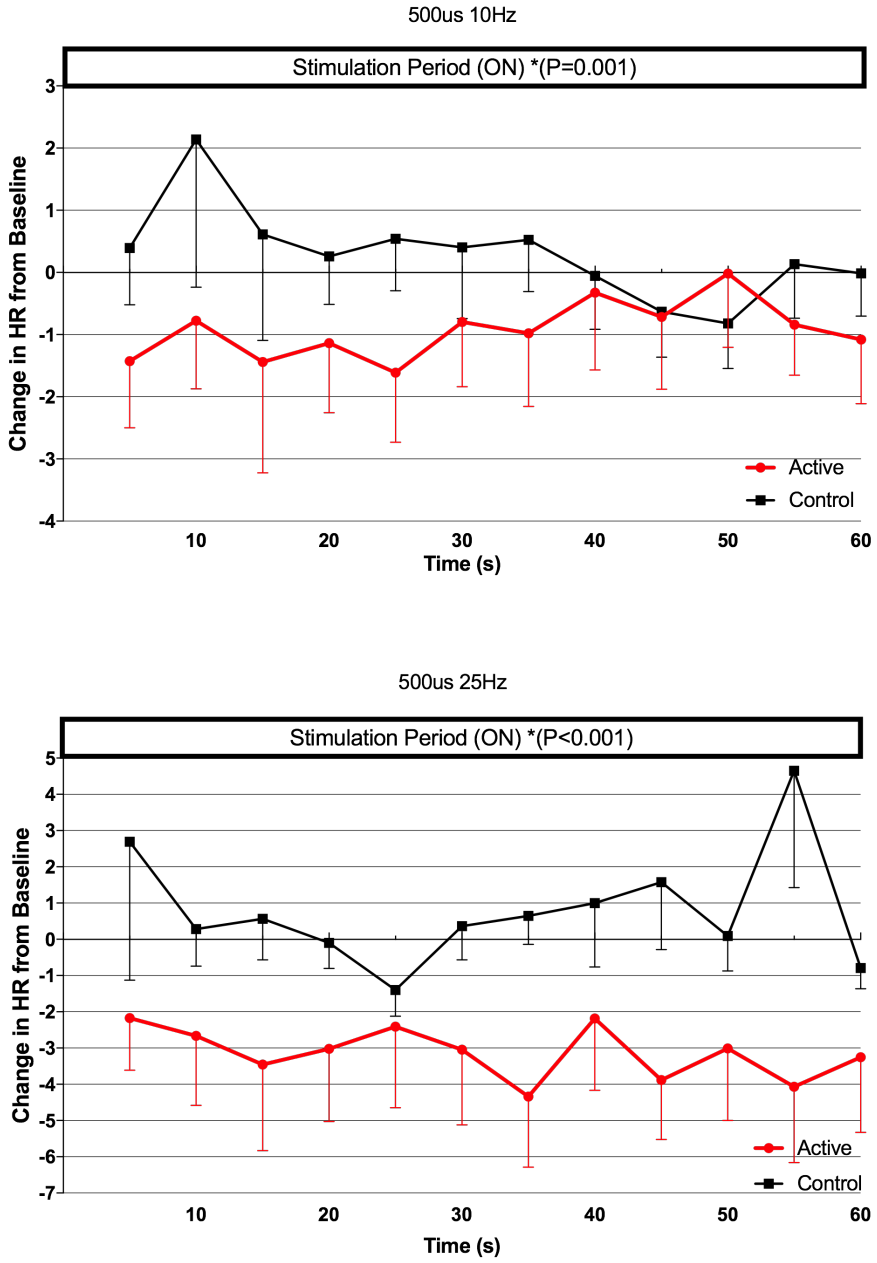


Figure 2-5 Parameters with largest bradycardia effect during stimulation. Active taVNS had significant parasympathetic activation compared to control in the following parameters (500us, 10Hz; 500us, 25Hz).

Table 2-4: Multivariate Analysis on Mean Change in HR for Recovery Period

Parameter		Mean Change in Recovery HR ± SD (Average over 60s Recovery Period)		Partial Eta Sq.	Condition Effect? (p value)
		Tragus (Active)	Earlobe (Control)		
100µs	1 Hz	3.859 ± 0.998	7.296 ± 0.998	0.017	Y (0.015)
	10 Hz	1.244 ± 0.547	4.077 ± 0.547	0.038	Y (<0.001)
	25 Hz	4.348 ± 0.581	5.2 ± 0.581	0.003	N
200µs	1 Hz	5.436 ± 0.672	5.76 ± 0.672	0.000	**N**
	10 Hz	3.591 ± 0.476	4.59 ± 0.476	0.007	N
	25 Hz	2.995 ± 0.600	5.792 ± 0.600	0.031	Y (0.001)
500µs	1 Hz	4.512 ± 00.647	5.755 ± 00.647	0.005	N
	10 Hz	2.703 ± 0.539	4.926 ± 0.539	0.025	Y (0.004)
	25 Hz	2.644 ± 0.601	5.474 ± 0.601	0.032	Y (0.006)

suppression of the parasympathetic rebound based on magnitude difference of peak spike suppression was 100μs, 10Hz and 500μs 10Hz.

Discussion

Our analysis reveal that one-minute stimulation sessions of the left tragus is a safe form of neuromodulation that has no major or significant acute bradycardia effect during the stimulation period compared to control, although active taVNS suppresses the post-stimulation tachycardia rebound associated with sympathetic recovery. When multivariate analyses were conducted, significant parameter-specific bradycardia effects and tachycardia suppression during and post stimulation were revealed. Conditions with more energy dense parameters had noticeably larger effects. Most notably, the parameters with highest bradycardia effects were those with higher pulse width and frequency (500μs 10Hz and 500μs 25Hz). The largest suppressors of the sympathetic rebound in HR post-stimulation were ones with 10Hz frequency (500μs, 10Hz; 100μs,10Hz)

We enrolled 15 healthy individuals in this parametric feasibility and safety trial exploring the effects of nine various taVNS parameters of different frequencies and pulse widths. To our knowledge, this is the first prospective trial to systematically test the physiological effects of various taVNS parameters. From a feasibility and safety perspective, a taVNS system with custom electrodes was built for conducting laboratory studies. There were no minor or major adverse effects observed throughout the duration of this trial, suggesting 1 minute taVNS periods at 200% perceptual threshold is safe and tolerable. This is very

similar to the safety of cervically implanted VNS (27, 33). We also determined that sensitivity based on perceptual thresholds varied by pulse width and stimulation location. As pulse width increases, less current is needed for the sensation to be perceived. The earlobe is significantly more sensitive (needs more current) than the tragus. The mean overall pain scores for active taVNS were recorded as 1.6 versus control stimulation of 1.1. These are considered perceivable but not painful, making taVNS rather painless.

Like all neuromodulation methods, the parameter space is vast and systematic parametric optimization trials are needed to determine optimal stimulation parameters. This trial suggests that individual parameters may be better than others at modulating vagal tone as measured by HR. Although there was no condition effect when all parameters were combined, there were clearly parameters that induced significant bradycardia associated with the parasympathetic nervous system, which is directly modulated by the vagus nerve (10). The more energy dense parameters caused larger decreases in HR compared to the lower frequency or smaller pulse width parameters. It is early to make a conclusion as to whether these effects are directly driven through the vagus nerve, although given the strict control region, these data are very encouraging.

The sympathetic rebound attenuation was an unexpected finding. It has been demonstrated in the prior studies that this sympathetic spike in HR occurs after stimuli(109, 110) and is thought to be a reorienting phenomenon. It is seen pharmacologically as well, demonstrated in a reciprocal effect of noradrenergic blockade

via beta-blockers (111). The reciprocal mechanisms are intended to maintain bodily homeostasis and rapid activation/deactivation of stimulation of either system has a strong reciprocal action that occurs afterwards. This effect was measured temporally, lasting approximately 15 seconds to peak sympathetic rebound and final recovery to baseline at 1minute. One prior taVNS trial suggests sympathetic nervous activity is reduced upon stimulation (86). It was not anticipated that active taVNS may be able to significantly attenuate this sympathetic rebound and it may be due to a sustained parasympathetic effect that persists beyond stimulation.

There is a dose confound revealed in these findings, as the PT for active and control sites were significantly different for each of the three pulse width settings. This occurred as a product of trying to control for stimulation pain levels by conducting a titration based on sensory perception. If we had controlled for stimulation current (i.e. given everyone identical stimulation parameters) we would have likely seen a confound of painfulness. Whether the current strength that is driving the condition effect rather than the pulse width or frequency is still unknown, but should be acknowledged as a limitation of this trial.

There have been several studies exploring the behavioral effects of taVNS, many of which are positive (78, 83, 112, 113) and prior literature has suggested that tragus stimulation directly modulates the vagus network via the ABVN. There have also been recent studies demonstrating stimulation of the ABCN has a direct effect on the afferent

projection of the vagus nerve, with similarities in blood oxygen level dependent (BOLD) as compared to cervically implanted VNS (91, 92). These fMRI findings suggest one possible mechanistic hypothesis for the immediate, sustained decrease in HR – stimulation is entering the afferent vagus system towards the central nervous system, activating the parasympathetic efferent cholinergic pathway targeting the viscera, including the heart.

This field is still in its infancy and there is a lack of consensus on parameters. There is a wide range of currents (ranging from sub-perceptual threshold to sub-painful threshold), frequency (ranging from 1Hz to 299Hz), pulse width (ranging from 20us-1ms) stimulation duration (ranging from brief pulses to 1 minute), and type of electrical stimulation (constant current/voltage/direct/alternating) (91, 92, 104-108, 112). It would be impossible to test all various parameters, but many groups have leaned on the parameters used in cervically implanted VNS as a guideline.

This study aimed to guide future taVNS trials in safety and HR effect of these parameters. It is important to determine stimulation current based on individual perceptual threshold, although it is still unknown as to what level (sub- or supra-threshold stimulation) works best. It is plausible to suggest a pulse width closer to chronaxie (approximately 500μs) (35) would optimally cause depolarization of nerves and shrink the wide range of potential parameters. And lastly, it is important to use a

stringent control region on the same ear of stimulation, since there appears to be a large physiological response due to ear lobe stimulation alone.

Limitations

We acknowledge that a sample size of 15 individuals may not be large enough to truly determine which parameters modulate HR the best. This initial trial was developed as a safety and feasibility trial, with the secondary effects of HR to be explored, as the parameter space was quite large. Larger controlled trials should further explore some of the parameters here that are suggested to be more optimal in modulating HR.

Secondly, it is impossible to truly say these outcomes are as a direct effect of stimulating the ABVN as the only way to definitively say that would require dissection and direct nerve stimulation. Based on prior literature and anatomical trials (81), we believe that stimulation of the tragus is directly stimulating the ABVN, whereas the earlobe has little to no ABVN innervation, and therefore less parasympathetic derived effects.

Lastly, we concede it is still unknown whether the effects seen are through the hypothesized afferent central targets of the vagus system (ear –> brain –> vagus -> body), or whether they are modulating parasympathetic response via direct efferent projections from the ear to the periphery (ear -> vagus -> heart). This is difficult to determine without systematic, parametric testing using combined taVNS and neuroimaging paradigms or with in-vivo microelectrode recording of nerve dissections in animal models.

Conclusion

taVNS administered for 1 minute at 200% perceptual threshold in these nine parameters is a feasible and safe neuromodulator technique. No significant adverse events were observed and overall, both active and control stimulation result in a minor bradycardia during stimulation, whereas active stimulation suppressed the sympathetic reciprocal effect post-stimulation. Further parametric exploration must be conducted to determine optimal taVNS parameters.

CHAPTER 3

STUDY 2: DETERMINING THE OPTIMAL taVNS PARAMTER WHICH ACTIVATES THE PARASYMPATHETIC NERVOUS SYSTEM

Study Summary

Background: An initial exploratory study conducted by our team (described in Chapter 2) investigated the effects of nine various taVNS parameters on HR and determined two optimal parameters which most likely activated the parasympathetic nervous system. This study follows-up on this initial trial by testing the winning parameters against each other and against control.

Objective: Determine which of the following parameters (500μs 10Hz or 500μs 25Hz) optimally activate the parasympathetic nervous system compared to control stimulation.

Methods: We enrolled 20 healthy individuals in a 2-visit follow-up trial exploring the HR effects of the two parameters optimally modulating HR determined by trial 1. Individuals attended two separate experimental visits (active, control) and received 10 sessions of 1-minute taVNS, flanked by a 60 second baseline and 90 second recovery period. HR was monitored continually throughout each experimental visit. Statistical

analysis was conducted on overall effect of condition (both parameters combined; active vs control) for the entire time course (120s) as well as a focused analysis on the independent stimulation period (60s) and recovery period (60s). Specific parametric analysis on each discrete parameter was conducted exploring the overall individual parameter effects (active vs control) similar to the method for all paramters.

Results: Active taVNS significantly decreased HR compared to control during stimulation (p=0.02). taVNS did not affect recovery sympathetic spikes as control and active stimulation had similar magnitude reorientation increases in HR. The overall effect was primarily driven by the strong bradycardia effect induced in the 500μs 10Hz parameter (p=0.032) although both active parameters decreased HR compared to control.

Conclusion: This confirmatory follow-up study determined that the optimal parameter to modulate the parasympathetic response activated via direct electrical stimulation of the auricular branch of the vagus nerve (ABVN) was 500μs, 10Hz. Both active parameters induced bradycardia compared to control, suggesting taVNS activates the parasympathetic nervous system.

Introduction

Study Aim 1 Findings

Chapter 2 of this dissertation described an initial parametric study we conducted in 15 healthy individuals. The goal of this first trial was three-fold. Initially, when developing a

novel form of neuromodulation, it is important to establish the safety profile of the method, as well as appease concerns of the MUSC IRB concerning participant safety. In 2014 when these trials were conceptually started, the literature was very sparse and MUSC had not conducted a prior taVNS study. There also was no system to administer it, so Bashar Badran (with the help of Alan Badran) developed stimulating electrodes targeting the requisite ear targets.

Secondly, the trial was aimed at determining whether there were parameter-specific effects of taVNS on HR. More precisely, we aimed to determine whether longer pulse widths and higher frequencies (more energy per pulse) would result in larger decreases in HR via the parasympathetic nervous system. Prior cervically implanted VNS/fMRI trials conducted by Nahas et al (51) demonstrated increased blood oxygen level dependent (BOLD) signal activation in the brain as the pulse width was serially increased from 130us to 500us. Before conducting studies in the MRI scanner with our taVNS system, we aimed to use HR as a surrogate marker of parasympathetic activity and show increased effects with higher pulse widths and frequencies.

Our final goal was to determine which of the nine parameters were optimal in modulating the parasympathetic system. We analyzed this data and compiled the measures to give us an overall perspective on optimal parameters. The effect size of the repeated measures ANOVA as well as whether there was a control effect were both unutilized in making the ultimate decision that 500us 10Hz and 500us 25Hz parameters optimal parameters at

modulating parasympathetic nervous system activity. The findings of the first study determined that taVNS was feasible, safe, and identified optimal parameters of the initial nine exploratory parameters.

Rationale Behind Study 2

This follow-up study aimed to be a confirmatory study on the parameters hypothesized to optimally modulate the parasympathetic nervous system. To do this, the best two parameters from study 1 (500μs 10Hz & 500μs 25Hz) were tested against each other and against control stimulation (earlobe). To positively determine this, the number of participants was increased to 20 for this follow-up trial and also increased the number of times the parameter was tested (five stimulation runs for each parameter, 10 total), unlike trial 1 which had only one stimulation run for each of the 9 parameters. We hypothesized that by making these changes, active taVNS will have a significant effect on HR during stimulation. More specifically active taVNS will induce bradycardia during stimulation as modulated by the parasympathetic nervous system activation that occurs. Given the nerve innervation of the human auricle (81), control stimulation should have minimal effect on activating the parasympathetic nervous system.

Methods

Overview

This follow-up study is resembles and is nearly identical to the design of the first HR study described in Chapter 2. This study conducted was a 2-visit, controlled, crossover

trial. Individuals came to the Medical University of South Carolina (MUSC) brain stimulation laboratory for two separate 45 minute experimental visits (active/control visits, counterbalanced design) (**Figure 3-1a**). Each visit was identical except for stimulation condition. This study was approved by the MUSC Institutional Review Board (IRB) and is registered on ClinicalTrials.org (NCT02835885).

Although similar to the study described in Chapter 2, there are several important major changes that differentiate this study from the prior study. The reader should draw their attention to the following differences:

1. Only 2 parameters are explored in this trial (500us 10Hz, 500us, 25Hz). See introduction for the rationale.
2. 10 stimulation rounds were administered each visit (5 for each parameter). This differs from study 1 in which each parameter was tested only once. This is intended to increase the power for statistical analysis.
3. Biopac System was used for Heart Rate using 2-channel electrocardiogram (ECG) rather than BVP as used in the Thought Technology system from study 1.
4. Methods that are repeated in this section will be indicated to refer reader back to the appropriate section of Chapter 2 to avoid repeating general methodology.

Participants and Inclusion Criteria

We enrolled 20 healthy adults (10 female) in this trial after meeting the same inclusion criteria listed in Chapter 2 (See Chapter 2, Methods for detailed description).

taVNS Stimulation System

We used the same stimulation system developed for the initial HR study described in the previous chapter. (See Chapter 2, Methods for detailed description and figure.)

taVNS Stimulation Paradigm & Parameters

Participants lay supine with their neck and head elevated in a comfortable position with a pillow. They were instructed to stay awake and maintain a still, comfortable position. Stimulation targets were prepped with alcohol swabs (70% isopropyl alcohol) to clean surface oils and decrease skin resistance.

Stimulation parameters of either 500μs 10Hz or 500μs 25Hz were used. Parameters were randomized and counterbalanced over each of the 10 rounds (5 rounds of each parameter). These parameters were chosen as the most likely candidates to best modulate heart rate from trial 1. Similarly to trial 1, the stimulation current (mA) was delivered at 200% of each participant's individual perceptual threshold (PT) of a 500μs pulse width. Each stimulation period lasted 1minute, flanked by a 60 second baseline and 90s recovery period. This is repeated 10 times, once for each stimulation parameter in a randomized

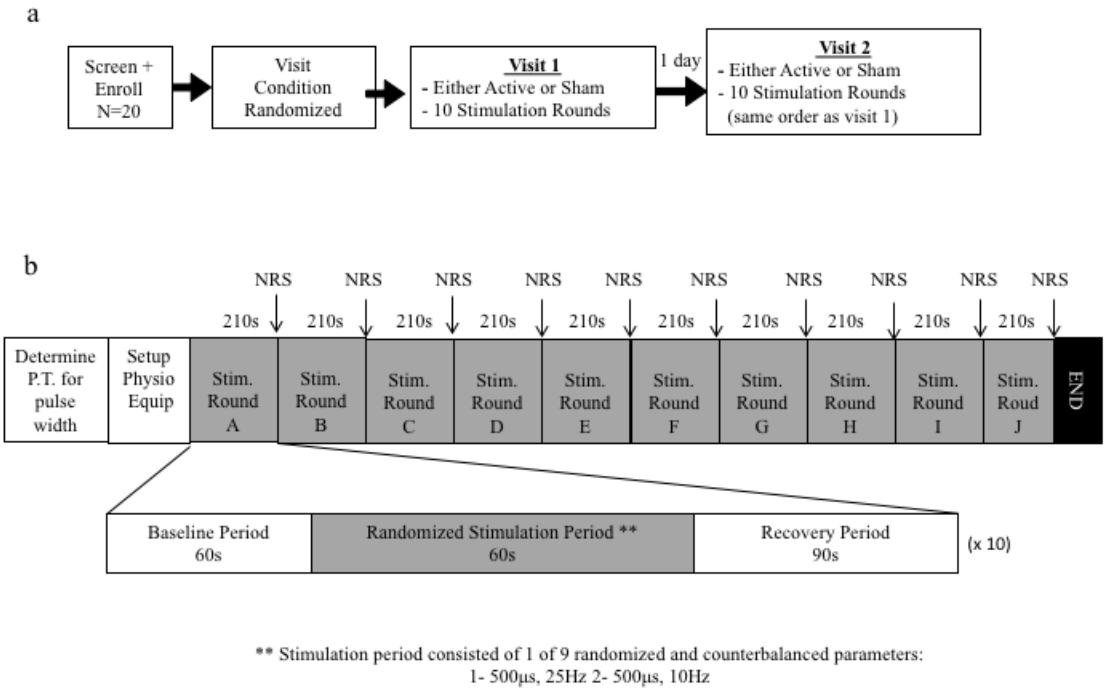


Figure 3-1 Overview of Study a) timeline demonstrating flow of participants through trial. **b)** Experimental visit timeline. Each participant attended two identical visits structured as presented in the figure.

order between subjects (orders were kept identical within both subject visits). See **Figure 3-1b** for an overview of experimental design.

Ear Stimulation Targets

This controlled trial employed identical stimulation sites on the left ear. Active taVNS was delivered to the left tragus, control stimulation to the left earlobe. For a detailed description and figure of these sites, refer to Chapter 2, Methods, Ear Stimulation Targets).

Safety and Tolerability Reporting

Participants were constantly monitored for major and minor adverse events during each stimulation session regardless of condition and were asked to report pain ratings at the completion of each of the ten stimulation periods using a NRS. For a detailed description of safety criteria and NRS refer to Chapter 2, Methods).

HR Data Acquisition, Preprocessing, Statistics

Physiological measures were recorded using a 2-channel Biopac ECG system (Biopac Systems Inc., USA), which measured HR using electrocardiogram electrodes attached to the subject's chest. AcqKnowledge 4.1 software was used for both online safety monitoring as well as offline analysis. All HR was consolidated into 5sec epoch bins and exported for analysis in IBM SPSS Statistics version 23 (IBM Corp, USA).

HR was scanned for usability, and participant 20 of this study reported excessive artifact in stimulation round C of the control visit, which was excluded from analysis. The remaining 9 stimulation rounds for this participant were kept in the analysis.

HR was analyzed in 5-second bins. There were 12 bins for stimulation period (totaling 60s); 12 bins for recovery (totaling 60s). The baseline used was the final 5-seconds before stimulation started. Change scores for the stimulation period were calculated as the difference in HR in beats per minute (BPM) during stimulation bin and baseline. Change scores for the recovery period were calculated from the final stimulation 5-second bin (bin 12).

A repeated measures ANOVA was conducted to determine the overall effect of stimulation condition (active vs. control) on change in HR over time throughout the entire time course (120s), as well as focused analysis on the stimulation period (60s) and recovery period (60s) independently. Secondly, individual parametric effects of HR by a multivariate analysis was conducted. Similar analyses were conducted for the stimulation and recovery period, and determined partial eta-squared was used to determine effect sizes.

Results

Participants, Perceptual Thresholds (PT), and Stimulation Current

20 healthy, right-handed individuals (10 female, 10 male, mean age 25.65 SD 5.53) were included in this study. All participants completed both visits without any dropouts. Mean perceptual thresholds (PT) are as follow (mean ± SD mA): 500μs (tragus- 1.045 ± 0.49; earlobe 1.02 ± 0.41). The current at which taVNS was delivered was again a scale multiplier of the PT (200%). Mean stimulation currents were as follow (mean ± SD mA): 500μs (tragus- 2.09 ± 0.97; earlobe 2.04 ± 0.82). Using a paired 2-tailed t-test, it was determined that there was no difference in perceptual threshold between the two stimulation sites. **Table 1** outlines mean PTs and stimulation data for all subjects.

Adverse Events and Pain Ratings

Similar to experiment one, there were no minor or major adverse events during experiment two. The Mean NRS scores for pain are described in **table 3-1**, and although there was a significant difference in pain between parametric-specific stimulation targets when analyzed using a paired t-test, these are not reflective of the minimal pain reflected in the ratings (mean pain difference between conditions less than one rating point, max mean rating 2.24). These pain ratings should be considered relatively painless for both conditions.

Effect of Stimulation Condition on HR (HR)

Experiment two successfully demonstrated replication of the physiological response to taVNS. As stimulation starts, there is an immediate bradycardia observed that persists throughout the entire stimulation period. Upon termination of stimulation tachycardia occurs for approximately 30s. The overall effect on HR over the 120-second period is not statistically significant. **Figure 3-2** shows the time course analysis of mean changes from baseline in the stimulation and recovery periods of all active vs. all control stimulation rounds.

To determine the effect of taVNS on HR during the stimulation period, the 60s stimulation period was analyzed by condition in a repeated measures ANOVA and demonstrates a strong active taVNS effect on bradycardia ($P<0.02$). Active taVNS produced a mean decrease in HR of 1.82 ± 0.174 (SD), whereas control stimulation only decreased a mean of 1.2BPM $\pm$ 0.178 (SD). A repeated measures ANOVA was also conducted on the recovery period, which did not meet significance.

Table 3-1: Mean PT, Stimulation Current, and Pain NRS ratings

	Tragus (Active)	Earlobe (Control)	**Significant? (p value)**
PT ± SD (mA)	1.045 ± 0.48	1.02 ± 0.41	N
Stim. Current ± SD (mA)	2.09 ± 0.97	2.04 ± 0.82	
Mean 500us 10Hz NRS Pain Rating ± SD	1.35 ± 0.68	2.24 ± 1.28	Y (P<0.01)
Mean 500us 25Hz NRS Pain Rating ± SD	1.32 ± 0.57	2.10 ± 1.17	Y (P<0.01)

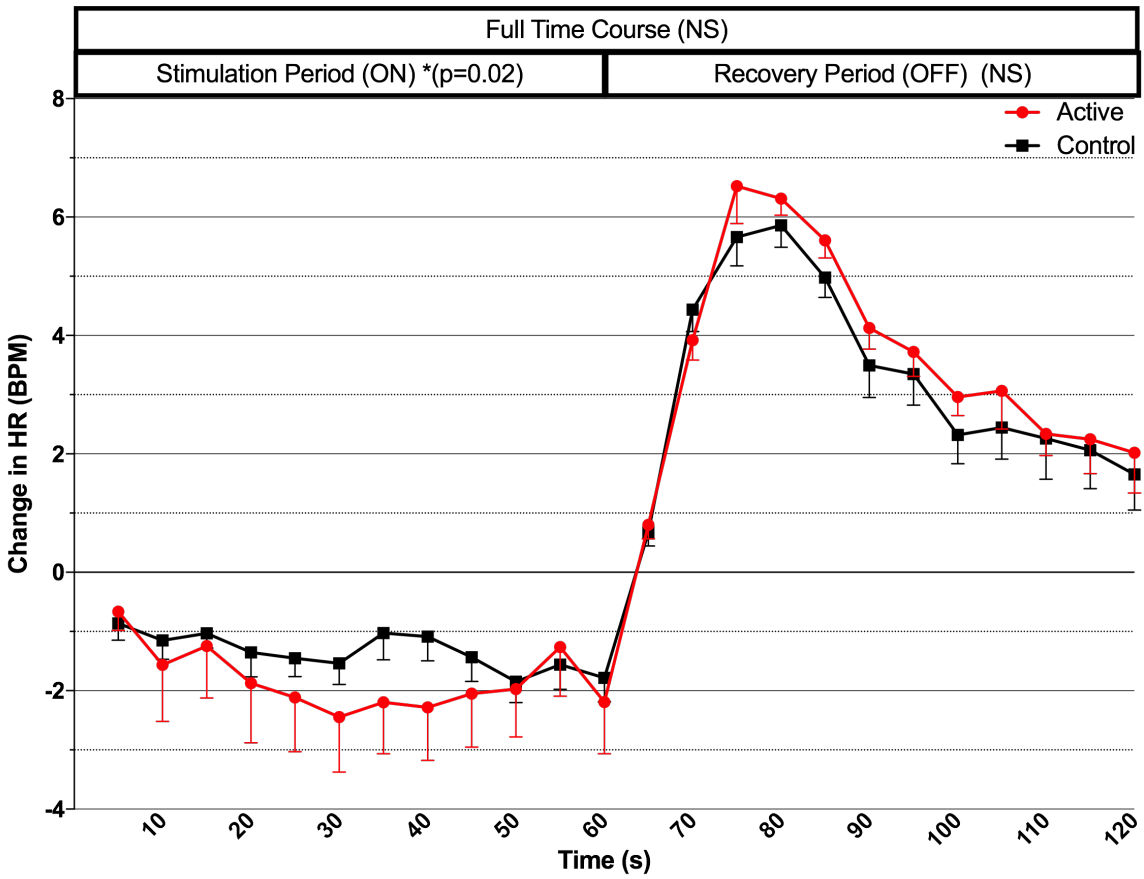


Figure 3-2 Mean change in HR over time (active v control). This figure presents the mean change of all both parameters tested in this trial. Active taVNS induces significant decrease in HR during stimulation (p=0.02), although the recovery HR spike upon termination is not significantly different from control.

Parameter-Specific Effects on HR

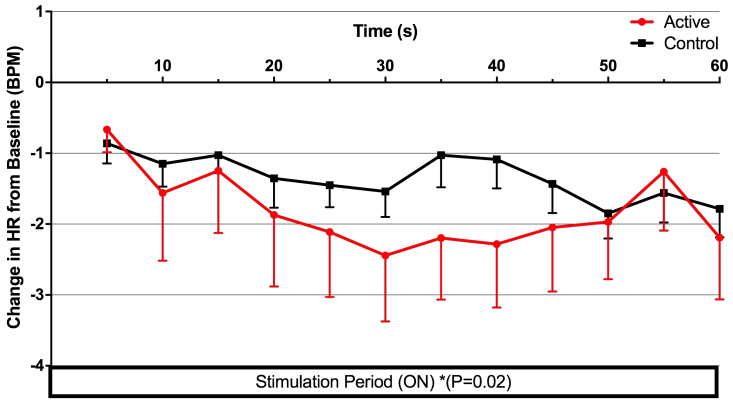
The grouped data can subsequently be split into 500μs, 10Hz and 500μs 25Hz parameters. Exploring the significant effect of bradycardia in the stimulation period in the overall analysis, a repeated measures ANOVA was conducted on the stimulation period by parameter, and the results are highlighted in **Figure 3-3.** Active taVNS at 500μs, 10Hz induces a significant bradycardia effect sustained throughout the entire stimulation period (P=0.032). Mean decrease for the active condition was -2.40BPM ± 0.275 vs. control, which only produced a -1.56BPM ± 0.275 change from baseline (**Table 3-2**). The 500μs 25Hz parameter showed a non-significant effect on bradycardia throughout the stimulation period.

Discussion

We enrolled 20 healthy individuals in this follow-up, confirmatory study aimed to determine the optimal taVNS parameter modulating parasympathetic nervous system activity. When compared against control stimulation, active taVNS significantly decreased HR, a surrogate marker of parasympathetic nervous system activity. When analyzed by parameter, 500μs 10Hz had the greatest effect on HR during the stimulation period. A prior taVNS trial suggested sympathetic nervous activity is reduced upon stimulation (86) and is briefly mentioned in Chapter 2. That study used a smaller pulse width, but higher frequency (200μs 30Hz). Whether the mechanism is the direct decrease of sympathetic system or rather the increase of the parasympathetic nervous system is still unknown.

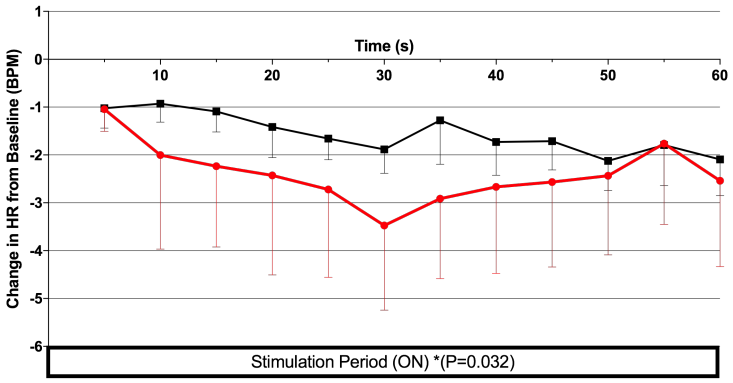
a

Overall Bradycardia Effect



b

500us, 10Hz bradycardia effect



c

500us, 25Hz bradycardia effect

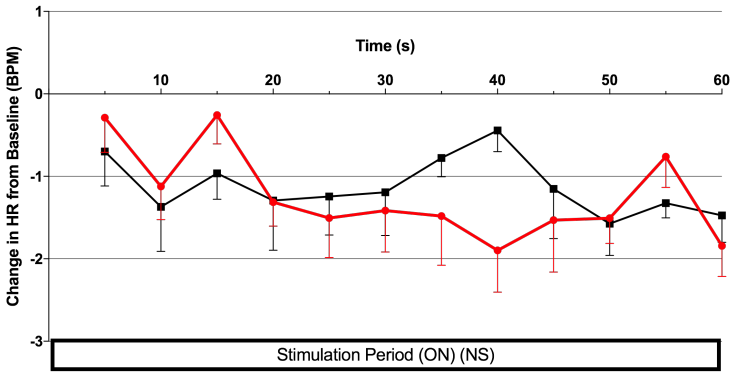


Figure 3-3

Figure 3-3 Parametric effects on bradycardia. a) Overall effect of active taVNS compared to control (p=0.02). b-c) Active taVNS at 500us 10Hz has a significant effect on HR (p=0.032) when compared to control whereas 500us 25Hz is not statistically different.

Table 3-2: Mean Decrease in HR During Stimulation Period (By Parameter)

Parameter		**Mean Change in HR from Baseline ± SEM** (Average over 60s Stimulation Period)		**Partial Eta Sq.**	**Condition Effect? (p value)**
		Tragus (Active)	Earlobe (Control)		
500μs	10 Hz	-2.40 ± 0.275.	-1.56 ± 0.275	0.01	Y (P=0.032)
	25 Hz	-1.244 ± .180	-1.036 ± 1.84	0.001	N

Unlike the initial nine-parameter trial described in Chapter 2, which demonstrated an attenuation of the sympathetic rebound in HR in the recovery period, this effect was not demonstrated in this study in either the overall or parametric study analysis. Both active and control stimulations had similar spikes in HR upon termination of stimulation that occurs as a described in the healthy functioning of the autonomic nervous system(109, 110). It is suspected that the reciprocal sympathetic spike in heart rate counteracts the parasympathetic activation via active taVNS, although in this trial, the active stimulation effect was much larger than the first trial, possibly causing a higher magnitude reciprocal spike. This directly contradicts the initial hypothesis generated from Study 1 in which it was believed there was a persisting parasympathetic activation that blunts the sympathetic spike. It is impossible to say that this is definitively what is occurring and future studies should be designed to directly focus on this balance between rapid sympathetic/parasympathetic activation.

The many trials exploring the positive neuropsychiatric effects of taVNS (78, 83, 112, 113) could not possible if taVNS had primarily an efferent parasympathetic effect. It must be due to increasing parasympathetic or decreasing sympathetic nervous system activity as modulated centrally in either cortical or subcortical brain structure. Recent fMRI trials seem to confirm this hypothesis (91, 92). One can reasonably conclude that taVNS has both afferent and efferent vagal effects although whether the modulation of

heart rate is directly efferent or whether the heart rate effect is due to a short loop traveled by the signal to the central hypothalamic cholinergic pathway is still to be determined.

It is unknown as to which is more important – pulse width or frequency. There is a lacking of data in the recent taVNS trials to answer such a question. The various behavioral effects are derived from many various pulse widths, frequencies and stimulation currents. (91, 92, 104-108, 112). This trial confirms that a pulse width of 500μs at both 10Hz and 25Hz, when combined, give an overall effect of bradycardia during stimulation and that the 10Hz parameter, when compared to control, induced a larger effect on HR. It is suspected that pulse width is more important than firing frequency, as that is required for neuronal depolarization near the nerve chronaxie levels (35) causing depolarization and signal transduction.

The bradycardia associated with control stimulation was again seen in this study, similarly in effect and magnitude to trial 1. A responsive control site is important to investigate the active site of stimulation with rigor, although it is plausible that there may be some transduction of electrical signal into superficial branches of the ABVN. Nerves are often branched and act as electrical conductors. It is highly plausible that there is some transduction of electricity from the earlobe to distal branches of the ABVN that may be spatially proximal to the site of stimulation. Even with stringent controls, the parasympathetic effect of taVNS is significant, demonstrating that less than 3 cm of distance can have different physiological effects on the human.

Conclusion

This follow-up study confirms that electrical stimulation of the a ABVN via taVNS at 500μs 10Hz is the optimal parameter for activating the parasympathetic nervous system and decreasing heart rate in healthy individuals. No significant adverse events were observed and overall. Active taVNS in an overall analysis demonstrated significant bradycardia during the stimulation period. This serves as a strong foundation that stimulation of the ABVN can induce efferent autonomic nervous system responses that can be measured using physiological recordings. It is important to conduct neuroimaging trails further exploring the central effect of taVNS to determine whether there are significant brain activations associated that maybe independent or driving these parasympathetic effects.

Synthesis of Experiments One and Two

The two prior experiments described are important both practically and conceptually. There is an ever-present push to drive therapeutics rapidly towards treating various disorders, whether central or peripheral. This rush introduces a high risk of failure, or the early dismissal of a therapy that may have a biologic effect if parametrically tested for optimal administration.

In the case of electrical stimulation, there are a variety of different parameter combinations that may have a biologic effect. This series of studies demonstrates 1) pulse

width matters. The higher the pulse width, the stronger of an effect described in the initial parametric trial. 2) Higher frequencies are not always better. 10Hz outperformed 25Hz in the follow-up confirmatory trial. 3) taVNS does have a biologic effect that seems to be driven through the vagus nerve.

The final confirmatory step to determine if taVNS has a direct brain effect or whether these are just peripheral vagal responses is to conduct a concurrent taVNS imaging trial exploring the brains response to stimulation. This was the third aim of the dissertation and will be presented in the subsequent chapter.

CHAPTER 4

STUDY 3: USING CONCURRENT taVNS/fMRI TO DETERMINE THE DIRECT BRAIN EFFECTS of taVNS

Study Summary

Background: Although there are numerous trials involving stimulating the auricular branch of the vagus nerve (ABVN), the exact brain regions activated are poorly understood. Electrical stimulation of the ABVN via transcutaneous auricular vagus nerve stimulation (taVNS) likely targets vagal afferent networks and is the theorized mechanism for taVNS.

Objective: We developed a concurrent taVNS/fMRI system to determine the direct brain effects of taVNS compared against control stimulation.

Methods: We enrolled 17 individuals in the two-visit controlled, crossover trial. Individuals attended two scanning visits in which they received taVNS at 500μs 25HZ delivered to either their left earlobe (control) or tragus (active). Whole brain analysis was performed using SPM 12 exploring the effect of the following groups: control stimulation only, active stimulation only, active>control, active <control (FWE corrected P<0.05). An ROI analysis was conducted on the midbrain and brainstem regions of all groups.

Results: Earlobe (control) stimulation produces BOLD signal activation in the contralateral somatosensory representation of the face region, whereas tragus (active) stimulation produces significant activations in the contralateral postcentral gyrus, bilateral insula, frontal cortex, right operculum, and left cerebellum. In the active vs. control contrast, active stimulation produces significant activations in the right caudate, bilateral anterior cingulate, cerebellum, left prefrontal cortex, and mid-cingulate.

Conclusion: These findings reveal the afferent projection of taVNS delivered to the tragus produces cortical and subcortical effects in regions of the brain known to be part of the afferent vagal pathway.

Introduction

Modern functional neuroimaging methods can be used to measure the neurophysiological effects of a stimulus or intervention. The most common of these methods are electroencephalography (EEG), positron emission tomography (PET), and functional magnetic imaging (fMRI). Each technique has its benefits and drawbacks. EEG has an extremely high temporal resolution on the scale of milliseconds and is great for capturing fast neuronal propagations, although it lacks spatial resolution and deductions can only reliably be made regarding cortical activity. PET and fMRI are similar as they both indirectly measure brain activity, although PET has a much lower imaging resolution than fMRI, uses radioactive isotopes, and is more expensive.

fMRI images the blood oxygen level dependent (BOLD) signal in the brain, which is considered and utilized as an indirect marker of neural activity (47). The BOLD signal measures the temporal changes of oxyhemoglobin and deoxyhemoglobin. As neuronal depolarization occurs, oxygen is consumed and increased blood flow is delivered to higher metabolism areas, bringing with it oxyhemoglobin, producing a stronger MR signal than its deoxygenated counterpart. It is this difference in magnetism is exploited in the fMRI method. In short, increased oxyhemoglobin is believed to reflect increased neuronal activation.

taVNS/fMRI Potential Roadblocks and Solutions

In order to successfully conduct these multimodal imaging trials in which stimulation electrodes like those used in cervically implanted VNS and taVNS and conductive wires are either implanted or externally placed in the magnetic field of the scanner, the following potential issues need to be considered:

1. Induced electrical current in the wire

In 1831, Michael Faraday (114) demonstrated the law of induction. Faraday's law states that electrical current is produced in conductive materials placed in or around a magnetic field. Electromagnetic induction is used in power generation and transmission and is used immensely in modern electronics. We also can reference Faraday's law for how transcranial magnetic stimulation (TMS) works (115) in stimulating the brain.

Unfortunately, induction poses a problem when placing conductive materials in the MRI scanner. The static and dynamic magnetic field of the scanner introduces the risk of inducing unwanted electrical current in the wire.

To circumvent these induced fields, resistors (5kΩ) of sufficient size must be placed in the lead wires of the electrode to block these currents while simultaneously being able to be surmounted by the power of the driving electrical stimulator. Without controlling for induced currents, unwanted stimulation of target sites may occur without intent or knowledge.

2. Electrode Heating

A potential safety concern of electrodes attached to the end of long wires placed in the MRI is the risk of heating these electrodes due to the interaction of the radio frequency (RF) electromagnetic field (116-119). This heating occurs at the end of the wire or electrode and can cause burns to the stimulation site, or even permanent irreversible damage if the electrodes are implanted in the case of VNS or deep brain stimulation (DBS) (49, 120). For this reason, sufficient testing must be conducted to determine safety of these paradigms.

Although there is no direct solution to this problem, special head coils for the fMRI image acquisition can be used in order to minimize the RF electromagnetic field and

minimize heating. The resistors placed in the lead wires also reduce the heating. It is important to ensure the conductive medium (e.g. gel, paste) used for stimulation can withstand heating without degrading.

3. MRI Artifact

Wires delivering electrical stimulation from outside the magnet room of the scanner (equipment room or control room) introduce RF into the scanner. The wire becomes a large antenna that may produce an RF overflow artifact causing a non-uniform appearance in part of the image or even the entire image. In order to minimize this artifact issue, any externally driven stimulation needs to be delivered through an RF filter and grounded to a panel so that the fMRI signal does not become distorted.

The prior three concerns were all addressed before conducting the final study described in this chapter. All developmental experimentation was conducted on MRI phantoms in order to optimize stimulation feasibility as well as reduce artifact and heating and ensures safety. Three pilot scans on healthy individuals were also conducted following phantom trials, those individuals were used for optimization of the taVNS method and were not enrolled into the prospective taVNS/fMRI trial.

Prior taVNS/fMRI Trials

There have been five imaging trials specifically designed to determine the afferent pathway of taVNS. The first taVNS/fMRI trial was conducted in 2007 by Kraus and

colleagues (91). They used fMRI to image 22 healthy individuals and aimed to determine whether stimulating the left tragus (active) or earlobe (control) at 20μs and 8 Hz for (30s ON, 60s OFF) produced short-term brain activations in a crossover design. Active stimulation produced decreases in limbic brain areas, including the amygdala, hippocampus, parahippocampal gyrus and the middle and superior temporal gyrus. They also demonstrated increased activation in the insula, precentral gyrus and the thalamus. Control stimulation revealed no significant BOLD responses in either direction. No brainstem activation was reported in either condition.

This same group conducted a follow-up trial in 16 healthy individuals exploring regional specificity of the induced BOLD response from taVNS. This study stimulated either the left auditory canal (8 subjects receiving anterior tragus stimulation, 8 subjects receiving posterior auditory ear canal stimulation) or the earlobe (all 16 subjects) in a crossover design. Stimulation parameters were identical to their prior trial (20μs and 8 Hz for 30s ON, 60s OFF). The results of the study are in line with their previous fMRI studies (92), showing robust BOLD signal decreases in limbic structures and the brain stem during as well as BOLD activation in frontal and insular cortex via electrical stimulation of the left anterior auditory canal. Interestingly, stimulation at the posterior wall seems to lead to unspecific changes of the BOLD signal within the brainstem and vagal afferent projections. Earlobe control stimulation again produced no major significant BOLD signal responses. This study suggests the anterior auditory canal (tragus) to be a much more effective ABVN target than the posterior wall.

In 2008, a very small taVNS trial was conducted by Dietrich and colleagues in four healthy males. The goal of this study was to test new parameter settings and a novel device they have developed. This open-label single arm (active only) study delivered 50s blocks of taVNS (250μs, 25HZ) to the left tragus. They authors suggest that taVNS induced increases in BOLD signal in the thalamus, prefrontal cortex and brainstem (90) although this was not described in any of the other four trials. This study is considered very inconclusive as it lacks effective control and based on the small sample size, but merits further exploration of the parameters employed.

In 2015, Frangos et al (93) conducted a taVNS/fMRI trial exploring the regional effects of electrical stimulation of two stimulation sites (cymba conchae vs. earlobe control; crossover design) in 12 healthy individuals. The stimulation parameters were 250μs, 25Hz, for one stimulation ON block lasting 7minutes. Their findings reveal conchae stimulation, compared to earlobe (control) stimulation, produced significant activation of vagal afferents, most notably the ipsilateral NTS, locus coeruleus (LC), dorsal raphe, amygdala, and nucleus accumbens. Findings also demonstrate deactivations in the hypothalamus and hippocampus. This trial differs from prior trials as the stimulation duration is highly unconventional and longer than any of the prior behavioral or imaging trials (7min stimulation of target sites with only one block each). No forebrain findings were discovered and imaging the deep mid brain and brain stem activation validity are still debated amongst imaging experts.

The most recent taVNS/fMRI trials were conducted by Yakunina et al. in 2016. This study explored brainstem activations (NTS and LC) of four different stimulation sites on the ear (inner tragus, posterior wall of ear canal, cymba conchae, earlobe control). Not only did it have the most stimulation sites of any study, it also recruited the highest number of subjects of any prior taVNS trial (37 healthy individuals)(89). Stimulation was delivered at 500μs, 25Hz for 6min ON blocks. Both earlobe and posterior ear canal stimulation sites produced the weakest NTS and LC activation, whereas the tragus and concha targets produced robust brainstem (vagal mediate) activations. When tested head to head, the concha was revealed to be the optimal stimulation site to activate the NTS and LC. This study suggests the concha may be a better target than the tragus.

It is important to note that all fMRI trials have widely varied methodology. They employ different stimulation parameters, stimulation durations, and whether a control was used or not. These studies, their differences and findings are summarized in **table 4-1**. The field of taVNS still lacks a consensus on many of these stimulation targets are highly debated within the small field.

Study Introduction and Hypothesis

Prior studies described in Chapters 2 and 3 of this dissertation attempted to determine whether optimal taVNS parameters could be discovered using parasympathetic decreases in HR as measures. Although it was determined that larger pulse widths and higher frequencies of parameters modulate the parasympathetic response greater than lower

parameters and control, it is still unknown whether taVNS has a direct effect on the afferent vagal pathway of the central nervous system. It has been suggested via fMRI studies that stimulation of the ABVN has afferent brain effects that are initiated via the brainstem (89, 93) although there have been no neck dissection studies looking at the connection of the ABVN to the main bundle or the brainstem. There is mixed, limited fMRI data on the afferent effects of taVNS, with studies varying in stimulation duration and parameter.

One of the optimal parameter candidates from the initial physiological trials described in Chapter 2 was used to explore the direct brain effects of taVNS using concurrent taVNS/fMRI. We hypothesize that by using parameters known to have a biologic effect in our prior trials relevant and significant brain activation changes in afferent vagal pathway areas as measured by BOLD fMRI.

Methods

Overview

We conducted a 2-visit, single blind, sham-controlled, crossover fMRI trial exploring the effects of active taVNS stimulation compared to earlobe stimulation (control). Participants attended 2 scanning visits, separated by at least 1 day apart to avoid any carryover effect. All scanning was conducted at the MUSC Center for Biomedical Imaging 30 Bee Street location. This study was approved by the MUSC Institutional Review Board (IRB) and is registered on ClinicalTrials.org (NCT02835885).

Table 4-1. Prior taVNS/fMRI Trials

taVNS/fMRI studies							
Year	**Author**	**n**	**Subjects**	**Site**	**Parameters**	**Control Condition**	**Findings**
2007	Kraus et al.	22	Healthy Controls	Outer ear Canal	20μs 8 Hz 30s stim	Yes Earlobe	−BOLD ↓ in limbic (amg, hp, parahp g) and the MTG, STG −BOLD ↑ in insula, PreCG, thal
2008	Dietrich et al.	4	Healthy Controls	Inner Tragus	250μs 25 Hz 50s stim	No	−BOLD ↑ in L LC, L >> R thal, L PFC, Bl PcG, L PCG, L insula −BOLD ↓ in R Nacc, R Cb
2013	Kraus et al.	16	Healthy Controls	L outer canal (ant vs post)	20μs 8 Hz 30s stim	Yes Earlobe	−Ant: BOLD ↓ in parahp g, PCC, R thal (pulvinar), LC, STN −Ant and Post: BOLD ↑ in insula; otherwise oppose signal directions
2015	Frangos et al.	12	Healthy Controls	L Conchae	250μs 25 Hz 7 min stim	Yes Earlobe	−BOLD ↑ signal in L NTS, Bl STN, DR, LC, cl PBA, Amg, Nacc, bl paracentral lobule −BOLD ↓ in hp, hypoth
2016	Yakunina et al.	37	Healthy Controls	4 areas	500 μs 25 Hz	Yes Earlobe	-BOLD ↑ in Conchae > Tragus > Canal in both NTS and LC
2017	Badran et al. (this study)	17	Healthy Controls	Tragus	500 μs 25 Hz	Yes Earlobe	-control = ↑BOLD Postcentral -tragus = ↑BOLD Postcentral & Afferent Vagal tragus>control= ↑BOLD Afferent Vagal

Bl- bilateral; L-left; R-right; Stim- Stimulation; AMS- Adjective Mood Scale; Brain areas: MFC-Middle Frontal Cortex; NTS-Nucleus of Solitary Tract; LC-Locus Coeruleus; thal-thalumus, PFC-prefrontal cortex, PreCG-Precentral gyrus; PostCG-Postcentral Gyrus; PCG-posterior cingulate gyrus; Nacc- Nucleus Accumbens; STN-spinal trigeminal nucleus; cl PBA- contralateral Parabrachial area; Cb- Cerebellum
*Inner tragus; inferoposterior wall of canal; cymba conchae

Participants and Inclusion Criteria

17 healthy individuals (8 female) were enrolled after meeting the following inclusion criteria: age 18-45, no personal or family history of seizure, mood, or cardiovascular disorders, no facial or ear pain, no recent ear trauma, no metal implants including pacemakers, not pregnant, no dependence on alcohol or recent illicit drug use, not on any pharmacological agents known to increase seizure risk (Bupropion, neuroleptics, albuterol, theophylline, antidepressants, thyroid medications, or stimulants). Participants were screened for MRI exclusionary criteria as well (metal in body and claustrophobia).

fMRI Scanning

All MRI scanning was conducted using a Siemens TIM Trio 3.0T system and the provided Siemens 32-Channel head coil. Individuals were positioned head-first supine on the bed of the scanner and foam pads were used to stabilize the head and minimize movement.

Each of the two visits lasted approximately 30-minutes in duration during which 3 functional sessions were acquired (**Figure 4-1a**). Following a localizer scan, a high resolution anatomical MPRAGE (TR: 1900ms; TE: 2.26ms; Voxel size: $1mm^3$; 208 slices, FA: 9 deg) was collected. Following the anatomical image, three separate functional scans were acquired, in which subjects received (either active or sham) concurrent taVNS. The order of active and sham stimulation was counterbalanced.

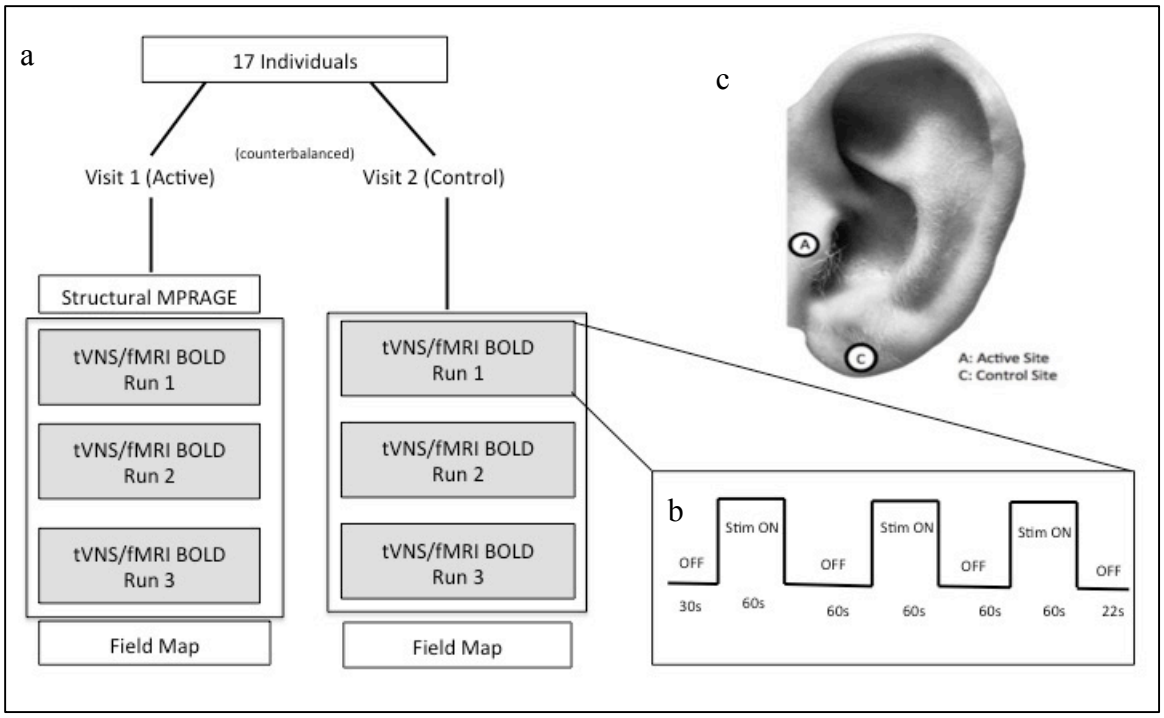


Figure 4-1 Imaging study design. a) Overview of scanning visits and MRI scans acquired. b) Block design of the concurrent taVNS/fMRI BOLD scans with time on and off. c) Ear stimulation targets (identical to prior two physiologic trials).

Lastly, a field map was acquired to correct for distortions due to magnetic field inhomogeneity.

The concurrent taVNS/fMRI scans were conducted using an echo-planar imaging (EPI) sequence (TR: 2800ms; 126 volumes, TA: 5:52s, TE: 35ms; Voxel size: 3.0mm^3; 47 slices, FA: 76 deg), with a block design (**Figure 4-1b)**. Each scan run was identical, consisting of an initial 30s "OFF" period with no stimulation, followed by 3, 60s "ON" periods in which electrical stimulation was delivered to the ear. The time between "ON" periods was 60s, followed by a final 22s "OFF" period after the final stimulation block. Each functional session lasted 6 minutes. The stimulation was synchronized with the start of each taVNS/fMRI BOLD sequence acquisition (from 0:00 and ran to 5:52 for each taVNS/fMRI run) and was triggered upon first fMRI volume acquisition in the equipment room using an automated stimulation system that delivered TTL pulses to the constant current stimulator at specific frequency and duration. Timing validation was confirmed with the console timer after each individual stimulation session. Upon completion of each taVNS/fMRI scan, individuals were asked through intercom how many stimulation blocks they felt in order to verify signal transmission into the scanner and all three "ON" blocks were delivered. They were also asked to rate their pain on a NRS from 1 (no pain) -10 (extreme pain)

Concurrent taVNS/fMRI System

Stimulation was delivered via custom developed stimulating electrodes pictured in **Figure 4-2a.** Computer assisted drawings of the electrode clamps (**Figure 4-2b**) were generated in SketchUp (Timble Navigation, USA) and subsequently 3D printed out of ABS plastic at the MUSC Brain Stimulation Laboratory (Flashforge Creator Pro, China). The round, unipolar stimulation electrodes were 1cm in diameter made of Ag/AgCl and affixed to the 3D printed clamps using cyanoacrylate. Copper was used for all wiring. Ten20 conductive paste was used as a conductor for the electrodes.

Constant current stimulation was delivered using a Digitimer DS7a set to <400V. Lead wires were attached to the Digitimer output and connected to a radio frequency (RF) patch panel in the wall between the equipment room and magnet room using a serial connector on both sides. **Figure 4-3** demonstrates the taVNS/fMRI setup visually. Wire was run from the patch panel in the magnet room towards the foot of the MRI scanner, where it was then run on top of the participant who was laying supine head first on the scanning table. ½ inch PVC piping was used to insulate the wires and rested on the participant's abdomen and the stimulation electrodes were clamped to the individual's tragus or earlobe depending on condition.

taVNS Parameters and Stimulation Targets

The parameters used for this fMRI trial were 500μs 25Hz (monophasic square waves) based on previous autonomic effects described in Chapters 2 and 3 of this

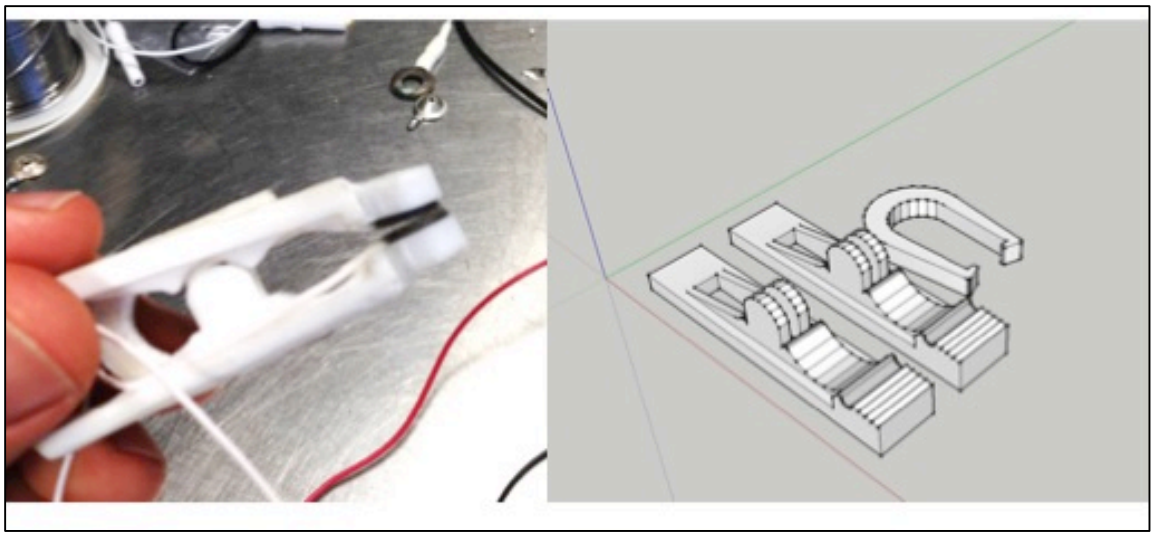


Figure 4-2 fMRI compatible electrodes a) picture of final taVNS electrodes that have been 3d printed and assembled b) CAD drawings of electrodes demonstrating the 3-piece design and "U" shaped spring clip. Ag/AgCl electrodes were affixed to the inside part of the electrode clips.

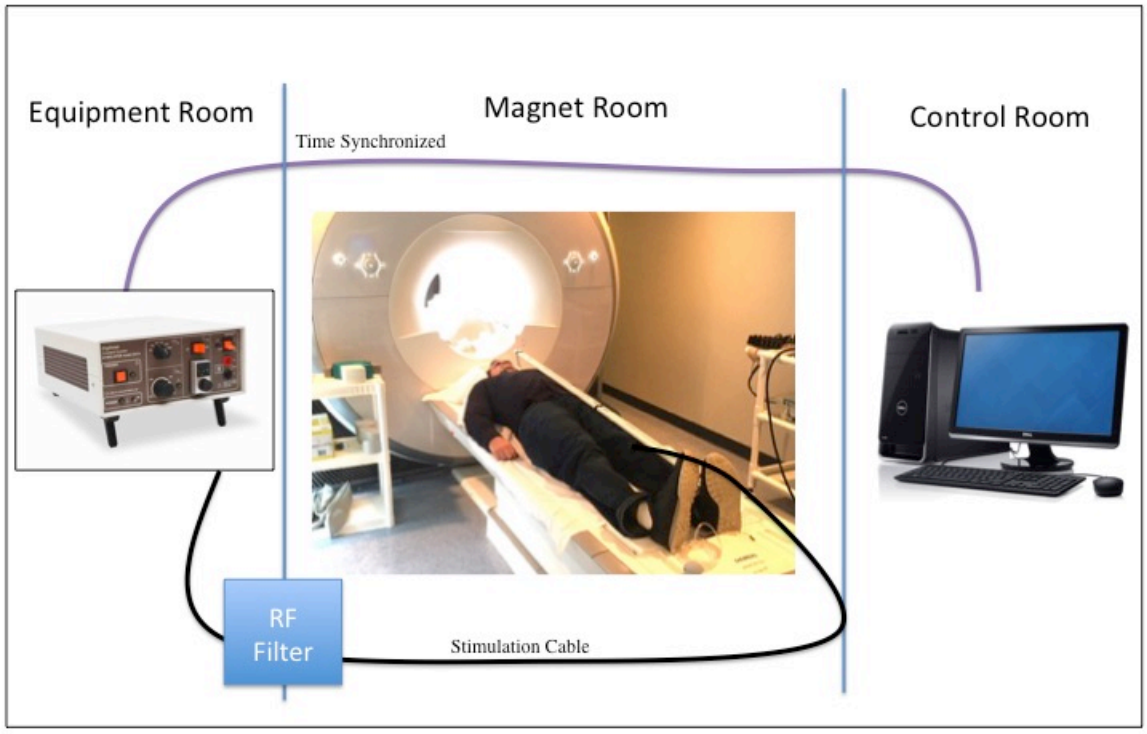


Figure 4-3 taVNS/fMRI Setup. This figure shows how taVNS is synchronized and delivered to the scanner. Timing is driven off the control room main console computer. Triggering of the direct current stimulator occurs in the equipment room which propagates an electrical stimulation current through a grounded RF filter and into the magnet room through a 10m cable that attaches to the participant's ear in the scanner.

dissertation. Stimulation current was set to 200% of perceptual threshold (PT) and the PT was determined while the subject had electrodes attached and was lying in the scanner. Perception of stimulation was relayed to the equipment room via intercom and the current modified until the participant felt the minimum perceptual level. Active stimulation was delivered to the left tragus, control stimulation to the left earlobe (**Figure 4-1c**).

Data Processing and Analysis

All images were converted from DICOM to NifTI using dcm2nii program. All further processing and analysis was performed in SPM 12 software (UCL) using MATLAB R2012a (The MathWorks Inc, Natick, MA). First, deformations required for normalization were derived from whole brain anatomical images using Segment. Skull stripped anatomical images were created from grey matter, white matter and CSF masks with Image Calculator to improve functional to anatomical coregistration. Next, the functional images were processed through Realign and Unwarp to reduce motion related variance and correct distortions due to magnetic field inhomogeneities. The mean image from realignment was coregistered to the skull stripped anatomical image using a normalized mutual information algorithm. The estimated coregistration parameters were combined with the forward deformations applied to the functional data in a single step to bring the data into MNI space. Finally, the data was smoothed using an 8mm FWHM Gaussian smoothing kernel. Estimated movement parameters were examined and no participants exceeded our movement threshold of one voxel.

For subject level general linear modeling, the three stimulation "ON" periods (onset times: 30s, 150s, 270s; duration 60s) were convolved with the canonical hemodynamic response provided by SPM. Estimated motion parameters were included in the model as nuisance regressors and the data was high pass filtered with a cutoff of 180 seconds. Each subject's contrast estimates for stimulation "ON" condition was combined into a second level model in which two separate group analysis were conducted (active group only, control group only) using a one-sample t-test (active: ON>OFF, ON<OFF; control: ON>OFF, ON<OFF). The duration of the "OFF" time was unmodeled and left as the implicit baseline. Additionally, an overall paired t-test contrast was also conducted (active ON>OFF > control ON>OFF; active ON<OFF > control ON<OFF). Lastly, a brain stem mask was created in order to explore if regional activations in this small area could be detected in each of the group analyses.

Results

Participants, Stimulation, and Tolerability

17 healthy, right-handed individuals (8 female, 9 male, mean age 25.8 SD 7.59) were included in this study. All participants completed both visits without any dropouts. Mean perceptual thresholds (PT) are as follows (mean ± SD mA): tragus 1.57 ± 0.48; earlobe 1.22 ± 0.58. The current at which taVNS was delivered was again a scale multiplier of the PT (200%). Mean stimulation currents were as follow (mean ± SD mA): tragus- 3.14 ± 0.99; earlobe 2.43 ± 1.16. Using a 2-tailed paired t-test, it was determined that there was no difference in perceptual threshold between the two stimulation sites (**table 4-2**).

Mean NRS scores were low, with the mean difference in pain rating between active and control being 0.6 on a subjective 1-10 scale in 0.5 increments (**table 4-2**). Although a paired t-test revealed this difference in pain ratings is significant, in practicality stimulation should be considered painless in both conditions, as maximum of 2.1 (mean active pain rating) on a 1-10 rating scale is insignificant pain.

Whole-Brain fMRI Analysis

Earlobe (control condition) Stimulation Only

In the control group analysis exploring the BOLD signal changes during left earlobe stimulation, statistically significant increases in BOLD signal associated with stimulation (ON>OFF) were only found in the right inferior postcentral gyrus, operculum, and insula (n=17, one sample t-test, cluster FWE $p<0.05$, cluster forming threshold $p<0.005$, extent threshold =100 voxels) (**Figure 4-4, Table 4-3**). There were areas in which a significant decrease of the BOLD signal below the baseline was found (ON<OFF contrast).

Table 4-2. Mean PT, current, and pain ratings during stimulation

	Tragus (Active)	Earlobe (Control)	**Significant? (p value)**
PT ± SD (mA)	1.57 ± 0.48	1.22 ± 0.58	N
Stim. Current ± SD (mA)	3.14 ± 0.99	2.43 ± 1.16	
Mean 500us 25Hz NRS Pain Rating ± SD	2.1 ± 0.87	1.43 ± 0.68	Y ($p<0.01$)

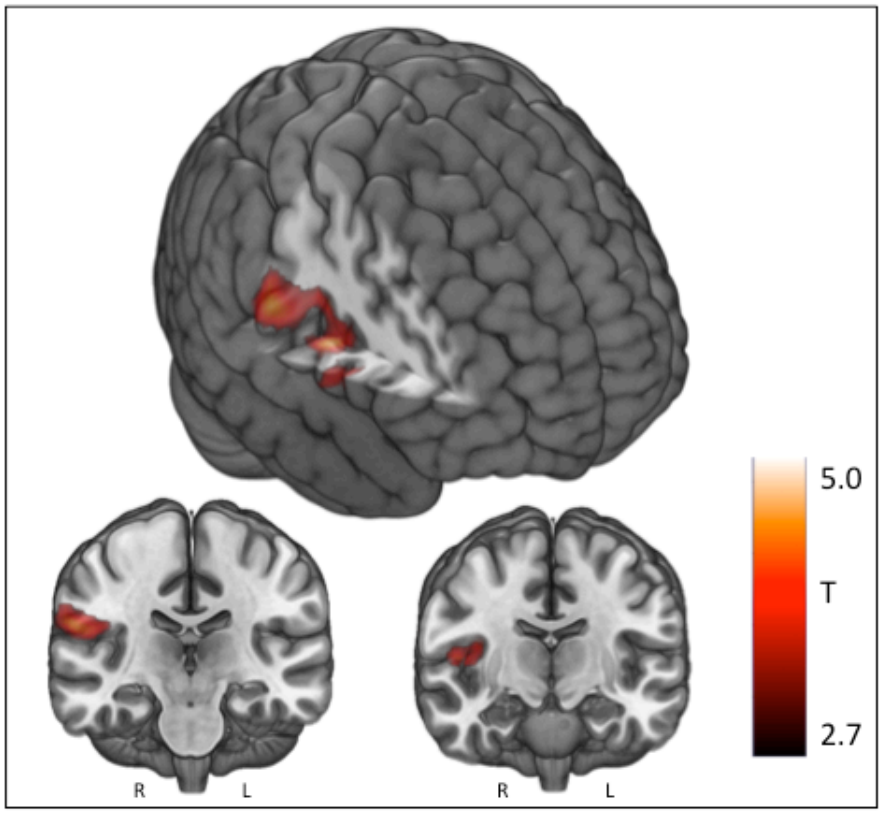


Figure 4-4 Earlobe (Control) Stimulation Only a) fMRI BOLD activations resulting from control stimulation only (compared to rest). (n=17, one sample t-test, cluster FWE $p<0.05$, cluster forming threshold $p<0.005$, extent threshold =100 voxels).

Tragus (active condition) Stimulation Only

In the active group analysis exploring the BOLD signal changes during left tragus stimulation condition, statistically significant increases in BOLD signal associated with stimulation (ON>OFF) were found in similar areas as earlobe stimulation (right postcentral gyrus, operculum, and insula) as well as other more wide spread areas such as the left insula, angular gyrus, cerebellum, and bilateral frontal lobes (n=17, one sample t-test, $p<0.05$ FWE corrected, cluster forming threshold $p<0.005$, extent threshold =100 voxels) **(figure 4-5, table 4-3)**. No significant deactivations were found (ON<OFF contrast).

Tragus (active) Greater Than Earlobe (control)

The effect of control stimulation was subtracted from the effect of active stimulation to analyze the group effect of taVNS compared to control. From this analysis, one can visualize the effects of active taVNS in two contrasts (ON>OFF, ON<OFF). When examining areas in which active stimulation was greater than control, significant clusters were found in the right mid cingulate, caudate, bilateral operculum, bilateral cerebellum, and bilateral anterior cingulate cortex (ACC) (paired t-test, $p<0.05$ FWE corrected, cluster forming threshold $p<0.005$, extent threshold =100 voxels) (**Figure 4-6, Table 4-3**). No areas were found in which active stimulation led to a lower response than control.

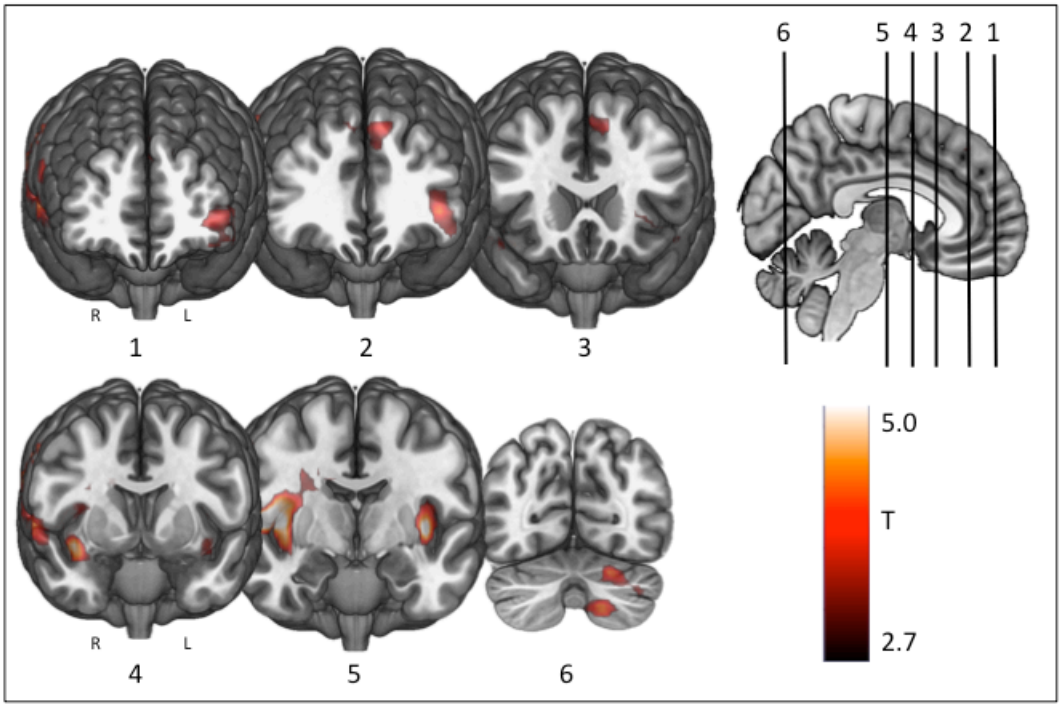


Figure 4-5 Tragus (Active) Stimulation Only a) fMRI BOLD activations resulting from tragus stimulation only (compared to rest) (n=17, one sample t-test, cluster FWE $p<0.05$, cluster forming threshold $p<0.005$, extent threshold =100 voxels).

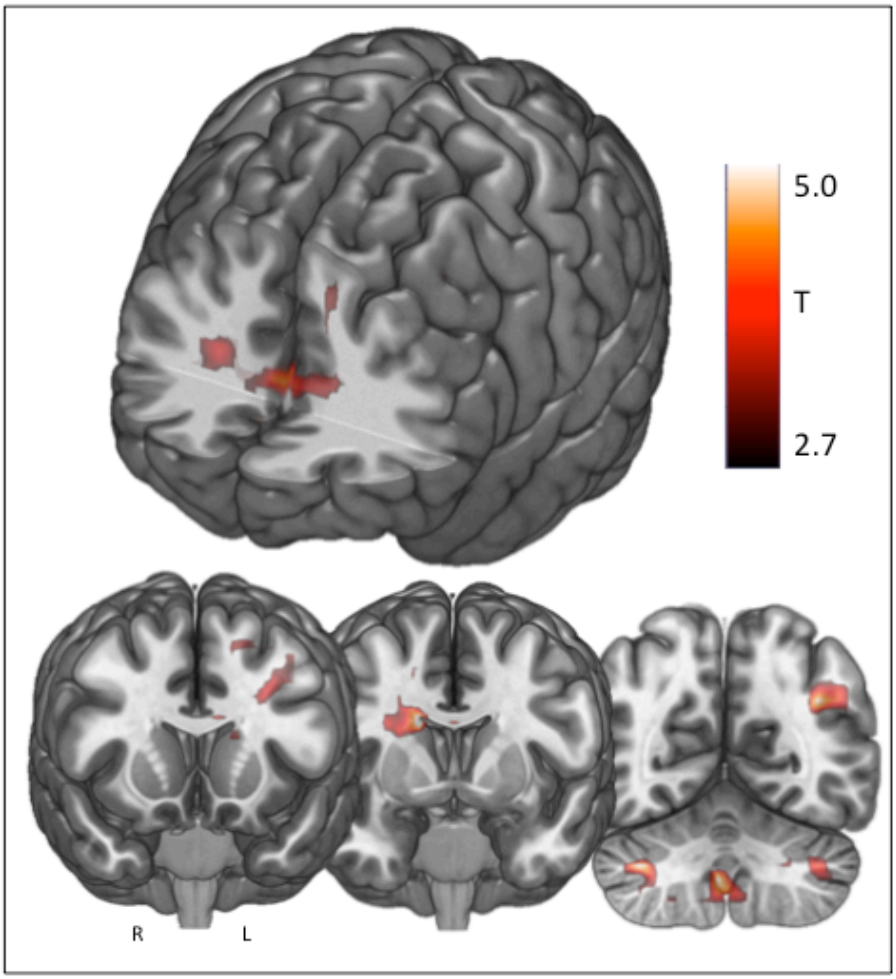


Figure 4-6 Active stimulation > control stimulation a) fMRI BOLD activations resulting from the contrast active > control stimulation only. (n=17, paired sample t-test, cluster FWE $p<0.05$, cluster forming threshold $p<0.005$, extent threshold =100 voxels).

Table 4-3. Results from GLM analyses for active and control stimulation.

Cluster Statistics			Cluster Locations	Peak Location (MNI)		
$p_{FWE\text{-}corr}$	# voxels	$p_{uncorrected}$		x	y	z
Left Earlobe (Control) Stimulation						
0.007	165	0.001	Right Central Operculm	48	-4	8
			Right Postcentral Gyrus	63	-16	23
			Right Insula	36	-13	17
Left Tragus (Active) Stimulation Only						
0	1491	0	Right Insula	36	-13	17
			Right Central Operculum	48	-7	8
			Right Postcentral Gyrus	60	-16	32
0.011	359	0.001		-36	-16	11
			Left Insula	-39	-7	2
				-39	2	-10
0.007	392	0	Left Angular Gyrus	-42	41	-1
			Left Inferior Frontal Gyrus	-48	44	-16
				-36	50	-16
0.077	221	0.005	Left Supplementary Motor Area	-9	23	47
			Left Superior Frontal Gyrus	-9	38	47
			Right Superior Frontal Gyrus	-9	35	47
0.082	217	0.006	Left Cerebellum	-15	-70	-46
Active Stimulation > Control Stimulation						
0.005	369	0		27	-7	35
			Right Caudate	15	2	23
			Right Mid Cingulate Gyrus	18	-4	38
0	698	0	Bilateral Cerebellum	3	-58	-43
				36	-58	-37
				-18	-67	-40
0.007	353	0	Bilateral Anterior Cingulate	3	35	11
			Left Anterior Cingulate	-12	26	17
			Left Mid Cingulate	-9	14	23
0.053	221	0.003	Left Mid Frontal Gyrus	-30	14	35
			Left Superior Frontal Gyrus	-12	26	44
			Left Superior Frontal Gyrus	-12	38	35

Brain stem analysis

A post-hoc, brainstem analysis was conducted using an explicit mask comprised of the pons, midbrain, and medulla (121). Using the same thresholds on this limited region, no significant findings were discovered in either condition (active only, control only) or in a direct comparison contrast (active vs control).

Discussion

Using this taVNS/fMRI system, we have demonstrated that active taVNS in healthy young adults at 500μs 25Hz produces significant BOLD activations throughout cortical, subcortical, and cerebellar brain regions associated with the afferent vagal pathway. In contrast, control stimulation of the earlobe exclusively produces a contralateral somatosensory BOLD signal response in the postcentral gyrus representation of the face. When control response is subtracted from the active response in the overall contrast of active>control, significant activations emerge throughout the cingulate gyrus (bilateral ACC, bilateral mid cingulate), frontal cortex (left middle and frontal gyrus), cerebellum, and right caudate.

We investigated the direct brain effects of taVNS to either the left tragus (active) or earlobe (control) using a novel taVNS/fMRI paradigm in 17 healthy individuals. Each participant attended two scanning sessions, in which both left tragus (active) and left earlobe (control) stimulation was administered in order to determine the afferent brain effects of electrical stimulation of the ABVN. Within this trial, we describe two effects:

1) the somatosensory cortical representation of the ear, and 2) the cortical and subcortical direct brain effects of stimulating the ABVN.

Penfield described the homuncular representation of the human primary sensory cortex (122), and notably the ear is omitted from these trials. To date there have only been two studies exploring ear somatosensory representation (123, 124), the first using magnetoencephalography (MEG) and the second using fMRI. Both describe the somatosensory response of the left ear being represented on the contralateral somatosensory cortex in the face and neck areas. The MEG findings demonstrated that somatosensory evoked magnetic fields (SEFs) were produced in response to slow (1Hz), ultra brief (0.05ms pulse width) electrical stimulation of the earlobe. The follow-up MRI findings by the same group confirm initial MEG findings that slow (2Hz), brief (0.5ms pulse width) electrical stimulation solely activates contralateral postcentral gyrus. Although our stimulation current was faster (25 Hz compared to 1 and 2 Hz), the pulse width was identical and our control stimulation findings replicate these two sequential trials conducted by Nihashi et al.

The afferent pathway of the ABVN is still poorly understood although it is hypothesized to activate the main vagal afferent pathway (via the NTS, LC, and upstream cortical projections as described in detail in Chapter 1) (13, 125-127). To date, excluding this trial, there have only been five taVNS/fMRI studies exploring the direct brain effect of electrical stimulation to the ear listed in **table 4-1**. The findings are widely variant as are

the methods. We have understood from prior heart rate trials described in Chapters 2 and 3 that pulse width and frequency affect the stimulation-induced parasympathetic effect of taVNS. This was also described in a VNS/fMRI trial exploring pulse width effect on BOLD (49) Unfortunately two of the prior trials explored 250μs pulse width stimulation (90, 93) and two others administered 20μs stimulation (91, 92). Our trial used a 500μs 25Hz parameter similar to Yakunina et al (89) with similar findings. Like the Yakunina group, we demonstrate tragus stimulation produced significant increased activation in the angular gyrus, caudate, cerebellum, cingulate, and frontal cortex. These regions in general are also found activated throughout the other smaller pulse width trials listed in **Table 4-1** and are afferent targets of the vagus nerve pathway, suggesting ABVN stimulation enters the vagal bundle and projects to the brain via the brainstem.

A major difference between these trials was the time of stimulation during scanning. Three studies stimulated for less than 1 minute (90-92), while the most recent two stimulated for six or seven minutes (89, 93). In our trial, stimulation was delivered for 1 minute blocks. The studies conducting long stimulation periods reported BOLD signal activations in the brainstem region, while the prior three trials did not. It is difficult to image small brainstem regions such as the LC and NTS without rapid, thin slice acquisition of that region. Even in perfect conditions the breathing, moving, and swallowing artifacts make imaging this region of the brain a challenge. It is plausible that we did not see any brainstem activations due to our short stimulation period of 1 minute

and possible future studies should consider longer stimulation periods and scans optimized for imaging the brainstem rather than whole brain scans.

Interestingly, the strong bilateral activation of the ACC and left DLPFC may reveal a potential mechanism for the anti-depressant effect of cervically implanted VNS as well as taVNS that has been described in the literature (33, 40, 88). It has been demonstrated that the ACC is involved in cognition and emotional processing (128, 129)and has been shown to play a key role in the depressions, expressing reduced glutamate release (130-132)and reduced glial cell density (133, 134) in pathologic conditions. It also has been used as a longitudinal predictor of treatment response in depression (135, 136). The left DLPFC has been demonstrated to be hypoactive in depression (137, 138) and is targeted with high frequency rTMS (139-142). Presented with significant BOLD activations in regions of the brain associated with major depression, it is reasonable to consider a bottom-up approach to treating MDD. Rather than pharmacological agents aimed at neurotransmitters, or rTMS which treats the cortex, taVNS can potentially target desired brain regions by entering the brain through cranial nerves and having a cortical effect driven from the brainstem.

Limitations

It is important to recognize some limitations in our trial. Firstly, we chose a 1 minute stimulation period was chosen to be consistent with our prior physiological trials. The safety profile of longer periods of stimulation was unknown and we did not want to risk

adverse events in the MRI scanner. Secondly, we acknowledge that there may residual cortical brain effects persisting in the 60s inter-stimulation rest blocks. This type of design was used to increase the power of our effect by increasing number of stimulation blocks while minimizing scanner drift of long stimulation trials. Lastly, the control region was chosen as the earlobe in line with prior taVNS trials, although it may not be the most reliable as some individuals may have ABVN projections spanning to the lobule of the ear.

Conclusion

These findings demonstrate taVNS delivered at 500μs 25Hz to the left tragus produces significant cortical effects in the vagal afferent pathway compared to earlobe stimulation. These findings are similar to prior taVNS trials. Furthermore, bilateral ACC and left prefrontal BOLD signal increases shed light on the ability to conduct bottom-up brain stimulation modalities in which stimulating cranial nerves can potentially be used as therapeutics. Future taVNS/fMRI trials should be conducted to explore the effect of parameter and stimulation duration on the BOLD signal response.

CHAPTER 5

CONCLUSIONS and FUTURE DIRECTIONS

Summary of Findings

This body of work aimed to address five goals. They are listed below along with their summarized overall findings in their respective subsections.

1. Develop a system that stimulates the ABVN at MUSC
2. Determine if taVNS is safe
3. Optimize stimulation parameters using heart rate as a biomarker
4. Develop and optimize a taVNS/fMRI method
5. Measure the direct brain effects of taVNS using BOLD fMRI

Develop a system that stimulates the ABVN at MUSC

This body of work began in 2014, and at that time there was one commercially available ABVN stimulation unit on the market sold under the name NEMOS© (Cerbomed GMBH, Germany). It is not sold in the United States, and there were several issues associated with using a commercially available device. Firstly, stimulation parameters (pulse width, frequency, duration) are not modifiable making it difficult to use them in

laboratory-controlled trials. Secondly, the stimulation electrodes target the cymba conchae of the ear, which is a different primary site of active stimulation than had been chosen for these trials (tragus stimulation/ear canal). As of 2017 this commercially available company has unfortunately declared bankruptcy and no longer sells these devices. For these reasons, it became important to develop a standalone, independent system at MUSC that has completely modifiable parameters and ability to stimulate various ear targets.

A stimulation electrode was built by Bashar and Alan Badran, made of 1cm diameter round cup electrodes (cupped in order to hold conductive gel) that were affixed to each other in a custom clip arrangement, forming what are essentially direct electrical current clamps that can stimulate either the tragus (active) or earlobe (control). These are pictured in the Methods section of Chapter 2.

In order to avoid filing an Investigational Device Exemption (IDE) with the FDA as required by the MUSC IRB, the constant current stimulator used was required to be FDA-cleared. The custom electrodes were modified to be compatible with the Digitimer DS7a stimulator, which was used for all three stimulation trials. This stimulator allowed for easy and reliable parametric modifications, most importantly changing pulse width, frequency, current, and stimulation duration. The custom electrodes paired with the stimulator allowed for a variety of experimental taVNS studies to be easily conducted.

Determine if taVNS is safe

Given the parasympathetic efferent projections of the vagus nerve described in Chapter 1, the primary safety concern was the potential of adverse cardiac events. Cervically implanted VNS has a long history of animal and human safety and feasibility trials(36, 42, 99, 100). taVNS is a relative newcomer, having been formally proposed in 2000 and lacking the large number of prospective human trials. There had been several prior reports that were published while the safety trials were conducted suggesting its safe use (84, 88, 143-145), although parameters were highly inconsistent and variable.

The first subjects of our initial HR trial were conducted in the electroconvulsive therapy suite at MUSC. Dr. Mark George was the first subject to receive stimulation from our custom taVNS system. Before the experimental visit started, Dr. George asked Bashar "Do you know how to call 9-1-1?" Retrospectively, that comment sounds rather egregious, but it accurately conveys the perceived safety risk and concern of the research team working with this new modality.

Our systematic testing of the effects of nine different taVNS parameters on immediate decrease in heart rate established that 1-minute stimulation sessions of tragus stimulation elicits a safe, relatively minor decrease in heart rate during stimulation. No adverse events were reported in any of the three trials conducted. Redness was perceived at the stimulation site that resolved after several minutes. taVNS was determined to be safe in our stimulation paradigms.

Optimize stimulation parameters using heart rate as a biomarker

We conducted two physiological trials exploring the effects of taVNS on HR described in Chapters 2 and 3 of this dissertation. The first trial in 15 healthy individuals revealed no overall effect of active taVNS (vs control) in heart rate during the stimulation period. Upon multivariate analysis of each of the nine individual parameters, significant effects on HR were discovered in several parameters, suggesting the parasympathetic activation via taVNS is parameter specific. The optimal parameters at modulating HR in this trial were 500μs 10Hz and 25Hz.

Following this initial study, we conducted a follow-up confirmatory study was conducted in 20 healthy individuals exploring the effects on HR of the two optimal parameters (500μs 10Hz, 500μs 25Hz) against each other and against control stimulation. In this trial, when both parameters were combined, active taVNS produced significant decreases in HR during stimulation period compared to control stimulation. Upon individual parameter analysis, both parameters produced decreases in HR during the stimulation period, although the optimal parameter determined to activate parasympathetic decrease in HR compared to control was 500μs 10Hz. These sequential trials suggest parametric-specific effects of taVNS on the parasympathetic nervous system possibly modulated by the ABVN and vagal pathway.

Develop and optimize taVNS/fMRI method

We developed a non-ferromagnetic MRI-compatible system that can safely and reliably stimulate the ABVN through the tragus and earlobe in order to conduct taVNS in the magnetic field of the fMRI scanner. As described in Chapter 4 were the three roadblocks (heating of electrodes, induced currents, MRI artifact) were surmounted using a variety of electronic and manufacturing achievements. Electrodes were fabricated out of 3D printed plastic and Ag/AgCl materials; 5kΩ resistors were placed in the copper stimulation wires (insulated with PVC); and a grounded RF filter attached to a patch panel in the wall adjacent to the scanner room was used to solve all these potential pain points.

After fabrication of all components, three separate quality control scans were conducted on MRI phantoms in order to confirm electrical stimulation in the MRI scanner did not disrupt the image acquisition and that heating was minimal. Subsequently two pilot scans were completed on healthy individuals to confirm scanning was feasible and safe. This development process created one of less than five MRI-compatible taVNS systems in the United States as of 2017.

Measure the direct brain effects of taVNS using BOLD fMRI

We conducted a two-visit trial in 17 healthy individuals in which participants received either active (tragus) or control (earlobe) taVNS stimulation (via our custom taVNS/fMRI system), while imaging the direct brain effects using fMRI. The control stimulation only group analysis revealed significant BOLD activation in exclusively the

primary somatosensory cortex while the active stimulation only group produced significant global cortical and subcortical BOLD activations. Of note, active taVNS activated the bilateral insula, bilateral frontal cortex, and similar somatosensory activation as control.

The main contrast explored the overall BOLD effects of taVNS significantly greater than control (active>control, paired t-test), which revealed significant activations in the bilateral anterior cingulate, bilateral cerebellum, left frontal cortex, and right caudate. These findings suggest taVNS stimulates the afferent vagal pathway, supporting the hypothesis that the ABVN that innervates the ear most likely joins the main vagal bundle and enters the brain to affect similar neuroanatomical structures. These data are in line with the few taVNS/fMRI studies in the literature. Of notable difference, this study did not discover any brainstem or midbrain activations, as suggested by two of the prior trials. Further discussion regarding this topic can be found in the Discussion section of Chapter 4.

Limitations

Heart Rate Trials

There are three limitations to consider and improve upon from these heart rate trials. Firstly, one cannot be certain that stimulating the tragus or any part of the ear is directly stimulating the ABVN. There is one prior study in which dissections of the ear reveal the underlying nerves (81). It is revealed that only 45% of the dissections in their sample

contained ABVN innervation in the tragus and ear canal. This plausibly can introduce the high level of variance between individual effects of taVNS, especially at low stimulation parameters. It is nearly impossible to determine the nerve innervation in individual participants before conducting a trial, so this limitation may persist until noninvasive subcutaneous nerve mapping technology becomes available.

Secondly, there is a decrease in heart rate that is sustained in the control group. It is still unknown what causes this bradycardia. One hypothesis is that this earlobe-induced bradycardia could be due to "electrical bleed" from the earlobe to closely innervating ABVN projections. The distance between the earlobe and tragus is less than 3cm on average, and of all possible controls, earlobe stimulation is arguably the most stringent. Studies like this require these active control sites to definitively conclude the findings.

Lastly, perhaps heart rate is not the most effective surrogate marker of vagal activity in taVNS trials, especially given the bidirectional communication of the vagal nerve. It is difficult to determine whether the effects on HR are due to direct efferent projections to the heart (ear->heart) or whether these effects are relayed up (afferent pathway) to the brainstem and loop back down to target efferent targets.

fMRI Trial

A major limitation in the fMRI trial is that the stimulation time (60s) may not have been long enough to capture the BOLD signal response to stimulation in the brainstem. This

trial was only the sixth taVNS/fMRI exploring the afferent effects of ABVN stimulation (See Chapter 4, Table 1), and only two of the prior studies (89, 93) demonstrated brainstem effects that suggest ABVN stimulation enters the brain via the NTS in an identical manner as the cervical vagus nerve. One trial stimulated an "ON" period of 6 minutes, and another trial stimulated an "ON" period for 7 minutes. The remaining trials with short stimulation periods reveal no brain stem activity. It is difficult to say that it is strictly due to stimulation time, as imaging that region of the brain is extremely difficult to image.

Future Directions

Further Optimization of Parameters

These trials, although highly promising, are not conclusive as to whether a higher pulse width of 500μs or higher frequencies of 10-25Hz are the optimal stimulation parameters for taVNS. Although these results demonstrate biologic effects on heart rate and central effects of the vagus afferent projections measured by fMRI, further parametric optimization needs to be conducted.

These physiological trials were conducted in a laboratory setting with individuals laying supine and a resting HR. This was considered a conservative position and paradigm for exploring effects of a parasympathetic modulator. Future parametric explorations should be conducted in high stress situations in which the sympathetic nervous system is active and attempt to modulate it by activating the reciprocal parasympathetic nervous system.

This may yield larger effect sizes and possibly decrease the amount of response earlobe stimulation produces. Potential autonomic blockade with pharmacological agents could also be used to knock out the active taVNS potential effect.

Conducting parametric explorations of taVNS will be integral in moving this technology forward. Similar to a pulse-width study conducted at MUSC with cervical VNS/fMRI (146, 147), various pulse widths and frequencies can be conducted in the MRI scanner and capture the neurophysiological signature of the stimulation. These fMRI studies are time and cost intensive, so it would be prudent to conduct trials exploring taVNS on surrogate markers of vagus activation or EEG before going into the fMRI scanner, similar to how these sequential HR and fMRI trials were conducted.

Aside from pulse width and frequency, duty cycle must be explored. Given the BOLD signal changes observed in longer stimulation periods (>6 minutes), perhaps taVNS is not as powerful as cervically implanted VNS. All taVNS trials are following the parameter space of cervically implanted VNS described in the early 1990's (23, 24, 148), although the mechanism and fibers could be completely different when delivered through the ear. There are only disperse nerve projections in the ear rather than the large bundle of the vagus nerve and it is plausible that rather than mimicking the short "ON" period of therapeutic VNS, longer "ON" periods must be explored in future therapeutic trials to potentially deliver identical signals to the brainstem.

Perhaps rather than pulse width, frequency, or stimulation time being the issues, the strength of electrical stimulation may have been under-dosed, possibly limiting the robustness of the effects Our studies reveal at 200% PT, taVNS is painless, rating on average a 2.1 on a 1-10 scale. Aside from safety concerns which were appeased during preliminary trials, future studies should potentially explore increasing dosage to 3-400% PT in order to determine whether stimulation intensity matters, or maintaining a lower PT and administering bilateral taVNS to double the signal entering the brain. The field of taVNS is still very new and these parameters must be worked out in order to have an effective noninvasive modality.

taVNS Induced Plasticity & Therapeutic Potential

A budding area of VNS research is the area known as targeted plasticity (66), championed by a team of researchers at the University of Texas, Dallas. This team has demonstrated that stimulating the cervical vagus nerve in conjunction with various therapies produces significant cortical reorganization properties as measured by single channel electrode recordings in rodents (70). Most notably, this pairing of VNS with specific audio tones has been shown to treat and reverse tinnitus (65, 68) and paired with rehabilitation paradigms to restore motor behavior in stroke (69, 149). These paradigms have been successfully translated from animal models to human clinical trials (71-73) and It is conceivable that these targeted plasticity findings can feasibly and easily be translatable with taVNS without the cost or risk of surgical implantation.

Pairing taVNS with specific therapy has yet to emerge as an area of study. There are two open label, small sample trials which attempted taVNS paired with tones to treat tinnitus (87, 150) with minimal effects. These findings could be more promising if the proper stimulation parameter optimization is conducted beforehand. Aside from paring taVNS with tones, there have been no other paired taVNS trials and this area needs to be explored as a potential treatment modality.

taVNS can be used in a similar manner as conventional VNS where pulses are delivered constantly for a long period of time (months to years). It is still unknown what the optimal dosing paradigm would be, as cervical VNS cycles between on and off periods constantly for many years until the battery needs to be replaced. Suggested daily use sessions on the scale of hours would likely be a starting point of therapeutic delivery. Many of the animal and human studies exploring the use of implantable VNS for central and peripheral disorders described in Chapter 1, such as epilepsy, depression, obesity, stroke, and heart disease could be explored with taVNS and easily translated to humans (98, 151-155). There have been some early trials exploring the use of various parameters of taVNS for the treatment of depression (88, 156, 157), autism (158) tinnitus (85) and pain (159-161) all revealing small effect sizes and mixed results.

The main problem encountered with taVNS as a take home therapy is the lack of practicality as a long term therapeutic modality. The majority of patients that will respond to cervically implanted VNS therapy for epilepsy or depression do so 12 months post-

implantation (162). This delayed response would require daily use of a taVNS device for multiple sessions per day, over a period of at least one year while maintaining patient compliance. More realistically, taVNS will most likely be used as an intermediary device that will predict response, guiding which individuals would make good candidates for surgical implantation rather than used as a long term therapeutic modality.

This body of work aims to take the first step in optimizing taVNS. For taVNS to become a possible future noninvasive therapeutic device in the future, further development is needed before rushing to clinical trials. Using physiological measures along with functional neuroimaging trials such as fMRI BOLD to determine optimal stimulation parameters will lead to more effective, noninvasive treatments for a variety of neuropsychiatric and peripheral disorders.

REFERENCES

1. Monkhouse S. Cranial nerves: functional anatomy: Cambridge University Press; 2005.
2. Gray H. Anatomy of the human body: Lea & Febiger; 1918.
3. Tracey KJ. Physiology and immunology of the cholinergic antiinflammatory pathway. The Journal of clinical investigation. 2007;117(2):289-96.
4. Foley JO, DuBois FS. Quantitative studies of the vagus nerve in the cat. I. The ratio of sensory to motor fibers. Journal of Comparative neurology. 1937;67(1):49-67.
5. Smith G, Jerome C, Cushin B, Eterno R, Simansky K. Abdominal vagotomy blocks the satiety effect of cholecystokinin in the rat. Science. 1981;213(4511):1036-7.
6. Smith GP, Jerome C, Norgren R. Afferent axons in abdominal vagus mediate satiety effect of cholecystokinin in rats. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 1985;249(5):R638-R41.
7. Pagani M, Lombardi F, Guzzetti S, Rimoldi O, Furlan R, Pizzinelli P, et al. Power spectral analysis of heart rate and arterial pressure variabilities as a marker of sympatho-vagal interaction in man and conscious dog. Circulation research. 1986;59(2):178-93.
8. Pomeranz B, Macaulay R, Caudill MA, Kutz I, Adam D, Gordon D, et al. Assessment of autonomic function in humans by heart rate spectral analysis. American Journal of Physiology-Heart and Circulatory Physiology. 1985;248(1):H151-H3.
9. Buijs RM, Chun SJ, Niijima A, Romijn HJ, Nagai K. Parasympathetic and sympathetic control of the pancreas: a role for the suprachiasmatic nucleus and other hypothalamic centers that are involved in the regulation of food intake. Journal of comparative neurology. 2001;431(4):405-23.
10. Benarroch EE, editor The central autonomic network: functional organization, dysfunction, and perspective. Mayo Clinic Proceedings; 1993: Elsevier.
11. Kalia M, Sullivan JM. Brainstem projections of sensory and motor components of the vagus nerve in the rat. Journal of Comparative Neurology. 1982;211(3):248-64.
12. Van Bockstaele EJ, Peoples J, Valentino RJ. Anatomic basis for differential regulation of the rostrolateral peri–locus coeruleus region by limbic afferents. Biological psychiatry. 1999;46(10):1352-63.
13. Krahl SE, Clark KB, Smith DC, Browning RA. Locus coeruleus lesions suppress the seizure‐attenuating effects of vagus nerve stimulation. Epilepsia. 1998;39(7):709-14.
14. Cheyuo C, Jacob A, Wu R, Zhou M, Coppa GF, Wang P. The parasympathetic nervous system in the quest for stroke therapeutics. Journal of Cerebral Blood Flow & Metabolism. 2011;31(5):1187-95.
15. Dong J, Debonnel G. Enhancement of the function of rat serotonin and norepinephrine neurons by sustained vagus nerve stimulation. Journal of psychiatry & neuroscience: JPN. 2009;34(4):272.
16. Loewi O. Über humorale übertragbarkeit der herznervenwirkung. Pflüger's Archiv für die gesamte Physiologie des Menschen und der Tiere. 1921;189(1):239-42.

17. Bailey P, Bremer F. A sensory cortical representation of the vagus nerve: with a note on the effects of low blood pressure on the cortical electrogram. Journal of Neurophysiology. 1938;1(5):405-12.
18. MacLean PD. The triune brain in evolution: Role in paleocerebral functions: Springer Science & Business Media; 1990.
19. Dell P, Olson R. * PROJECTIONS SECONDAIRES MESENCEPHALIQUES, DIENCEPHALIQUES ET AMYGDALIENNES DES AFFERENCES VISCERALES VAGALES. Comptes rendus des seances de la Societe de biologie et de ses filiales. 1951;145(13-1):1088-91.
20. Radna RJ, MacLean PD. Vagal elicitation of respiratory-type and other unit responses in basal limbic structures of squirrel monkeys. Brain Research. 1981;213(1):45-61.
21. Radna RJ, MacLean PD. Vagal elicitation of respiratory-type and other unit responses in striopallidum of squirrel monkeys. Brain research. 1981;213(1):29-44.
22. Zabara J, editor Time course of seizure control to brief, repetitive stimuli. Epilepsia; 1985: LIPPINCOTT-RAVEN PUBL 227 EAST WASHINGTON SQ, PHILADELPHIA, PA 19106.
23. Zabara J. Peripheral control of hypersynchronous discharge in epilepsy. Electroencephalography and Clinical Neurophysiology. 1985;61(3):S162.
24. Zabara J. Inhibition of experimental seizures in canines by repetitive vagal stimulation. Epilepsia. 1992;33(6):1005-12.
25. Corning JL. CONSIDERATIONS ON THE PATHOLOY AND THERAPEUTICS OF EPILEPSY. The Journal of Nervous and Mental Disease. 1883;10(2):243-8.
26. Corning JL. Electrization of the sympathetic and pneumogastric nerves, with simultaneous bilateral compression of the carotids1884.
27. Penry JK, Dean JC. Prevention of intractable partial seizures by intermittent vagal stimulation in humans: preliminary results. Epilepsia. 1990;31(s2):S40-S3.
28. Uthman B, Wilder B, Penry J, Dean C, Ramsay R, Reid S, et al. Treatment of epilepsy by stimulation of the vagus nerve. Neurology. 1993;43(7):1338-.
29. Rutecki P. Anatomical, physiological, and theoretical basis for the antiepileptic effect of vagus nerve stimulation. Epilepsia. 1990;31(s2):S1-S6.
30. Ben‐Menachem E, Mañon‐Espaillat R, Ristanovic R, Wilder B, Stefan H, Mirza W, et al. Vagus nerve stimulation for treatment of partial seizures: 1. A controlled study of effect on seizures. Epilepsia. 1994;35(3):616-26.
31. George R, Salinsky M, Kuzniecky R, Rosenfeld W, Bergen D, Tarver W, et al. Vagus Nerve Stimulation for Treatment of Partial Seizures: 3. Long‐Term Follow‐Up on First 67 Patients Exiting a Controlled Study. Epilepsia. 1994;35(3):637-43.
32. DeGiorgio C, Schachter S, Handforth A, Salinsky M, Thompson J, Uthman B, et al. Prospective long‐term study of vagus nerve stimulation for the treatment of refractory seizures. Epilepsia. 2000;41(9):1195-200.
33. Sackeim HA, Rush AJ, George MS, Marangell LB, Husain MM, Nahas Z, et al. Vagus nerve stimulation (VNS™) for treatment-resistant depression: efficacy, side effects, and predictors of outcome. Neuropsychopharmacology. 2001;25(5):713-28.

34. Ranck JB. Which elements are excited in electrical stimulation of mammalian central nervous system: a review. Brain research. 1975;98(3):417-40.
35. Irnich W. The chronaxie time and its practical importance. Pacing and Clinical Electrophysiology. 1980;3(3):292-301.
36. Agnew WF, McCreery DB. Considerations for safety with chronically implanted nerve electrodes. Epilepsia. 1990;31(s2):S27-S32.
37. McCreery DB, Agnew WF, Yuen TG, Bullara L. Charge density and charge per phase as cofactors in neural injury induced by electrical stimulation. IEEE Transactions on Biomedical Engineering. 1990;37(10):996-1001.
38. Boudjada A, Hernandez O, Meinnel J, Mani M, Paulus W. 1,3,5-Triiodo-2,4,6-trimethylbenzene at 293 K. Acta Crystallogr C. 2001;57(Pt 9):1106-8.
39. Harden CL, Pulver MC, Ravdin LD, Nikolov B, Halper JP, Labar DR. A pilot study of mood in epilepsy patients treated with vagus nerve stimulation. Epilepsy & Behavior. 2000;1(2):93-9.
40. Rush AJ, George MS, Sackeim HA, Marangell LB, Husain MM, Giller C, et al. Vagus nerve stimulation (VNS) for treatment-resistant depressions: a multicenter study. Biological psychiatry. 2000;47(4):276-86.
41. Nahas Z, Marangell LB, Husain MM, Rush AJ, Sackeim HA, Lisanby SH, et al. Two-year outcome of vagus nerve stimulation (VNS) for treatment of major depressive episodes. The Journal of clinical psychiatry. 2005;66(9):1097-104.
42. Schlaepfer T, Frick C, Zobel A, Maier W, Heuser I, Bajbouj M, et al. Vagus nerve stimulation for depression: efficacy and safety in a European study. Psychological medicine. 2008;38(05):651-61.
43. Rush AJ, Marangell LB, Sackeim HA, George MS, Brannan SK, Davis SM, et al. Vagus nerve stimulation for treatment-resistant depression: a randomized, controlled acute phase trial. Biological psychiatry. 2005;58(5):347-54.
44. George MS, Rush AJ, Marangell LB, Sackeim HA, Brannan SK, Davis SM, et al. A one-year comparison of vagus nerve stimulation with treatment as usual for treatment-resistant depression. Biological psychiatry. 2005;58(5):364-73.
45. Garnett E, Nahmias C, Scheffel A, Firnau G, Upton A. Regional cerebral blood flow in man manipulated by direct vagal stimulation. Pacing and Clinical Electrophysiology. 1992;15(10):1579-80.
46. Henry TR, Bakay RA, Votaw JR, Pennell PB, Epstein CM, Faber TL, et al. Brain blood flow alterations induced by therapeutic vagus nerve stimulation in partial epilepsy: I. Acute effects at high and low levels of stimulation. Epilepsia. 1998;39(9):983-90.
47. Ogawa S, Lee T-M, Kay AR, Tank DW. Brain magnetic resonance imaging with contrast dependent on blood oxygenation. Proceedings of the National Academy of Sciences. 1990;87(24):9868-72.
48. Bohning DE, Lomarev MP, Denslow S, Nahas Z, Shastri A, George MS. Feasibility of vagus nerve stimulation–synchronized blood oxygenation level–dependent functional MRI. Investigative Radiology. 2001;36(8):470-9.
49. Lomarev M, Denslow S, Nahas Z, Chae J-H, George MS, Bohning DE. Vagus nerve stimulation (VNS) synchronized BOLD fMRI suggests that VNS in depressed

adults has frequency/dose dependent effects. Journal of psychiatric research. 2002;36(4):219-27.
50. Mu Q, Bohning DE, Nahas Z, Walker J, Anderson B, Johnson KA, et al. Acute vagus nerve stimulation using different pulse widths produces varying brain effects. Biological psychiatry. 2004;55(8):816-25.
51. Nahas Z, Teneback C, Chae J-H, Mu Q, Molnar C, Kozel FA, et al. Serial vagus nerve stimulation functional MRI in treatment-resistant depression. Neuropsychopharmacology. 2007;32(8):1649-60.
52. Liu WC, Mosier K, Kalnin A, Marks D. BOLD fMRI activation induced by vagus nerve stimulation in seizure patients. Journal of Neurology, Neurosurgery & Psychiatry. 2003;74(6):811-3.
53. Sucholeiki R, Alsaadi TM, Morris Iii GL, Ulmer JL, Biswal B, Mueller WM. fMRI in patients implanted with a vagal nerve stimulator. Seizure. 2002;11(3):157-62.
54. Narayanan JT, Watts R, Haddad N, Labar DR, Li PM, Filippi CG. Cerebral activation during vagus nerve stimulation: a functional MR study. Epilepsia. 2002;43(12):1509-14.
55. Sobocki J, Thor P, Uson J, Diaz-Guemes I, Lipinski M, Calles C, et al. Microchip vagal pacing reduces food intake and body mass. Hepato-gastroenterology. 2000;48(42):1783-7.
56. Gil K, Bugajski A, Thor P. Electrical vagus nerve stimulation decreases food consumption and weight gain in rats fed a high-fat diet. Journal of physiology and pharmacology. 2011;62(6):637.
57. Banni S, Carta G, Murru E, Cordeddu L, Giordano E, Marrosu F, et al. Vagus nerve stimulation reduces body weight and fat mass in rats. PLoS One. 2012;7(9):e44813.
58. Val-Laillet D, Biraben A, Randuineau G, Malbert C-H. Chronic vagus nerve stimulation decreased weight gain, food consumption and sweet craving in adult obese minipigs. Appetite. 2010;55(2):245-52.
59. Sarr MG, Billington CJ, Brancatisano R, Brancatisano A, Toouli J, Kow L, et al. The EMPOWER study: randomized, prospective, double-blind, multicenter trial of vagal blockade to induce weight loss in morbid obesity. Obesity surgery. 2012;22(11):1771-82.
60. Ikramuddin S, Blackstone RP, Brancatisano A, Toouli J, Shah SN, Wolfe BM, et al. Effect of reversible intermittent intra-abdominal vagal nerve blockade on morbid obesity: the ReCharge randomized clinical trial. Jama. 2014;312(9):915-22.
61. Hotchkiss RS, Coopersmith CM, McDunn JE, Ferguson TA. The sepsis seesaw: tilting toward immunosuppression. Nature medicine. 2009;15(5):496-7.
62. Borovikova LV, Ivanova S, Zhang M, Yang H, Botchkina GI, Watkins LR, et al. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature. 2000;405(6785):458-62.
63. Zhang Y, Popović ZB, Bibevski S, Fakhry I, Sica DA, Van Wagoner DR, et al. Chronic Vagus Nerve Stimulation Improves Autonomic Control and Attenuates Systemic Inflammation and Heart Failure Progression in a Canine High-Rate Pacing ModelCLINICAL PERSPECTIVE. Circulation: Heart Failure. 2009;2(6):692-9.

64. Norena A, Eggermont J. Changes in spontaneous neural activity immediately after an acoustic trauma: implications for neural correlates of tinnitus. Hearing research. 2003;183(1):137-53.
65. Engineer ND, Møller AR, Kilgard MP. Directing neural plasticity to understand and treat tinnitus. Hearing research. 2013;295:58-66.
66. Engineer ND, Riley JR, Seale JD, Vrana WA, Shetake JA, Sudanagunta SP, et al. Reversing pathological neural activity using targeted plasticity. Nature. 2011;470(7332):101-4.
67. Kilgard MP. Harnessing plasticity to understand learning and treat disease. Trends in neurosciences. 2012;35(12):715-22.
68. Shetake JA, Engineer ND, Vrana WA, Wolf JT, Kilgard MP. Pairing tone trains with vagus nerve stimulation induces temporal plasticity in auditory cortex. Experimental neurology. 2012;233(1):342-9.
69. Khodaparast N, Hays SA, Sloan AM, Fayyaz T, Hulsey DR, Rennaker RL, et al. Vagus nerve stimulation delivered during motor rehabilitation improves recovery in a rat model of stroke. Neurorehabilitation and neural repair. 2014:1545968314521006.
70. Porter BA, Khodaparast N, Fayyaz T, Cheung RJ, Ahmed SS, Vrana WA, et al. Repeatedly pairing vagus nerve stimulation with a movement reorganizes primary motor cortex. Cerebral Cortex. 2012;22(10):2365-74.
71. Dawson J, Pierce D, Dixit A, Kimberley TJ, Robertson M, Tarver B, et al. Safety, feasibility, and efficacy of vagus nerve stimulation paired with upper-limb rehabilitation after ischemic stroke. Stroke. 2016;47(1):143-50.
72. De Ridder D, Kilgard M, Engineer N, Vanneste S. Placebo-controlled vagus nerve stimulation paired with tones in a patient with refractory tinnitus: a case report. Otology & Neurotology. 2015;36(4):575-80.
73. De Ridder D, Vanneste S, Engineer ND, Kilgard MP. Safety and efficacy of vagus nerve stimulation paired with tones for the treatment of tinnitus: a case series. Neuromodulation: Technology at the Neural Interface. 2014;17(2):170-9.
74. Ay I, Lu J, Ay H, Sorensen AG. Vagus nerve stimulation reduces infarct size in rat focal cerebral ischemia. Neuroscience letters. 2009;459(3):147-51.
75. Ay I, Sorensen AG, Ay H. Vagus nerve stimulation reduces infarct size in rat focal cerebral ischemia: an unlikely role for cerebral blood flow. Brain research. 2011;1392:110-5.
76. Handforth A, DeGiorgio C, Schachter S, Uthman B, Naritoku D, Tecoma E, et al. Vagus nerve stimulation therapy for partial-onset seizures A randomized active-control trial. Neurology. 1998;51(1):48-55.
77. Rainero I, De Martino P, Rubino E, Vaula G, Gentile S, Pinessi L. Non-invasive Vagal Nerve Stimulation for the Treatment of Headache Attacks in Patients with Chronic Migraine and Medication-Overuse Headache (P1. 262). Neurology. 2014;82(10 Supplement):P1. 262.
78. Magis D, Gérard P, Schoenen J. Transcutaneous Vagus Nerve Stimulation (tVNS) for headache prophylaxis: initial experience. The journal of headache and pain. 2013;14(S1):P198.

79. Nesbitt A, Marin J, Tomkins E, Ruttledge M, Goadsby P. Non-invasive vagus nerve stimulation for the treatment of cluster headache: a case series. The journal of headache and pain. 2013;14(1):P231.
80. Nesbitt AD, Marin JC, Tompkins E, Ruttledge MH, Goadsby PJ. Initial use of a novel noninvasive vagus nerve stimulator for cluster headache treatment. Neurology. 2015;84(12):1249-53.
81. Peuker ET, Filler TJ. The nerve supply of the human auricle. Clinical Anatomy. 2002;15(1):35-7.
82. Ventureyra EC. Transcutaneous vagus nerve stimulation for partial onset seizure therapy. Child's Nervous System. 2000;16(2):101-2.
83. Capone F, Assenza G, Di Pino G, Musumeci G, Ranieri F, Florio L, et al. The effect of transcutaneous vagus nerve stimulation on cortical excitability. J Neural Transm (Vienna). 2015;122(5):679-85.
84. Kreuzer PM, Landgrebe M, Husser O, Resch M, Schecklmann M, Geisreiter F, et al. Transcutaneous vagus nerve stimulation: retrospective assessment of cardiac safety in a pilot study. Front Psychiatry. 2012;3:70.
85. Kreuzer PM, Landgrebe M, Resch M, Husser O, Schecklmann M, Geisreiter F, et al. Feasibility, safety and efficacy of transcutaneous vagus nerve stimulation in chronic tinnitus: an open pilot study. Brain stimulation. 2014;7(5):740-7.
86. Clancy JA, Mary DA, Witte KK, Greenwood JP, Deuchars SA, Deuchars J. Non-invasive vagus nerve stimulation in healthy humans reduces sympathetic nerve activity. Brain stimulation. 2014;7(6):871-7.
87. Lehtimaki J, Hyvarinen P, Ylikoski M, Bergholm M, Makela JP, Aarnisalo A, et al. Transcutaneous vagus nerve stimulation in tinnitus: a pilot study. Acta Otolaryngol. 2013;133(4):378-82.
88. Hein E, Nowak M, Kiess O, Biermann T, Bayerlein K, Kornhuber J, et al. Auricular transcutaneous electrical nerve stimulation in depressed patients: a randomized controlled pilot study. Journal of Neural Transmission. 2013;120(5):821-7.
89. Yakunina N, Kim SS, Nam EC. Optimization of Transcutaneous Vagus Nerve Stimulation Using Functional MRI. Neuromodulation: Technology at the Neural Interface. 2016.
90. Dietrich S, Smith J, Scherzinger C, Hofmann-Preiß K, Freitag T, Eisenkolb A, et al. A novel transcutaneous vagus nerve stimulation leads to brainstem and cerebral activations measured by functional MRI/Funktionelle Magnetresonanztomographie zeigt Aktivierungen des Hirnstamms und weiterer zerebraler Strukturen unter transkutaner Vagusnervstimulation. Biomedizinische Technik/Biomedical Engineering. 2008;53(3):104-11.
91. Kraus T, Hösl K, Kiess O, Schanze A, Kornhuber J, Forster C. BOLD fMRI deactivation of limbic and temporal brain structures and mood enhancing effect by transcutaneous vagus nerve stimulation. Journal of neural transmission. 2007;114(11):1485-93.
92. Kraus T, Kiess O, Hösl K, Terekhin P, Kornhuber J, Forster C. CNS BOLD fMRI effects of sham-controlled transcutaneous electrical nerve stimulation in the left outer auditory canal–a pilot study. Brain stimulation. 2013;6(5):798-804.

93. Frangos E, Ellrich J, Komisaruk BR. Non-invasive access to the vagus nerve central projections via electrical stimulation of the external ear: fMRI evidence in humans. Brain stimulation. 2015;8(3):624-36.
94. Frangos E, Komisaruk BR. Access to Vagal Projections via Cutaneous Electrical Stimulation of the Neck: fMRI Evidence in Healthy Humans. Brain Stimulation. 2017;10(1):19-27.
95. Nilsson S. Autonomic nerve function in the vertebrates: Springer Science & Business Media; 2012.
96. Lim S, Anantharaman V, Teo W, Goh P, Tan A. Comparison of treatment of supraventricular tachycardia by Valsalva maneuver and carotid sinus massage. Annals of emergency medicine. 1998;31(1):30-5.
97. Schweitzer P, Teichholz LE. Carotid sinus massage. Its diagnostic and therapeutic value in arrhythmias. The American journal of medicine. 1985;78(4):645-54.
98. Premchand RK, Sharma K, Mittal S, Monteiro R, Dixit S, Libbus I, et al. Autonomic regulation therapy via left or right cervical vagus nerve stimulation in patients with chronic heart failure: results of the ANTHEM-HF trial. J Card Fail. 2014;20(11):808-16.
99. Holder LK, Wernicke JF, Tarver WB. Treatment of refractory partial seizures: preliminary results of a controlled study. Pacing Clin Electrophysiol. 1992;15(10 Pt 2):1557-71.
100. Ramsay RE, Uthman BM, Augustinsson LE, Upton AR, Naritoku D, Willis J, et al. Vagus nerve stimulation for treatment of partial seizures: 2. Safety, side effects, and tolerability. First International Vagus Nerve Stimulation Study Group. Epilepsia. 1994;35(3):627-36.
101. Setty AB, Vaughn BV, Quint SR, Robertson KR, Messenheimer JA. Heart period variability during vagal nerve stimulation. Seizure. 1998;7(3):213-7.
102. Frei MG, Osorio I. Left vagus nerve stimulation with the neurocybernetic prosthesis has complex effects on heart rate and on its variability in humans. Epilepsia. 2001;42(8):1007-16.
103. Lang J. Auris externa—Außenohr. Klinische Anatomie des Ohres: Springer; 1992. p. 1-30.
104. Fallgatter A, Neuhauser B, Herrmann M, Ehlis A-C, Wagener A, Scheuerpflug P, et al. Far field potentials from the brain stem after transcutaneous vagus nerve stimulation. Journal of neural transmission. 2003;110(12):1437-43.
105. Fallgatter AJ, Ehlis A-C, Ringel TM, Herrmann MJ. Age effect on far field potentials from the brain stem after transcutaneous vagus nerve stimulation. International journal of psychophysiology. 2005;56(1):37-43.
106. Polak T, Markulin F, Ehlis A-C, Langer JB, Ringel TM, Fallgatter AJ. Far field potentials from brain stem after transcutaneous vagus nerve stimulation: optimization of stimulation and recording parameters. Journal of neural transmission. 2009;116(10):1237-42.
107. Wang S-M, Peloquin C, Kain ZN. The use of auricular acupuncture to reduce preoperative anxiety. Anesthesia & Analgesia. 2001;93(5):1178-80.

108. Greif R, Laciny S, Mokhtarani M, Doufas AG, Bakhshandeh M, Dorfer L, et al. Transcutaneous electrical stimulation of an auricular acupuncture point decreases anesthetic requirement. The Journal of the American Society of Anesthesiologists. 2002;96(2):306-12.
109. Cobb JL, Santer RM. Electrophysiology of cardiac function in teleosts: cholinergically mediated inhibition and rebound excitation. J Physiol. 1973;230(3):561-73.
110. Koizumi K, Kollai M. Control of reciprocal and non-reciprocal action of vagal and sympathetic efferents: study of centrally induced reactions. J Auton Nerv Syst. 1981;3(2-4):483-501.
111. Paton JF, Boscan P, Pickering AE, Nalivaiko E. The yin and yang of cardiac autonomic control: vago-sympathetic interactions revisited. Brain Res Brain Res Rev. 2005;49(3):555-65.
112. Bauer S, Baier H, Baumgartner C, Bohlmann K, Fauser S, Graf W, et al. Transcutaneous vagus nerve stimulation (tVNS) for treatment of drug-resistant epilepsy: a randomized, double-blind clinical trial (cMPsE02). Brain stimulation. 2016;9(3):356-63.
113. Laqua R, Lotze M, Leutzow B, Usichenko T. fMRI evidence for a reduction in affective processing of thermal pain in responders of transcutaneous vagal nerve stimulation (TVNS). Clinical Neurophysiology. 2016;127(3):e9.
114. Faraday M. Experimental researches in electricity. Philosophical transactions of the Royal Society of London. 1832;122:125-62.
115. George MS. New methods of minimally invasive brain modulation as therapies in psychiatry: TMS, MST, VNS and DBS. Zhonghua Yi Xue Za Zhi (Taipei). 2002;65(8):349-60.
116. Konings MK, Bartels LW, Smits HF, Bakker CJ. Heating around intravascular guidewires by resonating RF waves. Journal of Magnetic Resonance Imaging. 2000;12(1):79-85.
117. Nitz WR, Oppelt A, Renz W, Manke C, Lenhart M, Link J. On the heating of linear conductive structures as guide wires and catheters in interventional MRI. Journal of Magnetic Resonance Imaging. 2001;13(1):105-14.
118. Pictet J, Meuli R, Wicky S, van der Klink JJ. Radiofrequency heating effects around resonant lengths of wire in MRI. Physics in medicine and biology. 2002;47(16):2973.
119. Park S, Kamondetdacha R, Amjad A, Nyenhuis J. MRI safety: RF-induced heating near straight wires. IEEE transactions on magnetics. 2005;41(10):4197-9.
120. Min HK, Hwang SC, Marsh MP, Kim I, Knight E, Striemer B, et al. Deep brain stimulation induces BOLD activation in motor and non-motor networks: an fMRI comparison study of STN and EN/GPi DBS in large animals. Neuroimage. 2012;63(3):1408-20.
121. Maldjian JA, Laurienti PJ, Kraft RA, Burdette JH. An automated method for neuroanatomic and cytoarchitectonic atlas-based interrogation of fMRI data sets. Neuroimage. 2003;19(3):1233-9.

122. Penfield W, Boldrey E. Somatic motor and sensory representation in the cerebral cortex of man as studied by electrical stimulation. Brain: A journal of neurology. 1937.
123. Nihashi T, Kakigi R, Kawakami O, Hoshiyama M, Itomi K, Nakanishi H, et al. Representation of the ear in human primary somatosensory cortex. Neuroimage. 2001;13(2):295-304.
124. Nihashi T, Kakigi R, Okada T, Sadato N, Kashikura K, Kajita Y, et al. Functional magnetic resonance imaging evidence for a representation of the ear in human primary somatosensory cortex: comparison with magnetoencephalography study. Neuroimage. 2002;17(3):1217-26.
125. Lulic D, Ahmadian A, Baaj AA, Benbadis SR, Vale FL. Vagus nerve stimulation. Neurosurg Focus. 2009;27(3):E5.
126. Valdés-Cruz A, Magdaleno-Madrigal VM, Martınez-Vargas D, Fernández-Mas R, Almazán-Alvarado S, Martınez A, et al. Chronic stimulation of the cat vagus nerve: effect on sleep and behavior. Progress in Neuro-Psychopharmacology and Biological Psychiatry. 2002;26(1):113-8.
127. Groves DA, Brown VJ. Vagal nerve stimulation: a review of its applications and potential mechanisms that mediate its clinical effects. Neuroscience & Biobehavioral Reviews. 2005;29(3):493-500.
128. Bush G, Luu P, Posner MI. Cognitive and emotional influences in anterior cingulate cortex. Trends in cognitive sciences. 2000;4(6):215-22.
129. MacDonald AW, Cohen JD, Stenger VA, Carter CS. Dissociating the role of the dorsolateral prefrontal and anterior cingulate cortex in cognitive control. Science. 2000;288(5472):1835-8.
130. Auer DP, Pütz B, Kraft E, Lipinski B, Schill J, Holsboer F. Reduced glutamate in the anterior cingulate cortex in depression: an in vivo proton magnetic resonance spectroscopy study. Biological psychiatry. 2000;47(4):305-13.
131. Rosenberg DR, Mirza Y, Russell A, Tang J, Smith JM, Banerjee SP, et al. Reduced anterior cingulate glutamatergic concentrations in childhood OCD and major depression versus healthy controls. Journal of the American Academy of Child & Adolescent Psychiatry. 2004;43(9):1146-53.
132. Rosenberg DR, MacMaster FP, Mirza Y, Smith JM, Easter PC, Banerjee SP, et al. Reduced anterior cingulate glutamate in pediatric major depression: a magnetic resonance spectroscopy study. Biological psychiatry. 2005;58(9):700-4.
133. Cotter D, Mackay D, Landau S, Kerwin R, Everall I. Reduced glial cell density and neuronal size in the anterior cingulate cortex in major depressive disorder. Archives of general psychiatry. 2001;58(6):545-53.
134. Cotter DR, Pariante CM, Everall IP. Glial cell abnormalities in major psychiatric disorders: the evidence and implications. Brain research bulletin. 2001;55(5):585-95.
135. Pizzagalli D, Pascual-Marqui RD, Nitschke JB, Oakes TR, Larson CL, Abercrombie HC, et al. Anterior cingulate activity as a predictor of degree of treatment response in major depression: evidence from brain electrical tomography analysis. American Journal of Psychiatry. 2001;158(3):405-15.

136. Mayberg HS, Brannan SK, Mahurin RK, Jerabek PA, Brickman JS, Tekell JL, et al. Cingulate function in depression: a potential predictor of treatment response. Neuroreport. 1997;8(4):1057-61.
137. Baxter LR, Schwartz JM, Phelps ME, Mazziotta JC, Guze BH, Selin CE, et al. Reduction of prefrontal cortex glucose metabolism common to three types of depression. Archives of general psychiatry. 1989;46(3):243-50.
138. Bench CJ, Friston KJ, Brown RG, Scott LC, Frackowiak RS, Dolan RJ. The anatomy of melancholia–focal abnormalities of cerebral blood flow in major depression. Psychological medicine. 1992;22(03):607-15.
139. George MS, Wassermann EM, Williams WA, Callahan A, Ketter TA, Basser P, et al. Daily repetitive transcranial magnetic stimulation (rTMS) improves mood in depression. Neuroreport. 1995;6(14):1853-6.
140. George MS, Wassermann EM, Kimbrell TA, Little JT, Williams WE, Danielson AL, et al. Mood improvement following daily left prefrontal repetitive transcranial magnetic stimulation in patients with depression: a placebo-controlled crossover trial. Am J Psychiatry. 1997;154(12):1752-6.
141. George MS, Nahas Z, Molloy M, Speer AM, Oliver NC, Li XB, et al. A controlled trial of daily left prefrontal cortex TMS for treating depression. Biol Psychiatry. 2000;48(10):962-70.
142. George MS. Transcranial magnetic stimulation for the treatment of depression. Expert Rev Neurother. 2010;10(11):1761-72.
143. Badran BW, Glusman CE, Badran AW, Austelle C, DeVries WH, Borckardt JJ, et al., editors. Development, Safety, and Tolerability of Transcutaneous Auricular Vagus Nerve Stimulation (taVNS), a Novel Form of Noninvasive Vagus Nerve Stimulation. NEUROPSYCHOPHARMACOLOGY; 2015: NATURE PUBLISHING GROUP MACMILLAN BUILDING, 4 CRINAN ST, LONDON N1 9XW, ENGLAND.
144. Badran B, Glusman C, Badran A, Austelle C, DeVries W, Borckhardt J, et al. The physiological and neurobiological effects of transcutaneous auricular vagus nerve stimulation (taVNS). Brain Stimulation: Basic, Translational, and Clinical Research in Neuromodulation. 2017;10(2):378.
145. Kreuzer PM, Landgrebe M, Resch M, Husser O, Schecklmann M, Geisreiter F, et al. Feasibility, safety and efficacy of transcutaneous vagus nerve stimulation in chronic tinnitus: an open pilot study. Brain Stimul. 2014;7(5):740-7.
146. Lomarev M, Denslow S, Nahas Z, Chae JH, George MS, Bohning DE. Vagus nerve stimulation (VNS) synchronized BOLD fMRI suggests that VNS in depressed adults has frequency/dose dependent effects. J Psychiatr Res. 2002;36(4):219-27.
147. Nahas Z, Teneback C, Chae JH, Mu Q, Molnar C, Kozel FA, et al. Serial vagus nerve stimulation functional MRI in treatment-resistant depression. Neuropsychopharmacology. 2007;32(8):1649-60.
148. George MS, Rush AJ, Sackeim HA, Marangell LB. Vagus nerve stimulation (VNS): utility in neuropsychiatric disorders. Int J Neuropsychopharmacol. 2003;6(1):73-83.

149. Khodaparast N, Hays SA, Sloan AM, Hulsey DR, Ruiz A, Pantoja M, et al. Vagus nerve stimulation during rehabilitative training improves forelimb strength following ischemic stroke. Neurobiology of disease. 2013;60:80-8.
150. Shim HJ, Kwak MY, An Y-H, Kim DH, Kim YJ, Kim HJ. Feasibility and safety of transcutaneous vagus nerve stimulation paired with notched music therapy for the treatment of chronic tinnitus. Journal of audiology & otology. 2015;19(3):159-67.
151. Bodenlos JS, Kose S, Borckardt JJ, Nahas Z, Shaw, O'Neil PM, et al. Vagus nerve stimulation and emotional responses to food among depressed patients. J Diabetes Sci Technol. 2007;1(5):771-9.
152. Bodenlos JS, Kose S, Borckardt JJ, Nahas Z, Shaw D, O'Neil PM, et al. Vagus nerve stimulation acutely alters food craving in adults with depression. Appetite. 2007;48(2):145-53.
153. Borckardt JJ, Kozel FA, Anderson B, Walker A, George MS. Vagus nerve stimulation affects pain perception in depressed adults. Pain Res Manag. 2005;10(1):9-14.
154. George MS, Sackeim HA, Marangell LB, Husain MM, Nahas Z, Lisanby SH, et al. Vagus nerve stimulation. A potential therapy for resistant depression? Psychiatr Clin North Am. 2000;23(4):757-83.
155. George MS, Ward HE, Jr., Ninan PT, Pollack M, Nahas Z, Anderson B, et al. A pilot study of vagus nerve stimulation (VNS) for treatment-resistant anxiety disorders. Brain Stimul. 2008;1(2):112-21.
156. Trevizol AP, Taiar I, Barros MD, Liquidatto B, Cordeiro Q, Shiozawa P. Transcutaneous vagus nerve stimulation (tVNS) protocol for the treatment of major depressive disorder: A case study assessing the auricular branch of the vagus nerve. Epilepsy & Behavior. 2015;53:166-7.
157. Zhang Z-X, Li C-R, Rong P-J, Bai Z-H, Hill AM, Jing Q, et al. Efficacy and Safety of Auricular Therapy for Depression. Medical Acupuncture. 2016;28(5):256-67.
158. Jin Y, Kong J. Transcutaneous vagus nerve stimulation: a promising method for treatment of autism spectrum disorders. Frontiers in Neuroscience. 2016;10.
159. Hacker H, Möller N, Gürtlerz N, Hahnenkamp K, Usichenko T. P044 The effect of transcutaneous vagal nerve stimulation on experimental heat pain–A crossover blinded sham-and placebo-controlled investigation. Clinical Neurophysiology. 2017;128(3):e28-e9.
160. Laqua R, Leutzow B, Wendt M, Usichenko T. Transcutaneous vagal nerve stimulation may elicit anti-and pro-nociceptive effects under experimentally-induced pain—A crossover placebo-controlled investigation. Autonomic Neuroscience. 2014;185:120-2.
161. Usichenko T, Laqua R, Leutzow B, Lotze M. Preliminary findings of cerebral responses on transcutaneous vagal nerve stimulation on experimental heat pain. Brain imaging and behavior. 2016:1-8.
162. Sackeim HA, Rush AJ, George MS, Marangell LB, Husain MM, Nahas Z, et al. Vagus nerve stimulation (VNS) for treatment-resistant depression: efficacy, side effects, and predictors of outcome. Neuropsychopharmacology. 2001;25(5):713-28.