The effects of non-invasive cranial nerve neuromodulation on the autonomic nervous system in human research participants

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Intellectual Property Declaration

The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

Dr Jennifer Clancy provided experimental recording data for 6 of the 8 heart patients included in Chapter 5. However, all data analysis was performed by the candidate.

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Abstract

The normal ageing process is underpinned by progressive autonomic nervous system dysfunction, which can lead to the development of conditions such as heart failure. In recent years there has been substantial interest in the therapeutic potential of electrical neuromodulatory therapies such as vagus nerve stimulation. However, vagus nerve stimulation is an invasive technique requiring the use of a surgical procedure and non-invasive methods could have greater clinical utility. This thesis investigated the cardiovascular autonomic effects of two non-invasive cranial nerve neuromodulatory techniques in humans: transcutaneous vagus nerve stimulation (tVNS) and non-invasive trigeminal nerve stimulation (TNS).

tVNS applied to the tragus of the ear to stimulate the auricular branch of the vagus nerve (ABVN) was found to increase heart rate variability and baroreflex sensitivity in healthy older participants (n = 18) and patients with heart failure (n = 8). Microneurography in aged volunteers (n = 5) showed this change in autonomic function may have been partly due to a reduction in muscle sympathetic nerve activity (MSNA). However a validation study of stimulation at different ear sites detected a similar change in HRV elicited by helix stimulation in a subset of healthy volunteers (n = 12), suggesting a role for the auriculotemporal (trigeminal) nerve, which also innervates the tragus, in the observed autonomic effects.

TNS applied to supraorbital region in healthy participants (n = 26) found no evidence however of changes in HRV or BRS, suggesting that this technique has a limited effect on cardiovascular autonomic function. Further clinical studies are needed to determine if tVNS applied to the tragus could be an effective adjunctive therapy for disorders where autonomic dysregulation is present. In addition, the precise mechanisms behind the autonomic effects of tVNS should be further investigated in animal studies in order to optimise the technique and inform future translational studies.

Publications

Papers

Murray AR., Atkinson L., Mahadi, MK., Deuchars, SA., Deuchars J. (2016) The strange case of the ear and the heart: the auricular vagus nerve and its influence on cardiac control. Autonomic Neuroscience: Basic and Clinical. 199 48 – 53.

Abstracts

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List of Abbreviations

ABVN	Auricular branch of the vagus nerve
ACE	Angiotensin converting enzyme
ACh	Acetylcholine
AF	Atrial fibrillation
ANS	Autonomic nervous system
ANOVA	Analysis of variance
ATN	Auriculotemporal nerve
BMI	Body mass index
BOLD	Blood oxygenation level dependent
BP	Blood pressure
BPM	Beats per minute
BRS	Baroreflex sensitivity
ChAT	Cholinergic acetyl transferase
CHF	Chronic heart failure
CN	Cranial nerve
CNS	Central nervous system
CRP	C-reactive protein
CTB	Cholera toxin B
CVLM	Caudal ventrolateral medulla
DVN	Dorsal vagal nuclei
ECG	Electrocardiogram
EEG	Electroencephalographic
FDA	Federal Drug Administration
FFT	Fast Fourier transform

fMRI	Functional magnetic resonance imaging
GABA	Gamma-aminobutyric acid
GAN	Great auricular nerve
GI	Gastrointestinal
GVA	General visceral afferent
GVE	General visceral efferent
HF	High frequency
HR	Heart rate
HRP	Horseradish peroxidase
HRT	Hormone replacement therapy
HRV	Heart rate variability
HSP	Heat shock protein
HTN	Hypertension
H-TNS	High frequency trigeminal nerve stimulation
IL-6	Interleukin-6
IML	Intermediolateral nucleus
LF	Low frequency
LLTS	Low level tragus stimulation
L-TNS	Low frequency trigeminal nerve stimulation
LV	Left ventricular
LVEDP	Left ventricular end-diastolic pressure
LVEF	Left ventricular ejection fraction
LVESVI	Left-ventricular end-systolic volume index
MDD	Major depressive disorder
MI	Myocardial infarction
MSNA	Muscle sympathetic nerve activity

MVC	Maximum voluntary contraction
NA	Nucleus ambiguus
N-N	Normal to normal heartbeat interval
NO	Nitric oxide
NTS	Nucleus tractus solitarius
nVNS	Non-invasive vagus nerve stimulation
NYHA	New York Heart Association
OSA	Obstructive sleep apnoea
Pa5	Paratrigeminal nucleus
PBS	Phosphate buffer saline
pNN50	Number of pairs of adjacent NN-intervals differing by > 50 ms
QoL	Quality of Life
RA	Rheumatoid arthritis
RHR	Resting heart rate
RMSSD	Square root of the sum of squares of differences between adjacent normal to normal heart beat intervals
RSA	Respiratory sinus arrhythmia
RVLM	Rostral ventrolateral medulla
SDANN	Standard deviation of the averages of normal to normal intervals in a 5 minute data recording
SDNN	Standard deviation of normal to normal intervals
SSNA	Skin sympathetic nerve activity
TENS	Transcutaneous electrical nerve stimulation
TNF-alpha	Tumour necrosis factor alpha
TNS	Trigeminal nerve stimulation

tVNS	Transcutaneous vagus nerve stimulation
V_1	Ophthalmic division of trigeminal nerve
V_2	Maxillary division of trigeminal nerve
V_3	Mandibular division of trigeminal nerve
VF	Ventricular fibrillation
VNS	Vagus nerve stimulation
VSEP	Vagus somatosensory evoked potential
WHBP	Working heart brainstem preparation

Chapter 1

General Introduction

1.1 The autonomic nervous system

The autonomic nervous system (ANS; from the Greek *"auto"* meaning *"self"* and *"nomos"* meaning *"law"* or *"governance"*) regulates physiological processes without conscious input. The term "autonomic nervous system" was invented by the physiologist John Newport Langley, who divided the ANS into three principal components: the sympathetic nervous system (derived from the Greek *"sympathes"* or *"affected by like feelings"*), the parasympathetic nervous system (from the Greek adjective *"para"* meaning *"beside"*) and the enteric nervous system which is found in the gastrointestinal (GI) tract (Langley, 1921). The parasympathetic and sympathetic subdivisions form a complex network of central structures within the brain and spinal cord associated with ascending and descending spinal pathways as well as peripheral nerves. These peripheral nerves allow the central nervous system (CNS) to exert an effect on a target or end-organ via efferent neurons, with afferent neurons providing sensory input from the end-organ to the CNS. This allows the autonomic nervous system to maintain homeostasis and modulate visceral function.

The sympathetic and parasympathetic nervous systems provide dual input to a number of different visceral organs throughout the body including the heart, lungs and GI tract. Both divisions are tonically active under normal circumstances: activity in one will influence activity in the other, allowing for subtle modulation of visceral function via integrated responses. External stimuli can also exert a profound influence on ANS activity, leading to a shift in autonomic predominance whereby one division becomes more active over the other. Increased activation of the sympathetic nervous system is referred to as the "flight, fight or fright" response, whereby the presence of a physical or emotional stressor leads to rapid physiological changes such as increased heart rate and blood pressure (Karemaker, 2017). Meanwhile, a "rest and recovery" state is associated with increased parasympathetic predominance, an example of which would be the reduction in heart rate at rest via tonic inhibition at the sinoatrial node (Jose and Collison, 1970, Karemaker, 2017). However, the increase in heart rate observed during physical activity is in fact due to parasympathetic withdrawal, which is then enhanced further by

increased sympathetic activation (Fagraeus and Linnarsson, 1976). This complexity in the interaction between the actions of the parasympathetic and sympathetic subdivisions of the ANS, coupled with the ability to rapidly respond to physiological stress without conscious input, is vital for homeostasis and normal visceral function. Nevertheless, a chronic imbalance in sympathetic and parasympathetic activation can have a progressive damaging effect on the structure and function of organs such as the heart, leading to pathological conditions such as ischaemic heart disease and heart failure.

1.1.1 The sympathetic nervous system

The sympathetic nervous system has a complex neuroanatomy with extensive distribution throughout the body to sites as wide-ranging as the heart, GI tract, smooth muscle of blood vessels, erector pili muscle and sweat glands. In mammals, the cell bodies of sympathetic preganglionic neurones are located in the lateral horns of the spinal cord from the thoracic to the upper lumber spinal segments, with the majority in the intermediolateral (IML) cell column (Gilbey and Spyer, 1993). Myelinated sympathetic preganglionic axons exit the spinal cord at their segment of origin along with somatic motor fibres in the ventral nerve roots (Deuchars and K. Lall, 2015). The sympathetic preganglionic neurones then divert into the paravertebral ganglia of the sympathetic chain via white *rami communicantes* or connecting branches. At this point the sympathetic preganglionic neurone axons may synapse upon entering the chain with a sympathetic postganglionic neurone, or travel in a rostral or caudal direction in the sympathetic chain before synapsing with a postganglionic neurone (Gilbey and Spyer, 1993). The non-myelinated sympathetic postganglionic neurones exit the sympathetic chain via grey *rami communicantes* and project to target end-organs by travelling in the spinal nerves or along major blood vessels such as the common carotid arteries. Some sympathetic preganglionic neurones do not synapse within the sympathetic chain and instead exit the chain as sympathetic splanchnic nerves, which project to

prevertebral ganglia in the abdomen including the coeliac, superior mesenteric and inferior mesenteric ganglia (Figure 1.1). These preganglionic neurones then synapse at the prevertebral ganglia with postganglionic neurones. In addition, there are substantial preganglionic projections to the chromaffin cells in the medulla of the adrenal gland which are involved in the secretion of norepinephrine and epinephrine into the bloodstream.

Norepinephrine and acetylcholine are the integral neurotransmitters in the sympathetic nervous system. Acetylcholine (ACh) is released from preganglionic neurones into the synaptic cleft where it binds with nicotinic receptors on sympathetic postganglionic neurones, facilitating the activation of these postganglionic neurones. These in turn release norepinephrine, which binds to alpha and beta adrenergic receptors on the target end-organs. For this reason, postganglionic neurones are described as being adrenergic, while preganglionic neurones are cholinergic. Postganglionic neurones also provide cholinergic innervation to the sweat glands of the skin, where activation is elicited by the binding of acetylcholine to muscarinic receptors.

1.1.2 The parasympathetic nervous system

Activation of the parasympathetic nervous system is integral to a wide range of processes including digestion, salivation, urination and lacrimation. The parasympathetic nervous system is traditionally considered to be organised into a craniosacral outflow as the parasympathetic preganglionic neurones arise from various cranial nerve (CN) nuclei in the brainstem as well as S2 – S4 segments in the sacral spinal cord (Langley, 1921, McCorry, 2007). Cranial nerves which contain parasympathetic efferent fibres are the oculomotor nerve (CN III), the facial nerve (CN VII), the glossopharyngeal nerve (CN IX) and the vagus nerve (CN X). The nucleus ambiguus (NA) and the dorsal vagal nucleus (DVN) of the medulla contain the cell bodies of vagal preganglionic neurones.

There has been recent controversy over whether the sacral component of the parasympathetic nervous system should be reclassified as part of the sympathetic nervous system. A study by Espinosa-Medina et al. investigated

markers and transcription factors in developing cranial and spinal preganglionic neurones at different mouse embryonic stages, specifically days E11.5, E13.5 and E16.5 of embryological development (Espinosa-Medina et al., 2016). The expression of several transcription factors was shared by both thoracolumbar preganglionic neurones and sacral preganglionic neurones but not cranial preganglionic neurones. Langley's traditional model of the craniosacral parasympathetic outflow was based on observations of the functional effects of neurostimulation, whereas the proposed new model by Espinosa-Medina et al. is based on developmental observations. As the latter offers no explanation for the distinct functional characteristics of the sacral autonomic outflow and the thoracolumbar sympathetic outflow, Langley's model should continue to be supported. Moreover, Espinosa-Medina et al. offer no explanation as to why sacral preganglionic neurones do not enter the sympathetic chain.

Like sympathetic preganglionic neurones, parasympathetic preganglionic neurones are cholinergic. However, parasympathetic preganglionic axons are generally much longer than those of sympathetic preganglionic neurones, as parasympathetic ganglia are situated near to the end-organ. Parasympathetic preganglionic neurones then synapse with parasympathetic post-ganglionic neurones, which have much shorter axons and also use ACh as a neurotransmitter. While ACh from parasympathetic preganglionic neurones binds to nicotinic receptors, ACh from the postganglionic neurones binds to muscarinic receptors at the target end-organ, similar to the cholinergic innervation of the sweat glands by sympathetic postganglionic neurones.

1.1.3 The enteric nervous system

The enteric nervous system (ENS) receives limited modulatory input from the parasympathetic and sympathetic nervous systems but can otherwise be considered to be functionally independent from these divisions. This complex self-regulatory capacity is facilitated by approximately 200 – 600 million neurons which are organised into two neuronal plexi spanning the course of the GI tract (Furness et al., 2014). The myenteric or Auerbach's plexus is located in between the circular and longitudinal muscle layers and coordinates their activity to generate peristaltic contractions. It extends from the smooth muscle in the lower pharynx to the internal anal sphincter (Furness et al. 2014). The submucosal or Meissner's plexus regulates secretion and is found between the circular muscle and the inner mucosa of the small and large intestine. While the ENS can function without input from the central nervous system, under normal circumstances there is considerable signalling from the gut to the brain via vagal afferents. This signalling can include information about sensations of satiety and nausea from the stomach, although much of the transmitted information is not consciously registered (Rao and Gershon, 2016).

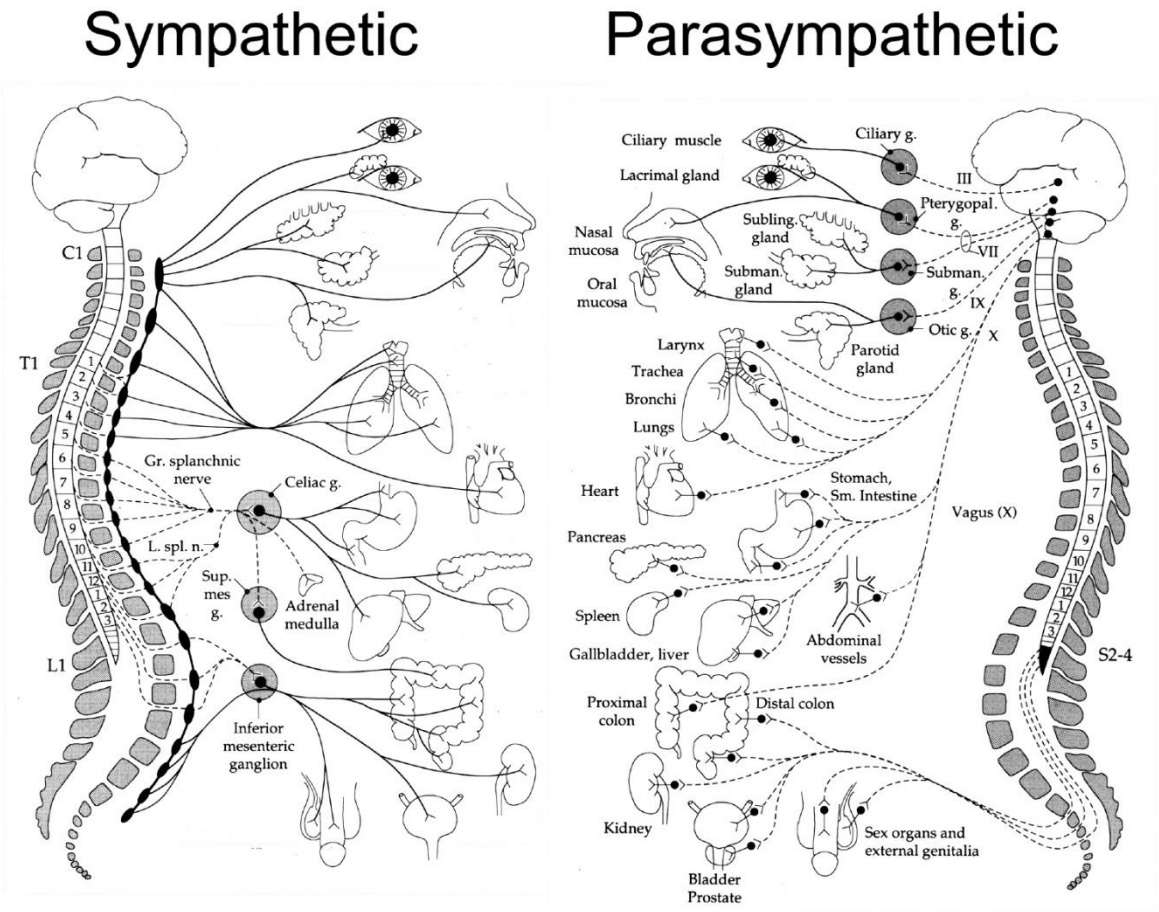


Figure 1.1 An overview of the sympathetic and parasympathetic divisions of the autonomic nervous system

Image taken from Karemaker et al. (2017).

1.1.4 Regulation of cardiovascular autonomic function

The brainstem medulla is of vital importance to the regulation of cardiovascular autonomic function as it is the site of major sympathetic and parasympathetic nuclei containing preganglionic cell bodies. These autonomic nuclei provide dynamic control of activity in the cardiovascular system in order to respond to changes in physiological demand. Visceral afferent information transmitted via the vagus and glossopharyngeal nerves is critical to the modulation of cardiovascular autonomic efferent outflow. In the first instance, cardiovascular afferent signals converge on the nucleus tractus solitarius (NTS) of the dorsomedial medulla.

The NTS is a major integration site for cardiovascular reflexes such as the arterial baroreceptor reflex, which is important for the regulation of arterial blood pressure (Dampney and Horiuchi, 2003). The arterial baroreflex is initiated by mechanosensitive afferent nerve endings in the aortic arch and carotid sinus which fire in response to the arterial wall distension caused by an increase in blood pressure (Andresen and Kunze, 1994). Afferent fibres in the aortic depressor (vagus) and carotid sinus (glossopharyngeal) nerves have been shown in cats to project to the NTS from baroreceptors in the aortic arch and carotid sinus respectively, as confirmed using retrograde transganglionic transport of horseradish peroxidase (HRP) (Ciriello et al., 1981). Some barosensitive NTS neurones project to the dorsal vagal motor nucleus (DVN) and nucleus ambiguus, key nuclei associated with thoracic and subdiaphragmatic vagal activity (Deuchars et al., 2000). The nucleus ambiguus contains cardioinhibitory vagal efferent preganglionic neurones and excitatory projections from the NTS can induce a rapid reduction in heart rate via these parasympathetic efferents (Izzo et al., 1993). These preganglionic neurones project to the cardiac plexi located in the epicardium (Singh et al., 1999) where they synapse with vagal postganglionic neurones which release acetylcholine at the sinoatrial node. Acetylcholine binds to muscarinic (M_2) receptors on cardiomyocytes in the sinoatrial node, with the subsequent hyperpolarisation leading to a reduction in heart rate (Neff et al., 1998, Hasan, 2013). This reduction in heart rate is accompanied by reduced cardiac output, thereby lowering blood pressure.

In addition, the reduction in blood pressure observed during activation of the arterial baroreflex is further enhanced by inhibition of the rostral ventrolateral medulla, the primary regulatory centre for central sympathetic outflow to the heart and vasculature (Kumada et al., 1990). The RVLM has a critical role in the maintenance of blood pressure and lesions at this site produce marked decreases in blood pressure at rest (Dampney and Moon, 1980). The RVLM has been shown to have direct excitatory projections to the intermediolateral (IML) cell column of the lateral horn of the spinal cord, where sympathetic preganglionic neurones arise (Ross et al., 1984a, Zagon and Smith, 1993). This was confirmed through work by Ross et al. in anaesthetised rats where electrical stimulation applied to the rostral ventrolateral medulla induced tachycardia, increased arterial pressure, inhibited the arterial baroreflex and greatly increased the plasma concentration of norepinephrine (five-fold increase) and epinephrine (seventeen-fold increase) (Ross et al., 1984b). Transection of the cervical spinal cord at C1 spinal level abolished this response (Ross et al., 1984b). Microinjection of the inhibitory amino-acid GABA into the RVLM in six rats elicited a dose-dependent depressor response which manifested in decreased arterial pressure in all animals and bradycardia in four out of six rats (Ross et al., 1984b).

GABAergic projections from the caudal ventrolateral medulla (CVLM) have been found to exert a strong inhibitory effect on the RVLM (Li et al., 1992). The CVLM receives glutamatergic projections from the NTS which fire in response to increased baroreceptor activation, which in turn causes a reciprocal inhibition of the RVLM via GABAergic projections from the CVLM and a decrease in sympathetic tone (Guyenet, 2006). Blessing et al. showed that this depressor response could be artificially induced using microinjection of glutamate into the CVLM and abolished through injection of a GABA antagonist into the RVLM (Blessing, 1988).

1.2 Assessment of cardiovascular autonomic activity

There are a variety of techniques that are used in research and clinical contexts to measure cardiovascular autonomic function in humans. Many of these techniques are by necessity indirect, as direct recordings of autonomic nervous system activity are invasive and difficult to perform in human research participants. As the heart is under the influence of both the parasympathetic and sympathetic nervous systems, the simplest and least specific marker of cardiovascular autonomic activity is resting heart rate. The normal range for resting heart rate is from 60 beats per minute (bpm) to 100 bpm depending on physical activity levels, although individuals with athletic training may have much lower resting heart rates. Resting heart rate is known to be a prognostic factor in the outcome of cardiovascular disease and individuals with high RHR (> 75 bpm) have a greatly increased risk of sudden death due to myocardial infarction (Jouven et al., 2005).

1.2.1 Heart rate variability

Heart rate variability analysis is one of the most common non-invasive methods of assessing cardiovascular autonomic function. Heart rate which is in normal sinus rhythm naturally fluctuates on a beat-to-beat basis due to the modulation of sinoatrial node activity by sympathetic and parasympathetic inputs. This modulation in heart rate can occur in response to a wide range of factors including changes associated with respiratory control, exercise intensity and baroreceptor activation. Heart rate variability is typically measured in clinical and research contexts using electrocardiography (ECG) recordings but can also be derived from photoplethysmographic recordings. However, the ECG method is considered to be more accurate as it allows for improved detection and identification of ectopic beats, which may disrupt HRV analysis algorithms (Shaffer et al., 2014). HRV can be assessed using a variety of different methods but the most commonly used measures are time-domain HRV and frequency-domain HRV (Malik, 1996).

Time-domain measures of HRV are simple statistical analyses of a sample of RR-interval data, most often 24 hours for longer-term recordings and 5

minutes for short-term recordings (Malik, 1996). Moreover, these analyses are based on the normal-to-normal or NN-intervals, the interbeat intervals between R-peaks which are in sinus rhythm and free from disruptions caused by signal artefacts or ectopic beats (Tarvainen et al., 2014). A common time-domain measure is SDNN (standard deviation of normal-to-normal intervals), which is associated with overall variation in the RR-interval data in either a long-term (24 hours) or short-term (5 minutes) context. In short-term recordings, SDNN is primarily derived from respiratory sinus arrhythmia (Shaffer et al., 2014). RMSSD (root mean square of successive differences in RR-intervals) and pNN50 (percentage of pairs of consecutive NN-intervals which differ by > 50 ms) are measures of short-term RR-interval variability which correlate with vagal activation (Malik, 1996).

Frequency-domain measures of HRV use power spectral density (PSD) analysis to separate the RR-interval signal into oscillating rhythms which are associated with discrete frequency bandwidths. This can be achieved through the use of PSD algorithms such as the Lomb-Scargle periodogram or fast Fourier transform (FFT). The separate frequency components can be used to describe how variance (power) distributes as a function of frequency. These frequency bands are: very low frequency power (< 0.04 Hz; VLF); low frequency power (0.04 – 0.15 Hz; LF) and high frequency power (0.15 – 0.4 Hz, HF). All variance within the entire frequency bandwidth is known as the total power. To date the VLF component has been poorly characterised but is thought to be influenced by changes in circadian rhythms, hormonal fluctuations and thermoregulation i.e. physiological processes which may occur over long time periods (Kleiger et al., 2005; Shaffer et al., 2014). The HF component has been associated with parasympathetic modulation of heart rate, whereas LF power reflects both parasympathetic and sympathetic modulation of heart rate (Akselrod et al., 1981; Shaffer et al., 2014). Akselrod and colleagues (1981) showed that parasympathetic blockade using glycopyrrolate (a muscarinic receptor antagonist) eliminated the HF power component of HRV in conscious dogs, while propranolol did not, indicating that HF power is generated by vagal activity (Akselrod et al., 1981). HF power is also known to correlate with RMSSD (Kleiger et al., 2005). The

LF power appears to originate from both parasympathetic and sympathetic activity, as evidenced by a propranolol-induced decrease in LF power in rats which was not completely abolished (Aubert et al., 1999). Atropine blockade which abolished the HF power component also failed to completely abolish the LF power component, suggesting that the LF power component may reflect both sympathetic and parasympathetic activity (Aubert et al., 1999). LF power and HF power are typically measured in absolute values of power (ms^2) but can also be converted to normalised units (n.u.) to reduce the impact of changes in total power on these components (Malik, 1996). The balance between these normalised units can then be represented using the LF/HF ratio, a combined reflection of sympathetic and parasympathetic modulation on sinoatrial node activity with a reduced LF/HF ratio signifying parasympathetic predominance (Malik, 1996).

1.2.2 Baroreflex sensitivity

Baroreflex sensitivity (BRS) is defined as the beat-to-beat change in the RR interval (in milliseconds) in response to a change in blood pressure (Swenne, 2013). Initial investigations into the assessment of BRS in man were conducted by Smyth et al. in a study which used the infusion of angiotensin or phenylephrine via an intra-arterial catheter to generate a brief rise in BP (Smyth et al., 1969). This technique caused a reflexive increase in RR interval to occur, corresponding to the decrease in heart rate. Angiotensin was replaced in later studies with phenylephrine, a vasoactive pharmacological agent which is an agonist for α_1-adrenergic receptors and thus has no effect on sinoatrial node activity (Ebert and Cowley, 1992, Rudas et al., 1999, La Rovere et al., 2008). However, the invasive cannulation required to monitor continuous beat-to-beat changes in BP and infuse the pharmacological agent limits the scope for applying this method of quantifying BRS. Non-invasive beat-to-beat measurement of blood pressure can be achieved using a non-invasive blood pressure (NIBP) system such as a Finapres or Finometer (Finapres Medical Systems B.V., Netherlands). These systems are equipped with a finger photoplethysmograph, a small finger inflatable finger cuff with a built-in infrared sensor which can detect

changes in arterial blood pressure in the digital arteries (Parati et al., 1989). This is based on the volume-clamp method developed by Penaz (Penaz, 1973). There is a strong correlation between BP measurements obtained using finger photoplethysmography and intra-arterial BP monitoring, offering a simple method of obtaining continuous beat-to-beat BP and quantifying BRS (Pinna et al., 2000).

Spontaneous fluctuations in blood pressure can also be used to measure BRS without the need for a pharmacological intervention. Spontaneous BRS can be measured using either the sequence method or by calculating spectral indices. These have the advantage of being completely non-invasive with no adverse effects caused by artificial perturbations in BP. The sequence method uses linear regression analysis of sequences of ≥ 3 cardiac cycles where there is a change in systolic blood pressure (an increase or decrease) coinciding with a simultaneous change in the duration of the RR-interval (Parati et al., 2000, La Rovere et al., 2008). Spectral analysis of BRS can include measurement of the alpha index or the averaged transfer gain function of systolic pressure variability and the RR interval variability (Robbe et al., 1987, Parati et al., 2000). However spectral methods of spontaneous BRS analysis should only be performed in individuals who are in sinus rhythm, as methods of spectral BRS analysis are highly sensitive to the data loss and estimation bias caused by ectopic beats (Pinna et al., 2005). The sequence method can avoid this issue through the automatic exclusion of sequences where there are observable haemodynamic changes caused by the presence of ectopic beats (Pinna et al., 2015).

1.2.3 Direct measures of cardiovascular autonomic function

While non-invasive indices such as HRV and BRS can provide useful insights into cardiovascular autonomic function, they only provide an indirect estimate of autonomic nervous system activity. At present there is no viable method of recording parasympathetic nervous system activity in humans but there are several techniques which can be used to assess sympathetic nervous

system activity. The first of these is the measurement of the plasma concentration of norepinephrine, the neurotransmitter released from sympathetic nerve terminals (Cohn et al., 1984, Esler, 1993). However this method may be confounded by decreased reuptake of norepinephrine from circulation and decreased metabolism (Charkoudian and Rabbitts, 2009). A more sophisticated approach is the norepinephrine spill-over technique, which involves the infusion of tritiated norepinephrine into a peripheral vein with simultaneous sampling of blood from a catheter inserted into the vasculature surrounding a target organ e.g. the venous coronary sinus for the heart or the renal vein for the kidney (Esler et al., 1984). This provides a regional estimation of sympathetic nerve activation which is not possible to achieve with plasma measurements of norepinephrine, but the invasiveness of norepinephrine spillover measurements limits the feasibility of this technique.

A less-invasive method is microneurography, which was first pioneered in the late 1960s (Hagbarth and Vallbo, 1968). Microneurography involves the use of high-impedance tungsten microelectrodes inserted percutaneously into a superficial nerve such as the common peroneal nerve to directly record action potentials in awake humans (Vallbo et al., 2004). This technique is most commonly used to record multi-unit bursts of vasoconstrictor muscle sympathetic nerve activity (MSNA) from groups of unmyelinated sympathetic postganglionic neurones innervating the intramuscular vasculature (Delius et al., 1972, Charkoudian and Rabbitts, 2009). The microelectrode is carefully adjusted within the nerve until a suitable signal-to-noise ratio is obtained. Macefield et al. refined the technique to permit recording of single MSNA units from individual vasoconstrictor sympathetic neurones, although this is more technically challenging than multi-unit recordings (Macefield et al., 1994, Macefield et al., 1999).

There is a strong association between the arterial baroreflex and muscle sympathetic nervous system activity, with stimulation of the carotid sinus nerve eliciting a decrease in MSNA bursts (Wallin et al., 1975). A decrease in blood pressure will elicit a reflexive increase in MSNA to induce peripheral vasoconstriction. This means that MSNA can be observed in a pulse

synchronous manner, allowing it to be differentiated from skin sympathetic nerve activity, which is not associated with the arterial baroreflex and does not fire in synchrony with the cardiac cycle (Charkoudian and Rabbitts, 2009). MSNA also shows substantial inter-individual variability, but it is possible to record repeated measurements in the same individual which are reproducible (Fagius and Wallin, 1993). In addition, MSNA has been shown to correlate with cardiac norepinephrine spill-over measurements obtained from the coronary sinus (Wallin et al., 1992). This means that microneurographic recordings of MSNA from a peripheral site such as the common peroneal nerve can provide a less-invasive but informative indicator of cardiac sympathetic activation.

1.3 Heart failure and autonomic dysregulation

Chronic heart failure (CHF) is an increasingly significant public health issue affecting an estimated 23 million people worldwide, with 5.3 million of those living in the USA (Bui et al., 2011). The majority of patients living with CHF are aged ≥ 65 years and this demographic constitutes 80% of all CHF-associated deaths, with the mortality rate 5 years after diagnosis reported to be greater in men than in women (Levy et al., 2002). The condition often develops from an existing cardiovascular disorder where cardiac output has been compromised e.g. due to pathological defects caused by myocardial infarction or coronary artery disease. Inadequate oxygen perfusion throughout the body leads to the activation of the sympathetic nervous system and renin-angiotensin system, which initially counteract the lack of oxygen in body tissues by increasing heart rate and blood pressure (Florea and Cohn, 2014). However, chronic sympathetic activation cannot be maintained without degenerative cardiotoxic effects, leading to the development of CHF. The progression of CHF can be determined by comparing patient symptoms to the New York Heart Association (NYHA) classification (Table 1.1).

Table 1.1 The New York Heart Association (NYHA) classification system

NYHA Classification	Patient Symptoms
I (Mild)	Asymptomatic with no limitations regarding physical activity
II (Mild)	No symptoms at rest but patient experiences dyspnoea, fatigue or palpitations following ordinary physical activity
III (Moderate)	No symptoms at rest but patient experiences dyspnoea, fatigue or palpitations after less than ordinary physical activity
IV (Severe)	Cardiac insufficiency symptoms at rest with patient unable to perform any physical activity without discomfort. Discomfort worsens with physical activity

There is a consensus that increased sympathoexcitation is an integral pathophysiological force behind CHF progression (Florea and Cohn, 2014). Early evidence for increased sympathetic activation in CHF came from analysis of norepinephrine levels in the urine of a group of heart failure patients (n = 110) compared to samples from healthy controls (n = 13) (Chidsey et al., 1965). This has further been established by the observation of increases in the plasma concentration of norepinephrine as CHF progresses (Thomas and Marks, 1978). MSNA is also significantly increased in CHF patients and has been shown to diminish one month after successful cardiac transplantation (Leimbach et al., 1986, Rundqvist et al., 1997). Macefield et al. obtained multi-unit MSNA recordings in 8 CHF patients (NYHA class II to IV) and observed that MSNA burst incidence (bursts per

100 heartbeats) was increased when compared to healthy controls (Macefield et al., 1999). Barretto et al. (2009) also measured MSNA via microneurography in 122 heart failure patients (NYHA class II to IV) and found that MSNA levels greater than 49 bursts per minute indicated an increased mortality rate over one year (Barretto et al., 2009). This increased incidence of sympathetic activation also corresponds with observed reductions in HRV and BRS in CHF patients (Mortara et al., 1997, Nolan et al., 1998). In their prospective study of 433 CHF patients, Nolan et al. showed that reductions in 24 hour time-domain measures of HRV such as the SDNN could provide a useful CHF prognostic assessment (Nolan et al., 1998).

Pharmacological treatment strategies for CHF include beta-blockers, which can selectively or non-selectively block cardiac β_1 and β_2-adrenergic receptors in order to reduce heart rate, lower blood pressure and thus alleviate the effects of chronic sympathetic activation (Brophy et al., 2001). Patients with myocardial infarction (MI) who received propranolol, a non-selective beta-blocker, showed a significant decrease in LF/HF ratio compared to a control group of patients who received a placebo (Lampert et al., 2003). This effect suggests that a change in autonomic nervous system activity towards parasympathetic predominance occurred in the propranolol-treated patients. Further evidence of this has been shown with carvedilol, another non-selective beta-blocker which has been shown in 36 CHF patients to increase BRS, increase the HF power component of frequency-domain HRV and reduce cardiac norepinephrine spill-over after 4 months of treatment (Kubo et al., 2005). In this way, the increased vagal activation demonstrated by increased HF power and BRS could be as a result of removal by beta-blockade of the inhibitory effects of elevated cardiac norepinephrine on vagus nerve activity at the sinoatrial node. However, the mortality rate for CHF remains relatively high at around 10% per annum despite the use of pharmacological treatments such as beta-blockers (Nolan et al., 1998). This persistently high mortality rate underscores the need for further development of novel therapeutic interventions.

Increased sympathetic activation is not solely responsible for CHF progression and parasympathetic withdrawal has also been recognised as a key pathophysiological feature of the condition (Triposkiadis et al., 2009, Sabbah et al., 2011). The results of a comparative study by Eckberg et al. of healthy volunteers (n = 23) and CHF patients (n = 22) showed that the patients had impaired parasympathetic activity (Eckberg et al., 1971). Atropine was used to induce parasympathetic blockade in both groups and the patients displayed an attenuated increase in heart rate, suggesting that the patients had a significantly lower level of parasympathetic activity at baseline compared to the controls (Eckberg et al., 1971). This parasympathetic withdrawal would suggest that neuromodulation applied with the intent of activating the parasympathetic nervous system may be a potential method of altering the autonomic dysregulation associated with CHF. One way in which this may be achieved is via electrical stimulation of the vagus nerve.

1.4 The vagus nerve

As the 10th and longest cranial nerve, the vagus nerve is notable for providing the main parasympathetic innervation to the thoracic and abdominal viscera. Arising bilaterally as 8 - 10 rootlets between the inferior olive and the inferior cerebellar peduncle of the medulla, the vagus nerve merges into a common trunk that traverses the jugular foramen to exit the cranium (Berthoud and Neuhuber, 2000). Located within the jugular foramen is the jugular (or superior) ganglion, which contains somatic sensory afferent cell bodies (Ruffoli et al., 2011). The larger nodose (inferior) ganglion lies distal to the jugular foramen and contains the cell bodies of visceral afferent fibres (Ruffoli et al., 2011). After exiting the cranium, the right and left vagus nerves descend inferiorly through the neck within the carotid sheath, providing innervation to the soft palate, pharynx and muscles of the larynx prior to entering the thorax (Berthoud and Neuhuber, 2000). At this point, the ‘wandering’ nature of the vagus becomes apparent through its complex

distribution to the heart, lungs, liver, adrenal medulla and the gastrointestinal tract up to the splenic flexure of the colon (Berthoud and Neuhuber, 2000).

While the traditional view is that in humans vagus nerve innervation terminates at the splenic flexure of the colon, there is evidence that vagal afferents may also innervate the pelvic organs. In rats, labelled neuronal cell bodies were observed in the nodose ganglia following injection of HRP into the ovaries (Burden et al., 1983) and the uterine walls (Ortega-Villalobos et al., 1990, Collins et al., 1999). There is evidence that the uterine cervix is also innervated by the vagus nerve (Collins et al., 1999). Transection of both the vagus nerve and the sacral pelvic nerves in pregnant rats delayed parturition and the associated remodelling of the cervix (Clyde et al., 2011). The vagal innervation of the large intestine varies across species, electrical stimulation of the cervical vagus nerve in rats has been shown to elicit peristaltic contractions in the distal colon (Tong et al., 2010).

At a histological level, the vagus is a mixed nerve which has been shown in cats to consist of 65% to 80% somatic afferent fibres together with a smaller amount of visceral and general efferent fibres from the nucleus ambiguus and dorsal motor nucleus of the vagus (Foley and DuBois, 1937, Ruffoli et al., 2011). This imbalanced ratio suggests that the vagus is an important sensory relay for the regulation of visceral function. Moreover, there are several distinct types of vagal afferent fibres: general visceral afferents which transmit information from viscera, mucosa and aortic baroreceptors, as well as special sensory afferents, which are involved with taste sensation at the root of the tongue and epiglottis. In addition to the visceral afferents, somatic sensory afferents convey sensation from the external ear, pharynx, larynx, trachea, cranial meninges and oesophagus to the spinal trigeminal nucleus, with their cell bodies located in the jugular ganglion.

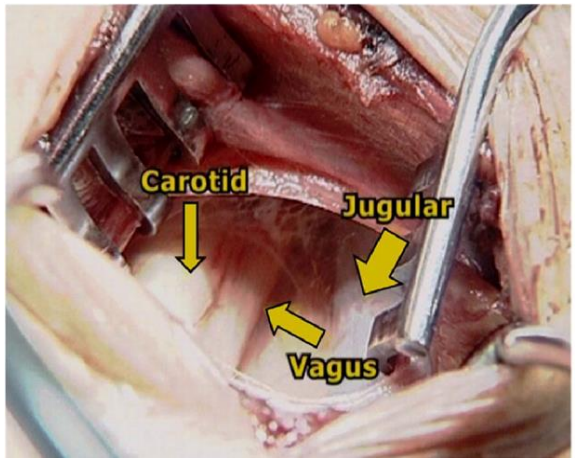
1.4.1 Vagus nerve stimulation

The widespread parasympathetic visceral innervation supplied by the vagus nerve, as well as its substantial projections to cortical and subcortical sites, has led to much interest in the therapeutic effects of modulating vagal activity (Clancy et al., 2013). Vagus nerve stimulation involves the surgical implantation of a bipolar electrode cuff around one of the cervical vagus nerves, most commonly the left (Groves and Brown, 2005). This is due to the fact that the right vagus nerve provides innervation to the sinoatrial node, with the risk that stimulation may elicit bradycardia or asystole, while the left vagus nerve innervates the atrioventricular node and thus does not generate the same cardiovascular effects (Randall et al., 1985). Left-sided VNS has to date been most commonly implanted in treatment-resistant epilepsy patients and has been approved for clinical use in Europe since 1994 and the USA since 1997 (Revesz et al., 2016).

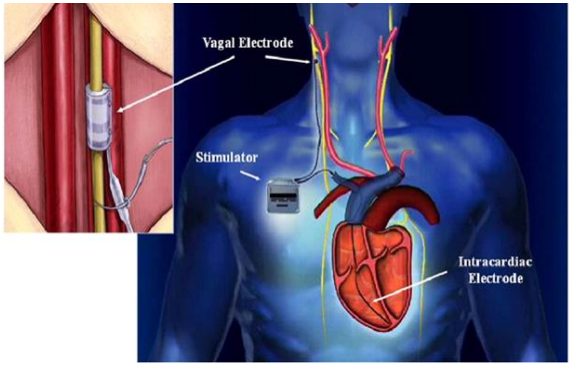
Figure 1.2 An example of an implanted vagus nerve stimulation (VNS) system. The right or left cervical vagus nerve is isolated and fitted with a bipolar electrode (A). All VNS systems include a battery generator (B) and some designs include an additional intracardiac sensing lead inserted into the right ventricle of the heart. An X-ray is shown of a patient implanted with a VNS system (C). Images sourced from Schwartz et al. (2008).

Figure 1.2 An example of an implanted vagus nerve stimulation (VNS) system.

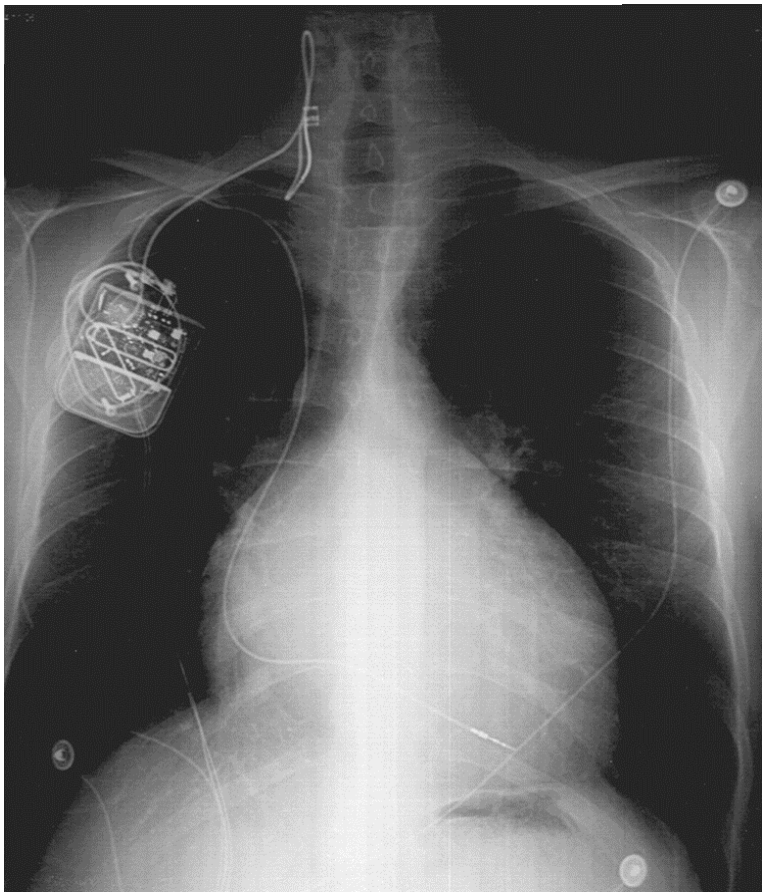
A



B



C



1.4.2 VNS for the treatment of epilepsy

The first documented attempt at electrical stimulation of the cervical vagus nerves was by the American neurologist James L. Corning, who developed a device in the mid-1880s which combined bilateral compression of the carotid arteries with transcutaneous direct electrical current (Corning, 1884). At the time it was believed that epileptic seizures were the result of "cerebral hyperaemia" and Corning believed this method would reduce cerebral blood flow and thus reduce seizure frequency through stimulation of efferent fibres in the vagus nerve (Corning, 1884). He observed transient bradycardia as a result of stimulation and presented anecdotal evidence that this method of VNS was effective in suppressing seizures (Corning, 1884). However, Corning's contemporaries proved to be sceptical of his claims and VNS was largely forgotten until the mid-20th century (Lanska, 2002). Work done in the 1930s using the encéphale isolé technique in vagotomised cats showed that VNS applied to the central cut end of the cervical vagus could decrease the frequency of strychnine-induced seizures (Schweitzer and Wright, 1937) and generate electrical potentials in the orbital region of the frontal lobe (Bailey, 1938). Zanchetti et al. found that VNS (2.0V, 50Hz, 0.5ms) in anaesthetised encéphale isolé cats evoked widespread desynchronization of cortical activity measured using EEG (Zanchetti et al., 1952). Moreover, this study demonstrated that the change in cortical activity was due to the activation of vagal afferents, as the cervical vagus nerve was sectioned and stimulation was applied to the central cut end of the nerve (Zanchetti et al., 1952). Later work by Zabara et al. found that VNS had an effect on seizure control when stimulation of the cervical vagus attenuated strychnine-induced motor seizures and pentylenetetrazol-induced tremors (Zabara, 1992). This seizure suppression was proposed to be due to a VNS-induced desynchronization of cortical activity, with the seizure suppressive effect persisting following the end of stimulation (Zabara, 1992).

Following on from this preclinical evidence, the first human trial of VNS was performed on four treatment-resistant epilepsy patients with refractory partial seizures (Penry and Dean, 1990). Six to twelve months after a stimulator device was surgically implanted subcutaneously below the clavicle and

connected via an electrode lead to the left cervical vagus, two of the patients experienced a complete absence of seizures, with a third patient reporting a 40% reduction in seizure frequency (Penry and Dean, 1990). A larger trial conducted by DeGiorgio et al. in 164 refractory epilepsy patients observed a median reduction in seizure frequency of 45% after 12 months of VNS (DeGiorgio et al., 2000). A retrospective review by Elliott et al. which focused on the clinical outcomes of 65 treatment resistant epilepsy patients 10 years post-VNS implantation showed improvements in seizure control with increasing long-term duration of VNS therapy (Elliott et al., 2011). A plateau in mean seizure reduction was observed at the 2 year mark, after which further reductions were modest (Elliott et al., 2011).

Despite the proven therapeutic efficacy of VNS in patients with treatment-resistant epilepsy, the mechanisms underlying the seizure-suppressive effects of this technique are yet be fully clarified. However, the activation of vagal afferents is known to be critical to the efficacy of VNS. The afferents in the vagus nerve are categorised into myelinated A-fibres and B-fibres as well as unmyelinated C-fibres, all of which have different amplitude-duration thresholds for excitation (Erlanger, 1930, Groves and Brown, 2005). The unmyelinated C-fibres have the highest excitation threshold (> 2.0mA), whereas the excitation thresholds are lower for A-fibres and B-fibres (ranging from 0.02 – 0.2mA and 0.4 – 0.6mA respectively). However, A-fibres and B-fibres are the most likely to be activated by the VNS stimulation parameters as the current is typically at a lower intensity (20 – 30Hz) than the C-fibre threshold (DeGiorgio et al., 2000, Ruffoli et al., 2011). This was directly influenced by preclinical evidence from a study of VNS in a rat model of pentylenetetrazol-induced seizures (Krahl et al., 2001). This study showed that VNS could reduce seizure severity following the selective capsaicin-induced destruction of vagal C-fibres, providing further evidence that the seizure-suppressive effects of VNS are due to vagal A-fibre and B-fibre activation (Krahl et al., 2001).

1.4.3 VNS for the treatment of major depressive disorder (MDD)

Improvements in mood were observed in many epilepsy patients who received VNS treatment. This phenomenon inspired a pilot study by Harden et al. which demonstrated that VNS did have a quantifiable effect on mood that was unrelated to its anti-convulsant effects (Harden et al., 2000). Three months of VNS in patients with epilepsy (n = 20) led to significant decreases in mood scale scores compared to a control group receiving standard care (n = 20). Moreover, improvements in mood were observed in patients for whom there was little improvement in seizure frequency (Harden et al., 2000). This effect led to a number of clinical trials to assess the efficacy of VNS in major depressive disorder (MDD) and VNS has subsequently been approved by the US Food for Drug Administration (FDA) for the treatment of MDD.

MDD is a highly disabling condition which is treatment-resistant in around 10 30% of patients (Al-Harbi, 2012). An early randomised controlled trial of VNS in treatment-resistant MDD patients showed no significant differences between the VNS (n = 112) and control groups after 10 weeks (Rush et al., 2005a). However, a significant VNS-induced improvement in mood was observed from 12 weeks onwards and this sustained at 12 months follow-up, suggesting that VNS is more effective over longer time-scales (Rush et al., 2005b). Electrophysiological investigation in rats has shown that VNS increases the firing rate in the neurones of the locus coeruleus, suggesting that VNS can alter noradrenergic signalling: a potential explanation for the anti-depressive effects observed with VNS (Dorr and Debonnel, 2006).

1.4.4 VNS for the treatment of chronic heart failure (CHF)

Given the role of the vagus nerve with regards to cardiac parasympathetic innervation, there has been interest in the potential modulatory effects of implanted VNS on cardiac function. In particular, several preclinical animal studies provided evidence that VNS may have a therapeutic effect in heart failure. Early work by Vanoli et al. in a canine model of healed myocardial infarction (MI) showed that VNS applied to the cervical vagus nerve could elicit a significant reduction in the incidence of ventricular fibrillation during

exercise testing compared to controls (Vanoli et al., 1991). Following initial exercise and ischaemia testing to identify animals susceptible to episodes of exercise-induced ventricular fibrillation, 22 out of 24 (92%) of the control group animals experienced ventricular fibrillation during a second trial of exercise testing compared with just 3 out of 30 (10%) of animals in the VNS treatment group (Vanoli et al., 1991). Additional testing without VNS in the treatment group found the incidence rate of ventricular fibrillation increased to 87% of animals (Vanoli et al., 1991). This profound reduction in the incidence of ventricular fibrillation would suggest that VNS applied to the cervical vagus nerve has an anti-arrhythmic effect.

Following on from this work, Li et al. tested right-sided VNS in a rat model of chronic heart failure following left ventricular MI (Li et al., 2004). A marked reduction in mortality rate was found in rats treated with 6 weeks of VNS compared to an untreated control group (3 deaths out of 22 for treatment group; 15 deaths out of 30 for control group). This was accompanied by a significant reduction in left ventricular end diastolic pressure (LVEDP; treatment group = 17.1 ± 5.9 mmHg; control group = 23.5 ± 4.2 mmHg) as well as lower plasma norepinephrine levels in the treatment group (426 ± 102 pg/mL compared to 1182 ± 260 pg/mL in control group). This latter finding suggested that 6 weeks of VNS i.e. vagal activation was capable of generating a reciprocal decrease in sympathetic activation. As the progression of CHF is underpinned by a sustained increase in sympathetic activation and concurrent withdrawal of parasympathetic activity, this work by Li et al. was one of the first to demonstrate that VNS can exert a modulatory effect on the cardiovascular autonomic nervous system (Li et al., 2004). A later study by Zhang et al. further assessed the autonomic effects of right-sided VNS in a canine model of heart failure induced by rapid (220bpm over 4 weeks) ventricular pacing (Zhang et al., 2009). The LF/HF ratio decreased in the VNS group after 4 weeks of stimulation compared to unstimulated controls, suggesting a shift in autonomic nervous system activity towards parasympathetic predominance which was reinforced by an increase in HF power and a decrease in LF power. In addition, plasma norepinephrine and C-reactive protein levels were also found to be lower in the VNS-treated

group compared to control animals, further supporting previous evidence that VNS is capable of reducing sympathetic nervous system activity (Li et al., 2004, Zhang et al., 2009). A VNS-induced increase in baroreflex sensitivity at 4 weeks of stimulation which was sustained at the 8 week time-point was also identified, suggesting an enhancement in vagal control had occurred in the VNS group over the course of treatment (Zhang et al., 2009).

The results of these animal studies combined with the established safety profile of cervical VNS in human patient groups encouraged investigation into the feasibility and tolerability of VNS in CHF patients. The first of four landmark clinical trials to assess the therapeutic efficacy of VNS in heart failure was the CardioFit™ non-randomised proof-of-concept phase II safety and feasibility trial (Schwartz et al., 2008, De Ferrari et al., 2011). 32 CHF patients on optimal medical therapy (e.g. β-blockers, loop diuretics) with a NYHA functional classification of II – III and LVEF ≤ 35% were implanted with the CardioFit™ VNS system (BioControl Medical Ltd, Israel). This system was a “closed-loop” device with a bipolar electrode cuff positioned around the right cervical vagus nerve and an intracardiac sensing lead in the right ventricle to monitor heart rate and deliver stimulation at a fixed point in the cardiac cycle (Schwartz et al., 2008, Byku and Mann, 2016). In addition, 19 patients had already been fitted with an implanted cardioverter defibrillator device prior to enrolment (De Ferrari et al., 2011). Six months of chronic right-sided VNS led to a significant increase in LVEF and a significant reduction in left ventricular end systolic volume. Improved quality of life scores and 6-minute walk test distances were also identified at 6 months and 19 out of 32 patients were found to have improved by at least one NYHA class (De Ferrari et al., 2011). Crucially, the VNS-induced effects could still be observed in 23 patients who were assessed at 12 months such as an increase in LVEF from 21% at baseline to 34%, providing strong evidence against a placebo effect (De Ferrari et al., 2011). The promising outcomes from this trial led to a series of larger-scale clinical trials of VNS for the treatment of chronic heart failure (Premchand et al., 2014, Zannad et al., 2015, Gold et al., 2016).

The first of these larger trials was the ANTHEM-HF (Autonomic Neural Regulation Therapy to Enhance Myocardial Function in Heart Failure) multicentre open-label feasibility study, which compared the safety and efficacy of left-sided VNS to right-sided VNS in chronic heart failure patients over 6 months (Premchand et al., 2014). 60 CHF patients on optimal medical therapy with a NYHA functional classification of II – III and LVEF ≤ 40% were recruited to the study and implanted with an "open-loop" VNS system. This differed from the CardioFit™ VNS system in that it did not have an intracardiac sensing lead and stimulation was delivered at a lower intensity (2.0mA on average compared to 4.1mA for the CardioFit trial) (De Ferrari et al., 2011, Premchand et al., 2014, Byku and Mann, 2016). The ANTHEM-HF trial reported a significant 4.5% increase in LVEF but no significant effect on left ventricular end-systolic volume, the trial's other primary efficacy end-point. As with the CardioFit trial, quality of life scores and exercise tolerance were improved following 6 months VNS (De Ferrari et al., 2011, Premchand et al., 2014). Heart rate variability assessed using SDNN was also reported to be higher after 6 months VNS. No significant differences between left and right-sided VNS were reported (Premchand et al., 2014). An extended follow-up at 12 months in 49 consenting patients found that there were no serious adverse effects as a result of VNS or hardware problems, although some patients reported mild dysphonia or pain (Premchand et al., 2016). Importantly, the effects observed after 6 months of either left or right sided VNS were found to have persisted at the 12 month time-point and there were no differences in outcomes between the stimulation sites (Premchand et al., 2016).

Another study which used a similar "open-loop" VNS system was the NECTAR-HF (Neural Cardiac Therapy for Heart Failure) phase II randomised controlled trial (Zannad et al., 2015). This 12 month trial differed from ANTHEM-HF and CardioFit in that it included a sham VNS group as part of the study design. This trial recruited 96 CHF patients with NYHA class II – III, LVEF ≤ 35% and left ventricular end-systolic diameter > 55mm. The patients were all implanted with right-sided VNS and randomised 2:1 to receive either active VNS (20Hz, 10s on/50s off, 300μs) or sham stimulation

(stimulator switched off after initial titration visit) for the first 6 months of the trial. All patients then received active VNS treatment for the remaining 6 months of the trial. However, the current level achieved through titration in the active VNS group was 1.42 ± 0.80mA, lower than CardioFit or ANTHEM-HF trials. At 6 months no significant difference was observed between the active VNS and sham VNS groups for LV end-systolic diameter, the primary efficacy endpoint (Zannad et al., 2015). Moreover, no significant differences were observed between groups at the 6 month time-point for LVEF, LV end-systolic volume, exercise capacity (peak VO_2) or measures of HRV obtained from 24 hour ECG Holter recordings, with the exception of SDANN which was found to have increased in the active VNS group. A significant improvement in quality of life scores was also reported in the active VNS group compared to the sham stimulation and 62% of the active VNS patients improved their NYHA class by at least one point compared to 45% of controls (Zannad et al., 2015). While NECTAR-HF was the first and only randomised control trial of right-sided VNS for CHF in man, an important limitation of this trial was ineffective blinding of patients: 70% of patients in the active VNS group correctly guessed their randomisation compared to 31% in the control group. This may have been due to participants perceiving sensations or side-effects such as coughing associated with active VNS, which would have made it difficult to maintain adequate blinding (Zannad et al., 2015).

The largest of the four clinical trials for VNS in CHF was the INOVATE-HF (Increase of Vagal Tone in Heart Failure) phase III randomised controlled trial in 707 CHF patients (NYHA class III; LVEF < 40% and LV end-diastolic diameter of 50 – 80 mm). These patients were randomised 3:2 to receive either implanted right sided active VNS plus optimum medical therapy or optimum medical therapy alone (control group) (Gold et al., 2016). A key feature of INOVATE-HF was the use of the original CardioFit™ VNS system with right ventricular sensing lead. However, following titration the stimulus on-time in the INOVATE-HF trial (5.1 ± 0.8s) was less than in the CardioFit trial (7.1 ± 4.8s) (De Ferrari et al., 2011, Gold et al., 2016). At the outset of the trial the primary efficacy end-point was stated to be a composite of all-cause mortality or the time to first unplanned hospitalisation due to a heart

failure-associated event which included changes to a patient's medication regimen (Hauptman et al., 2012). Additional primary safety endpoints were >75% freedom from complications associated with VNS in the first 90 days following device implantation and comparison between the VNS and control groups for all-cause mortality and complications at one year follow-up. Patients enrolled in INOVATE-HF were followed up for an average of 16 months and in some patients up to 4.3 years. However, the trial was stopped early due to statistical futility in the VNS group following an interim analysis, with no significant difference in the primary efficacy outcome between the VNS and control groups (Gold et al., 2016). Death or unplanned CHF-related hospitalisation occurred in 30.3% of the VNS group versus 25.8% of the control group ($p = 0.37$), with no improvement in left ventricular end-systolic volume index (LVESVi) as a result of VNS (Gold et al., 2016). However, as with previous trials there were significant improvements in quality of life, NYHA class and 6-minute walking distance after 6 and 12 months of VNS treatment, although as the control group received optimum medical therapy rather than sham stimulation it is unclear if these improvements were the result of a placebo effect (Gold et al., 2016).

1.4.5 Complications associated with VNS

VNS requires a surgical procedure to implant the electrodes and a subcutaneous battery pack in the chest wall, increasing the risk of post-operative side-effects such as dysphonia, which can occur in up to 62% of patients (Handforth et al., 1998, Elliott et al., 2011). Other common adverse effects include stimulation-induced coughing, dyspnoea and neck pain (Handforth et al., 1998). In the longer term, the incidence of adverse effects decreases over time with 20% of patients experiencing dysphonia five years after VNS implantation and only 5% experiencing other adverse effects (Ben-Menachem et al., 2015). There are also a number of hardware issues associated with VNS systems. Patients must avoid machines which emit radiofrequency energy such as cardiac defibrillators or high-field MRI scanners, as there is a risk that the VNS system may overheat and damage

the vagus nerve and other surrounding tissues (Fahy, 2010). Hardware failure may also require revision surgery to replace faulty electrodes, leaving patients at risk of experiencing a relapse in their original symptoms (Dlouhy et al., 2012, Spuck et al., 2010).

1.4.6 Non-invasive methods of vagus nerve stimulation

1.4.6.1 Non-invasive cervical vagus nerve stimulation (gammaCore™)

Given the risk of complications associated with implanted VNS systems, there has been emerging interest in non-invasive methods of vagus nerve stimulation (nVNS). One recent development is the handheld gammaCore™ device (electroCore LLC, USA) which uses transcutaneous electrical nerve stimulation applied to the anterolateral surface of the neck via two stainless steel contact electrodes (Figure 1.3) to stimulate the cervical vagus nerve without the need for a surgical procedure (Goadsby et al., 2014). This method of non-invasive VNS has been shown in a recent fMRI study with 13 healthy volunteers to activate the NTS ipsilateral to the stimulation site as well other areas associated with vagal central projections such as the insula and thalamus (Frangos and Komisaruk, 2017). Activations were observed following cessation of nVNS in sites such as the dorsal raphe nuclei and periaqueductal grey, suggesting that the effects of nVNS can persist after stimulation has ceased (Frangos and Komisaruk, 2017). This pattern of activation is similar to that observed in fMRI studies of epilepsy and MDD patients with implanted VNS (Ko et al., 1996, Narayanan et al., 2002, Conway et al., 2012).

A recent randomised sham-controlled trial with 150 patients with episodic or chronic cluster headache patients found that 36% of the episodic cluster headache patients responded to nVNS with a substantial reduction in pain compared to sham, whereas this effect was not observe in the chronic cluster headache group compared to sham (Silberstein et al., 2016). However another randomised controlled trial of the gammaCore™ system in chronic

cluster headache patients found that nVNS may be more effective in preventing cluster headaches when combined with a standard care protocol compared to a patient group who received standard care alone (Gaul et al., 2016). Nonetheless, this new method of cervical VNS, while non-invasive, is not without its limitations. At present the gammaCore™ system is only available pre-charged with a maximum of 300 stimulation cycles, meaning that a new device needs to be purchased at a cost of £550 to continue treatment (Miller et al., 2016). The electrical current from the device must also pass through the tissue overlying the cervical vagus which includes branches of the facial nerve as well as nerves originating from the cervical plexus. The activation of other nerve pathways apart from the vagus nerve may be responsible for the reports of facial drooping and muscle twitches in a small proportion of patients receiving nVNS (Silberstein et al., 2016).

Figure 1.3 The gammaCore™ system for non-invasive cervical vagus nerve stimulation

Image sourced from Miller et al. (2016).

1.4.6.2 The auricular branch of the vagus nerve

An alternative site of interest for non-invasive vagus nerve stimulation is the auricular branch of the vagus nerve (ABVN), the only branch of the vagus nerve with a cutaneous distribution. The ABVN is thought to be a vestigial remnant of an embryonic nerve which supplied the first branchial arch (Gupta et al., 1986). In an evolutionary context it may be derived from the cutaneous nerve which supplies the lateral line organ in aquatic vertebrates such as fish, which use this sensory organ to perceive vibrations and movement in their surroundings (Engel, 1979). In humans, the ABVN innervates the skin overlying several cartilaginous structures on the external ear such as the tragus, concha and cymba concha as well as the walls of the external acoustic meatus (Peuker and Filler, 2002). The ABVN is mainly composed of thick myelinated afferent fibres and a cadaveric study by Safi et al. reported averages of 384 axons in the left ABVN and 362 axons in the right ABVN (Safi et al., 2016). The cell bodies of the ABVN afferents are located in the jugular ganglion (Safi et al., 2016). The ABVN contributes to the cutaneous innervation of the external ear along with branches from the great auricular nerve, the auriculotemporal nerve (V_3) and the lesser occipital nerve (Figure 1.4).

Peuker and Filler in their study of 14 dissected ears from 7 human cadavers found that the ABVN was the only source of innervation to the cymba concha (in 100% of specimens dissected; Table 1.2), with this nerve providing additional innervation to the antihelix (73%), tragus (45%) and cavity of concha (45%) (Peuker and Filler, 2002). An interesting clinical case report which corresponds with these results described the intracranial sectioning of the root of the vagus nerve in a tongue cancer patient to relieve refractory pain in the external ear (Fay, 1927). It was noted that his heart rate decreased to 40 beats per minute during the sectioning procedure, while the patient later experienced a loss of sensation in the posterior wall of the external auditory meatus as well as the concha, antihelix and antitragus (Fay, 1927). This pattern of paraesthesia corresponds approximately to the distribution of the ABVN as observed by Peuker and Filler (Peuker and Filler,

2002). However, there remains some doubt as to the exact arrangement and variability of the ABVN terminal distribution.

	ABVN	GAN	ATN
Crus of helix	20%	-	80%
Spine of helix	-	9%	91%
Tail of helix	-	100%	-
Scapha	-	100%	-
Crura of antihelix	9%	91%	-
Antihelix	73%	9%	18%
Antitragus	-	100%	-
Tragus	45%	46%	9%
Cymba concha	100%	-	-
Cavity of conchae	45%	55%	-
Lobule of auricle (earlobe)	-	100%	-

Table 1.2 Cutaneous distribution of auricular nerves reported by Peuker and Filler (2002).

ABVN = auricular branch of the vagus nerve; GAN = great auricular nerve; ATN = auriculotemporal nerve.

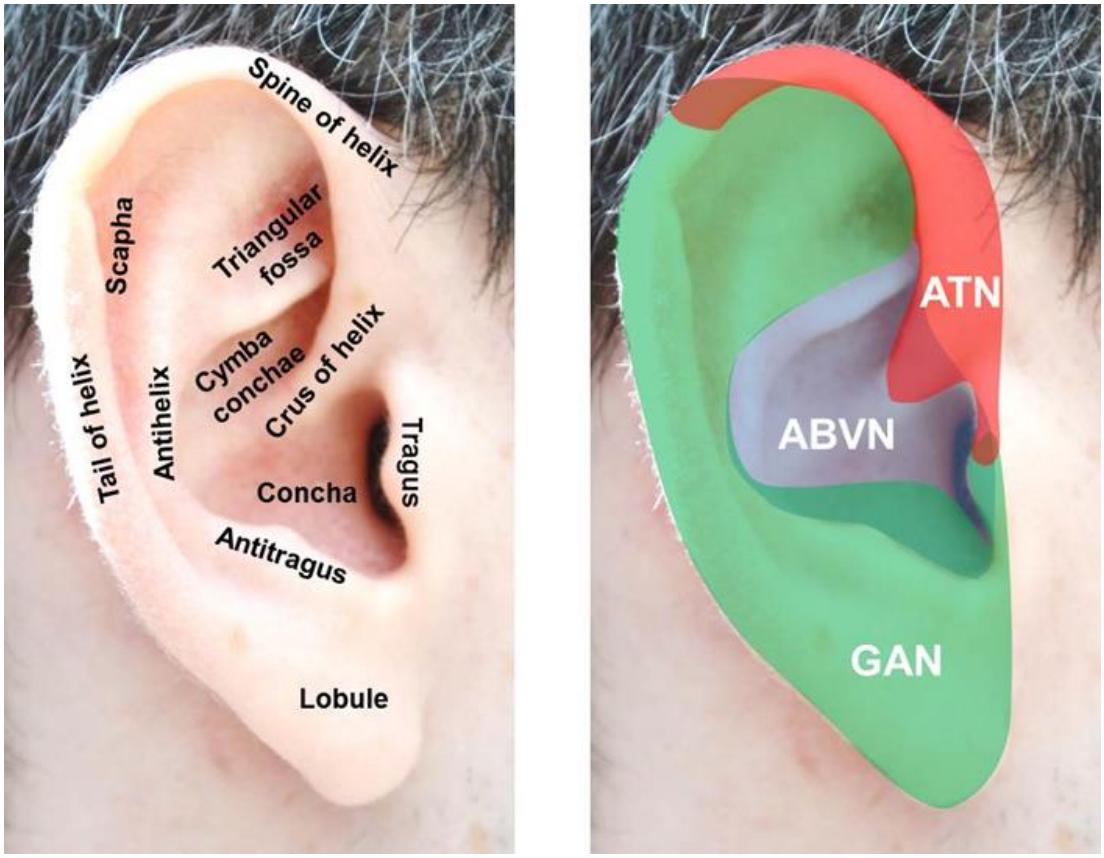


Figure 1.4 Anatomical landmarks and cutaneous innervation of the external ear.

Three auricular nerves contribute to the cutaneous innervation of the lateral aspect of the external ear: the auricular branch of the vagus nerve (ABVN), the great auricular nerve (GAN) and the auriculotemporal nerve (ATN). There is a variable degree of overlap in the distribution of these cutaneous nerves. Images sourced from Murray et al. (2016).

1.4.6.3 Central projections of the ABVN

To date there has been just one study with exclusive focus on tracing the ABVN central projections to the brainstem, where isolated sections of the ABVN were removed from the mastoid canaliculus of the temporal bone in seven cats and the transganglionic neuronal tracer horseradish peroxidase (HRP) applied to the central cut end of the nerve (Nomura and Mizuno, 1984). The study found vagal afferent cell bodies in the jugular ganglion but not the nodose ganglion of the vagus nerve, with the ABVN afferent fibres projecting to the principal sensory trigeminal nucleus, spinal trigeminal nucleus, nucleus tractus solitarius and cuneate nucleus (Nomura and Mizuno, 1984).

A later study by Chien et al. which isolated the rostral, middle and caudal internal auricular nerves in 23 dogs found the middle and caudal nerves to be composed principally of vagal fibres (88% and 79%) when treated with a conjugate of cholera toxin subunit B and HRP, with the neuronal cell bodies shown to be located mostly in the jugular ganglion with some retrograde labelling present in the geniculate ganglion of the facial nerve (Chien et al., 1996). The vagal nerve fibres of the middle auricular nerve were found to project to the lateral medulla, specifically the spinal trigeminal nucleus and nucleus tractus solitarius, with HRP-labelled caudal internal auricular nerve terminals located in the paratrigeminal nucleus (Chien et al., 1996). That both studies by Nomura et al. (1984) and Chien et al. (1996) demonstrated ABVN projections to the NTS is significant because general visceral afferents from the vagus nerve also terminate at this site, suggesting the NTS may act as a relay for the ABVN to indirectly influence the autonomic activity of visceral organs such as the heart, lungs, liver and stomach. Further studies have found evidence of baroreceptor afferents projecting to a similar region as the ABVN on the caudal NTS suggesting that the ABVN could play a role in influencing the baroreceptor reflex (Ciriello and Calaresu, 1981, He et al., 2013b)

1.4.6.4 Auricular reflexes

There are a number of reports of auricular reflexes which highlight the link between the vagal innervation of the external ear and its widespread distribution to the thoracic and abdominal viscera (Murray et al., 2016). Two traditional names for the ABVN are the Arnold's nerve and the Alderman's nerve and these names have been used to describe auricular reflexes. In particular, the Arnold ear-cough reflex was named after the German anatomist Friedrich Arnold, who noted that manual stimulation of the posterior wall of the external acoustic meatus could lead to coughing in some patients. This reflex is present in approximately 1.7% to 4.2% of the adult population (Bloustine et al., 1976, Gupta et al., 1986, Tekdemir et al., 1998) and in one third of those individuals can be elicited by stimulating the anterior wall of the external acoustic meatus as well (Bloustine et al., 1976). A recent cadaveric study of the myelinated axons in the ABVN in humans found that one cadaver had a substantially greater proportion of thickly-myelinated A beta class axons in the left ABVN compared to the right ABVN (1007 versus 289 axons ≥ 7μm) (Safi et al., 2016). This study proposed that the prevalence of the ear-cough reflex may be associated with the number of A-beta class axons in the ABVN, although no pre-mortem history of ear-cough reflex was confirmed in this individual (Safi et al., 2016).

Related to the ear-cough reflex is gastroauricular phenomenon, which was reported in one case study as an itching sensation in the left external acoustic meatus which coincided with gastric reflux, both of which were relieved by ingesting bicarbonate of soda (Engel, 1979). In a related case study, unilateral ear pain secondary to gastric reflux was resolved with administration of ranitidine, a H_2 histamine receptor antagonist used to decrease stomach acid production (Blau, 1989). A different form of auricular reflex response was reported in a case study involving a female patient who perceived a severe pain inside the left ear which coincided with menstruation (Engel, 1979). More recently, Thakar et al. described auricular syncope in a young female patient with epilepsy following manual stimulation of the posterior wall of the left external acoustic meatus with a cotton-tipped ear probe. Stimulation evoked a bradycardiac response with an observed

decrease in heart rate of around 12 bpm. Interestingly, this response could not be elicited via stimulation of the anterior wall of the ear canal or stimulation of any point in the right ear canal (Thakar et al., 2008). In addition, there have been reports of pain referred to the ear region as a result of angina and myocardial infarction, highlighting a potential neurological association between the ear and the heart via visceral afferents (Rothwell, 1993).

1.4.6.5 Transcutaneous vagus nerve stimulation (tVNS) of the ABVN

Given the considerable limitations associated with electrical stimulation of the cervical vagus nerve, stimulation of the ABVN (tVNS) may offer a convenient non-invasive alternative to VNS. Fallgatter et al. showed that it was possible to use transcutaneous electrical stimulation applied to the tragus of the external ear to generate vagal sensory evoked potentials (VSEPs) recorded using EEG in 6 healthy subjects (Fallgatter et al., 2003). These VSEPs were only reported for stimulation of the tragus and not for other sites such as the earlobe, scapha and helix (Fallgatter et al., 2003). Later fMRI studies demonstrated that tVNS (8Hz, 20μs) applied to the tragus could generate comparable pattern of cortical activation to invasive VNS (Kraus et al., 2007, Kraus et al., 2013). A later fMRI study in 12 healthy volunteers showed that tVNS applied to the left cymba concha could induce significant activation in the area of the medulla corresponding to the location of the NTS, with further activation in sites such as the dorsal raphe and locus coeruleus (Frangos et al., 2015).

Following on from the VNS for the treatment of refractory epilepsy clinical trials, several studies have investigated the anti-convulsant efficacy of tVNS (Stefan et al., 2012, He et al., 2013a, Bauer et al., 2016). A proof-of-concept trial by Stefan et al. observed a reduction in seizure frequency after 9 months of tVNS in 5 out of the 7 patients who continued stimulation until this time-point (Stefan et al., 2012). He et al. showed that TENS (20Hz, 0.4 – 1.0mA, 30 minutes stimulation 3 times per day) applied using electrode clips to the concha and cymba concha in 13 paediatric epilepsy patients caused a

significant reduction in seizure frequency after 24 weeks (He et al., 2013a). However, a more recent randomised controlled trial of tVNS in epilepsy patients found no difference in seizure frequency between the treatment group which received tVNS at 25Hz and the control group which received tVNS at 1Hz (Bauer et al., 2016). This may have been due to the study being too underpowered, as seizure frequency was shown to decrease by 23.4% in the 25Hz tVNS group and increase by 2.9% in the control group (Bauer et al., 2016).

Meanwhile, promising results have been observed in trials of tVNS in patients with treatment-resistant MDD (Hein et al., 2013, Rong et al., 2016). tVNS delivered for 15 minutes per day for 2 weeks using a device with electrodes inserted into the external acoustic meatus (NET-1000, Auri-Stim Medical Inc., USA) showed improvements in mood scores compared to sham stimulation (Hein et al., 2013). A later non-randomised clinical trial in patients with mild to moderate MDD compared a treatment group (n = 91) who received tVNS applied to the cymba concha and concha with a sham stimulation group who received earlobe stimulation (n = 69). Compared to sham, tVNS could reduce depressive symptoms as well as symptoms of anxiety after 4 weeks of stimulation (Rong et al., 2016). An fMRI study of tVNS applied to the concha and cymba concha in patients with MDD (n = 17) compared to a control patient group (n = 21) who received stimulation of the scapha (innervated by cervical spinal afferents) showed that 4 weeks of tVNS induced significant activation in the anterior insula compared to controls (Fang et al., 2017). The anterior insula is a cortical site known to be integral for the processing of emotional stimuli and is further implicated in the neuropathology of MDD (Sprengelmeyer et al., 2011). In this way, Fang et al. proposed that tVNS-induced anterior insula activation assessed using fMRI may be a biomarker to identify MDD patients who might respond to longer-term treatment with tVNS (Fang et al., 2017).

Another condition for which there is evidence that tVNS may have therapeutic potential is chronic tinnitus. Tinnitus is associated with pathological changes in the central auditory system due to factors such as noise exposure (Steinmetz et al., 2009) or ageing (Ferreira et al., 2009) and

affects up to 16% of the general adult population (Nondahl et al., 2011, McCormack et al., 2014). Invasive VNS paired with acoustic stimulation (tones outside of the frequencies associated with tinnitus) has been shown to produce improvements in tinnitus severity in a rat model of tinnitus (Engineer et al., 2011) and in clinical pilot studies (Vanneste and De Ridder, 2012, De Ridder et al., 2014). Initial safety data for the use of tVNS as a treatment for tinnitus showed that tVNS was safe for use in patients without cardiac disease (Kreuzer et al., 2012, Kreuzer et al., 2014). However the efficacy of tVNS seemed to be dependent on the use of paired acoustic stimulation, as tVNS alone had a minimal effect on tinnitus symptoms. Another small-scale study of tVNS for the treatment of tinnitus included paired acoustic stimulation as part of the treatment protocol (Lehtimaki et al., 2013). Lehtimaki et al. recruited 10 patients with chronic tinnitus to receive daily tVNS applied to the left tragus (25Hz) at the same time as listening to an edited piece of classical music over 10 days, with each individual session lasting 45 - 60 minutes. The music was filtered to remove frequencies corresponding the individual's tinnitus frequencies (Lehtimaki et al., 2013). Significant improvements in mood were observed as a result of tVNS and the loudness of the tinnitus sounds was reported to have diminished (Lehtimaki et al., 2013). The study also reported that tVNS modulated the activity of evoked auditory cortical potentials as assessed by magnetoencephalography (Lehtimaki et al., 2013).

1.4.6.6 The cardiac effects of tVNS

The retrospective assessments of cardiac safety performed in studies with tinnitus patients found that tVNS caused no adverse cardiac effects in patients with no prior medical history of cardiac pathology (Kreuzer et al., 2012, Kreuzer et al., 2014). Kreuzer et al. reported a decrease in heart rate after 30 minutes of tVNS, but no adverse events or side-effects occurred as a direct result of stimulation (Kreuzer et al., 2012, Kreuzer et al., 2014). However, the investigations of invasive VNS for the treatment cardiovascular disease states such as CHF has also led to interest in the potential cardiac therapeutic benefits of tVNS.

Two early tVNS studies were performed in coronary artery disease (CAD) patients with angina pectoris who were due to receive a coronary artery bypass graft (Zamotrinsky et al., 1997, Zamotrinsky et al., 2001). Electroacupuncture was used to deliver tVNS to the inferior concha of both ears for 15 minutes daily over the course of 10 days. This electrical stimulation was observed to reduce the incidence of angina pectoris and the reduction in symptom severity persisted for several weeks following the course of treatment. In addition, there was also an observed decrease in the use of oral vasodilator medication such as glycerol trinitrate (Zamotrinsky et al., 1997, Zamotrinsky et al., 2001). Biopsies of atrial tissue taken during the coronary artery bypass procedure showed that patients who had undergone tVNS had decreased tissue concentrations of heat shock protein (HSP) 70 as well as reduced levels of ATP in the atrial tissue (Zamotrinsky et al., 1997). Following the surgical procedure, the patients who had received tVNS had improved clinical outcomes compared to a control group, with the tVNS-treated patients having a reduced prevalence of acute heart failure (Zamotrinsky et al., 2001). In a later study of a 10-day course of tVNS with coronary artery disease patients, Popov et al. found a significant reduction in the severity of angina attacks in approximately 62.5% of the patients (Popov et al., 2013). While this study did not demonstrate any improvements in clinical outcomes of tVNS, an improvement in general quality of life was observed (Popov et al., 2013). However, further analysis of patients who were seen to respond to tVNS showed a significant reduction in the LF/HF

ratio from baseline following tVNS, suggesting that in this cohort of patients treatment with tVNS may have generated an increase in vagal activation (Popov et al., 2013). A larger 15 day trial of daily tVNS (up to 30 minutes per day) in patients with chronic heart failure and severe left ventricular dysfunction (n = 51) found significant improvements in clinical outcomes for the majority of patients who received tVNS compared to a control group (Afanasiev et al., 2016). These improvements included significantly increased LVEF and a decrease in LV end-systolic volume, as well as reduced dyspnoea and fatigue. Moreover, these improvements were accompanied by a significant increase in exercise tolerance as assessed by increased distance covered during a 6 minute walk test (Afanasiev et al., 2016). In addition tVNS was observed to evoke bradycardia in the patients who received tVNS as well as increased levels of heat shock proteins HSP60 and HSP70 in patients who had an initial heart rate of less than 80 bpm. Patients with a heart rate greater than 80 bpm exhibited increased levels of HSP70 alone (Afanasiev et al., 2016). In patients with paroxysmal atrial fibrillation, one hour of low level tVNS performed under general anaesthesia led to a significant decrease in the duration of atrial fibrillation episodes induced by burst atrial pacing (Stavrakis et al., 2015). Moreover, tVNS elicited significantly reduction in plasma levels of the inflammatory cytokines such as tumour necrosis factor alpha (TNF-alpha), interleukin-6 (IL-6) and C-reactive protein (Stavrakis et al., 2015). Given the promising outcomes of these clinical studies and the possibility that tVNS may have a cardioprotective effect, further investigation is warranted to identify which cardiovascular disease states may potentially benefit most from tVNS.

The observed efficacy of tVNS in patients with cardiac disease has been reinforced by preclinical evidence in animal models. In a canine model of atrial fibrillation induced under general anaesthesia, tVNS (20Hz, pulse width 1ms, duty cycle 5s on/5s off) applied using electrodes clipped onto the right tragus had a significant effect on atrial remodelling and inhibited the induction of atrial fibrillation elicited by 3 hours of rapid atrial pacing prior to stimulation (Yu et al., 2013). Additional studies where tVNS the right or left tragus suppressed atrial fibrillation when performed alongside 9 hours of atrial

pacing by preventing the loss of the atrial connexin gap junction proteins Cx40 and Cx43 (Chen et al., 2015b, Chen et al., 2015a). Another study using the same stimulation parameters in conscious dogs with healed myocardial infarction showed that 90 days of tVNS (4 hours per day) applied bilaterally to the tragus could increase LVEF, reduce left ventricular dilatation and improve left ventricular contractility (Wang et al., 2014). In addition, tVNS was shown to reduce the plasma concentration of norepinephrine compared to controls, suggesting that the cardiac effects observed as result of chronic tVNS were accompanied by a reduction in sympathetic nerve activity (Wang et al., 2014).

1.4.6.7 The effects of tVNS on cardiovascular autonomic function

A number of studies have examined the effects of tVNS on measures of cardiovascular autonomic function in humans. La Marca et al. identified a significant increase in respiratory sinus arrhythmia (an indirect marker of parasympathetic nervous system activity) following auricular acupuncture in 14 healthy male volunteers (La Marca et al., 2010). This effect was observed only with electroacupuncture and no significant changes in RSA were observed in these volunteers during manual acupuncture, sham acupuncture (using a false needle) or during quiet rest with no needle insertion (La Marca et al., 2010). In 15 female patients with chronic pelvic pain, no significant change in heart rate or heart rate variability was detected as a result of tVNS gated to the exhalation phase of respiration (Napadow et al., 2012). A study in 34 healthy volunteers by Clancy et al. found that 15 minutes of tVNS (10 – 50mA, 30Hz, 200μs) applied bilaterally to the tragus led to a decrease in the LF/HF ratio from baseline (Clancy et al., 2014). This was further accompanied by a decrease in MSNA measured using microelectrodes inserted into the common peroneal nerve in 10 volunteers, suggesting that the change in autonomic function was due to a decrease in sympathetic nervous system activity (Clancy et al., 2014). Further work using the same stimulation parameters in a cohort of young healthy male volunteers (n = 13) showed that tVNS could not only decrease the LF/HF ratio but also evoke an

increase in spontaneous baroreflex sensitivity (BRS) measured using the sequence technique (Antonino et al., 2017). This effect was not observed in either sham tVNS (electrical current switched off) or earlobe stimulation (Antonino et al., 2017).

However, a recent study by De Couck et al. did not find a significant effect on cardiovascular autonomic function as a result of tVNS applied to cymba concha of the ear (De Couck et al., 2017). This study occurred in two stages and the first stage compared the effects on HRV of 10 minutes tVNS applied to the left cymba concha to tVNS applied to the right cymba concha in a cohort of 30 healthy volunteers. Right-sided tVNS increased SDNN from baseline whereas left-sided tVNS caused no significant changes in HRV. However, when right-sided tVNS was investigated in the second stage over the course of one hour, no significant changes in HRV were observed after correction for confounding variables (De Couck et al., 2017). A key difference between this study and the studies by Clancy et al. and Antonino et al. is that De Couck et al. employed different stimulation parameters (250μs, 25Hz, duty cycle of 30s on/ 30s off) in addition to stimulating the cymba concha rather than the tragus. As both of these sites are known to receive innervation from the ABVN, the choice of stimulation site should in theory not matter so long as the electrodes are still positioned within the range of the ABVN dermatome (Peuker and Filler, 2002). However, the presence of other nerves innervating the tragus but not the cymba concha such as the auriculotemporal nerve and great auricular nerve may suggest that tVNS applied to the tragus could also be activating trigeminal and cervical spinal afferent pathways (Peuker and Filler, 2002)

1.5 Non-invasive trigeminal nerve stimulation (TNS)

While the invasive and non-invasive forms of vagus nerve stimulation have shown promise as adjunctive treatments for a wide range of disorders, other forms of electrical neuromodulatory therapies are currently under investigation. One of these is non-invasive trigeminal nerve stimulation applied to the supraorbital branches (V_1) located under the skin of the forehead (DeGiorgio et al., 2006). This alternative method of non-invasive cranial nerve neuromodulation has been trialled in several conditions such as treatment resistant epilepsy (DeGiorgio et al., 2006, DeGiorgio et al., 2013), MDD (Cook et al., 2013) and migraine (Schoenen et al., 2013).

1.5.1 Neuroanatomy of the trigeminal nerve

While the vagus nerve is the longest and most widely-distributed of the 12 cranial nerves, it is the trigeminal nerve (cranial nerve V) which is physically the largest (Go et al., 2001). It emerges bilaterally as a single large sensory root and a smaller motor root from the ventrolateral aspect of the pons, which both cross anteriorly through the prepontine cistern and enter Meckel's cave, a cerebrospinal fluid-filled extension of the subarachnoid space (Borges and Casselman, 2010). At this point lies the trigeminal sensory ganglion, containing somatic afferent cell bodies and from here the three major branches of the trigeminal nerve divide: the ophthalmic (V_1), maxillary (V_2) and mandibular (V_3) divisions. The respective divisions then exit the skull and are distributed to the face via the superior orbital fissure (V_1), the foramen rotundum (V_2) and foramen ovale (V_3) (Go et al., 2001).

As with the vagus, the trigeminal nerve is a mixed nerve but the motor efferent component is confined to the V_3 division in order to innervate the anterior belly of the digastric muscle, tensor veli palatini, tensor tympani and the four muscles of mastication: temporalis, masseter and the medial and lateral pterygoids (Go et al., 2001). The V_1 and V_2 divisions are composed exclusively of somatic afferents which transmit information about orofacial touch, pain and temperature sensation, as well as intracranial sensation from the dura mater. V_3 also transmits cutaneous sensory information from the

external ear via the auriculotemporal nerve, where its receptive field overlaps with the auricular branch of the vagus nerve (Peuker and Filler, 2002).

The trigeminal ganglion, located in Meckel's cave, projects to a bilaterally-arranged trigeminal tract containing three sensory nuclei – the mesencephalic nucleus, major trigeminal sensory nucleus and spinal trigeminal nucleus – as well as a smaller motor nucleus (Borges and Casselman, 2010). The trigeminal sensory nuclei have reciprocal projections to other brainstem structures such as the paratrigeminal nucleus, NTS, locus coeruleus and the extensive network of diffuse nuclei known as the reticular formation (Grzanna et al., 1987, Caous et al., 2001). These sites are known from studies in animal models of epilepsy to be critical to the inhibition of seizures and there is evidence that electrical stimulation of the trigeminal nerve and associated nuclei can suppress seizure activity. In a feline model of chronic seizures induced by stimulating electrodes implanted in the amydala, stimulation of the NTS delayed or suppressed the onset of seizure activity (Magdaleno-Madrigal et al., 2002). Similar effects were observed with stimulation of the locus coeruleus in rats following induction of seizures by intracerebral infusion of 6-hydroxydopamine and electrical stimulation of the amydala (Jimenez-Rivera et al., 1987). The locus coeruleus in particular is known to be involved in catecholaminergic neurotransmitter synthesis (e.g. norepinephrine) and is known to be a critical pathway for the anticonvulsant efficacy of implanted VNS (Krahl et al., 1998).

1.5.2 Non-invasive TNS for the treatment of intractable epilepsy

The anti-epileptic effects of implanted VNS formed the basis for an investigation by Fanselow et al. (2000) into the therapeutic benefits of trigeminal nerve stimulation in a rat model of generalised tonic-clonic seizures (Zabara, 1992, Penry and Dean, 1990, Fanselow et al., 2000). Intraperitoneal injection of pentylenetetrazol was used to induce acute seizures in awake adult female rats (n = 8). The duration and severity of the seizures was assessed using field potentials recorded from the thalamus and somatosensory cortex via implanted microwire electrode arrays. Stimulating electrode cuffs were also implanted around the infraorbital nerves (V_2 division) to deliver either unilateral or bilateral stimulation. Fanselow and colleagues found continuous unilateral stimulation of the infraorbital nerve reduced seizure activity (a reduction of around 36% to 58% in seizure frequency), with the effect becoming stronger with increasing levels of current (3 – 11mA in 2mA increments). For these experiments the stimulus pulse duration and frequency were kept constant (0.5ms and 333Hz respectively). However a frequency-dependent effect was also observed when current was held at 9mA, with stimulation at greater frequencies (100Hz, 125Hz, 200Hz and 333Hz) leading to fewer seizures than lower frequencies (≤ 50Hz) or control (no stimulation). Bilateral stimulation was also found to be more effective at reducing seizure activity than unilateral stimulation, with the additional benefit of being able to apply a lower level of current using bilateral stimulation to achieve a similar level of seizure reduction (Fanselow et al., 2000).

This study by Fanselow et al. informed a proof-of-concept clinical study which investigated the safety and efficacy of TNS applied bilaterally to the infraorbital and supraorbital nerves in seven intractable epilepsy patients (DeGiorgio et al., 2003, DeGiorgio et al., 2006). Stimulation was well-tolerated and four patients experienced a ≥50% reduction in the frequency of their seizures after three months of chronic TNS (≥12 hours stimulation per day), an effect which was maintained at six months. The patients also reported that they preferred to apply TNS to the supraorbital stimulation site rather than the infraorbital site as the electrodes could be concealed using a

hat (DeGiorgio et al., 2006). It is nonetheless important to note that none of the observed reductions were reported as significant, likely due to the small number of patients recruited to the study (DeGiorgio et al., 2006). A subsequent, larger scale phase II randomised clinical trial (n = 42) of supraorbital TNS for intractable epilepsy compared a treatment group which received TNS based on stimulation parameters used in the previous studies (120Hz, pulse duration <250μs, ≥12 hours stimulation per day) against a group which received lower-intensity stimulation (2Hz, 50μs, duty cycle 2 seconds on and 90 seconds off). This trial reported a significant 25% decrease in seizure frequency at 12 weeks in the treatment group (n = 23) compared to a 1.2% increase in seizure frequency in the low intensity stimulation group (n = 19). After 18 weeks, 40.5% of the treatment group exhibited a ≥ 50% reduction in seizure frequency compared to 15.6% of the low intensity stimulation group at the same time point (DeGiorgio et al., 2013). A follow-up study with the same patient cohort after 12 months of stimulation found that 7 of the 19 patients assigned to the treatment group experienced a ≥ 50% reduction in seizure frequency while only 4 out of 16 patients in the low-intensity stimulation group achieved the same level of seizure reduction (Soss et al., 2015).

The potential mechanism behind this reduction in seizure activity was hypothesised to be due to the cortical desynchronisation produced by TNS, a change in cortical excitability which can disrupt seizure activity, which may be due to a generalised activation of midbrain nuclei and not solely through activation of the trigeminal tract. Furthermore this would indicate that multiple cranial nerves other than the vagus may be used to elicit the anti-epileptic effects described by invasive VNS and tVNS studies (Fanselow et al., 2000, Stefan et al., 2012, Rong et al., 2014).

1.5.3 Non-invasive TNS for treatment-resistant major depressive disorder

The phase II randomised clinical trial of supraorbital TNS for intractable epilepsy (n = 42 patients) also reported an improvement in mood in the treatment group compared to the active control (low intensity stimulation) group, as assessed using the Beck Depression Inventory (DeGiorgio et al., 2013). Given the neuroanatomy of the trigeminal nerve and the documented effects of VNS on mood, a proof-of-concept study of supraorbital TNS in patients with treatment-resistant major depressive disorder (MDD) was performed (Schrader et al., 2011, Cook et al., 2013). Eleven MDD patients received 8 hours of TNS each night while asleep over the course of 8 weeks, with fortnightly assessment of depressive symptoms using the 28-item Hamilton Depression Rating Scale ($HDRS_{28}$). TNS was well-tolerated in these patients apart from mild skin irritation due to the electrode pads. By week 8, six of 11 patients had experienced a significant reduction of ≥ 50% in depressive symptoms from baseline, with remission of symptoms in 4 of 11 patients (Cook et al., 2013). A similar trend was observed in a second study in 12 adult patients with comorbid MDD and posttraumatic stress disorder assessed using the $HDRS_{17}$. Five out of 12 patients experienced a ≥ 50% reduction in depression severity, of which three achieved remission of symptoms (Cook et al., 2016). A significant improvement in quality of life scores was also identified at 8 weeks using the Q-LES-Q Quality of Life Enjoyment and Satisfaction Questionnaire (Cook et al., 2016).

1.5.4 Non-invasive TNS for the treatment of migraine

The limited treatment and preventative options available to patients with chronic migraine has inspired several trials to assess the efficacy of TNS for this disabling primary headache condition. An initial pilot study of TNS over 3 months in episodic migraine patients (n = 9) found a significant reduction from baseline in the frequency of migraine attacks per month using the Cefaly supraorbital nerve stimulation system (STX-Med., Belgium) to deliver TNS (Schoenen et al., 2013). Schoenen et al. followed this study with a

randomised sham-controlled trial where 67 migraine patients (≥ 2 migraine attacks per month) received either active TNS (250μs, 60Hz) or sham stimulation (30μs, 1Hz) for 20 minutes each day over 3 months (Schoenen et al., 2013). In the first month of treatment, the number of days per month where a migraine occurred (monthly migraine days) decreased by 20% for both the active TNS and sham TNS groups. However, only the active TNS group exhibited a further decrease in monthly migraine days by the end of the trial, with a significant decrease at 3 months for active TNS of 29.7% (Schoenen et al., 2013). In addition this trial did not report any serious or adverse side-effects as a result of TNS and the few contraindications for use (unsuitable for patients with recent brain or facial trauma) are in marked contrast to the frequent adverse effects of existing pharmacological anti-migraine treatments (Schoenen et al., 2013).

A more recent controlled trial by Przeklasa-Muszynska et al. (2017) compared the effects of ten 20 minute sessions of supraorbital TNS in a cohort of patient with either migraine with aura, migraine without aura or another primary headache condition (n = 57). The effects of TNS in these patients were then compared with another group of migraine patients who received optimum pharmacological therapy (n = 30). Patients who received TNS reported a significant reduction in pain intensity (36 – 37%) migraine attacks, whereas no decrease in pain intensity was observed for the control group (Przeklasa-Muszynska et al., 2017). A study of the acute effects of TNS in 30 migraine patients who had the stimulus applied during a migraine attack found that 76% of patients experienced a ≥ 50% reduction in pain intensity after one hour of stimulation (Chou et al., 2017).

1.5.5 Cardiovascular effects of trigeminal nerve stimulation

Almost all of the clinical trials of supraorbital TNS included non-invasive assessments of heart rate and blood pressure to monitor for adverse cardiovascular effects in response to stimulation. DeGiorgio et al. (2006) assessed HR, blood pressure and ECG changes every five minutes when TNS was applied for one hour on the first day of stimulation. No significant changes in any of these measures were reported as a result of TNS during this observation period or at a subsequent observation period 24 hours later (DeGiorgio et al., 2006). The later phase II clinical trial of TNS in epilepsy also failed to detect any significant changes in heart rate, diastolic BP or systolic BP either between or within groups (treatment versus control) over the course of the 18 week stimulation period (DeGiorgio et al., 2013). The TNS for MDD trials also failed to detect any significant changes in HR or BP, although Cook et al. (2016) detected a small but insignificant decrease in HR from 76.7bpm to 74.9bpm after 30 min of stimulation at the first visit to the clinic (Cook et al., 2013, Cook et al., 2016). The lack of any observed TNS-induced changes in cardiovascular function is perceived to be a major benefit to this form of neuromodulation, which broadens its potential clinical utility compared to invasive techniques such as implanted VNS. However, a safety study in epilepsy patients by Pop et al. did detect a small (4%) significant decrease in heart rate after 15 minutes of TNS applied to the supraorbital nerves, suggesting that some changes in cardiovascular autonomic function may occur as a result of TNS (Pop et al., 2011).

There is preclinical evidence that TNS may indeed be able to have an effect on the cardiovascular system. In a rat model of traumatic brain injury (n = 12), intermittent TNS (25 Hz, 1 second on/1 second off duty cycle, 1.0V, 1.0 ms) applied directly to the anterior ethmoidal nerve (V_1) caused a significant increase in heart rate from baseline during the first minute of stimulation (340 ± 26 bpm to 351 ± 26 bpm) as well as a significant increase in mean arterial pressure (118.6 ± 10.7 to 129.5 ± 9.1 mmHg). Both HR and MAP returned to baseline levels within 10 minutes of cessation of the stimulus (Chiluwal et al., 2017). In the same study, electroacupuncture stimulation applied for 1 minute to the V_1 dermatome at a higher frequency and intensity (100Hz, 1

second on/1 second off duty cycle, 3.0V, 0.5ms) elicited a similar increase in HR and MAP as direct stimulation of the anterior ethmoidal nerve (Chiluwal et al., 2017). This may have been due to increased activation in the RVLM as a result of TNS applied to V_1, which corresponds with the additional observation of a 70.4% increase in cerebral blood flow due to TNS-induced cerebral vasodilation.

1.6 General hypothesis

Non-invasive cranial nerve stimulation will alter autonomic nervous system activity. Both transcutaneous vagus nerve stimulation and trigeminal nerve stimulation will induce changes in cardiovascular autonomic function.

1.7 Aims and Objectives

The primary aim of this thesis was the investigation of two non-invasive methods of electrical cranial nerve neuromodulation – transcutaneous vagus nerve stimulation (tVNS) and transcutaneous trigeminal nerve stimulation (TNS). The objectives for the tVNS studies included in this thesis were:

1. To investigate the effects on HRV and BRS of tVNS applied to the tragus and compare these effects with those elicited through electrical stimulation of the cymba concha (ABVN), earlobe (cervical spinal afferent innervation) and helix (trigeminal nerve) in healthy volunteers.
2. To determine the effects of tVNS applied to the tragus on HRV, BRS and MSNA in an aged healthy volunteers
3. To determine the effects of tVNS on HRV and BRS in a cohort of heart failure patients

The objectives for the TNS study were:

1. To investigate and compare the effects on HRV and BRS of low frequency (L-TNS) and high frequency TNS (H-TNS).

Chapter 2

General Methods

2.1 Human research participants

All experiments with human research participants conformed to the standards outlined in the Declaration of Helsinki. Informed written consent was obtained voluntarily from all research participants and their data were anonymised and stored securely according to the Data Protection Act (1998). Most experiments were carried out in a dedicated human physiology study room at University of Leeds between the hours of 8am and 11am in order to minimise the impact of circadian rhythm variations on the autonomic nervous system. For some participants it was not possible to perform experiments during this time or in the same room and these exceptions are highlighted in the appropriate chapters. The ambient temperature of the study rooms where experiments took place was maintained at 21 ± 2°C.

Prior to taking part in experiments, all participants were asked to complete a basic health questionnaire. Physical activity level was assessed using the Godin Leisure Time Exercise Questionnaire (Amireault and Godin, 2015). Inclusion criteria varied according to each study and these criteria are detailed in the respective chapters. Exclusion criteria were a prior medical history of hypertension, cardiac disease, diabetes mellitus or epilepsy. Female participants were asked to indicate if they were receiving hormonal replacement therapy (HRT) for treatment of menopausal symptoms, as HRT has been shown to independently alter cardiac autonomic activity (Yildirir et al., 2001). All participants were required to abstain from caffeine, alcohol, nicotine and strenuous exercise for a minimum of 12 hours prior to their visit. They were further required to consume a light breakfast and use the toilet prior to attending for the experiment.

2.2 Experimental protocol

2.2.1 General protocol

Participants reclined semi-supine on a couch for the duration of each experiment while recordings of their heart rate, finger arterial blood pressure and respiration rate were obtained. In Chapter 4 a smaller cohort of participants returned for an additional visit where microneurography was performed to record MSNA in addition to the non-invasive measures. Participants rested for 10 minutes during experimental set-up and before recordings commenced. Three 10 minute recordings were obtained at each visit: at baseline (pre-intervention), during the final 10 minutes of the 15 minute stimulation period and during recovery (post-intervention). Brachial blood pressure was determined using a digital monitor (Omron Intellisense M7, Omron Healthcare, Netherlands) at the end of each recording to validate the finger arterial blood pressure measurements. The number of experiments the participants were asked to attend depended on the particular study and this information is detailed in each chapter.

2.2.3 Transcutaneous vagus nerve stimulation

In Chapters 3, 4 and 5 tVNS was applied for 15 minutes to the tragus and anterior wall of the external acoustic meatus via a TENS machine (V-TENS Plus, Body Clock Health Care Ltd, United Kingdom; Figure 2.1) with customised auricular electrode clips (Auricular Clips, Body Clock Health Care Ltd, UK; Figure 2.1). In all tVNS experiments the participants wore the electrode clips throughout the three experimental recordings obtained at each visit. Following the baseline recording, the electrical current was increased by small increments until the participant's threshold of sensory perception was reached and the individual could perceive the stimulus, often reported as a 'pin-prick' or 'tingling' sensation in the tragus (10-50mA; Table 2.1). The current was then decreased until the stimulus was borderline perceptible and not uncomfortable.

2.2.3.1 Sham tVNS

Chapter 4 included a sham tVNS visit in addition to an active tVNS visit. The sham tVNS protocol involved positioning the electrode clips on the tragus of the ear and titrating the stimulus amplitude as normal until the participant could perceive any sensation from the clips. The participant was then informed that the amplitude would be reduced to prevent any discomfort and the electrode leads were disconnected from the TENS machine without the participant's knowledge.

2.2.3.2 Stimulation tolerability questionnaire

In Chapter 5, the heart failure patients were invited to complete a tolerability questionnaire following completion of their experiment. The purpose of this questionnaire was to assess their experience of receiving short-term tVNS and identify potential discomfort and other stimulus-associated sensations. Each question on the questionnaire was accompanied by a Likert-type scale ranging from 0 ("not at all") to 5 ("extremely"). Patients were further asked to rate the comfort of the couch itself and also to report any anxiety or chest palpitations they might have experienced during the experiment. Space was provided on the questionnaire for additional comments from the participants.

2.2.4 Trigeminal nerve stimulation

In Chapter 6, TNS was applied for 15 minutes to supraorbital nerve branches of the ophthalmic division (V1) of the trigeminal nerve using two adhesive TENS pads positioned approximately one inch apart (see Figure 2.2). Participants wore the electrodes throughout all three experimental recordings. Following the baseline recording, the electrical current was increased by small increments until the participant's threshold of sensory perception was reached and the individual could perceive the stimulus, often reported as a 'tingling' sensation running superiorly from the forehead across the scalp, following the course of the supraorbital nerve branches (10-50mA).

The current was then decreased until the stimulus was borderline perceptible and not uncomfortable.

2.2.4.1 Stimulation parameters for TNS

Two active TNS parameters were investigated in Chapter 6 – low frequency TNS (L-TNS, 30Hz, 200µs, continuous current) and high-frequency TNS (H-TNS, 120Hz, 250µs, 30s on/30s off duty cycle). The L-TNS parameters were identical to those used for tVNS and were delivered using a V-TENS Plus TENS device (V-TENS Plus, Body Clock Health Care Ltd, United Kingdom). The H-TNS parameters were derived from clinical studies by DeGiorgio and colleagues (UCLA, USA) and were delivered using an EMS DE7500 device which was capable of delivering the current on an adjustable on/off duty cycle (DeGiorgio et al., 2006).

2.2.4.1 Sham TNS

For the sham TNS experiments in Chapter 6 (n=10), participants were told that a separate set of stimulation parameters would be tested involving a reduction in the current below the participant's level of sensory perception. The parameters described in the previous section for L-TNS were used again in the initial sham TNS titration stage. The amplitude was briefly (10-30 seconds) titrated up to the threshold of sensory perception before being reduced and the electrode leads were then disconnected from the TENS machine without the participant's knowledge.

Table 2.1 Overview of the stimulation parameters included in this thesis.

Intervention	Stimulation site	Method	Stimulation parameters
tVNS	Tragus of external ear (bilateral)	Two auricular electrode clips with V-TENS Plus device	15 minutes, 10-50mA, 30Hz, 200μs, continuous current
Sham tVNS	Tragus of external ear (bilateral)	Two auricular electrode clips with V-TENS Plus device	10-20 seconds exposure to continuous current (10-50mA, 30Hz, 200μs) followed by 15 minutes of no current (electrodes disconnected from TENS machine)
L-TNS	Supraorbital region	Two adhesive TENS electrodes with V-TENS Plus device	15 minutes,10-50mA, 30Hz, 200μs, continuous current
H-TNS	Supraorbital region	Two adhesive TENS electrodes with EMS DE7500 device	15 minutes 10-50mA, 120Hz, 250μs, 30 seconds on/ 30 seconds off duty cycle
Sham TNS	Supraorbital region	Two adhesive TENS electrodes with V-TENS Plus device	10-20 seconds exposure to continuous current (10-50mA, 30Hz, 200μs) followed by 15 minutes of no current (electrodes disconnected from TENS machine)

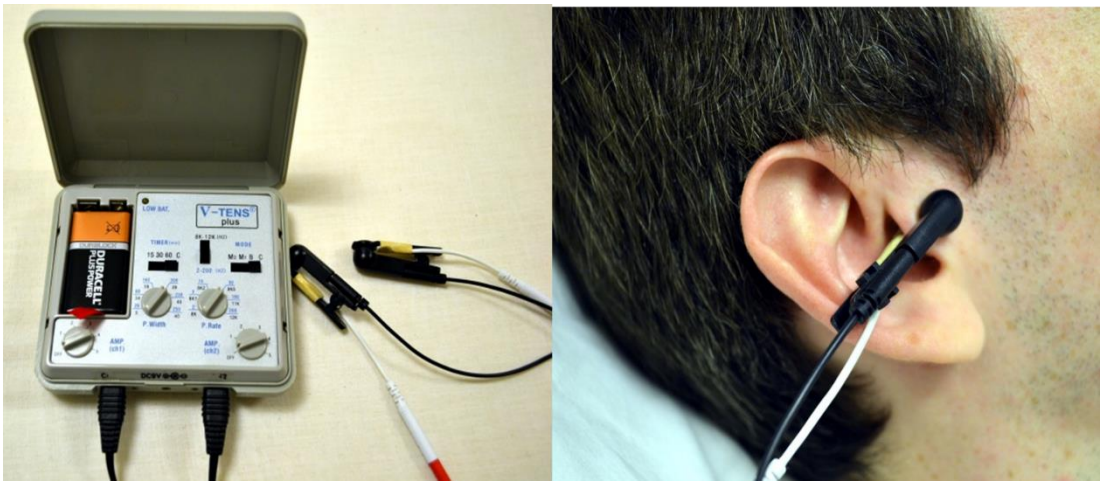


Figure 2.1 Transcutaneous vagus nerve stimulation (tVNS) applied to the tragus of the ear. The technique uses a TENS machine and modified auricular electrode clips to stimulate cutaneous nerve endings in the tragus.

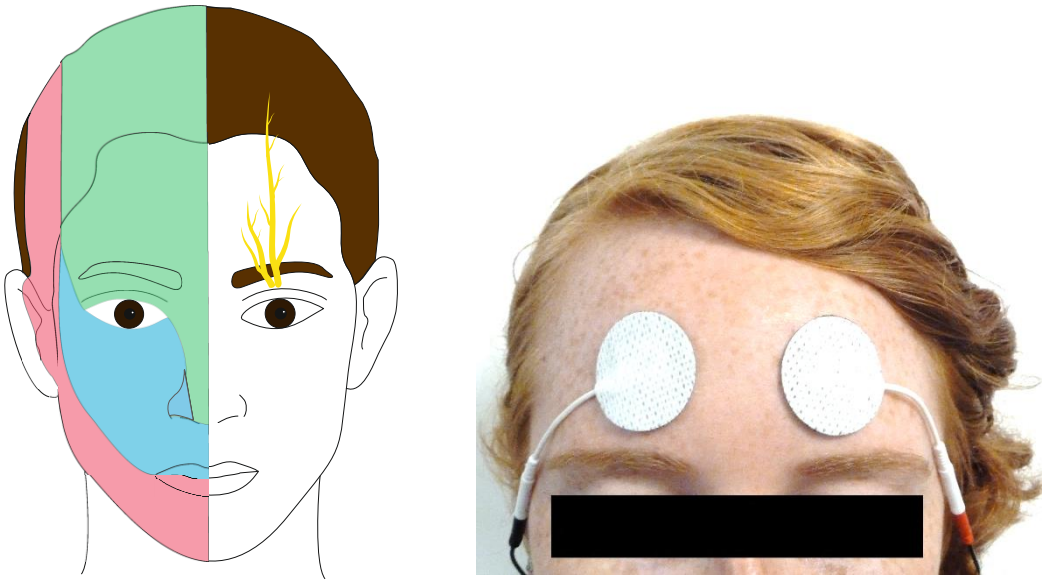


Figure 2.2 Non-invasive trigeminal nerve stimulation applied to the supraorbital nerves of the forehead. This technique uses a TENS machine and adhesive electrode pads to stimulate the supraorbital branches of V_1 (dermatome shaded in green).

2.4 Heart rate variability

Heart rate was recorded using a three lead ECG where electrodes (Ambu BlueSensor SP, UK) were placed on the right and left costal margins and the right clavicle in order to detect a prominent R peak (lead II) for HRV analysis with Spike2 software (Cambridge Electronic Design, UK). The ECG was monitored throughout all experiments for the presence of signal artefacts or ectopic beats (premature ventricular contractions or ventricular extrasystoles).

Offline analysis was carried out using Spike2 (version 7.1; CED, UK) and Labchart (version 8.1.5; ADInstruments, Bella Vista, Australia) software. Mean heart rate was derived from the final five minute segment of each ECG recording. The R-R intervals from this time period were used to produce a tachogram which was inspected manually to ensure all R-peaks had been detected. Occasional R-R interval abnormalities due to ectopic beats (≤ 2 events in the five minute ECG segment) were corrected by averaging the R-R intervals immediately before and after the event. The R-R interval tachogram was resampled at 5 Hz a DC removal process applied to change the channel offset to zero. The tachogram then underwent 512 point Fast Fourier Transform with a Hanning window (50% overlap) to calculate the HRV power spectrum. The LF power and HF power components were normalised as a percentage of the total frequency power (0 - 0.4 Hz) minus very low frequency power (0 – 0.04 Hz) to calculate the LF/HF power ratio.

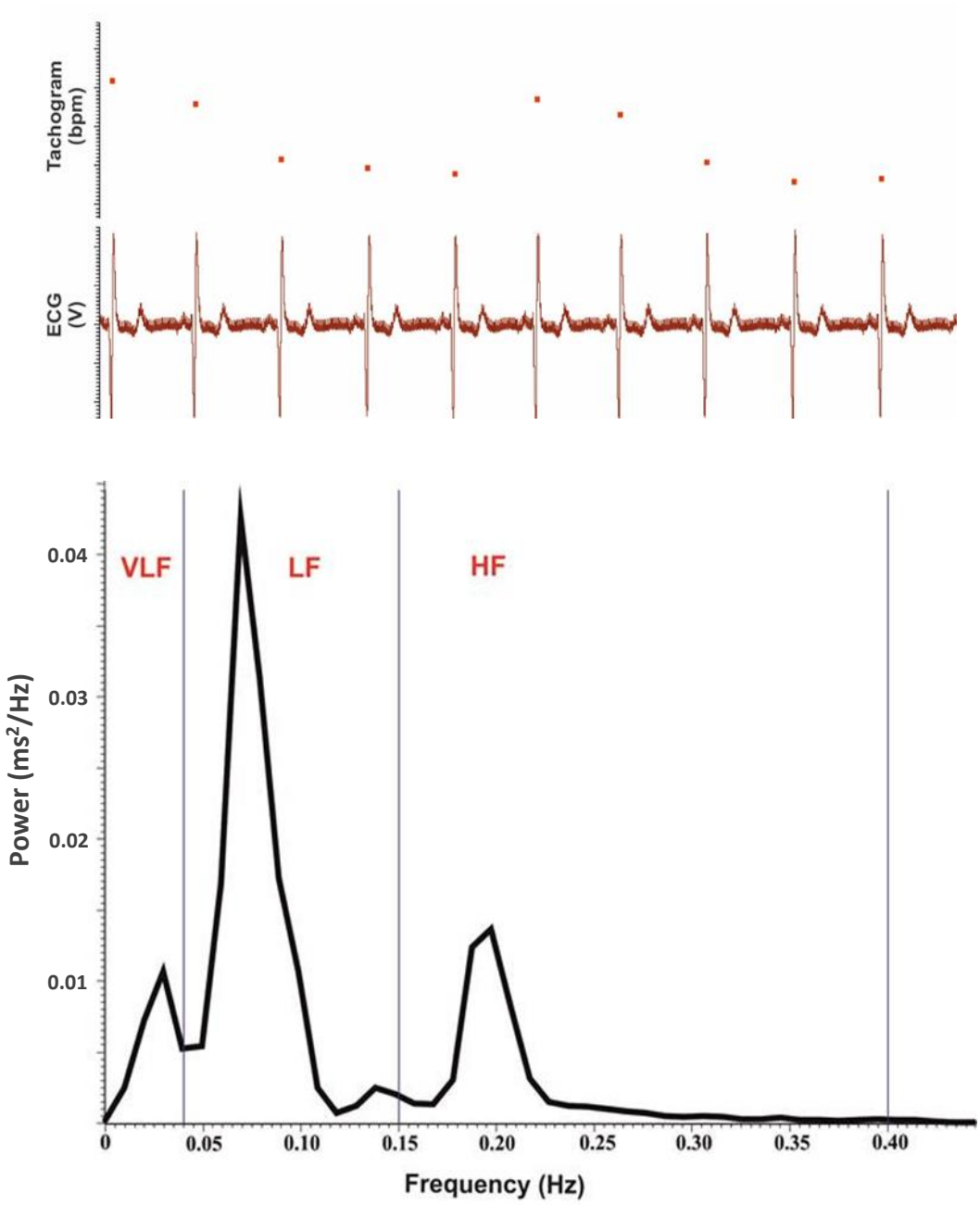


Figure 2.3: An example of heart rate variability analysis. An R-R interval tachogram is derived from the ECG channel (Lead II). Fast Fourier transform (512 point) is used to calculate the HRV power spectrum and obtain values for the VLF (very low frequency), LF (low frequency) and HF (high frequency) components.

2.5 Respiration

Participants were allowed to breath spontaneously during all experiments, but their respiration rate was closely monitored using a piezo-electric transducer (Pneumotrace, UFI, USA). A respiration rate of < 8 breaths per minute has a negative effect on the reliability of frequency-domain heart rate variability analysis, as the HF power peak (which is associated with respiration rate) may merge with the LF power peak (Malliani, 2005). Participants were thus assessed at the start of their first experiment to ensure they had a minimum respiration rate of ≥ 10 breaths per minute. Where participants failed to meet the minimum respiration rate, their breathing was paced using a custom metronome set at 12 breaths per minute.

2.6 Finger arterial blood pressure

A Finometer PRO (Finometer Medical Systems B.V., Arnhem, Netherlands) with finger cuff attachment and height-correction unit was used for continuous photoplethysmographic recording of finger arterial blood pressure in all experiments. The Finometer PRO device uses the volume clamp method (Penaz, 1973), whereby an inflatable cuff is positioned around the middle phalanx of digit three on one hand. Changes in the intensity of infrared light emitted by the finger cuff are detected using a sensor to identify changes in blood volume (photoplethysmography). The systolic and diastolic values obtained were validated against readings from a digital brachial blood pressure monitor on the same arm. These brachial values were determined before and after each experimental recording to ensure Finometer accuracy within 10 mmHg. In the event of increased disparity in blood pressure values between the two sites, one or more measures were taken:

- The finger cuff was positioned on another finger or the opposite hand
- The participant's hand was wrapped in a towel for warmth
- The hand was immersed in warm water for five minutes

If these steps failed to correct for the difference in BP between the two sites, finger arterial pressure was not recorded for that participant. Automatic calibration of finger arterial blood pressure was performed continuously using PhysioCal software. Offline analysis was performed using Spike2 software and values for systolic pressure, diastolic pressure and mean arterial pressure were derived from the same five minute time period of the experimental recording as the R-R interval tachogram.

2.7 Baroreflex sensitivity

The sequence method was used to provide a spontaneous estimate of spontaneous cardiac baroreflex sensitivity (BRS). The RR interval and systolic BP waveforms were exported into Labchart (version 8.1.5, ADInstruments, Bella Vista, Australia) and custom scripts were used to detect sequences of three or more consecutive cardiac cycles where systolic pressure and R-R interval changed in the same direction e.g. an increase in systolic pressure of at least 1 mmHg coinciding with an increase in R-R interval. The slope of each individual sequence was calculated using linear regression analysis and slopes were averaged over 5 minutes to determine BRS. Minimum thresholds of 1 mmHg for systolic pressure and 2 ms for R-R interval were applied and all individual sequences were required to have an R^2 value ≥ 0.85 to be accepted for inclusion. All accepted sequences in the last five minute data block from each recording were averaged to calculate a mean value for BRS (ms/mmHg).

2.8 Microneurography

In Chapter 4, seven participants who had previously received active tVNS and experienced a decrease in LF/HF ratio were asked to return for an additional visit where microneurography was performed along with tVNS. Simultaneous recording of single-unit muscle sympathetic nerve activity (MSNA) was obtained from the common peroneal nerve of the leg (Figure 2.4). Foam cushions and pillows were used to support the lower limb and

keep the knee joint flexed. The skin overlying the common peroneal nerve was cleaned using an antibacterial wipe and the nerve was palpated at the level of the neck of the fibula. If the skin was intact and free of lesions, an active recording tungsten microelectrode (FHC Inc., USA) insulated with a high impedance epoxy resin coat was inserted percutaneously into the nerve. A second reference electrode was inserted subcutaneously 2 cm away from the recording electrode.

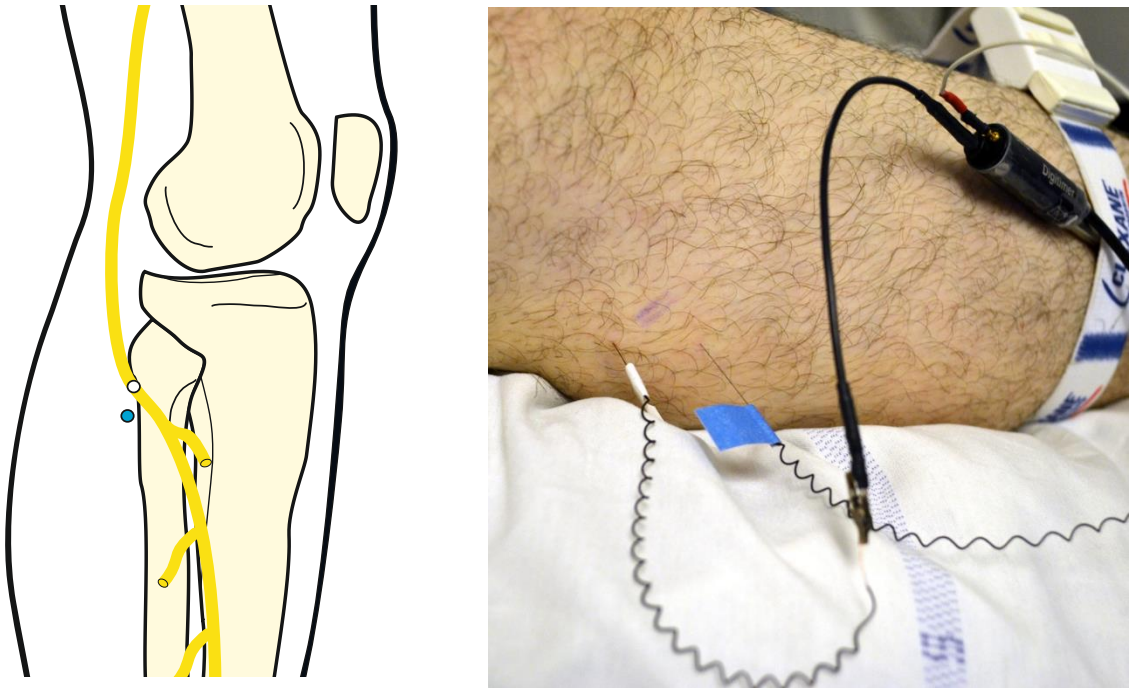


Figure 2.4 An example of the needle-insertion for microneurography.

Microneurography was performed using two tungsten microelectrode needles: one inserted percutaneously into the common peroneal nerve (active electrode, white colour) and the second inserted into the subcutaneous fascia 2cm distal (reference electrode, blue colour). The course of the common peroneal nerve around the fibular neck was manually palpated and marked with a surgical pen.

The microelectrodes were connected to a headstage (Neurolog NL100AK, UK) and an AC pre-amplifier with x50 k amplication (Neurolog NL104A, UK). A bandpass filter was applied (0.7 – 2.0 kHz) and a Humbug Noise Eliminator unit (Quest Scientific, Canada) was used to remove 50 Hz mains interference. The signal was sampled at 16 kHz, digitised using a Power 1401 (CED, UK) and displayed on a laptop using Spike2 (version 7.1) to allow the raw neurogram to be inspected in real time. The recording electrode was manipulated within the nerve until a candidate action potential was detected. Manipulations were small and restricted to 45 minutes following microelectrode insertion according to microneurography guidelines (Mano et al., 2006). A candidate single-unit was deemed to have originated from a sympathetic vasoconstrictor axon if it fulfilled the following criteria:

1. There was no change in single-unit activity in response to brushing the skin of the lateral surface of the leg and dorsum of the foot
2. The unit occurred only during diastole on the finger arterial blood pressure trace
3. There was an increase in single unit activity during the second half of the cold pressor test (one hand submerged in 4 degrees celsius ice water for one minute) or isometric handgrip test (handgrip squeezed by one hand at 50% maximal voluntary contraction for two minutes).

Units which fulfilled these criteria were manually inspected offline and superimposed to determine if they were genuine muscle sympathetic vasoconstrictor units from a single axon i.e. they shared a near-identical amplitude and morphology (Figure 2.5). Single-unit MSNA frequency (units detected per minute) and MSNA incidence (units detected per 100 heartbeats) were calculated to assess changes in sympathetic vasoconstrictor activity as a result of active tVNS. Due to high baseline variability between subjects, the baseline data were normalised to 1 and stimulation and recovery were normalised to baseline.

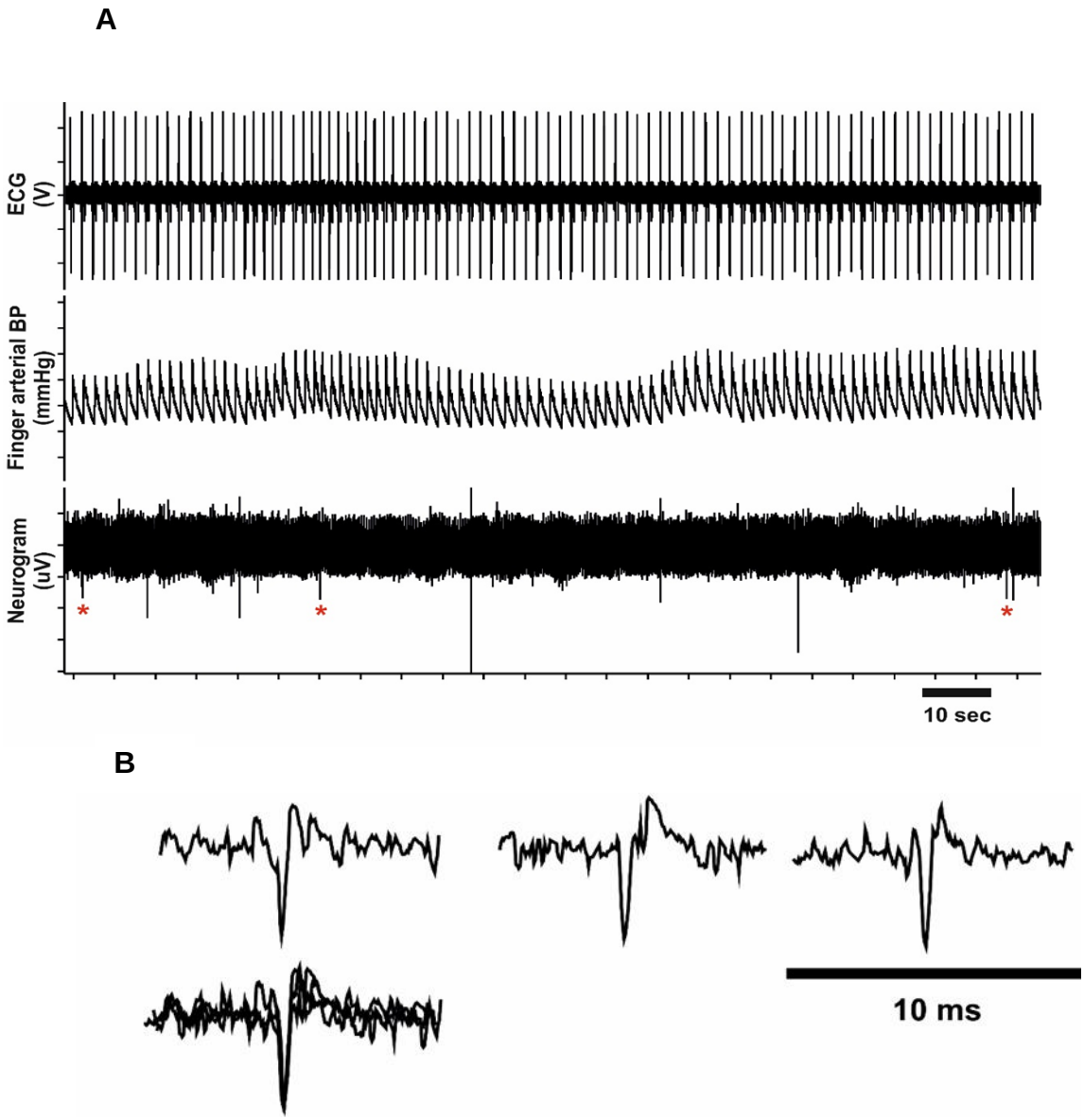


Figure 2.5 An example of a raw neurogram from one volunteer, with associated ECG and finger arterial blood pressure (BP) traces. Single-unit MSNA from one vasoconstrictor sympathetic axon is marked with an asterisk (A). Each candidate unit was inspected and superimposed to confirm the detection of MSNA (B).

2.8.1 Cold pressor test

Prior to the start of the test, participants were asked to rest quietly while baseline data were recorded for one minute. At the end of this minute, participants were then asked to submerge their left hand up to the wrist in ice water (4°C). Cold pressor test data were then recorded for one minute unless the participant reported too much discomfort. At the end of the test the participant's hand was removed from the water, wrapped in a towel and the participant rested while a further minute of recovery data was obtained.

The cold pressor test can be used to discriminate between MSNA and skin sympathetic nerve activity (SSNA). The cold pressor test evokes an increase in blood pressure that correlates well with an increase in MSNA firing rate (Fagius et al., 1989, Kregel et al., 1992). In addition, there is a time delay of approximately 30 seconds between the onset of the test and the onset of MSNA firing – the last 30 seconds of the test was thus compared to the baseline period to determine the participant's response to the cold pressor test. The slight variation in the onset of MSNA firing may be due to the participant's perceived level of discomfort (Victor et al., 1987, Kregel et al., 1992)) whereas skin sympathetic activity does not change in this respect.

2.8.2 Isometric handgrip test

Maximal voluntary contraction (MVC) was determined during the experimental set up by asking the participant to squeeze a handgrip as hard as possible for 10 seconds. The handgrip was connected to a dynamometer (MIE Medical Research Ltd, UK) which provided a numerical display. As with the cold pressor test, baseline data were recorded for one minute and the participant was asked to squeeze the handgrip at 50% of their MVC level. The participant was asked to maintain this level as best as they could for two minutes in total. Participants were asked to keep all muscles apart from those in their upper limb involved in the test as relaxed as possible and they were further instructed to maintain a normal breathing rhythm throughout the test. After two minutes the participants were asked to release the handgrip and a further one minute recovery period was recorded. The isometric

handgrip test evokes a rapid and continuous increase in blood pressure, initially mediated by tachycardia and subsequently sustained by an increase in MSNA (Seals et al., 1988). This exercise is useful in differentiating between muscle and skin SNA as SSNA increases abruptly at the start of exercise and remains constant whereas the increase in MSNA is delayed for 30 - 60 seconds and then gradually increases throughout the exercise (Seals et al., 1988, Saito et al., 1990). This time delay in firing meant that only the second minute of each isometric handgrip test was compared to the one minute baseline recording.

2.9 Data acquisition

Three separate data channels (ECG, finger arterial pressure and respiration) were fed into a data amplification system (Neurolog NL104A, CED, UK). An additional data channel was included for experiments in Chapter 4 for microneurographic recording of nerve activity. Data channels were sampled at 16 kHz and stored on hard drives. Channels were independently calibrated before digitisation and storage on PCs. Data channels were then displayed on monitors using Spike2 (CED, UK) software.

2.10 Statistical analysis

All statistical analyses were performed using SPSS (version 24). Shapiro-Wilk tests were performed on each variable to assess normality. Participant subgroup characteristics were compared using independent t-tests or Mann Whitney U-tests. One-way repeated measures ANOVA was used to analyse effect of time (baseline, stimulation and recovery) for each group and Bonferroni post-hoc correction applied. Linear mixed model analysis was performed to compare the effect of time (baseline, stimulation and recovery) between different visits (e.g. stimulation sites or stimulation parameters) where sample sizes of groups were unequal. Two-way repeated measures ANOVA was used to analyse the effect of time (baseline, stimulation and recovery) between different visits where sample sizes of groups were equal.

Non-parametric Friedman test was used to analyse effect of time for groups where data were not normally distributed and post-hoc analysis was performed using Wilcoxon-signed rank tests with Bonferroni correction applied. Spearman's Rank correlation test was used to identify possible relationships between variables and linear regression was used to further explore these correlations. All data are presented as group mean ± standard error of the mean (S.E.M).

Chapter 3

An investigation of the autonomic effects of transcutaneous electrical nerve stimulation applied to different sites on the external ear in healthy human research participants

3.1 Introduction

Transcutaneous vagus nerve stimulation (tVNS), involving the use of either percutaneous electroacupuncture or specialised surface electrodes to transmit current across the skin, has been shown to be safe in both healthy volunteers and in a clinical context (Murray et al., 2016). However, there is a high degree of heterogeneity in the literature in terms of stimulus parameters and sites of application on the ear. The principle sites of interest for delivering electrical stimulation to the ABVN have been the tragus (Busch et al., 2013, Kraus et al., 2013, Clancy et al., 2014, Weise et al., 2015, Stavrakis et al., 2015, Zhou et al., 2016), the concha (He et al., 2013a, Liu et al., 2013, Ay et al., 2015b, Fang et al., 2015), and the cymba concha (Kreuzer et al., 2014, Frangos et al., 2015). These regions are known to correspond with the ABVN dermatome, but there is overlap at the tragus and concha from the great auricular nerve which is derived from the second and third cervical spinal nerves which contribute to the cervical plexus (Peuker and Filler, 2002). In addition, the lateral (outer) side of the tragus is known to receive innervation from the anterior auricular branch of the auriculotemporal nerve, which originates from the mandibular (V_3) division of the trigeminal nerve (Shankland, 2001, Peuker and Filler, 2002). While the cutaneous innervation supplied by these nerves overlaps at the tragus, the cadaveric study by Peuker and Filler (2002) found a number of specific locations on the external ear where these nerves were the sole or predominate innervation. Examples of these locations are the cymba concha for the ABVN, the earlobe for the GAN and the spine of the helix for the auriculotemporal nerve.

3.1.1 Overview of the great auricular nerve and its central projections

The GAN is a superficial sensory branch of the cervical plexus which emerges through the superficial cervical fascia at the posterior border of sternocleidomastoid and courses superiorly over the lateral surface of the muscle towards the mandibular angle. As it ascends it divides into an anterior branch which supplies the skin overlying the parotid gland and a posterior branch which provides cutaneous innervation to the mastoid

process and inferior portion of the external ear (Ginsberg and Eicher, 2000). In humans, cadaveric dissection of the external ear has located branches of the GAN primarily in the earlobe, tail of helix and scapha as well as the posterior aspect of the ear (Peuker and Filler, 2002, Yang et al., 2015).

Following application of HRP to the central cut end of the GAN in rabbits, HRP labelling was identified in the afferent cell bodies of the great auricular nerves in the ipsilateral C2 and to a lesser extent C3 dorsal root ganglia, as well as the ipsilateral superior cervical ganglia (Liu and Hu, 1988). In the spinal cord, transganglionically-labelled GAN fibres were found in the cuneate fasciculus, laminae I – IV at C2 level and laminae II – IV at C3 level. From here, GAN fibres were found to project to medullary nuclei such as the medial and lateral cuneate nuclei, the NTS and the spinal trigeminal nucleus (Liu and Hu, 1988). The convergence of GAN fibres on the NTS is of particular interest as electrical neuromodulation applied to branches of the GAN may thus be able to influence autonomic function. However, an fMRI study in 12 healthy volunteers by Frangos et al. found that electrical stimulation of the left earlobe using the Cerbomed NEMOS® system produced no activation in cortical or subcortical sites associated with autonomic nervous system activity (Frangos et al., 2015). Activation was localised instead to spinal trigeminal nucleus and cuneate nucleus, consistent with previous HRP-labelling in rabbits (Liu and Hu, 1988). Further activation was observed in the contralateral insula and contralateral primary somatosensory cortex associated with the head and facial region (Frangos et al., 2015). This activation pattern was in marked contrast to that produced by electrical stimulation of the left cymba concha, which is innervated exclusively by the ABVN (Peuker and Filler, 2002). Widespread activation was observed in the region of the left medulla corresponding to the location of the NTS, known from neuroanatomical tracing work with cats to be consistent with the afferent distribution of the ABVN (Nomura and Mizuno, 1984, Frangos et al., 2015).

3.1.2 Overview of the auriculotemporal nerve and its central projections

The auriculotemporal nerve is a sensory nerve originating from the posterior division of mandibular (V_3) trigeminal nerve, with afferent cell bodies located in the dorsal half of the trigeminal ganglion (Jacquin et al., 1983, Shankland, 2001). It emerges from the infratemporal fossa deep to the parotid gland and ascends posterior to the temporomandibular joint and anterior to the tragus of the external ear. From this point it travels superiorly along the lateral aspect of the cranium deep to the superficial temporal artery (Shankland, 2001). Along its course the auriculotemporal nerve divides into eight major branches which provide cutaneous innervation to a large area of the lateral aspect of the head, with two branches supplying innervation to the external ear. These are the anterior auricular nerve, whose branches supply innervation to the skin of the tragus along with part of the helix, and the external acoustic meatus nerve (Shankland, 2001). In addition, the auriculotemporal nerve is known to have communications with the facial nerve trunk which allow auriculotemporal afferent fibres to innervate upper facial muscles such as orbicularis oculi and receive proprioceptive information during muscle movement (Cobo et al., 2017).

The central projections of the auriculotemporal nerve have been demonstrated in the rat (Jacquin et al., 1983, Takemura et al., 1987). Jacquin et al. compared projections of different sensory branches of the mandibular division by applying HRP to the central cut end of the auriculotemporal nerve in 5 adult rats (Jacquin et al., 1983). Widespread labelling was identified throughout the trigeminal nuclei including the ipsilateral principal trigeminal nucleus, subnucleus oralis, subnucleus interpolaris and the dorsal horn of C1 – C4. A later study by Takemura et al. which also compared the central projections of mandibular nerve branches using HRP reported a similar pattern of labelling at these sites when HRP was applied to the left auriculotemporal nerve in 4 adult rats (Takemura et al., 1987). Interestingly, Jacquin et al. identified sparse labelled projections from the auriculotemporal nerve to the NTS, although Takemura et al. did not observe any HRP labelling at this site. Both studies reported labelling in the NTS when HRP was applied to the lingual nerve and inferior alveolar nerve

(Jacquin et al., 1983, Takemura et al., 1987). These findings suggest that neuronal components of the mandibular division may be able to influence activity in the NTS, although it is not clear if stimulation of auriculotemporal nerve afferents can exert a modulatory role on NTS activation and thus autonomic nervous system activity.

In recent years there has been interest in using implanted electrodes to stimulate the auriculotemporal nerve as a treatment for pain disorders such as chronic migraine and temporomandibular joint syndrome (Simopoulos et al., 2010, Rodriguez-Lopez et al., 2015). However, there is limited information on the wider physiological effects of electrical stimulation of the auriculotemporal nerve beyond its potential as an analgesic therapy. A study where the auriculotemporal nerve in rats was stimulated to identify the effects of selective muscarinic M_1 and M_2 receptor antagonists on parotid salivary secretion showed no change in femoral intra-arterial blood pressure when stimulation was performed prior to application of the pharmacological agents (Tobin, 1998). A comparison of the autonomic effects of manual auricular acupuncture versus electroacupuncture at different ear sites in rat found that manual acupuncture of the A_1 site at the apex of the helix, innervated by the auriculotemporal nerve, elicited a significant depressor response in mean arterial pressure (Gao et al., 2008). This effect was similar to that observed with high frequency (100Hz) electroacupuncture at the same site.

3.1.3 Knowledge gap

There have been a number of studies which have compared the effects of transcutaneous electrical stimulation at different sites on the ear in man. Kraus et al. compared electrical stimulation of the inner tragus and posterior wall of the external acoustic meatus with earlobe stimulation, which was considered by the study authors to be a sham stimulation site (Kraus et al., 2013). The activation pattern observed by Frangos et al. suggests that stimulation of the ABVN dermatome (cymba concha) may be able to influence autonomic centres such as the NTS (Frangos et al., 2015). However, earlobe stimulation (great auricular nerve afferents) did not activate

the same sites as cymba concha stimulation. A more recent study by Yakunina et al. applied electrical stimulation to the cymba concha and earlobe as well as the inner tragus and inferoposterior wall of the external acoustic meatus in 37 healthy volunteers. Cymba concha stimulation produced the strongest activation in the area of the medulla corresponding to the NTS (Yakunina et al., 2017).

The present study employed non-invasive measures of cardiovascular autonomic function (heart rate variability and baroreflex sensitivity) to compare the effects of transcutaneous electrical nerve stimulation (TENS) applied to both ears at four different locations: tragus, cymba concha, helix and earlobe (Figure 3.1).

3.2 Hypothesis

Transcutaneous electrical stimulation of the tragus of the external ear will have a similar effect on cardiovascular autonomic function as cymba concha stimulation as both of these areas are innervated by the ABVN. The autonomic effects of tragus and cymba concha stimulation will differ from those observed with earlobe and helix stimulation.

3.3 Aims and objectives

The present study investigated the cardiovascular autonomic effects of transcutaneous electrical nerve stimulation (TENS) at four different sites on the external ear. The study aimed to identify stimulation-induced changes in cardiovascular autonomic activity in healthy participants using non-invasive measurements of heart rate and finger arterial blood pressure.

3.4 Materials and Methods

3.4.1 General protocol

University of Leeds ethical approval was secured (Ethics Reference: BIOSCI 16-009) and the all experiments conformed to the standards outlined in the Declaration of Helsinki. Informed written consent was obtained voluntarily by all research participants and their data were anonymised and stored securely according to the Data Protection Act (1998). All experiments were carried out in a dedicated human physiology study room at University of Leeds. These experiments occurred between the hours of 8am and 10am in order to minimise the impact of circadian rhythm variations on the autonomic nervous system. The ambient temperature of the study room was maintained at 21 ± 2°C. Inclusion criteria were male or female volunteers aged ≥ 18 years old. Exclusion criteria were a prior medical history of hypertension, cardiac disease, diabetes mellitus or epilepsy. Female participants were asked to indicate if they were receiving hormonal replacement therapy (HRT) for treatment of menopausal symptoms, as HRT has been shown to independently alter cardiac autonomic activity (Yildirir et al., 2001). All participants were required to abstain from caffeine, alcohol, nicotine and strenuous exercise for a minimum of 12 hours prior to their visit. They were further required to consume a light breakfast and use the toilet prior to attending for the experiment.

All participants were asked to attend for four visits to apply bilateral transcutaneous electrical nerve stimulation (TENS) to one of the following sites per visit in a randomised order: the tragus, the cymba concha, the helix and the earlobe. Both left and right ears were stimulated simultaneously at these sites. Stimulation of the tragus, helix and earlobe was delivered using a V-TENS Plus (Body Clock, U.K.) device and customised auricular electrode clips as used previously for tragus stimulation in healthy human volunteers (Clancy et al., 2014). The protocol for delivering stimulation was the same as described for active tVNS in Chapter 2 with the exception of cymba concha stimulation. As the auricular clips were not able to reach the cymba concha

without causing potential discomfort to the participant, two carbon fibre electrodes used to manufacture the auricular clips were applied separately to the skin of the cymba concha and positioned approximately 5 millimetres apart (Figure 3.1). The electrodes were held securely in place for the duration of the experiment using a non-conductive silicone putty ear plug moulded to the shape of the participant's cymba concha. This putty was then held in position using a lightweight headset to ensure good contact between the electrodes and the skin.

Participants were asked to report the sensations described during the initial titration of the stimulus at each site. Once the stimulus could be clearly detected by the participant, the current was reduced to a level where it was remained perceptible without any discomfort. Participants were asked to inform the investigator if they perceived a change in the strength of the current, which was then immediately adjusted back to a comfortable but perceptible level.

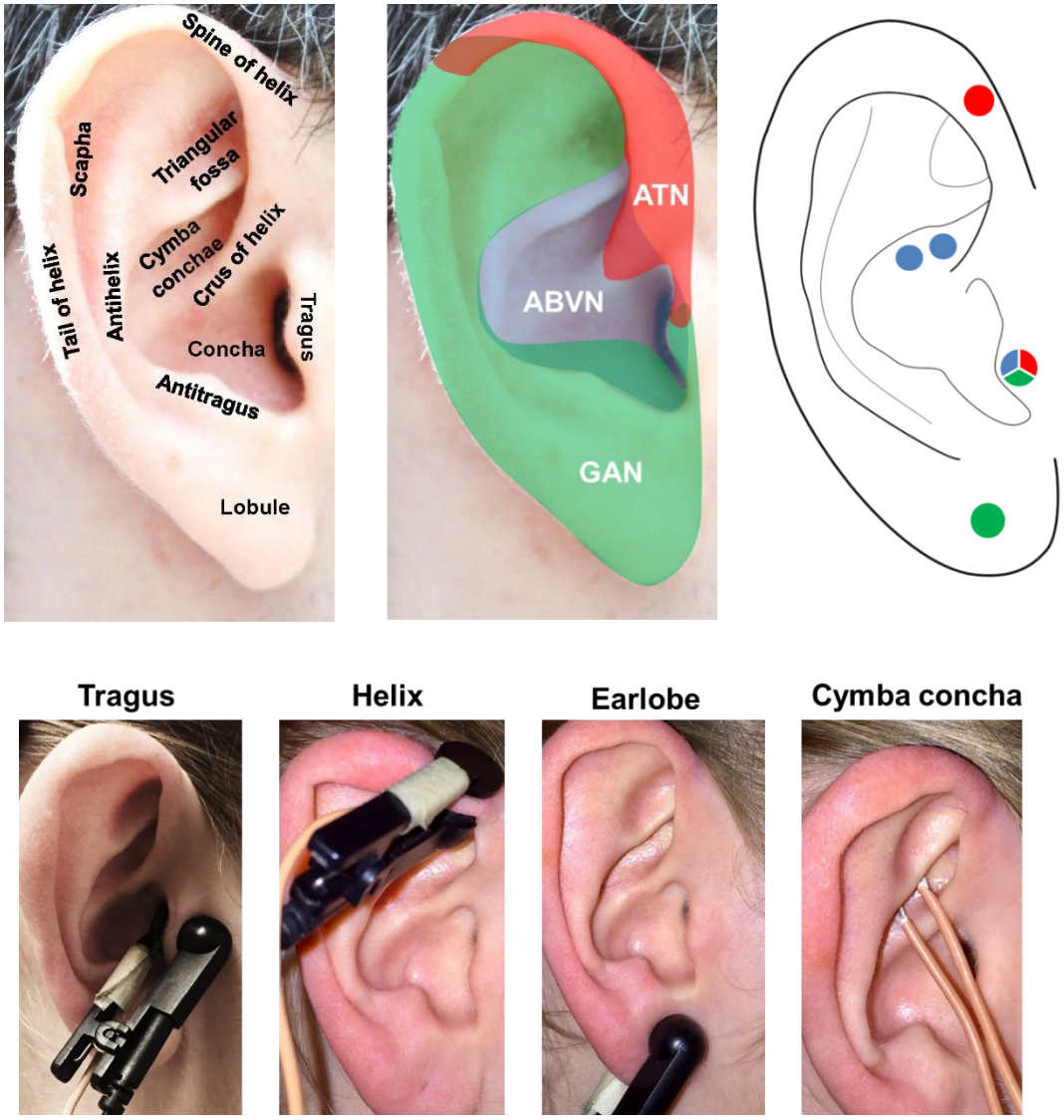


Figure 3.1 Overview of the stimulation sites used in the present study. Four sites were chosen based on the cutaneous distribution of the auricular nerves described by Peuker and Filler (Peuker and Filler, 2002). The stimulation sites were the helix (auriculotemporal nerve or ATN), earlobe (great auricular nerve or GAN), cymba concha (ABVN) and the tragus (which can be innervated by the ATN, GAN and ABVN).

3.4.2 Cardiovascular autonomic measurements and data acquisition

Recordings of heart rate, respiration, blood pressure and MSNA were obtained as described in Chapter 2. The data acquisition protocol and analyses of HRV and BRS were performed as described in Chapter 2. In some participants it was not possible to obtain reliable finger arterial blood pressure recordings or to detect BRS sequences and the sample sizes for these analyses are included in Table 3.10 for BP and Table 3.7 for BRS.

3.4.3 Statistical Analysis

All statistical analyses for this study were performed using SPSS (version 24). Shapiro-Wilk tests were performed on each variable to assess normality. Participant subgroup characteristics were compared using independent t-tests or Mann Whitney U-tests. One-way repeated measures ANOVA was used to analyse effect of time (baseline, stimulation and recovery) for each visit (active tVNS or sham tVNS) and Bonferroni post-hoc correction applied. Non-parametric Friedman test with Bonferroni correction for multiple pairwise comparison tests was used where data were not normally distributed. Friedman test with Bonferroni correction for multiple pairwise comparison tests was also used to compare effects between stimulation parameters (tragus, earlobe, helix and cymba concha) at a specific time-point for measures of HRV e.g. at baseline. Two-way repeated measures ANOVA was used to analyse the effect of stimulation (tragus, earlobe, helix and cymba concha) on BRS over time (baseline, stimulation, recovery). All data are presented as group mean ± standard error of the mean (S.E.M).

3.5 Results

3.5.1 Baseline characteristics

24 participants with no previous medical history of cardiovascular disease, diabetes or epilepsy were enrolled at the study and written informed consent was obtained at the first visit. One male participant (age = 28 years) was excluded from the study due to a slow respiration rate (< 8 breaths per minute) and non-compliance with a breathing metronome following coaching. One female participant (age = 62 years) was excluded from the study due to post-menopausal hot flashes (2 - 3 per visit, mean duration = 2 minutes) which produced extensive signal artefacts in the ECG channel and disrupted the HRV and BRS analyses. The baseline characteristics of the remaining 22 participants (n = 11 female and n = 11 male) included in the study are presented in Table 3.1. There were no significant differences in age, BMI or physical activity level as assessed by the Godin Leisure-Time Exercise questionnaire. There was no significant difference in LF/HF ratio at baseline between the male and female participants (male = 1.32 ± 0.22; female participants = 1.36 ± 0.27). No significant differences were detected at baseline between male and female participants for other measures of HRV, mean HR or mean BP.

Three participants (2 male, 1 female) were found prior to experimental recording at their first visit to have a respiratory rate < 10 breaths per minute. These participants were consequently required to breath to a metronome set at 12 breaths per minute at all four visits, as a respiration rate < 8 breaths per minute can affect frequency-domain HRV analysis (Malliani, 2005). However, the participants who were allowed to continue spontaneous breathing (n = 19) showed no significant changes in respiration as a result of stimulation at any of the external ear sites (repeated measures ANOVA; $p > 0.05$).

	N	Age (yrs)	BMI (kg/m^2)	Mean BP (mmHg)	Mean HR (bpm)	Baseline LF/HF ratio
All participants	22	32.86 ± 2.04 (range = 24 – 59)	24.66 ± 0.84	84.83 ± 2.79	67.05 ± 2.18	1.34 ± 0.18
Male participants	11	33.73 ± 3.42 (range = 26 – 59)	24.12 ± 0.90	81.88 ± 3.79	64.39 ± 2.58	1.32 ± 0.22
Female participants	11	32.00 ± 2.37 (range = 24 – 47)	25.20 ± 1.44	88.08 ± 3.47	69.72 ± 3.44	1.36 ± 0.27

Table 3.1 Baseline characteristics of participants enrolled in the study. No significant differences were detected between male and female participants in terms of baseline characteristics ($p < 0.05$).

3.5.2 Heart rate variability

When the 22 included participants were compared at baseline between the stimulation parameters, no significant differences were detected between parameters at each time point (baseline, stimulation and recovery) for any measure of frequency-domain HRV (Friedman test, $p > 0.05$). This may have been due to the relatively small sample size and the skewed non-normal distribution of data. HRV was then analysed for each stimulation parameter in all 22 participants (Table 3.2). However, the sole significant change detected was an increase in LF power which occurred following earlobe stimulation during the recovery period (Friedman test, $p = 0.048$), but

without any simultaneous significant difference from baseline for HF power (Friedman test, p = 0.186) or LF/HF ratio (Friedman test, p = 0.544) at this time-point.

To determine if the lack of significant changes in frequency-domain HRV were due to a high degree of inter-individual variation in resting cardiac autonomic activity, the dataset of 22 participants was split into participants who responded with a decrease in LF/HF ratio during tragus stimulation (tragus responders; n = 12; Figure 3.2, Table 3.3), implying either a reduction in sympathetic nervous system activity or an increase in parasympathetic predominance, and participants who exhibited no change or an increase in LF/HF ratio (tragus non-responders; n = 10; Figure 3.2, Table 3.4). The participants who responded to tragus stimulation had a greater LF/HF ratio during the baseline recording of their tragus stimulation visit than the non-responder group (responders = 1.68 ± 0.24; non-responders = 0.99 ± 0.20; p = 0.036). This disparity in baseline values corresponded with increased values of normalised LF power (p = 0.036) and normalised HF power (p = 0.036) at baseline in the tragus responder group compared to non-responders. A significant decrease in LF/HF ratio was observed during tragus stimulation in the responder group from baseline (repeated measures ANOVA; p = 0.001) whereas a significant increase in LF/HF ratio occurred in the non-responder group during tragus stimulation (repeated measures ANOVA; p = 0.032). Normalised LF power decreased during tragus stimulation in the responder group (repeated measures ANOVA; p = 0.001), while normalised HF power increased during stimulation (repeated measures ANOVA; p = 0.001). For the non-responder group, normalised LF power was observed to increase during tragus stimulation (Friedman test; p = 0.030) and remained elevated during the recovery period (Friedman test; p = 0.016), while a significant decrease in normalised HF power from baseline was detected during tragus stimulation (Friedman; p = 0.030) and during recovery (Friedman test; p = 0.016).

When data from the other stimulation sites was analysed for the tragus responder group, a significant decrease in LF/HF ratio of a similar magnitude to tragus stimulation was also observed during helix stimulation (Friedman

test; helix visit baseline LF/HF ratio = 1.71 ± 0.33; helix stimulation LF/HF ratio = 0.95 ± 0.16; p = 0.013) but not for earlobe or cymba concha stimulation at the same time point. This was accompanied by a decrease in normalised LF power and an increase in normalised HF power during helix stimulation (Friedman test; p = 0.013 for both normalised LF power and normalised HF power; Figure 3.3). No significant changes in LF/HF ratio were observed in the tragus non-responders during helix stimulation. No significant changes were observed in either response group for LF/HF ratio during earlobe stimulation (Figure 3.5) or cymba concha stimulation (Figure 3.6). However an increase in HF power was observed in the tragus responder group in the recovery period after stimulation of the cymba concha (Friedman test; p = 0.013; Figure 3.5) but this effect was not reflected in a change in normalised HF power (repeated measures ANOVA; p = 0.790).

The dataset of all 22 participants was also divided into male and female participants to determine if there were any sex-associated differences in cardiac autonomic control and subsequent responses to stimulation. No significant differences were observed between male and female participants at baseline for any measure of HRV and the male and female participant data were then analysed separately to identify within-group trends (Table 3.5 and Table 3.6). The only observed sex-associated effect was a significant increase in LF power in female participants during the recovery period following earlobe stimulation (Friedman test; p =0.025). No changes in HRV were detected for male participants. This was likely due to the small sample size (n = 11 in both groups).

Table 3.2 HRV data for all participants (n = 22) for the different stimulation sites. The sole significant change observed in data from all participants was an increase in LF power from baseline after earlobe stimulation (Friedman test, p = 0.048).

Table 3.3 HRV data for the tragus responder subgroup (n = 12) for the different stimulation sites. A significant decrease in LF/HF ratio was observed during tragus stimulation in the responder group from baseline (repeated measures ANOVA; p = 0.001). Normalised LF power decreased during tragus stimulation in the responder group (repeated measures ANOVA; p = 0.001), while normalised HF power increased during stimulation (repeated measures ANOVA; p = 0.001). Helix stimulation also caused a decrease in LF/HF ratio (Friedman test; p = 0.013), normalised LF power (Friedman test; p = 0.013) and normalised HF power (Friedman test; p =0.013).

Table 3.4 HRV data for the tragus non-responder subgroup (n = 10) for the different stimulation sites. A significant increase in LF/HF ratio from baseline was observed during tragus stimulation in the tragus non-responder group (repeated measures ANOVA; p = 0.001).

Table 3.5 HRV data for the female participants (n = 11) at the different auricular stimulation sites. A significant increase in LF power was observed from baseline in the recovery period following earlobe stimulation (Friedman test; p = 0.025).

Table 3.6 HRV data for the male participants (n = 11) at the different auricular stimulation sites. No significant changes were detected for any stimulation site.

Table 3.2 HRV data for all participants (n = 22) for the different stimulation sites.

	Tragus			Helix			Cymba Concha			Earlobe		
	Baseline	**Stim**	**Recovery**	**Baseline**	**Stim**	**Recovery**	**Baseline**	**Stim**	**Recovery**	**Baseline**	**Stim**	**Recovery**
Total Power (ms^2)	3374.78 ± 803.03	3612.78 ±672.12	4073.61 ± 974.66	3357.06 ± 873.76	3080.57 ±555.87	4250.17 ±1106.51	3477.01 ± 605.59	4346.53 ±1029.14	5983.04 ± 1241.34	3090.46 ± 558.21	3681.98 ± 739.27	4244.56 ± 963.37
LF Power (ms^2)	1409.90 ± 388.29	1452.56 ±321.30	1703.56 ± 435.66	1454.18 ± 450.32	1234.35 ±266.93	1720.29 ± 450.32	1452.28 ± 302.26	1941.35 ±532.93	1741.53 ± 493.05	1356.53 ±297.72*	1503.93 ± 359.05	1871.81 ± 460.26*
HF Power (ms^2)	1491.41 ± 347.31	1620.31 ± 347.31	1590.45 ± 391.12	1394.16 ± 329.64	1399.86 ± 334.83	1796.01 ± 592.71	1552.45 ± 291.29	1639.99 ± 401.69	1805.80 ± 498.43	1333.89 ± 251.64	1523.90 ± 306.89	1723.32 ± 398.17
Normalised LF Power	0.52 ± 0.04	0.50 ± 0.04	0.51 ± 0.04	0.51 ± 0.04	0.50 ± 0.04	0.54 ± 0.04	0.52 ± 0.04	0.52 ± 0.03	0.51 ± 0.04	0.53 ± 0.03	0.51 ± 0.04	0.53 ± 0.04
Normalised HF power	0.48 ± 0.04	0.50 ± 0.04	0.49 ± 0.04	0.49 ± 0.04	0.50 ± 0.04	0.46 ± 0.04	0.48 ± 0.04	0.48 ± 0.03	0.49 ± 0.04	0.47 ± 0.04	0.49 ± 0.04	0.47 ± 0.04
LF/HF	1.33 ± 0.17	1.21 ± 0.16	1.32 ± 0.18	1.38 ± 0.21	1.29 ± 0.22	1.56 ± 0.26	1.37 ± 0.19	1.36 ± 0.21	1.25 ± 0.16	1.42 ± 0.21	1.38 ± 0.24	1.54 ± 0.27

Table 3.3 HRV data for the tragus responder subgroup (n = 12) at different stimulation sites.

	Tragus			Helix			Cymba Concha			Earlobe		
	Baseline	**Stim**	**Recovery**	**Baseline**	**Stim**	**Recovery**	**Baseline**	**Stim**	**Recovery**	**Baseline**	**Stim**	**Recovery**
Total Power (ms^2)	3866.08 ± 1419.63	4039.45 ± 1090.60	4984.97 ± 1712.94	4296.14 ± 1538.01	3781.52 ± 884.88	5783.56 ± 1918.17	3798.96 ± 964.94	5371.84 ± 1684.05	5898.28 ± 1729.82	3489.95 ± 965.92	4158.59 ± 1152.05	5326.03 ± 1619.89
LF Power (ms^2)	1817.49 ± 682.94	1482.31 ± 435.80	1981.71 ± 735.91	1974.61 ± 795.75	1315.13 ± 360.75	2364.37 ± 779.44	1597.66 ± 480.31	2646.84 ± 928.47	2331.25 ± 831.97	1663.00 ± 513.75	1726.35 ± 532.21	2323.99 ± 736.48
HF Power (ms^2)	1627.74 ± 554.12	1905.10 ± 557.51	2593.35 ± 1030.58	1627.74 ± 554.12	1905.10 ± 557.61	2593.35 ± 1030.58	1552.99 ± 449.89	1997.99 ± 674.85	2464.67 ± 861.62	1336.11 ± 406.29	1745.62 ± 482.57	2236.70 ± 662.65
Normalised LF Power	0.56 ± 0.06*	0.45 ± 0.05*	0.54 ± 0.05	0.56 ± 0.06*	0.45 ± 0.05*	0.54 ± 0.05	0.55 ± 0.05	0.52 ± 0.04	0.52 ± 0.05	0.58 ± 0.04	0.51 ± 0.05	0.52 ± 0.05
Normalised HF power	0.44 ± 0.06*	0.55 ± 0.05*	0.46 ± 0.05	0.44 ± 0.06*	0.55 ± 0.05*	0.46 ± 0.05	0.45 ± 0.05	0.48 ± 0.05	0.48 ± 0.05	0.42 ± 0.04	0.49 ± 0.05	0.48 ± 0.05
LF/HF	1.68 ± 0.24*	1.03 ± 0.16*	1.21 ± 0.23	1.71 ± 0.33*	0.95 ± 0.16*	1.50 ± 0.33	1.54 ± 0.26	1.35 ± 0.26	1.33 ± 0.23	1.72 ± 0.31	1.34 ± 0.28	1.39 ± 0.31

Table 3.4 HRV data for the tragus non-responder subgroup (n = 10) at different stimulation sites.

	Tragus			Helix			Cymba Concha			Earlobe		
	Baseline	**Stim**	**Recovery**	**Baseline**	**Stim**	**Recovery**	**Baseline**	**Stim**	**Recovery**	**Baseline**	**Stim**	**Recovery**
Total Power (ms^2)	2785.35 ± 532.59	3100.77 ± 721.88	2979.98 ± 573.92	2230.17 ± 437.09	2239.43 ± 541.85	2410.32 ± 446.11	3090.68 ± 697.57	2845.09 ± 523.60	2484.44 ± 584.69	2611.08 ± 429.37	3110.05 ± 890.10	2946.80 ± 756.63
LF Power (ms^2)	970.79 ± 202.89	1416.86 ± 501.11	1369.78 ± 396.64	829.66 ± 181.07	1137.42 ± 415.89	947.40 ± 215.47	1277.82 ± 352.76	1094.76 ± 218.16	1033.88 ± 361.56	988.76 ± 200.86	1237.03 ± 481.48	1329.20 ± 481.60
HF Power (ms^2)	1477.41 ± 397.74	1286.71 ± 333.95	1183.07 ± 276.05	1113.87 ± 302.39	793.56 ± 211.58	839.21 ± 243.24	1551.81 ± 372.43	1210.39 ± 350.32	1015.15 ± 243.11	1331.22 ± 286.65	1257.84 ± 357.05	1107.27 ± 304.16
Norm LF Power	0.43 ± 0.06*	0.53 ± 0.07*	0.53 ± 0.06	0.46 ± 0.05	0.55 ± 0.06	0.54 ± 0.06	0.48 ± 0.05	0.51 ± 0.05	0.49 ± 0.05	0.46 ± 0.05	0.51 ± 0.06	0.55 ± 0.06
Norm HF power	0.57 ± 0.06*	0.47 ± 0.07*	0.47 ± 0.06	0.54 ± 0.05	0.45 ± 0.06	0.46 ± 0.06	0.52 ± 0.05	0.49 ± 0.05	0.51 ± 0.05	0.54 ± 0.05	0.49 ± 0.06	0.45 ± 0.06
LF/HF	0.93 ± 0.20*	1.46 ± 0.29*	1.46 ± 0.29	0.99 ± 0.20	1.69 ± 0.43	1.62 ± 0.42	1.15 ± 0.27	1.37 ± 0.36	1.15 ± 0.22	1.06 ± 0.22	1.43 ± 0.41	1.72 ± 0.47

Table 3.5 HRV data for the female participants (n = 11) at the different auricular stimulation sites.

	Tragus			Helix			Cymba Concha			Earlobe		
	Baseline	**Stim**	**Recovery**	**Baseline**	**Stim**	**Recovery**	**Baseline**	**Stim**	**Recovery**	**Baseline**	**Stim**	**Recovery**
Total Power (ms^2)	4234.57 ± 1525.09	3738.23 ± 1078.66	4652.79 ± 1771.65	3772.64 ± 1502.44	3433.63 ± 915.90	5435.72 ± 2096.47	3347.21 ± 934.52	4900.25 ± 1648.17	4715.09 ± 1787.47	3303.22 ± 988.40	4072.52 ± 1074.72	4893.44 ± 1615.48
LF Power (ms^2)	1639.37 ± 745.60	1159.18 ± 406.70	1761.32 ± 770.51	1636.16 ± 742.95	1158.52 ± 347.17	2140.70 ± 864.38	1225.45 ± 396.27	2154.00 ± 893.32	1681.92 ± 853.50	**1355.95 ± 534.28***	1478.14 ± 442.25	**1869.43 ± 690.77***
HF Power (ms^2)	2061.15 ± 697.95	2120.21 ± 618.13	2267.17 ± 722.82	1609.37 ± 572.63	1807.61 ± 595.91	2735.49 ± 1124.16	1703.16 ± 473.55	2238.43 ± 750.75	2512.41 ± 930.15	1568.12 ± 444.25	1900.18 ± 529.11	2178.77 ± 685.97
Norm LF Power	0.49 ± 0.06	0.42 ± 0.05	0.44 ± 0.06	0.53 ± 0.05	0.44 ± 0.05	0.49 ± 0.06	0.49 ± 0.06	0.47 ± 0.05	0.42 ± 0.06	0.50 ± 0.06	0.50 ± 0.06	0.50 ± 0.07
Norm HF power	0.51 ± 0.06	0.58 ± 0.05	0.56 ± 0.06	0.47 ± 0.05	0.56 ± 0.05	0.51 ± 0.06	0.51 ± 0.06	0.53 ± 0.05	0.58 ± 0.06	0.50 ± 0.06	0.50 ± 0.06	0.50 ± 0.07
LF/HF	1.27 ± 0.26	0.89 ± 0.20	1.02 ± 0.25	1.42 ± 0.29	0.92 ± 0.17	1.38 ± 0.38	1.25 ± 0.27	1.11 ± 0.25	0.99 ± 0.25	1.31 ± 0.29	1.44 ± 0.42	1.60 ± 0.51

Table 3.6 HRV data for the male participants (n = 11) at the different auricular stimulation sites.

	Tragus			Helix			Cymba Concha			Earlobe		
	Baseline	**Stim**	**Recovery**	**Baseline**	**Stim**	**Recovery**	**Baseline**	**Stim**	**Recovery**	**Baseline**	**Stim**	**Recovery**
Total Power (ms^2)	2514.99 ± 484.44	3487.33 ± 854.80	3494.43 ± 885.43	2941.49 ± 956.38	2727.50 ± 658.76	3064.81 ± 682.67	3606.82 ± 814.63	3546.38 ± 1065.85	3977.98 ± 1107.31	2877.71 ± 568.10	3291.44 ± 1053.51	3595.68 ± 1097.26
LF Power (ms^2)	1180.42 ± 258.43	1745.93 ± 500.95	1645.80 ± 450.54	1272.21 ± 541.38	1310.19 ± 421.39	1299.88 ± 289.96	1679.11 ± 465.19	1728.69 ± 621.12	1801.14 ± 540.23	1357.11 ± 294.65	1529.73 ± 587.98	1874.19 ± 642.31
HF Power (ms^2)	921.67 ± 184.64	1120.40 ± 273.04	913.73 ± 168.66	1178.95 ± 345.26	992.11 ± 287.66	856.54 ± 187.67	1401.75 ± 357.17	1041.55 ± 205.98	1099.19 ± 279.96	1099.65 ± 238.75	1147.63 ± 295.44	1267.87 ± 392.21
Norm LF Power	0.55 ± 0.04	0.58 ± 0.04	0.59 ± 0.03	0.50 ± 0.06	0.55 ± 0.06	0.59 ± 0.04	0.55 ± 0.05	0.56 ± 0.04	0.59 ± 0.03	0.56 ± 0.04	0.52 ± 0.05	0.57 ± 0.03
Norm HF power	0.45 ± 0.04	0.42 ± 0.04	0.41 ± 0.03	0.50 ± 0.06	0.45 ± 0.06	0.41 ± 0.04	0.45 ± 0.05	0.44 ± 0.04	0.41 ± 0.03	0.44 ± 0.04	0.48 ± 0.05	0.43 ± 0.03
LF/HF	1.40 ± 0.24	1.56 ± 0.21	1.62 ± 0.24	1.34 ± 0.31	1.66 ± 0.39	1.74 ± 0.35	1.49 ± 0.27	1.60 ± 0.34	1.51 ± 0.16	1.53 ± 0.30	1.31 ± 0.24	1.48 ± 0.18

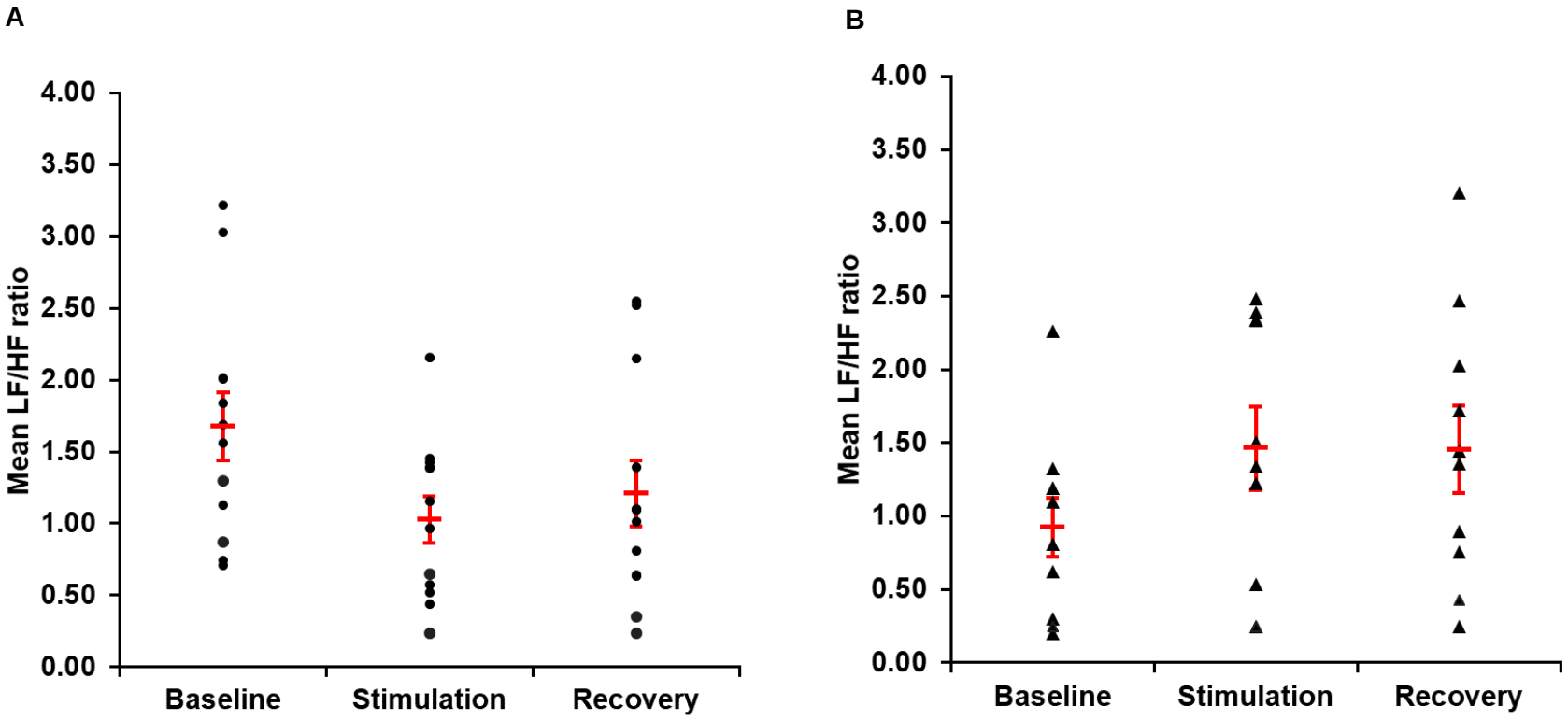


Figure 3.2: LF/HF ratio response trends for tragus stimulation. Participants were divided into the tragus responder subgroup (A; decrease in LF/HF ratio during to tragus stimulation; n = 12) and the tragus non-responder subgroup (B; no change or an increase in LF/HF ratio during tragus stimulation; n = 10) for the different stimulation sites. A significant decrease in LF/HF ratio was observed for the tragus responders during tragus stimulation ($p = 0.001$), whereas a significant increase in LF/HF ratio occurred in the tragus non-responders ($p = 0.032$).

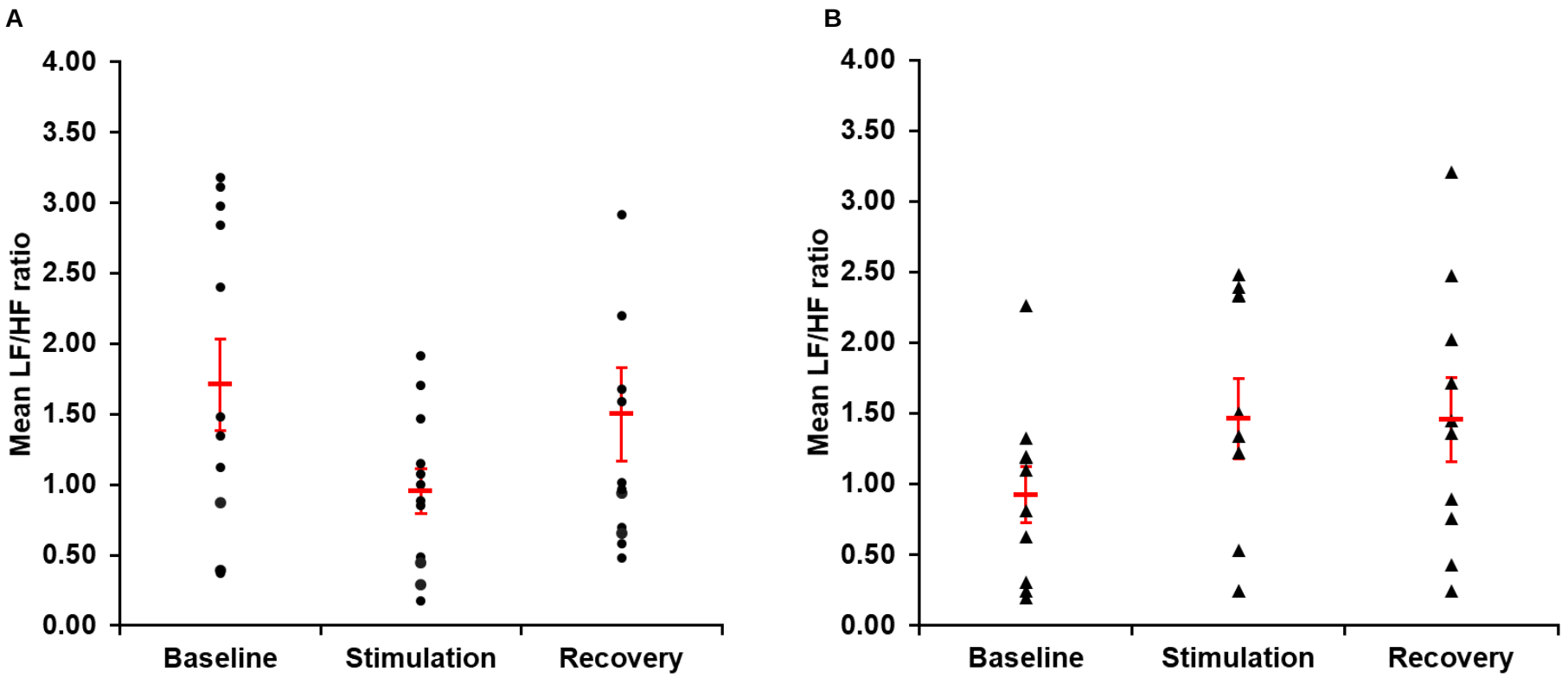


Figure 3.3: LF/HF ratio response trends in the tragus responder (A) and tragus non-responder (B) subgroups during helix stimulation. A significant decrease in LF/HF ratio occurred due to helix stimulation ($p = 0.013$). No significant changes in LF/HF ratio were observed in the tragus non-responders during helix stimulation.

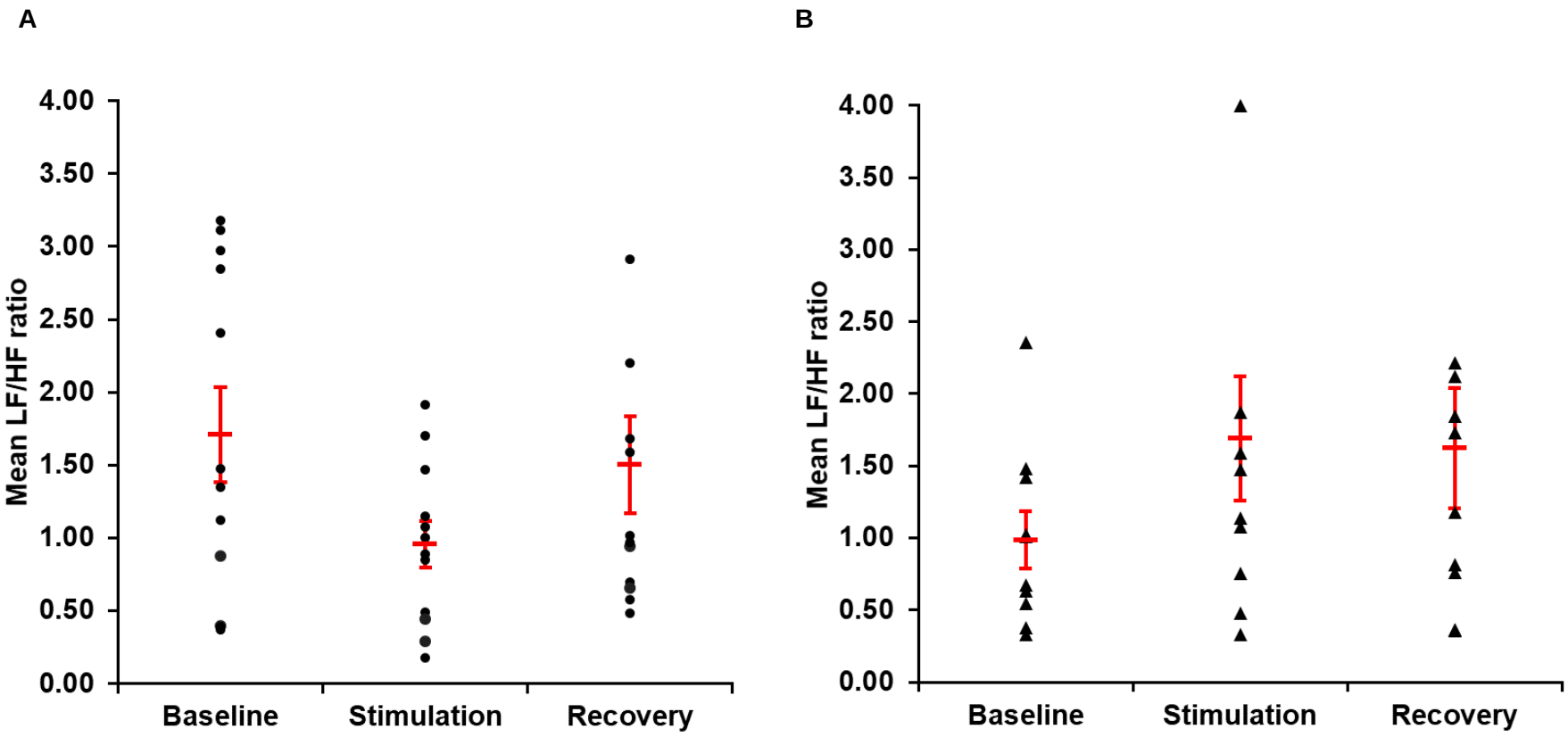


Figure 3.4: LF/HF ratio response trends in the tragus responder (A) and tragus non-responder (B) subgroups during earlobe stimulation. No significant changes in LF/HF ratio were observed during earlobe stimulation.

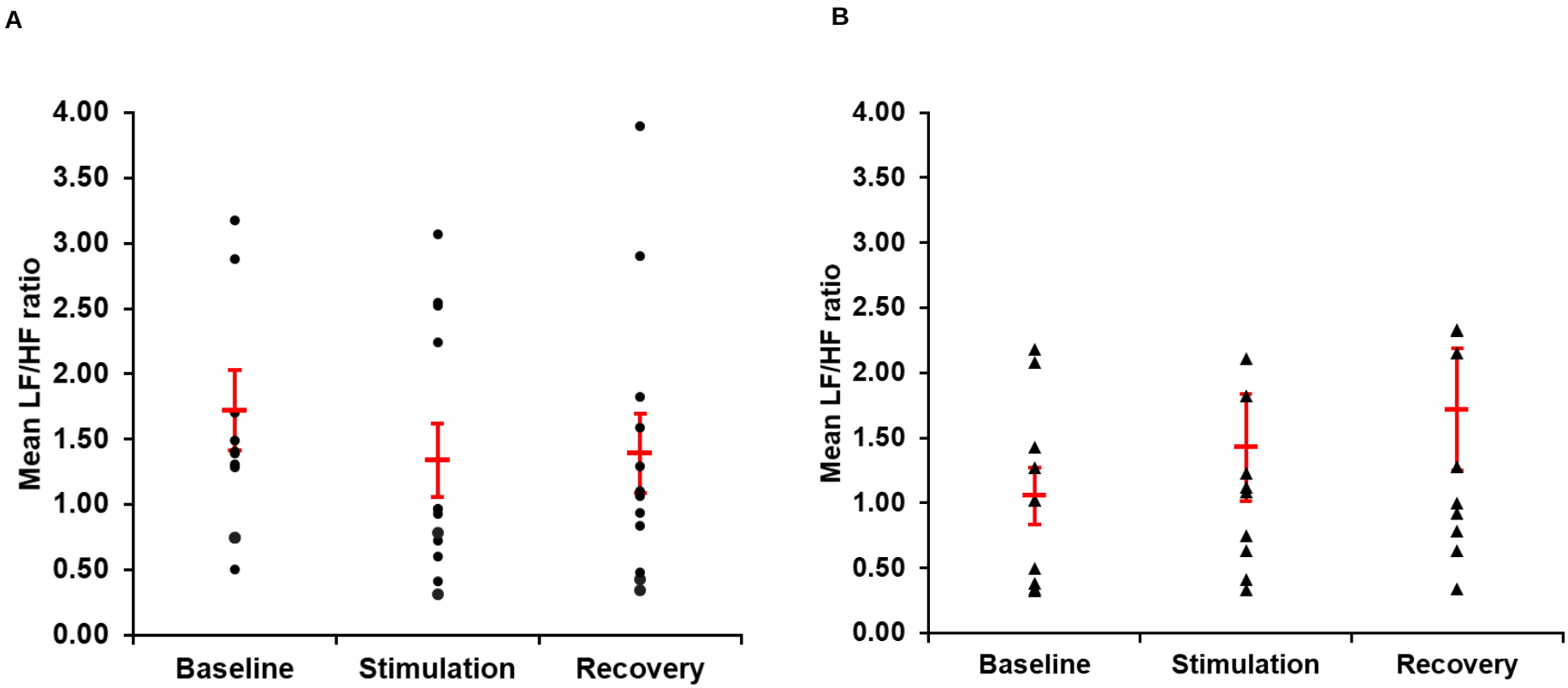


Figure 3.5: LF/HF ratio response trends in the tragus responder (A) and tragus non-responder (B) subgroups during cymba concha stimulation. No significant changes in LF/HF ratio were observed during cymba concha stimulation.

3.5.3 Baroreflex sensitivity

BRS data was obtained from 14 out of 22 participants and baroreflex sequences had to be detected in each recording (baseline, stimulation and recovery) at all four visits in order to include that participant's data in the subsequent analysis. Data were initially analysed irrespective of response or sex and no significant difference was observed at any time-point (baseline, stimulation or recovery) between the stimulation sites (two-way repeated measures ANOVA; $p = 0.418$). A significant increase in BRS was detected during tragus stimulation (one-way repeated measures ANOVA; $p = 0.039$; Table 3.7). Repeated measures ANOVA failed to detect any significant change in BRS during cymba concha stimulation ($p = 0.191$), helix stimulation ($p = 0.612$) or earlobe stimulation ($p = 0.742$). The BRS data was separated into male participants ($n = 9$) and female participants ($n = 5$), although no differences were observed between groups for the different stimulation sites (independent t-tests; $p > 0.05$ for all) or within groups for any stimulation site.

Eleven of the 14 participants for whom BRS data was available had previously been included in the tragus responders group (determined by a decrease in the LF/HF ratio). In these participants, a significant increase in BRS was detected during tragus stimulation (repeated measures ANOVA; $p = 0.009$; Table 3.8) but during the recovery period BRS had begun to decrease to baseline level ($p = 0.370$). No significant changes from baseline were identified as a result of stimulation at the cymba concha, helix or earlobe. BRS data was obtained for only 3 participants in the tragus non-responder (no change or increase in LF/HF ratio during tragus stimulation) group and this data was not analysed.

Table 3.7 BRS data for all participants (n = 14) during stimulation at different sites on the ear. A significant increase in BRS from baseline was observed during tragus stimulation (p = 0.001), but no other significant differences were observed (p > 0.05). Units = ms/mmHg.

	N	Baseline	Stimulation	Recovery	p (Baseline - Stimulation)	p (Baseline - Recovery)
Tragus	14	13.60 ± 1.04	16.55 ± 1.51	15.36 ± 1.24	0.039	0.688
Helix	14	15.00 ± 1.59	15.57 ± 1.22	16.26 ± 1.58	1.0	0.565
Cymba Concha	14	15.02 ± 1.79	15.51 ± 1.24	17.56 ± 1.64	1.0	1.0
Earlobe	14	14.92 ± 1.35	14.82 ± 1.80	15.60 ± 1.85	1.0	1.0

Table 3.8 BRS data for participants in the tragus responder subgroup (n = 11) during stimulation at different sites on the ear. A significant increase in BRS from baseline was observed during tragus stimulation ($p = 0.009$), but no other significant differences were observed ($p > 0.05$). Units = ms/mmHg.

	N	Baseline	Stimulation	Recovery	*p* (Baseline - Stimulation)	*p* (Baseline - Recovery)
Tragus	11	13.11 ± 1.17	17.07 ± 1.73	15.86 ± 1.45	0.009	0.370
Helix	11	14.70 ± 1.84	15.91 ± 1.23	16.60 ± 1.62	0.995	1.0
Cymba Concha	11	14.42 ± 2.05	15.63 ± 1.35	18.03 ± 1.75	1.0	0.389
Earlobe	11	14.76 ± 1.59	14.92 ± 1.67	14.96 ± 1.60	1.0	1.0

3.5.4 Heart rate and blood pressure

Heart rate data was obtained for all 22 participants and the baseline values for mean HR were not significantly different for each stimulation site (Friedman test; $p = 0.106$). A significant decrease in mean HR was observed during tragus stimulation (Figure 3.6; repeated measures ANOVA; $p < 0.001$) and during the recovery period ($p < 0.001$). Mean HR also decreased during earlobe stimulation (repeated measures ANOVA; $p < 0.001$), helix stimulation (repeated measures ANOVA; $p = 0.001$) and cymba concha stimulation (Friedman test; $p = 0.048$). This decrease in mean HR was sustained into the post-stimulation recovery period for all stimulation sites (Figure 3.6; $p < 0.001$ for cymba concha, helix and earlobe stimulation).

When the dataset was divided into male and female participants, one-way repeated measures ANOVA revealed a significant decrease in mean HR during tragus stimulation for both male ($p = 0.015$) and female ($p = 0.033$) participants. This decrease in heart rate from baseline was sustained during the post-stimulation recovery period (male $p = 0.038$; female $p = 0.008$; see Table 3.9). A significant decrease in mean HR also occurred during cymba concha stimulation (repeated measures ANOVA; male $p = 0.013$; female $p = 0.019$) and during earlobe stimulation (repeated measures ANOVA; male $p = 0.001$; female $p = 0.008$). However, a significant decrease in mean HR was detected during helix stimulation only in the female participants (repeated measures ANOVA; $p = 0.007$). There was no significant change in mean HR during helix stimulation for the male participants (Friedman test; $p = 0.529$).

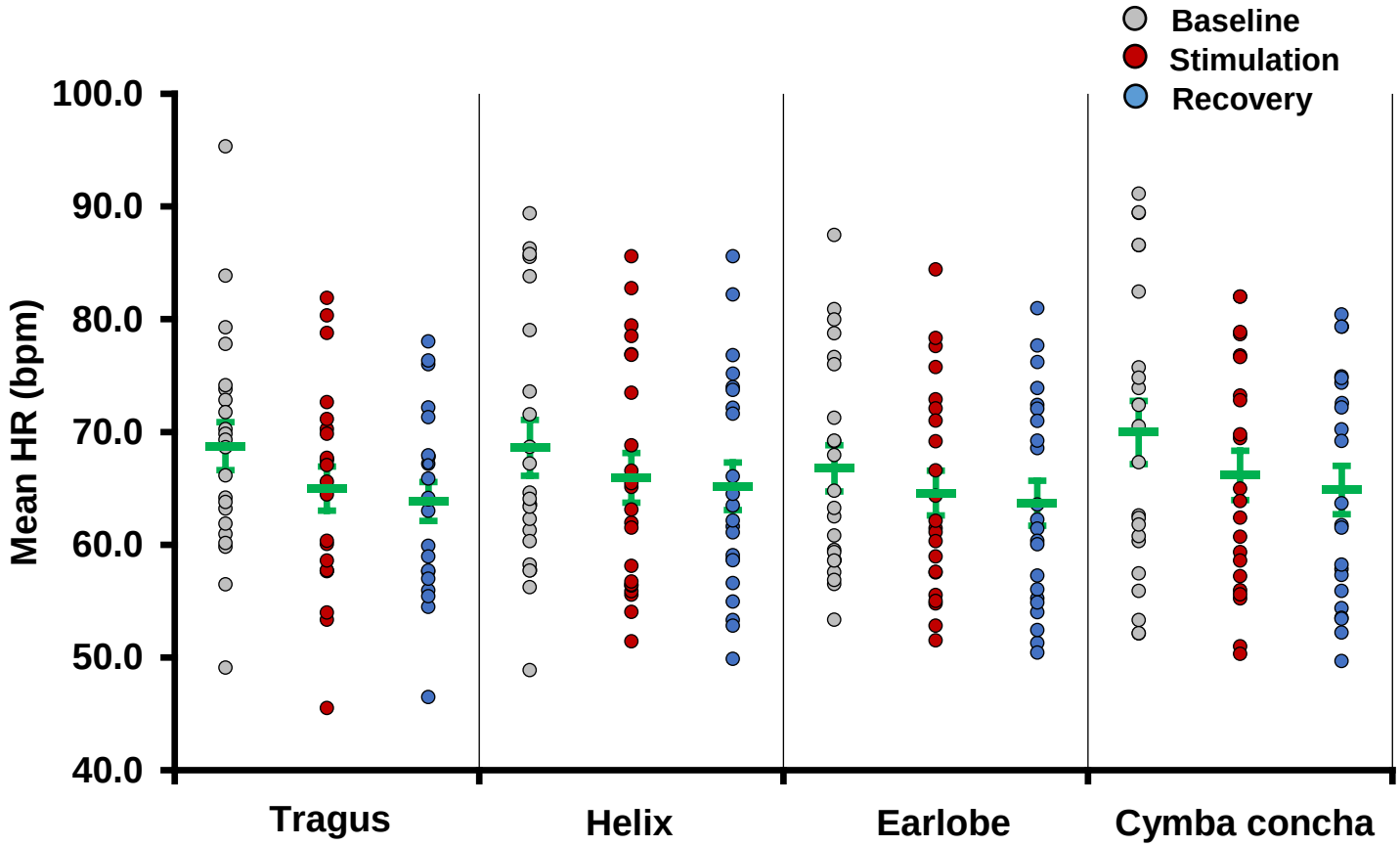


Figure 3.6 Participant HR responses as a result of stimulation at different sites on the ear (n = 22). Group mean and SEM shown in green. A small but significant decrease in mean HR from baseline was observed during stimulation at all sites ($p < 0.05$) and was sustained into the recovery period for all stimulation sites ($p < 0.05$).

	Male participants (n = 11)			Female participants (n = 11)		
	Baseline	**Stimulation**	**Recovery**	**Baseline**	**Stimulation**	**Recovery**
Tragus	**64.4 ± 2.5*†**	**61.30 ± 2.9***	**61.3 ± 2.6†**	**73.1*† ± 3.0**	**68.7* ± 2.3**	**66.4 ± 2.2†**
Helix	64.4 ± 2.6	62.7 ± 2.8	62.4 ± 2.7	**72.8*† ± 3.9**	**69.2 ± 3.2***	**68.0 ± 3.1†**
Cymba Concha	**64.1 ± 3.0*†**	**61.8 ± 2.7***	**60.3 ± 2.6†**	**75.8 ± 4.1*†**	**70.5 ± 3.0***	**69.4 ± 2.9†**
Earlobe	**62.3 ± 1.8*†**	**60.4 ± 2.1***	**59.5 ± 2.2†**	**71.2 ± 3.3*†**	**68.8 ± 3.0***	**67.9 ± 2.9†**

Table 3.9 Male and female participant HR responses as a result of stimulation at different sites on the ear. Both male and female participants showed a small but significant decrease in mean HR from baseline during stimulation at the tragus, earlobe and cymba concha ($p < 0.05$), which was sustained into the recovery period ($p < 0.05$). A similar decrease in mean HR due to helix stimulation was only observed in female participants.

Table 3.10: BP responses during stimulation at different sites on the ear in participants with successful finger arterial pressure recordings (n = 21). Significant increases in systolic BP, diastolic BP and mean BP were recorded in the recovery period after stimulation was applied to the tragus, cymba concha and helix ($p < 0.05$). No significant changes were observed as a result of earlobe stimulation ($p < 0.05$).

	Tragus			**Helix**			**Cymba Concha**			**Earlobe**		
	Baseline	**Stim**	**Rec**	**Baseline**	**Stim**	**Rec**	**Baseline**	**Stim**	**Rec**	**Baseline**	**Stim**	**Rec**
Systolic BP (mmHg)	**115.47 ± 3.33***	118.80 ± 3.11	**125.82 ± 3.34***	**113.59 ± 2.92***	119.05 ± 3.59	**126.65 ± 3.53***	**117.55 ± 3.56***	119.20 ± 2.92	**124.05 ± 2.91***	120.42 ± 2.73	122.33 ± 3.00	124.33 ± 2.60
Diastolic BP (mmHg)	**63.86 ± 2.20***	66.12 ± 2.53	**67.81 ± 2.64***	**63.90 ± 2.31***	67.00 ± 2.54	**70.58 ± 2.60***	**64.74 ± 2.37***	64.60 ± 1.89	**67.08 ± 1.94***	69.27 ± 2.63	68.25 ± 2.24	69.54 ± 2.29
Mean BP (mmHg)	**81.07 ± 2.40***	81.07 ± 2.56	**87.15 ± 2.73***	**80.46 ± 2.42***	84.35 ± 2.73	**89.36 ± 2.79***	**82.34 ± 2.69***	82.96 ± 2.69	**86.07 ± 2.12***	86.32 ± 2.49	86.47 ± 2.37	87.80 ± 2.24

Table 3.11: BP responses measured using a brachial BP cuff immediately after the baseline period and immediately after the stimulation period at different sites on the ear (n = 21). A small but significant decrease was observed from baseline at the end of tragus stimulation in brachial systolic BP (paired t-test; p = 0.039), brachial diastolic BP (paired t-test; p = 0.027) and brachial mean BP (paired t-test; p = 0.012).

	Tragus		Helix		Cymba Concha		Earlobe	
	Post-baseline	Post-stimulation	Post-baseline	Post-stimulation	Post-baseline	Post-stimulation	Post-baseline	Post-stimulation
Systolic BP (mmHg)	**118.95 ± 1.66***	**115.90 ± 1.66***	115.10 ± 1.17	118.57 ± 1.70	116.95 ± 1.85	116.71 ± 2.19	118.00 ± 1.77	117.35 ± 1.79
Diastolic BP (mmHg)	**68.14 ± 1.27***	**65.17 ± 1.14***	64.24 ± 1.25	65.19 ± 1.10	74.00 ± 1.21	74.14 ± 1.41	74.38 ± 1.37	74.00 ± 1.50
Mean BP (mmHg)	**85.75 ± 1.14***	**82.11 ± 1.14***	81.19 ± 1.20	82.98 ± 1.10	88.32 ± 1.25	88.33 ± 1.49	88.92 ± 1.34	88.45 ± 1.44

Finger arterial blood pressure was recorded in all but one participant (n = 21) across all four visits. Small but significant increases in systolic BP, diastolic BP and mean BP were recorded after stimulation applied to the tragus, cymba concha and helix (see Table 3.10, with the exception of earlobe stimulation, where there was no significant change in blood pressure over time. To investigate if this effect was due to recording BP using a finger arterial NIBP device, data was compared with BP data obtained using a digital brachial BP monitor in the two 5 minute rest periods between the baseline, stimulation and recovery recordings (Table 3.11). A significant decrease was observed from baseline at the end of tragus stimulation in brachial systolic BP (paired t-test; $p = 0.039$), brachial diastolic BP (paired t-test; $p = 0.027$) and brachial mean BP (paired t-test; $p = 0.012$). Significant differences were detected between finger arterial diastolic pressure and brachial systolic pressure at baseline and at the end of stimulation for all four stimulation sites ($p < 0.05$). This was also reflected in significant differences between finger arterial diastolic pressure and brachial diastolic pressure at baseline and at the end of stimulation for all four stimulation sites ($p < 0.05$). No significant differences were detected between methods of measurement for systolic BP.

3.5.5 Reported sensations during electrical stimulation

During the brief stimulus titration stage at each visit where the electrical current was adjusted to a comfortable but perceptible level, the participants were asked to describe as best they could the sensations associated with the electrical stimulation. The only further prompting from the investigator was to ask the participant to report if the sensation was localised to the area immediately in contact with the electrode or if the stimulus could be perceived elsewhere. The electrical current was typically described at all stimulation sites as being a “tingling”, “tickling”, “pins and needles” or “sharp” sensation before the current was adjusted to a lower level, with the sensation being localised to the stimulation site. However, a number of participants reported sensations at other sites on the ear or on nearby facial regions. The sensation evoked by helix stimulation was reported by some participants to travel posteriorly along the helix of the ear from the stimulation site (n = 5) or travel inferiorly towards the tragus on one or both ears (n = 3) or along the ipsilateral mandible (n = 2). In two participants the sensation caused by earlobe stimulation was felt to radiate inferiorly along the neck, while one participant reported an itching sensation along their left zygomatic arch which was not present on the right zygomatic arch. Tragus stimulation was reported in three participants to produce a tingling sensation which radiated inferiorly along the mandible on one or both sides. Lastly, in two participants cymba concha stimulation evoked a tingling or tickling sensation which radiated laterally from the cymba concha towards the helix of the ear and one participant reported a “vague sensation of current” travelling inferiorly from the ear along the neck. One participant reported a sensation of “warmth” in their chest for the duration of cymba concha stimulation, which ceased when the current was switched off.

3.6 Discussion

The results of the present study of transcutaneous electrical nerve stimulation applied to different sites on the external ear are mixed with regards to the effects on cardiovascular autonomic function. On the one hand, this study provides further evidence that transcutaneous electrical nerve stimulation applied to the tragus of the ear can induce a change in autonomic function similar to that described by Clancy et al., whereby tragus stimulation in healthy adults elicited a decrease in LF/HF ratio (Clancy et al., 2014). However, in the present study this effect was only observed in 12 out of 22 individuals recruited (54.5%), who were termed tragus responders as they exhibited a decrease in LF/HF ratio as opposed to the tragus non-responder group who did not have this response to tragus stimulation. Clancy et al. reported a significant decrease in LF/HF ratio for the whole group as a result of tragus stimulation although that study had a larger sample size (n = 34 versus n = 22 in the present study). In addition, Clancy et al. showed a decrease in MSNA from baseline as a result of tragus stimulation, characterising the change in cardiovascular autonomic function as sympathoinhibitory (Clancy et al., 2014).

Where the present study provides additional insight is firstly through the significant increase in spontaneous BRS observed during tragus stimulation in 14 participants who had detectable BRS sequences. Clancy et al. did not report BRS data although recordings of continuous beat-to-beat finger arterial pressure were obtained as part of the experimental protocol (Clancy et al., 2014). In addition, the observed increase in BRS in the present study only occurred during tragus stimulation, with no significant differences in BRS detected as a result of stimulation applied to the cymba concha, helix or earlobe. This outcome would suggest that tragus stimulation may have a unique effect on cardiovascular autonomic function compared to stimulation at the other three sites. A recent study by Antonino et al. in 13 young (mean age = 23 ± 1 years) healthy male volunteers also assessed the effect of 15 minutes of tragus stimulation on spontaneous BRS using the sequence method and observed a significant increase in BRS along with a simultaneous decrease in LF/HF ratio (Antonino et al., 2017). Moreover,

Antonino et al. compared tragus stimulation with 15 minutes control stimulation applied to the earlobe in the same volunteers and found no significant difference in BRS during earlobe stimulation (earlobe baseline BRS = 13.6 ± 1 ms/mmHg; earlobe stimulation BRS = 13.9 ± 1ms/mmHg; p = 0.23). This is consistent with the findings of the present study, where earlobe stimulation elicited no significant effect on BRS (Table 3.7). Antonino et al. attributed the increase in BRS during tragus stimulation to activation of ABVN afferents (Antonino et al., 2017).

However, the present study expands further on the effects of auricular nerve stimulation on BRS by including stimulation of the helix and cymba concha, with no significant change in BRS for either stimulation site. While the sample size of participants with detectable spontaneous BRS sequences was small (n = 14) the lack of an effect at sites such as the cymba concha is interesting. If the increase in BRS observed during tragus stimulation was due to activation of ABVN afferents alone, a significant increase in BRS should also have been observed during cymba concha stimulation using the same stimulation parameters. This may imply that the cardiovascular autonomic effects of tragus stimulation are due to simultaneous activation of multiple neural pathways rather than solely activation of ABVN afferents. It is possible that the cardiovascular autonomic effects reported by the present study, Clancy et al. and Antonino et al. were influenced by the activation of trigeminal and cervical spinal afferent pathways, with subsequent effects on medullary nuclei such as the nucleus tractus solitarius (NTS). However, there is less convincing evidence in the present study for the activation of cervical spinal pathways as part of the mechanism of tVNS, as earlobe stimulation did not significantly alter LF/HF ratio or BRS.

The lack of an effect on HRV or BRS as a result of cymba concha stimulation using the same stimulation parameters as tragus stimulation is an unexpected finding. If the activation of ABVN afferents is responsible for the cardiovascular autonomic effects of tVNS, then a similar decrease in LF/HF ratio and an increase in BRS should have been observed in the tragus responder subgroup as with tragus stimulation. However, the present study was not the first to investigate the effects of cymba concha stimulation on

HRV. Napadow et al. (2012) assessed the effects of cymba concha stimulation on HRV in female patients with chronic pelvic pain and found no significant change in LF/HF ratio. The recently published study by De Couck et al. also applied tVNS to the cymba concha, with no significant effect on measures of HRV such as LF/HF ratio (De Couck et al., 2017). However the stimulation parameters used by De Couck et al. (250μs, 25Hz, 30s on/off cycle) differed from the present study. Moreover, De Couck et al. did not stimulate both cymba conchae simultaneously, instead performing alternating 10 minute periods of unilateral right or left cymba concha stimulation at the same visit along with sham stimulation. Nevertheless, the results of the present study appear to be consistent with both Napadow et al. and De Couck et al. regarding the lack of changes in HRV as a result of cymba concha stimulation.

3.6.1 Central mechanisms

An interesting result from the present study is that the participants who showed a response to tragus stimulation (tragus responders; n = 12) in the form of a decrease in LF/HF ratio had a similar response to helix stimulation but not to stimulation of the cymba concha or earlobe. This may implicate afferent fibres in the auriculotemporal nerve rather than the ABVN as being responsible for the change in cardiovascular autonomic function seen with tragus stimulation. One explanation for this may be auriculotemporal afferent fibre projections to the NTS, a key relay point for the processing of cardiovascular reflexes and the control of autonomic outflow. A previous neuroanatomical tracing study by Jacquin et al. showed some evidence of auriculotemporal nerve projections to the NTS in rats, although a later study by Takemura et al. did not report any projections to this site (Jacquin et al., 1983, Takemura et al., 1987). In addition, Chien et al. reported some HRP-labelled nerve terminals in the canine NTS from the rostral internal auricular nerve, the canine equivalent of the auriculotemporal nerve (Chien et al., 1996). The study also observed HRP-labelling from the middle and caudal internal auricular nerves in the NTS and the majority of neurons were found

to have their cell bodies in the jugular ganglion of the vagus nerve, consistent with the ABVN (Chien et al., 1996). Evidence of NTS activation has also been found in an fMRI study where electrical stimulation was applied to the inner surface of the tragus in human volunteers (Kraus et al., 2013). In this way, activation of the NTS by afferent fibres from one or both of the ABVN or the auriculotemporal nerve could be responsible for the autonomic effects of tragus stimulation. Activation of the NTS could then also activate the nucleus ambiguus and dorsal motor nucleus of the vagus to elicit an increase in parasympathetic outflow via the vagus nerve (Izzo et al., 1993). This is consistent with the observation that the tragus responder subgroup of participants exhibited a significant increase in normalised HF power during both tragus and helix stimulation, with an additional increase in BRS observed during tragus stimulation. However, as it is not possible to perform direct recordings of vagus nerve activity in humans, further preclinical *in vivo* experiments are necessary to clarify the effects of tragus stimulation on parasympathetic activity.

A limitation of the present study is the lack of a direct method of assessing stimulation-induced changes in sympathetic nervous system activity. One approach may be to use microneurography to record MSNA, as MSNA has been previously shown in healthy volunteers to decrease as a result of tragus stimulation (Clancy et al., 2014). The effects observed in the present study could also in part be due to activation of the caudal ventrolateral medulla (CVLM) via the NTS, which in turn would lead to inhibition of the rostral ventrolateral medulla (RVLM) and a subsequent generalised reduction in sympathetic activity (Guyenet, 2006). Some evidence of this may potentially be found in the decrease in both LF power and LF/HF ratio observed during both tragus stimulation and helix stimulation in the tragus responder group. Nevertheless, without a direct measurement of sympathetic nervous system activity it is difficult to establish if this change occurred due to a reduction in sympathetic activity as well as an increase in parasympathetic predominance.

3.6.2 Response to tragus stimulation

One reason for the variable response rate to tragus stimulation in the healthy participants included in this study is evidence that the auriculotemporal nerve is not found at the tragus in all individuals. The cadaveric dissection study by Peuker and Filler found the auriculotemporal nerve at the tragus in only 55% of the 14 ears investigated, with the GAN providing sole innervation to the tragus in the remaining 45% (Peuker and Filler, 2002). The response rate to tragus stimulation in terms of a decrease in LF/HF ratio in the present study was 54.5% of participants, which may be related to the reported innervation pattern at the tragus. In the present study, no significant changes in HRV or BRS were observed for great auricular nerve stimulation at the earlobe. Thus, individuals with a greater distribution or exclusive innervation of the GAN at the tragus may not exhibit the same stimulation-induced cardiovascular autonomic effects as individuals with a greater distribution of ABVN or auriculotemporal nerve fibres at the tragus. Nevertheless, it is difficult to establish which nerve may be responsible for the autonomic effects of tragus stimulation without further clarification of anatomical variations of the cutaneous nerves of the external ear.

3.6.3 Distribution of the cutaneous nerves of the external ear

A potential explanation for the outcomes of the present study is that the distribution of the cutaneous nerves of the external ear described by Peuker and Filler may not be entirely accurate (Peuker and Filler, 2002). In that study, the ramifications of the ABVN, GAN and auriculotemporal nerve were dissected in 7 human cadavers (14 ears in total) with the aid of magnifying glasses and the results depicted on a generic template of the external ear. This approach may have led to inconsistencies in the innervation pattern due to inter-individual variation in the shape and size of the cadaveric ears and no data pertaining to this is reported as part of the study (Peuker and Filler, 2002). The small sample size of that study may be an additional issue, as some relatively common variations in the cutaneous innervation of the external ear may not have been present in the cadaveric specimens selected

for dissection. This is especially key with regards to the cymba concha, as Peuker and Filler reported it to be innervated exclusively by the ABVN. As such, this warrants further human cadaveric studies with a larger sample size of cadaveric specimens to investigate the auricular cutaneous distribution of the ABVN, GAN and auriculotemporal nerve. Nevertheless, the fine nature of the terminal branches of these nerves makes dissection technically challenging. An alternative approach may be to use a neuronal tracer suitable for fixed cadaveric tissue such as DiI, a carbocyanine dye which can travel through fixed neuronal tissue in both an anterograde and retrograde direction (Lanciego and Wouterlood, 2011). DiI applied to the cut peripheral end of nerves such as the ABVN could be used to visualise the cutaneous innervation of the ear using an epifluorescence microscope.

An approach which may allow the cutaneous distribution of the auricular nerves of the ear to be investigated in human volunteers could be the use of direct administration of local anaesthetic agents. Auriculotemporal nerve blockade can be used in clinical settings to alleviate temporomandibular joint pain or dysfunction, as the auriculotemporal nerve provides the principal sensory innervation to the temporomandibular joint (Donlon et al., 1984, Nascimento et al., 2013). In addition, other nerve blocks applied to the branches of the mandibular division of the trigeminal nerve such as the inferior alveolar nerve can cause a transient loss of sensation to the external ear (Ngeow and Chai, 2009). The auriculotemporal nerve is in close proximity to the superficial temporal artery and the course of this vessel can be closely identified using Doppler ultrasound. This approach allows for selective blockade of the auriculotemporal nerve via injection of a local anaesthetic agent at a site 1cm superior to the tragus, which has been performed in patients undergoing awake craniotomy (Bebawy et al., 2014). Pinprick-testing could then be used to assess sensory loss ipsilateral to the anaesthetic injection site and compared to sensitivity at the same point on the contralateral ear. A similar method has been previously performed in 20 human volunteers to assess the cutaneous innervation of the great auricular nerve (Thallaj et al., 2010). The great auricular nerve itself was visualised by an anaesthetist using an ultrasound probe and the nerve was injected with

0.1ml mepivacaine 1% as it emerged from the posterior border of sternocleidomastoid. When compared to the contralateral side, GAN blockade led to sensory loss in the earlobe, tail of helix and the antitragus in all individuals, consistent with the innervation pattern observed in previous cadaveric dissection work for these sites (Peuker and Filler, 2002, Thallaj et al., 2010). However, 7 out of 20 participants reported anaesthesia at the spine of the helix, which was the stimulation site chosen in the present study to stimulate the auriculotemporal nerve, as Peuker and Filler reported it being the sole innervation of the spine of the helix in 91% of cadaveric specimens examined (Peuker and Filler, 2002, Thallaj et al., 2010). This inconsistency further reinforces the need for larger scale studies which assess variation in the cutaneous innervation of the ear either through cadaveric dissection or through nerve blockade studies in human volunteers.

3.6.4 Conclusion

Stimulation of the tragus and helix evoked a similar response in normalised LF power, normalised HF power and LF/HF ratio in a subset of healthy volunteers. This suggests that the effects on cardiovascular autonomic function induced by tragus stimulation may be due in part to the influence of the auriculotemporal nerve. However, further *in-vivo* investigation in animals as well as greater clarification of the cutaneous innervation of the external ear in humans is needed before this influence can be confirmed. As such, tragus stimulation will be referred to as tVNS applied to the tragus for the remainder of this thesis.

Chapter 4

The autonomic effects of transcutaneous vagus nerve stimulation (tVNS) applied to the tragus of the external ear in older healthy human research participants

4.1 Introduction

There are now over 10 million people aged 65 and older living in the UK, with this figure expected to increase to 15.5 million by 2030 and 19 million by 2050 (Cracknell, 2010). Age is a critical factor in cardiovascular health and it is estimated that cardiovascular diseases such as myocardial infarction and stroke will cause 40% of deaths in those aged 65 and over by 2030 (North and Sinclair, 2012). Moreover, ageing is strongly associated with changes in autonomic nervous system activity, in particular autonomic control of the heart (Kuo et al., 1999). The autonomic nervous system can be altered with increasing age towards elevated sympathetic nerve activity and reduced parasympathetic activity, hastening the progression of cardiovascular disease (Abhishekh et al., 2013).

It is well-established that there is an age-associated decline in heart rate variability across both time domain and frequency domain measures (Umetani et al., 1998, Antelmi et al., 2004, Zhang, 2007, Zulfiqar et al., 2010, Abhishekh et al., 2013) with a reduction in HRV signifying corresponding with an increased risk of mortality (Tsuji et al., 1994)(Tsuji et al., 1994). Antelmi et al. found that time-domain measures of HRV such as RMSSD and pRR50, which reflect parasympathetic modulation, decreased with age in a cohort of 653 patients aged 14 - 82 years with no history of heart disease (Antelmi et al., 2004). Meanwhile, the LF/HF ratio was observed to increase with age across both sexes, although it was consistently greater in men (Antelmi et al., 2004). Abhishekh et al. also identified a positive correlation between age and LF/HF ratio ($R = 0.19$; $p < 0.01$) (Abhishekh et al., 2013). A previous study by Clancy et al. with a younger cohort of healthy volunteers (n = 48; 24 male, 24 female; age range = 20 – 62 years) observed a trend whereby a higher LF/HF ratio at baseline was associated with both a greater response to tVNS applied to the tragus ($R^2 = 0.58$; $p < 0.0005$) and increasing age ($R2 = 0.19$; $p = 0.013$), similar to the correlation observed by Abhishekh et al. (Abhishekh et al., 2013, Clancy et al., 2014). The study by Clancy et al. included only two individuals aged 60 years and older but linear regression analysis identified two trends whereby age was associated with increased LF/HF ratio at baseline and the response to tVNS was also associated with a higher

baseline LF/HF ratio. These correlations suggest that the stimulation parameters employed for tVNS could be more effective at altering autonomic nervous system activity in older individuals.

4.1.1 Knowledge gap

There are no other studies in the literature which specifically assess the autonomic effects of auricular tVNS in older healthy adults with no prior medical history of cardiovascular disease. La Marca and colleagues (2010) found that auricular electroacupuncture of the concha led to increased respiratory sinus arrhythmia (a non-invasive index of cardiac vagus nerve activity) in a cohort of healthy men (n = 14), although the age range for these volunteers was 20 – 40 years with a mean age of 28.2 years (La Marca et al., 2010). More recently, De Couck et al. (2017) looked at the effects on tVNS applied to the cymba concha of the ear (Cerbomed device, Germany) on measures of heart rate variability in two studies with healthy male and female participants: one study involving a comparison of the autonomic effects of 10 minutes left-sided tVNS versus right-sided tVNS (n = 30; mean age = 37 years; age range = 23 - 58 years) and a second study with slightly older participants looking at the effects of right-sided tVNS over one hour (n = 30, mean age = 44 years; age range = 30 – 65 years) (De Couck et al., 2017). The first study by De Couck et al. failed to detect any significant change in HRV apart from an increase in SDNN from baseline during right-sided tVNS. As the first study identified minimal effects as a result of 10 minutes tVNS and also in comparison with sham stimulation using the earlobe, the second study by De Couck et al. trialled right-sided tVNS over one hour. This study found that tVNS increased the LF/HF ratio after one hour, suggesting an alteration in cardiac autonomic activity towards elevated sympathetic nerve activity and/or reduced vagal tone (De Couck et al., 2017). The exact number of these participants aged 60 and older is not reported, although both this study and the stimulation site optimisation trial with participants aged <60 years did divide the volunteers into groups older and younger than 40 years (De Couck et al., 2017). A consistent finding in both studies was that

increased age was associated with an increased LF/HF ratio at baseline, but neither study saw any tVNS-induced alterations in HRV related to the age of the participants. Thus, the lack of information on the autonomic effects of tVNS in research participants aged 60 years and older, coupled with the contradictory nature of the results of previous studies in younger individuals, reveals a clear knowledge gap.

4.2 Hypothesis

Transcutaneous electrical stimulation of the tragus of the ear (tVNS) will alter cardiovascular autonomic activity in healthy aged volunteers free from cardiovascular disease, with an increased LF/HF ratio at baseline associated with a greater response to tVNS.

4.3 Aims and objectives

The present study investigated the effects of tVNS on cardiovascular autonomic activity in healthy participants aged 60 years and older by non-invasive measurements of heart rate and finger arterial blood pressure. Microneurography was then performed at an additional visit to record changes in single-unit muscle sympathetic nerve activity as a result of tVNS.

4.4 Materials and methods

4.4.1 General protocol

University of Leeds ethical approval was secured (Ethics Reference: BIOSCI 13-025) and the study conformed to the standards outlined in the Declaration of Helsinki. Informed written consent was obtained voluntarily by all research participants and their data were anonymised and stored securely according to the Data Protection Act (1998). All experiments were carried out in a dedicated human physiology study room at University of Leeds. These experiments occurred between the hours of 9am and 11.30am in order to minimise the impact of circadian rhythm variations on the autonomic nervous system. The ambient temperature of the study room was maintained at 21 ± 2°C.

27 healthy participants were recruited to the study and all participants were asked to complete a basic health questionnaire. Physical activity level was assessed using the Godin Leisure Time Exercise Questionnaire (Amireault and Godin, 2015). Inclusion criteria were male or female volunteers aged ≥ 60 years old. Exclusion criteria were a prior medical history of hypertension, cardiac disease, diabetes mellitus or epilepsy. Female participants were asked to indicate if they were receiving hormonal replacement therapy (HRT) for treatment of menopausal symptoms, as HRT has been shown to independently alter cardiac autonomic activity (Yildirir et al., 2001). All participants were required to abstain from caffeine, alcohol, nicotine and strenuous exercise for a minimum of 12 hours prior to their visit. They were further required to consume a light breakfast and use the toilet prior to attending for the experiment. Participants were asked to attend for two visits to receive active tVNS and then sham tVNS at an additional follow-up visit. The protocols for delivering these stimulation parameters are outlined in Chapter 2. The active tVNS data from included older participants was further compared to active tVNS data obtained from the younger participants aged <60 years (n = 22) who took part in the experiments outlined in Chapter 3.

4.4.2 Cardiovascular autonomic measurements and data acquisition

Recordings of heart rate, respiration, blood pressure and MSNA were obtained as described in Chapter 2. In some participants it was not possible to obtain reliable finger arterial blood pressure recordings or to detect BRS sequences and the sample sizes for these analyses are included in Table 4.6 for BP and Table 4.4 for BRS. To characterise if the change in the LF/HF ratio observed during tVNS was due to a decrease in sympathetic activity, seven participants who responded with a >10% decrease in LF/HF ratio during active tVNS (tVNS responders, n = 13) agreed to return for an additional visit. At this third visit, simultaneous recording of single-unit muscle sympathetic nerve activity (MSNA) was obtained from the common peroneal nerve of the leg (Figure 2.4). Single-unit MSNA frequency (units detected per minute) and MSNA incidence (units detected per 100 heartbeats) were calculated to assess changes in sympathetic vasoconstrictor activity as a result of active tVNS. Microneurography was not performed during sham tVNS as it was inappropriate to implement this invasive technique when sham tVNS had been observed to have no effect on LF/HF ratio or mean BRS at the first active tVNS visit.

4.4.3 Statistical Analysis

All statistical analyses for this study were performed using SPSS (version 24). Shapiro-Wilk tests were performed on each variable to assess normality. Participant subgroup characteristics were compared using independent t-tests or Mann Whitney U-tests. Linear mixed model analysis was performed to compare the effect of time (baseline, stimulation and recovery) between the active tVNS and sham tVNS visits. One-way repeated measures ANOVA was used to analyse effect of time (baseline, stimulation and recovery) for each visit (active tVNS or sham tVNS) and Bonferroni post-hoc tests performed. Two-way repeated measures ANOVA was used to analyse the effect of time (baseline, stimulation and recovery) in the subgroup of participants (n = 7) who attended for two active tVNS visits (active tVNS alone and active tVNS with microneurography). Non-parametric

Friedman test was used where data were not normally distributed with Bonferroni correction applied to adjust significance based on the number of pairwise comparisons within the test. Spearman's Rank correlation test was used to identify possible relationships between variables and linear regression was used to further explore these correlations. All data are presented as group mean ± standard error of the mean (S.E.M).

4.5 Results

4.5.1 Baseline characteristics of participants

27 participants with no previous medical history of cardiovascular disease or diabetes were enrolled at the study and written informed consent was obtained at the first visit. Seven of the 27 participants (n = 5 male, n = 2 female) were excluded from the study due to the presence of frequent ventricular extrasystoles. One volunteer (female, age = 88 years) was excluded due to poor ECG signal despite repeated attempts at repositioning the ECG electrodes and one volunteer (male, age = 62 years) was excluded due to an inability to stay awake during the recordings. The baseline characteristics of the remaining 18 participants (n = 12 female and n = 6 male) included in the study are presented in Table 4.1. Male participants had a higher baseline LF/HF ratio than female participants (male mean LF/HF ratio = 2.0 ± 0.16; female = 1.54 ± 0.29; p = 0.016). None of the female volunteers reported receiving hormonal replacement therapy during their involvement with the study. There were no significant differences in BMI or in physical activity level as assessed by the Godin Leisure Time Exercise Questionnaire in any of the study subgroups.

The baseline characteristics of the 18 older participants were then compared to mean data from the younger participants (< 60 years old, n = 22) in Chapter 3. The older participants had significantly reduced mean values at baseline for the HRV measures total power, VLF power, LF power and HF power but not normalised LF, normalised HF or LF/HF ratio (Table 2; $p < 0.05$). No significant differences in systolic BP, diastolic BP or mean BP were detected between the younger and older groups ($p > 0.05$). The older participants had significantly reduced mean BRS at baseline compared to the younger participants ($p < 0.001$; older baseline mean BRS = 8.40 ± 0.72 ms/mmHg; younger baseline mean BRS = 17.29 ± 1.07 ms/mmHg), but there was no difference in mean heart rate between the two groups ($p = 0.393$).

Table 4.1: Baseline characteristics of the older healthy participants.

	N	Age (yrs)	BMI (kg/m^2)	Godin activity score	Mean Blood Pressure	Heart Rate (bpm)	Baseline LF/HF		BRS N	Baseline BRS (ms/mmHg)
All participants	18	67.5 ± 1.56	26.0 ± 4.16	17.0 ± 3.79	84.35 ± 2.20	64.94 ± 1.86	1.69 ± 0.21		16	8.40 ± 0.75
Male participants	6	68.3 ± 3.05	25.82 ± 2.40	17.5 ± 7.97	84.72 ± 1.76	68.67 ± 4.42	**2.0 ± 0.16***		6	9.04 ± 1.35
Female participants	12	67.08 ± 1.75	26.03 ± 1.39	16.58 ± 4.06	84.15 ± 3.26	65 ± 1.67	**1.54 ± 0.29***		10	8.02 ± 0.84
tVNS responders	13 (F=7)	68.31 ± 1.95	26.21 ± 1.21	15.77 ± 4.84	86.12 ± 4.88	64.69 ± 3.46	**2.02 ± 0.23***		13	8.06 ± 0.86
tVNS non-responders	5 (F=5)	65.4 ± 2.09	25.32 ± 1.55	19.80 ± 5.07	83.33 ± 5.01	64.6 ± 2.20	**0.86 ± 0.17***		3	9.90 ± 0.88

	Older participants (≥ 60 years old)	Younger participants (< 60 years old)	*p*
N	18	22	-
Mean age (years)	67.50 ± 1.60 Age range = 60 – 84	32.86 ± 2.04 Age range = 24 - 59	-
Total power (ms^2)	**654.44 ± 82.10***	**3374.78 ± 803.03***	**< 0.001**
LF power (ms^2)	**348.71 ± 99.39***	**1409.90 ± 388.29***	**< 0.001**
HF power (ms^2)	**264.86 ± 101.58***	**1491.41 ± 373.58***	**< 0.001**
Normalised LF power (n.u.)	0.58 ± 0.04	0.52 ± 0.04	0.138
Normalised HF power (n.u.)	0.42 ± 0.04	0.48 ± 0.04	0.118
LF/HF ratio	1.69 ± 0.22	1.33 ± 0.18	0.081
BRS (ms/mmHg)	**8.40 ± 0.72***	**13.60 ± 1.04***	**< 0.001**

Table 4.2: Baseline characteristics of the older healthy participants compared with younger healthy participants from Chapter 3.

Significant differences between the older and younger healthy participants were observed for BRS as well as the HRV measures total power, LF power and HF power.

4.5.2 tVNS significantly improved heart rate variability in healthy older adults

18 participants attended for the active tVNS visit and 14 of those 18 participants attended for a sham tVNS visit a minimum of one week later. Baseline mean LF/HF ratio values were similar between the active and sham tVNS visits ($p = 0.118$, see Table 4.3). A significant decrease in overall mean LF/HF ratio from baseline was observed during active tVNS (repeated measures ANOVA; $p = 0.003$) but there was no significant change from baseline during sham tVNS (Figure 4.1 and Figure 4.2). Active tVNS also elicited a significant decrease in normalised LF power (Friedman test; $p = 0.023$) and an increase in normalised HF power (Friedman test; $p = 0.014$) from baseline, but no significant changes were observed for sham tVNS ($p > 0.05$).

As in Chapter 3, the participants at the active tVNS visit were further divided into subgroups based on their response to active tVNS: tVNS responders (decrease in LF/HF ratio during active tVNS, n = 13) and tVNS non-responders (no change or an increase in LF/HF ratio during active tVNS, n = 5; Figure 4.1 and Figure 4.2). All six male participants in the study were included in the tVNS responder subgroup. Mean baseline LF/HF ratio was significantly higher for tVNS responders than non-responders (independent t-test; $p = 0.011$; responder mean LF/HF ratio = 2.02 ± 0.23; non-responder mean LF/HF ratio = 0.86 ± 0.17) and the responder subgroup exhibited a decrease in LF/HF ratio during active tVNS (repeated measures ANOVA; $p = 0.001$; mean percentage change = - 34.27%). The mean LF/HF ratio of the tVNS responder subgroup approached baseline level once stimulation had ceased (Figure 4.1 and Figure 4.2). A significant decrease in normalised LF power (repeated measures ANOVA; $p < 0.001$) and an increase in normalised HF power (repeated measures ANOVA; $p < 0.001$) was also observed from baseline during active tVNS. The tVNS non-responder subgroup (n = 5) were all female and the group mean LF/HF ratio did not deviate significantly from baseline during or after active tVNS ($p > 0.05$; Figure 4.1).

Table 4.3: HRV responses to active tVNS and sham tVNS in older healthy participants Active tVNS elicited a significant decreases in normalised LF and LF/HF ratio from baseline ($p < 0.05$). A significant increase in normalised HF power was observed in the recovery period following active tVNS ($p = 0.014$). No significant changes were observed as a result of sham tVNS ($p > 0.05$).

	Active tVNS (n = 18)			Sham tVNS (n = 14)		
	Baseline	**Stimulation**	**Recovery**	**Baseline**	**Stimulation**	**Recovery**
Total power (ms^2)	654.44 ± 79.79	625.74 ± 81.54	860.43 ± 131.01	570.47 ± 69.11	718.17 ± 144.10	749.53 ± 113.07
LF power (ms^2)	348.71 ± 96.59	278.95 ± 70.32	454.62 ± 124.36	230.93 ± 38.71	276.21 ± 41.48	321.67 ± 49.02
HF power (ms^2)	264.86 ± 98.71	272.52 ± 73.26	331.42 ± 97.29	168.77 ± 57.04	201.26 ± 30.61	199.80 ± 30.71
Normalised LF power (n.u.)	**0.58 ± 0.04***	**0.50 ± 0.03***	0.59 ± 0.03	0.56 ± 0.04	0.58 ± 0.04	0.61 ± 0.04
Normalised HF power (n.u.)	**0.42 ± 0.04***	**0.50 ± 0.03***	0.42 ± 0.03	0.44 ± 0.04	0.42 ± 0.04	0.39 ± 0.04
LF/HF	**1.69 ± 0.21***	**1.16 ± 0.11***	1.63 ± 0.20	1.53 ± 0.21	1.67 ± 0.24	1.84 ± 0.24

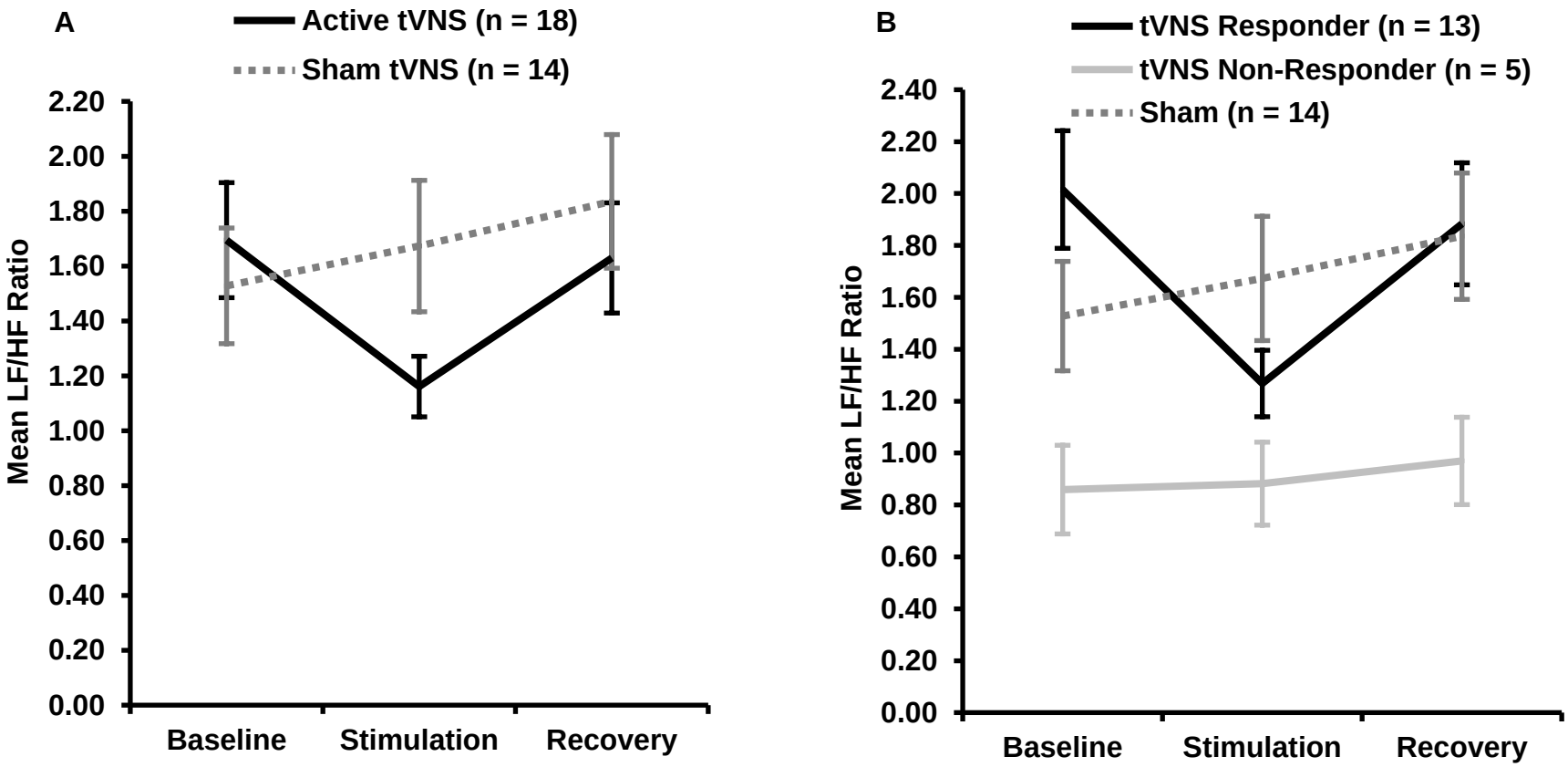


Figure 4.1 HRV responses to active tVNS and sham tVNS in older healthy participants: (A) Active tVNS elicited a significant decrease in LF/HF ratio from baseline ($p = 0.003$) while no significant change was observed as a result of sham tVNS ($p > 0.05$). (B) 13 participants experienced a decrease in LF/HF ratio during tVNS (tVNS responder; mean change = -34.27%; female = 7, male = 6). No change in LF/HF was observed for 5 female subjects (tVNS non-responder).

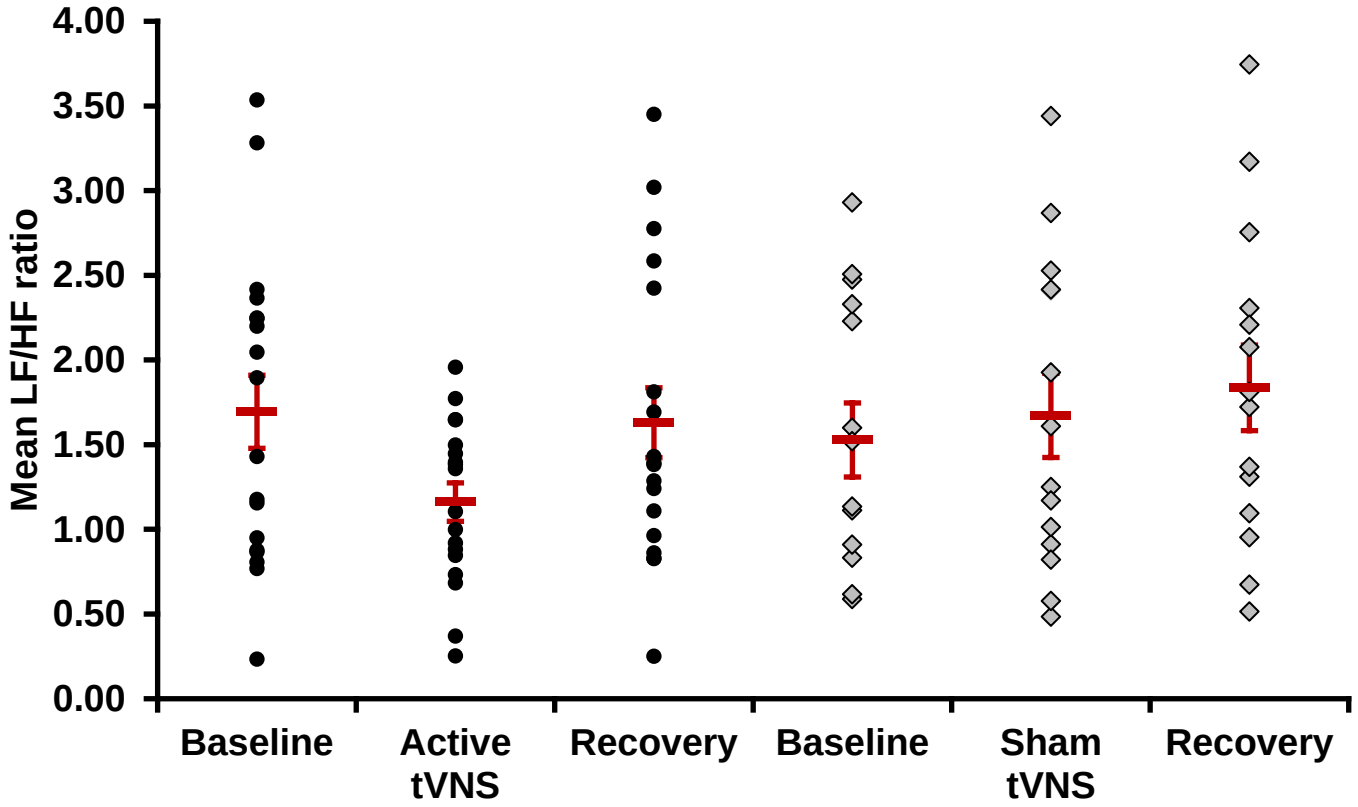


Figure 4.2 Individual HRV responses to active tVNS and sham tVNS in older healthy participants.

4.5.3 The LF/HF ratio at baseline is correlated with the change in LF/HF ratio during active tVNS

A relationship between baseline LF/HF ratio and the change in LF/HF during active tVNS was identified by linear regression analysis, indicating that the response to active tVNS can be predicted based on the baseline LF/HF ratio (R^2 = 0.79; p = 0.001; Figure 4.3). There was no correlation between baseline LF/HF ratio and age (R^2 = 0.16; p = 0.052) or with any other variable.

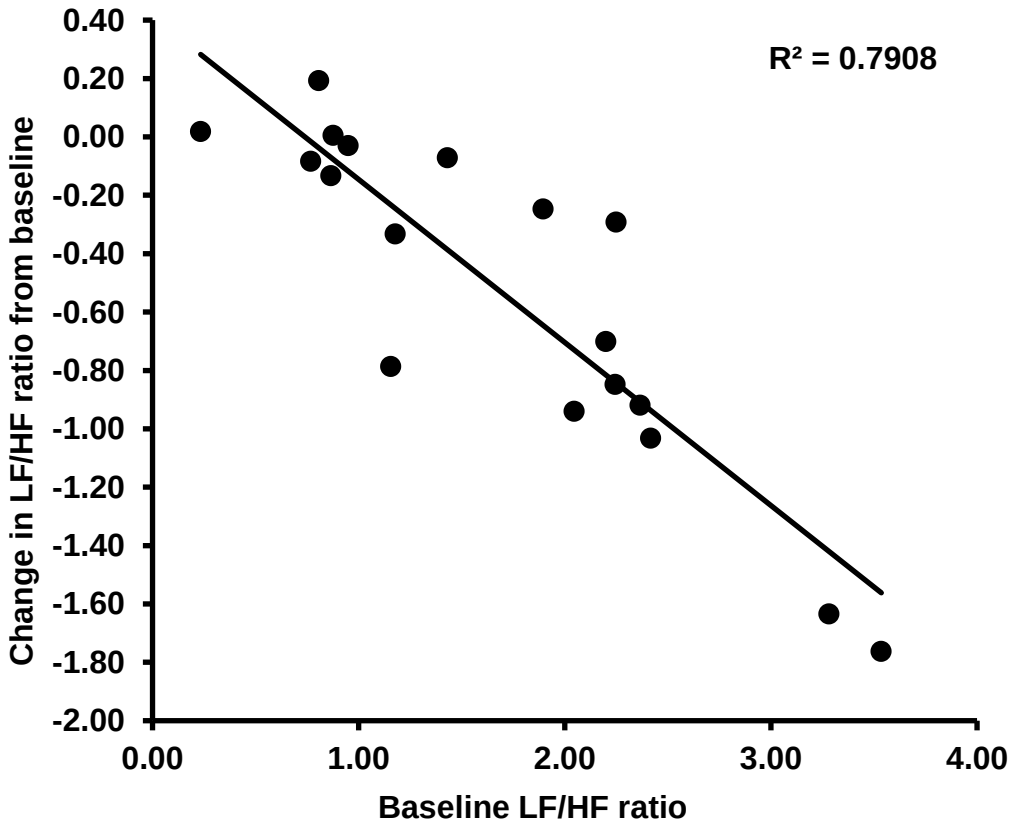


Figure 4.3 Relationship between baseline LF/HF ratio and change in LF/HF ratio from baseline.

4.5.4 The tVNS-induced changes in LF/HF ratio were reproducible in tVNS responders

Seven participants (male = 3, female = 4) from the tVNS responder subgroup returned for a second active tVNS visit where microneurography was performed to assess changes in single-unit MSNA. The group mean change in LF/HF ratio observed as a result of active tVNS in these participants was comparable between their first and second active tVNS visits (Figure 4.4) but no statistically significant changes were detected.

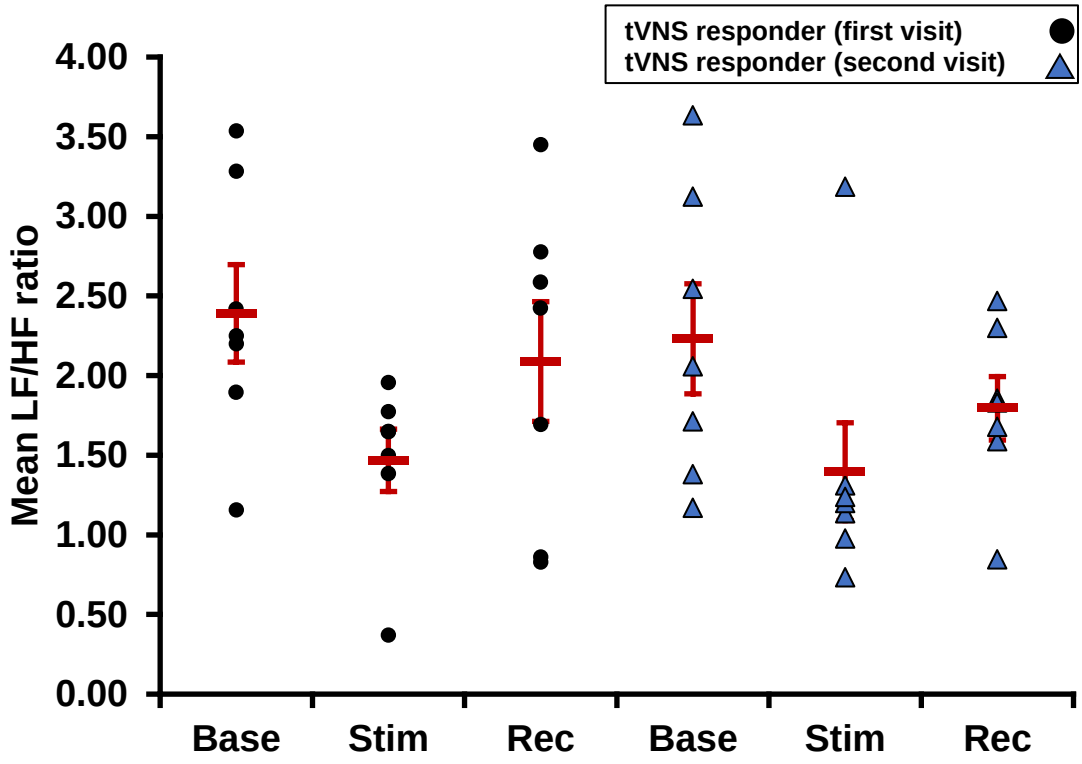


Figure 4.4 LF/HF ratio change observed as a result of active tVNS in participants who returned for microneurography (n = 7). A decrease in LF/HF ratio was observed due to active tVNS at both visits in all participants except one. This male participant did not show a decrease in LF/HF ratio at the second visit (baseline LF/HF = 1.38; stimulation = 1.31).

4.5.5 tVNS increased baroreflex sensitivity in healthy older adults

When all participants (responders and non-responders) were considered in analyses, there was a small but significant increase in BRS from baseline during active tVNS (n = 16; p = 0.031; see Figure 4.5 and Table 4.4), which was sustained in the recovery period (p = 0.002). This effect was not observed as a result of sham tVNS (n = 10; p = 0.520; Figure 4.5 and Table 4.4). The baseline BRS values for sham and active tVNS did not differ significantly (p = 0.701). No significant change in BRS was detected in the male participants during or after active tVNS (baseline BRS = 9.04 ± 1.35; tVNS BRS = 12.22 ± 1.75; n = 6; p = 0.096). This may have been due to the small sample size and substantial inter-individual variability. A significant increase in BRS was observed from baseline in female participants (n = 10; baseline BRS = 8.02 ± 0.84) in the recovery period (recovery BRS = 10.58 ± 1.20) but not during tVNS (BRS = 9.25 ± 1.01).

In participants who responded to tVNS with a decrease in LF/HF ratio (n = 13), there was an increase in BRS which remained elevated in the recovery period (see Table 4.4). Linear regression analysis showed that the mean LF/HF ratio in the recovery period for the tVNS responder group was inversely related to the mean BRS in these participants at the same time-point (R^2 = 0.406; p = 0.019). In the seven tVNS responders who returned for an additional visit where active tVNS and microneurography were performed, there was no difference in baseline BRS between this visit and the original active tVNS visit (first visit = 8.06 ± 1.28; second visit = 8.67 ± 0.84; p = 0.403). However, the small sample size at this additional visit (n = 6) meant that no significant change was detected (p= 0.194; baseline BRS value = 8.67 ± 0.84 ms/mmHg; stimulation BRS value = 9.95 ± 0.86 ms/mmHg).

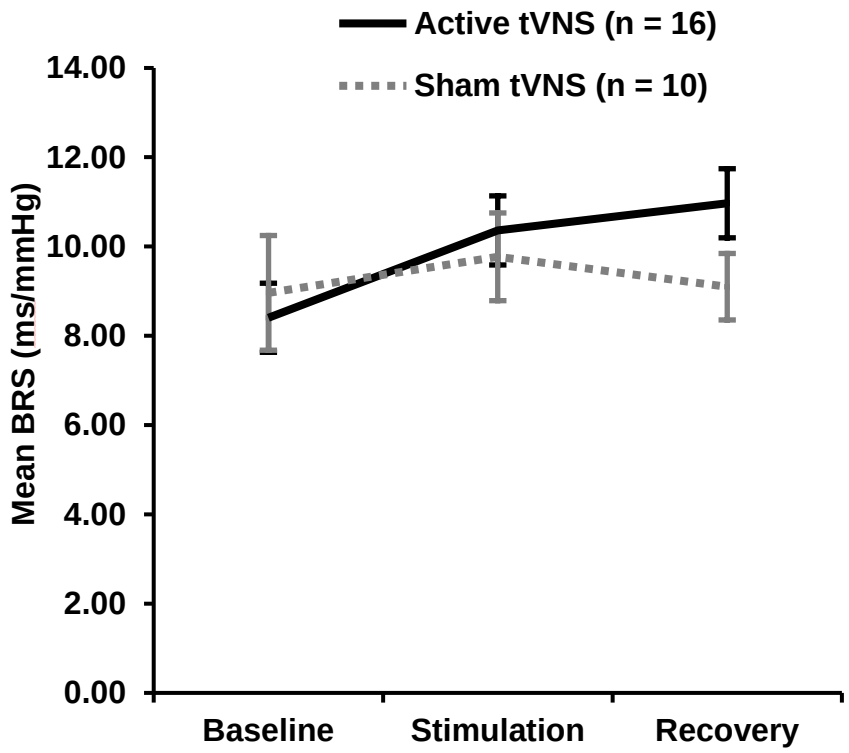


Figure 4.5 BRS response to active tVNS and sham tVNS in the older participants.

A significant increase in BRS was observed from baseline during active tVNS ($p = 0.031$), which was sustained into the recovery period ($p = 0.002$). Sham tVNS had no significant effect on BRS ($p = 0.520$).

	N	Baseline	Stimulation	Recovery	*p* (Baseline - Stimulation)	*p* (Baseline - Recovery)
Active tVNS	16	8.40 ± 0.75	10.36 ± 1.01	10.97 ± 1.04	**0.031**	**0.011**
Sham tVNS	10	8.96 ± 1.28	9.77 ± 0.98	9.10 ± 0.74	0.805	1.0
tVNS responders	13	8.06 ± 0.86	10.30 ± 1.16	10.73 ± 1.25	**0.011**	**0.004**
tVNS non-responders	3	9.90 ± 0.88	10.61 ± 1.34	12.00 ± 0.64	1.0	1.0
Active tVNS + microneurography	7	8.67 ± 0.84	9.95 ± 0.86	8.22 ± 0.93	0.078	1.0

Table 4.4: BRS responses in healthy older participants.

Active tVNS elicited a significant increase in BRS from baseline, which was sustained in the recovery period. Units = ms/mmHg.

	N	Baseline	Stimulation	Recovery	*p* (Baseline - Stimulation)	*p* (Baseline - Recovery)
Male participants	6	9.04 ± 1.47	12.22 ± 1.91	11.62 ± 2.03	0.140	0.505
Female participants	10	8.02 ± 0.84	9.25 ± 1.01	10.58 ± 1.20	0.392	**0.029***

Table 4.5: Comparison of BRS responses in male and female healthy older participants.

Female participants showed a significant increase in BRS from baseline in the post-stimulation recovery period. No significant changes were detected in the male participants. Units = ms/mmHg.

4.5.6 tVNS reduced muscle sympathetic nerve activity in healthy older adults

Simultaneous microneurography recording of single-unit MSNA from the common peroneal nerve was performed in five out of seven participants who returned for a second active tVNS session. Active tVNS elicited a significant decrease in mean MSNA frequency ($p = 0.043$; Figure 4.6 and Table 4.6) and mean MSNA incidence ($p = 0.043$; Figure 4.6), alongside a decrease in LF/HF ratio. All five participants experienced a decrease in single-unit MSNA frequency and incidence from baseline as a result of active tVNS, which remained below baseline level in four out of five participants after active tVNS ceased. Single-unit MSNA in the fifth participant returned to that individual's baseline level of MSNA frequency and MSNA incidence after active tVNS ceased, although the LF/HF ratio for this participant remained below baseline level.

	Baseline	Stimulation	Recovery
MSNA Frequency (units/min)	**0.60 ± 0.17*†**	**0.30 ± 0.14***	**0.16 ± 0.04†**
Normalised MSNA Frequency (units/min)	**1.00*†**	**0.40 ± 0.14***	**0.40 ± 0.16†**
MSNA Incidence (units/min)	**1.06 ± 0.31*†**	**0.56 ± 0.27***	**0.28 ± 0.06†**
Normalised MSNA Incidence (units/min)	**1.00*†**	**0.39 ± 0.14***	**0.41 ± 0.16†**

Table 4.6: Effect of active tVNS on MSNA in healthy older participants.

Active tVNS elicited a significant decrease from baseline in mean MSNA frequency ($p = 0.043$) and mean MSNA incidence ($p = 0.043$). Data normalised to 1.00 due to high degree of inter-individual variability in the small sample ($n = 5$).

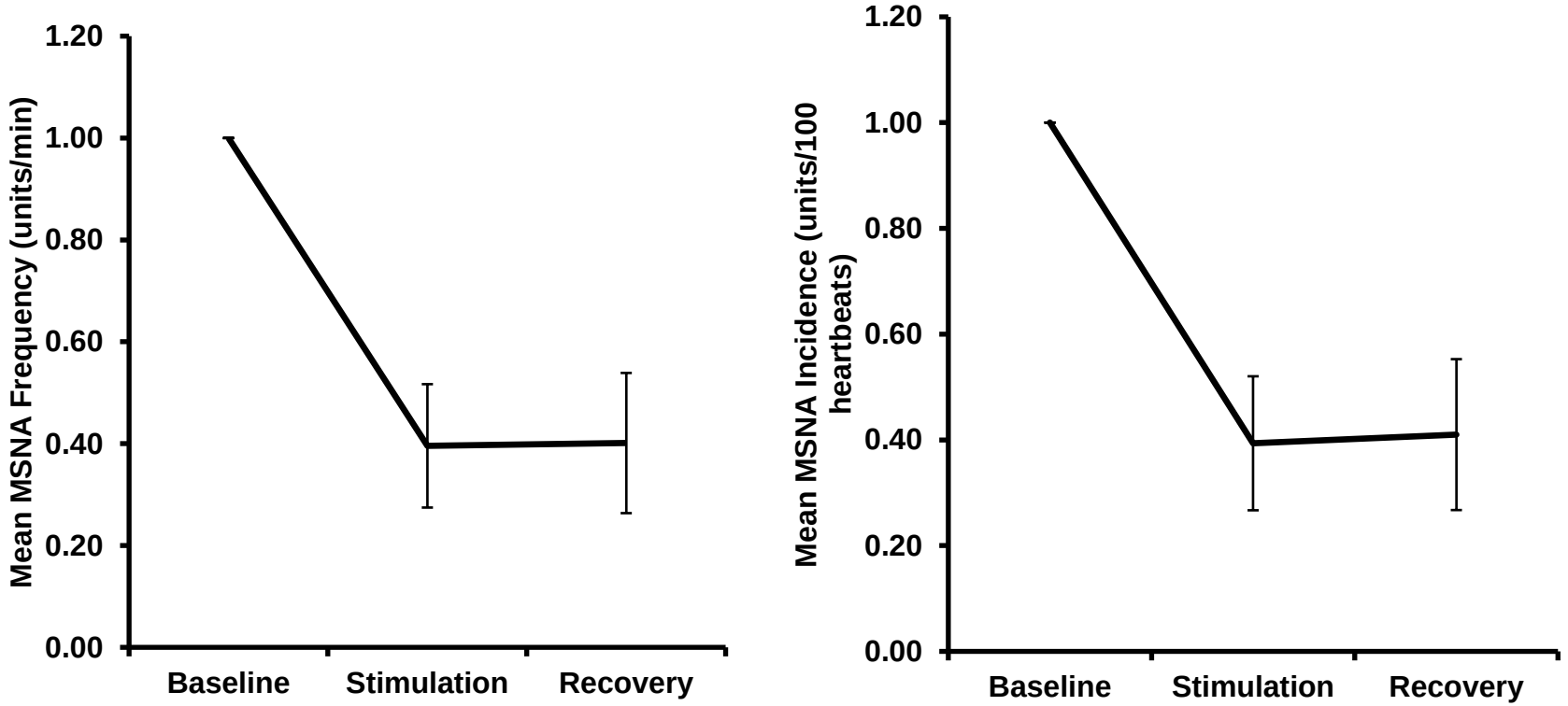


Figure 4.6 Effect of active tVNS on MSNA frequency and incidence in healthy older participants (normalised data).

A significant decrease in both MSNA frequency and MSNA incidence were detected as a result of active tVNS ($p < 0.05$). Data have been normalised to 1.00.

4.5.7 Effects of active and sham tVNS on heart rate and blood pressure

A slight but significant decrease in mean heart rate from baseline was observed during active tVNS (baseline mean heart rate = 66.4 ± 1.6 beats per minute; during tVNS = 65 ± 1.8 beats per minute; p = 0.007), which persisted once active tVNS had ceased (mean heart rate after tVNS = 63 ± 1.9; p = 0.01 compared with mean heart rate at baseline). This trend was not observed in the participants who returned for an active tVNS and microneurography experiment (n = 7). There was no change in mean heart rate from baseline during sham tVNS, but heart rate decreased in the post-intervention recovery period (p = 0.004). An increase in mean systolic blood pressure (p = 0.006) and mean diastolic blood pressure (p = 0.034) was further detected in the recovery period after sham tVNS (Table 4.7).

	Active tVNS (n = 17)				Sham tVNS (n = 11)		
	Baseline	**Stimulation**	**Recovery**		**Baseline**	**Stimulation**	**Recovery**
Systolic BP (mmHg)	122.71 ± 2.64	126.24 ± 2.25	122.76 ± 3.18		**116.45 ± 4.19***	116.36 ± 4.32	**123.64 ± 4.22***
Diastolic BP (mmHg)	65.18 ± 2.31	67.35 ± 1.71	66.41 ± 1.61		**68.09 ± 1.60***	66.82 ± 1.66	**70.91 ± 1.79***
Mean BP (mmHg)	84.35 ± 2.20	86.98 ± 1.54	85.20 ± 1.88		84.21 ± 1.74	83.33 ± 1.99	88.48 ± 1.92

Table 4.7: BP responses to active and sham tVNS in healthy older participants.

Active tVNS did not have a significant effect on systolic, diastolic or mean BP ($p > 0.05$) but an increase in systolic BP ($p = 0.034$) and diastolic BP ($p = 0.034$) from baseline was observed in the recovery period following sham tVNS.

4.6 Discussion

This study demonstrates that transcutaneous vagus nerve stimulation applied to the tragus of the ear can induce changes in autonomic nervous system activity in healthy adults aged 60 years and older. Active tVNS significantly decreased the LF/HF ratio from baseline and this observation was accompanied by a decrease in single-unit MSNA when measured in a subset of subjects during active tVNS and microneurography, characterising this change in autonomic activity as one involving a decrease in central sympathetic outflow. However, a small but significant increase in BRS was also observed during active tVNS, suggesting that there may be a concurrent increase in vagal tone as a result of stimulation too. As aging is a risk factor for cardiovascular dysfunction, tVNS may thus be beneficial to cardiovascular health in older people (North and Sinclair, 2012).

The results of this study contrast with Napadow et al. (2012) where tVNS applied to the cymba concha had no effect on HRV, although that study also used different stimulation parameters (Napadow et al., 2012). However, the present study lends some support to the conclusion of La Marca et al. (2010) that tVNS is able to enhance vagal tone, although that study focused on respiratory sinus arrhythmia rather than HRV or BRS and also used electroacupuncture of the left inferior concha of the ear rather than bilateral cutaneous surface electrodes positioned on the tragus (La Marca et al., 2010). The high frequency spectral power component is considered to be a quantitative marker of respiratory sinus arrhythmia (Pagani et al., 1986, Pomeranz et al., 1985) and although the present study failed to see any change in HF power as a result of active tVNS, an increase in normalised HF was detected. This disparity in outcomes between HF power and normalised HF power may have been due to the high variability in HF power between the older participants, which would have been adjusted and minimised by normalisation. This change in normalised HF also coincided with a small but significant increase in mean BRS from baseline in the older participants, further suggesting a potential increase in vagal tone.

The present study only agrees with the two studies by De Couck et al. (2017) insofar as their >40 year old age groups had depressed values of LF and HF power at baseline compared to younger participants, as well as a greater value for LF/HF ratio in the two >40 year old groups(De Couck et al., 2017). No other age-related trends in HRV were associated with this study, although the different stimulation sites (right or left cymba concha) and different stimulation parameters (1mA, 10 minutes tVNS and 1 hour tVNS) to the present study may be responsible for the contrasting results.

4.6.1 tVNS effects on cardiovascular function

The present study corroborates the conclusions of Clancy et al. (2014) in that tVNS is able to induce a decrease in both LF/HF ratio and single-unit MSNA frequency and incidence in healthy individuals (Clancy et al., 2014). In addition, the present study provides further insight into this short-term change in autonomic function through the detection of a small but significant increase in spontaneous baroreflex sensitivity as measured using the sequence method. This is particularly important given the association between lower values of BRS and prognosis following myocardial infarction and also in heart failure (La Rovere et al., 2011). Thus, tVNS may be a method of potentially increasing BRS where it may be impaired – the participants in the present study had a much lower mean BRS value at baseline than the younger participant data taken from Chapter 3, although the younger volunteers overall (n = 22) did not show a change in BRS as a result of tVNS applied to the tragus. Cardiovagal BRS is known to be inversely correlated with age and the reductions in BRS may involve any aspect of the cardiac baroreflex arc such as the afferent limb or the efferent limb i.e. the vagus nerve (Laitinen et al., 1998; Monahan et al., 2007). However it is not possible to directly record from the vagus nerve in humans and further in-vivo studies investigating the effects of tVNS applied to the tragus on spontaneous BRS are needed.

There remains uncertainty as to the residual autonomic effects of tVNS once stimulation has ceased. Clancy et al. (2014) observed a sustained decrease

in LF/HF ratio and MSNA following cessation of current, but the present study observed this effect for MSNA alone. The mean LF/HF ratio returned to baseline level within 15 minutes of the stimulus being switched off. This suggests that the cardiac autonomic modulation induced by tVNS may be short-lived in older individuals, but the reduction in vasoconstrictor sympathetic nerve activity to vascular smooth muscle (specifically to the smooth muscle of arteries supplying the leg skeletal musculature) may be able to persist for a longer duration. However, the observed change in LF/HF ratio in response to tVNS can be reproduced, as demonstrated by data from the tVNS responder subjects (n = 7) who returned for an additional microneurography visit. Their group mean LF/HF response to tVNS was similar between both active tVNS visits. Further studies are needed to elucidate the autonomic effects of chronic tVNS in older individuals and determine if the autonomic modulation induced in the short-term by this technique can be maintained over time.

There has been a number of previous studies performed over a longer time-scale using an electroacupuncture form of tVNS in patients with coronary artery disease (Popov et al., 2013, Zamotrinsky et al., 1997, Zamotrinsky et al., 2001). These studies are especially relevant to the present study given the known association between ageing and an increased incidence of coronary artery disease (Jousilahti et al., 1999). Current treatment strategies in coronary artery disease (CAD) patients with heart failure include β-adrenergic blockade to counter the early adverse effects of cardiac sympathoexcitation via the modulation of heart rate (Azevedo et al., 2001). Zamotrinsky and colleagues trialled a variation of tVNS involving electroacupuncture in patients with CAD who were awaiting coronary artery bypass surgery. Bilateral electrical stimulation of the concha for 15 minutes over 10 days led to a decrease in vasodilator use and a reduction in the frequency of angina pectoris attacks, with effects persisting up to three weeks after tVNS (Zamotrinsky et al., 1997, Zamotrinsky et al., 2001). Patients in the tVNS group had a significantly reduced incidence of acute heart failure post-surgery compared to a control group (Zamotrinsky et al., 2001). A later study by Popov and colleagues (2013) used a similar method

of tVNS over 10 days in a cohort of 48 male CAD patients (mean age = 53.5 ± 4.1 years). The study found that 62.5% of patients (n = 30) experienced a reduction in the severity of their angina attacks with a concurrent and significant decrease in LF/HF ratio (baseline mean LF/HF ratio = 2.43 ± 0.78; after tVNS = 1.25 ± 0.25; p = 0.001), although the remaining patients experienced no significant immediate change in HRV (baseline mean LF/HF ratio = 1.85 ± 0.20; after tVNS = 1.71 ± 0.21) (Popov et al., 2013). A follow-up examination one month later found improvements in the well-being of the responder group with a sustained decrease in LF/HF ratio (mean LF/HF ratio = 1.02 ± 0.03), although the non-responder patients also exhibited a significant decrease in LF/HF ratio one month on (mean LF/HF ratio = 1.56 ± 0.15; p = 0.043). The autonomic effects of chronic tVNS therefore may not manifest immediately after the course of stimulation has finished. Future studies with older healthy individuals free from cardiovascular disease should also include a follow-up period after tVNS has ceased, to ascertain if there are comparable changes in autonomic indices such as the LF/HF ratio and especially MSNA, given the sustained decrease in single-unit MSNA observed with short-term tVNS in the present study.

4.6.2 tVNS as a therapeutic adjunct for age-associated autonomic dysfunction

The results of the present study suggest that tVNS may be able to exert a short-term modulatory effect on the autonomic nervous system in older healthy individuals, suggesting that it may be a potential non-invasive method of counteracting the deleterious changes in autonomic nervous system activity that coincide with normal ageing. This autonomic dysfunction can manifest in chronic cardiovascular conditions such as heart failure, which increases in prevalence with age (Levy et al., 2002, Bleumink et al., 2004). However, autonomic dysfunction identified using non-invasive measures such as heart rate variability and baroreflex sensitivity is also associated with an increased risk of developing psychological disorders such as major depression (Broadley et al., 2005, Kemp et al., 2010) . A study by Kamphuis

et al. in a cohort of elderly men (n = 2285) free from cardiovascular disease found an association between depressive symptoms and increased resting heart rate, a sign of potential autonomic dysfunction (Kamphuis et al., 2007). The association between autonomic dysregulation and the risk of developing poor mental health in old age could be a potential research direction to explore with tVNS applied to the tragus, as the changes in cardiovascular autonomic function observed with tVNS may elicit a prophylactic effect.

In addition, problems with memory and cognition are common aspects of ageing which can have a profound effect on an individual's quality of life. Episodic memory, for example the ability to equate a face with a name, is one particular form of memory which declines with age (Ronnlund et al., 2005). There is evidence that tVNS applied to the tragus may have a beneficial effect on episodic memory performance in healthy older people (Jacobs et al., 2015). The application of 17 minutes tVNS was found to attenuate a decline in performance in a verbal word-learning task which was otherwise observed when stimulation was applied to the earlobe as a control (Jacobs et al., 2015). This finding suggests that tVNS may be a potential method of ameliorating episodic memory loss, reducing the impact of this particularly distressing condition on elderly individuals.

4.7 Conclusion

The present study supports the use of tVNS as a method of counteracting the detrimental age-associated changes in autonomic nervous system activity, specifically through the reduction of sympathetic nerve activity as measured using MSNA and the increase in baroreflex sensitivity. Further studies are needed to determine the long-term autonomic effects of tVNS in an aged cohort.

Chapter 5

The autonomic effects of transcutaneous vagus nerve stimulation applied to the tragus in chronic heart failure patients

5.1 Introduction

Vagus nerve stimulation for the treatment of heart failure was first trialled by Schwartz et al., who surgically implanted a modified VNS system (CardioFit™ model 5000, BioControl Medical Ltd.) in eight male heart failure patients who had already received optimum medical therapy such as beta-blockers and ACE-inhibitors for at least one month (Schwartz et al., 2008). In contrast to previous VNS trials in drug-refractory epilepsy, this single-centre phase II feasibility study implanted the stimulating electrodes around the right cervical vagus nerve rather than the left. An intracardiac sensing lead was also inserted into the right ventricle to monitor heart rate, allowing 1ms VNS pulses to be delivered at a fixed time delay (70ms) from the onset of the R wave to prevent bradycardia (Schwartz et al., 2008). At six months follow-up, there was a significant reduction in end-systolic left ventricular volume (baseline = 208 ± 71ml; six months = 190 ± 83ml; $p = 0.001$) as well as small yet significant decrease in resting heart rate (baseline = 87 ± 15bpm; six months = 82 ± 12bpm; $p = 0.02$). This preliminary study also found significant improvements in both symptoms and quality of life as assessed by the New York Heart Association (NYHA) classification and the Minnesota Living with Heart Failure® QoL questionnaire. An expanded multi-centre phase II trial of the CardioFit system with an additional 24 heart failure patients again demonstrated improved quality of life scores and NYHA classification at six months follow-up, plus an increase in left ventricular ejection fraction (baseline = 21.1 ± 7.5%; one year = 34.1 ± 12.5%; $p<0.0001$) and a reduction in heart rate (baseline = 85 ± 14bpm; one year = 76 ± 11bpm; $p < 0.003$) in 23 patients at one year follow-up (De Ferrari et al., 2011).

Since publication of the results of this study, subsequent larger-scale trials have found VNS to be safe in heart failure but have reported varied outcomes in terms of efficacy (Premchand et al., 2014, Zannad et al., 2015, Gold et al., 2016). Although measures such as NYHA classification and quality of life scores improved in all these later studies, only the ANTHEM-HF trial (n=60) reported improved measures of cardiac function after six months. These included increased LVEF (increase after 6 months = 4.5%), reduced left ventricular end-systolic volume (LVESV; change after 6 months = -4.1ml) and

decreased left ventricular end-systolic diameter (LVESD; change after 6 months = -1.7mm). SDNN, a time domain measure of heart rate variability, was also found to increase by 17ms after six months of VNS regardless of whether it was applied to the left or right cervical vagus nerve. However, both the randomised controlled NECTAR-HF (n=87) and INOVATE-HF (n=707) trials failed to detect differences in echocardiographic measures of cardiac function between their respective active and control groups.

Stimulation of the auricular branch of the vagus nerve, via the tragus and lateral wall of the external acoustic meatus, may be a feasible non-invasive alternative to cervical VNS. tVNS has been investigated as a potential therapy for epilepsy, depression, pain and tinnitus but has not been investigated as a potential therapy to alter autonomic function in heart failure. Following on from the promising results of a previous tVNS study in healthy human research participants (Clancy et al., 2014), which showed that 15 minutes of tVNS could improve HRV and also reduce MSNA, ethical approval was obtained to trial tVNS in a small group of patients with chronic heart failure. Once recruited these patients were compared to age-matched and sex-matched healthy controls free from diagnosed cardiovascular disease.

5.2 Hypothesis

Transcutaneous vagus nerve stimulation applied to the tragus of the external ear will alter cardiovascular autonomic function in heart failure patients. Based on the results of the previous studies of tVNS in healthy human volunteers, tVNS will decrease LF/HF ratio and increase BRS, signifying a shift in cardiovascular autonomic activity towards parasympathetic predominance.

5.3 Aims and Objectives

The aim of this study was to investigate the effects of tVNS on cardiovascular autonomic function in a cohort of patients with chronic heart failure. The objectives were:

1. To determine the effects of tVNS on heart rate variability and baroreflex sensitivity in heart failure patients.

2. To investigate the tolerability of short-term tVNS in these patients.

5.4. Methods

5.4.1 Research subjects

National Research Ethics Service ethical approval was secured to recruit heart failure patients (Ethics Reference: 12/YH/0354 and 14/YH/0178) and University of Leeds ethical approval was secured to recruit healthy volunteers as controls (Ethics Reference: BIOSCI 13-025) the study conformed to the standards outlined in the Declaration of Helsinki. Informed written consent was obtained voluntarily from all research participants and their data were anonymised and stored securely according to the Data Protection Act (1998). The heart failure patients were recruited from a weekly heart failure outpatient clinic at Leeds General Infirmary and eligible patients were identified and screened by the clinical care team. Inclusion criteria for heart failure patients were:

- Male or female with diagnosis of heart failure
- Aged 18 years and older
- In sinus rhythm with a low ectopy rate (≤2 events per five minutes)

Exclusion criteria for heart failure patients were:

- Cardiac arrhythmia
- Bradycardia
- Postural hypotension
- Unable to transfer onto a couch without assistance

All heart failure patients were receiving optimal medical therapy at the time of participation in the study including ACE-inhibitors, angiotensin receptor blockers, beta-blockers and loop diuretics. Three of the included heart failure patients also had cardiac resynchronisation therapy (CRT) using an implanted pacing device but none of these patients were atrially-paced. None of the female participants (patients or controls) reported receiving hormonal replacement therapy during their involvement with the study. Participants reclined semi-supine on a couch for the duration of each experiment while recordings of their heart rate, finger arterial blood pressure and respiration rate were obtained as previously described in Chapter 2.

5.4.2 Experimental protocol

All experiments with the heart failure patients were carried out in a vacant clinical consultation room or in the Human Physiology lab of the Cardiovascular Clinical Research Facility at Leeds General Infirmary. However, the time of day when experiments took place was not controlled as most of the patients were recruited directly from the outpatient clinic. In addition, for the patients it was not possible to control for the influence of medication, nicotine, caffeine, or exercise levels prior to participation in the study. All experiments with healthy controls occurred under the controlled conditions detailed in Chapter 4. Participants reclined semi-supine on a couch for the duration of each experiment while recordings of their heart rate, finger arterial blood pressure and respiration rate were obtained as previously described in Chapter 2.

5.4.3 Measurements

Ten minute recordings of heart rate (lead II ECG), respiration and finger arterial blood pressure were obtained at baseline, during active tVNS and following cessation of stimulation (recovery period) as described in Chapter 2. The heart failure patients were further asked to complete a stimulation tolerability questionnaire (Chapter 2).

5.4.4 Data analysis

Offline analysis was carried out using Spike2 (version 7.1; CED, UK) and Labchart (version 8.1.5; ADInstruments, Bella Vista, Australia) software. Five minute samples of data from each recording were analysed in Spike2 to obtain frequency-domain HRV and mean blood pressure. The same five minute samples of the R-R interval tachogram were then exported from Spike2 as a text file and imported into Labchart, where spontaneous baroreflex sensitivity was calculated using custom scripts in Labchart (version 8.1.5, ADInstruments, Bella Vista, Australia) from each five minute sample of data using the sequence method.

5.5.7 Statistical Analysis

All statistical analyses for this study were performed using SPSS (version 24). Shapiro-Wilk tests were performed on each variable to assess normality. The effect of tVNS compared to baseline on HRV, BRS, heart rate, blood pressure and respiration was analysed using paired t-tests or Wilcoxon signed ranks tests. Independent t-tests and Mann-Whitney U-tests were used to compare baseline characteristics of the heart failure patients to a cohort of age and sex-matched healthy controls. Levene's test was used to assess the equality of variances for variables compared between groups and correction applied where equal variances could not be assumed.

5.6. Results

5.6.1 Baseline characteristics

A total of 28 heart failure patients were recruited for this study but 20 were excluded for the following reasons:

- 14 had frequent ectopic beats or other cardiac arrhythmia
- 2 had frequent coughing during the experiment
- 2 had pre-existing pain conditions (arthritis of back and shoulder) that made lying on the couch for long periods of time uncomfortable
- 1 had poor ECG signal-to-noise ratio and numerous ECG signal artefacts
- 1 reported pain in their left arm

The baseline characteristics of the included eight patients alongside eight healthy age-matched controls selected from previous studies are presented in Table 5.1. Compared to the patients, the healthy controls were not significantly different at baseline with respect to the LF/HF ratio (patient mean LF/HF = 3.09 ± 0.61, healthy control mean LF/HF = 1.99 ± 0.52). However, the patients had a reduced LF power and HF power at baseline compared to healthy controls (Table 5.2). There were no significant differences in baseline mean HR between the two groups, but mean systolic blood pressure, mean diastolic blood pressure and mean blood pressure were all lower in the patients at baseline compared to the controls (Table 5.3). BRS was also greater in the healthy controls than the patients at baseline (healthy controls BRS = 9.34 ± 1.16ms/mmHg, patient BRS = 5.36 ± 1.18 ms/mmHg).

	Patients	Controls	*p*
Number	8	8	-
Age (years)	66.25 ± 4.16	64.0 ± 3.56	0.69
LVEF (%)	29.63 ± 3.63	-	-
NYHA classification	2 class I; 4 class II; 1 class III; 1 class IV	-	-
Months since CHF diagnosis	27.0 ± 7.12	-	-
Baseline LF/HF ratio	3.09 ± 0.62	1.99 ± 0.52	0.20
Mean heart rate (bpm)	68.29 ± 3.34	65.15 ± 3.64	0.38
Baseline BRS (ms/mmHg)	**5.36 ± 1.18***	**9.34 ± 1.17***	0.03
Mean BP (mmHg)	**64.85 ± 5.81***	**84.02 ± 2.21***	0.01

Table 5.1: Baseline characteristics of heart failure patients and healthy controls. The patients had a significantly reduced baseline BRS (p = 0.03) and mean BP (p = 0.01).

5.6.2 tVNS reduced LF/HF ratio and increased BRS in heart failure patients

A significant decrease in LF/HF ratio was observed in the heart failure patients compared to baseline ($p = 0.05$; Table 5.2). This effect was not observed in the healthy controls ($p = 0.121$) and may be due to the small sample size. No significant changes in LF power or HF power alone were detected for either group. BRS increased in the heart failure patients from baseline ($p = 0.017$; Table 5.3) but not in the controls ($p = 0.494$).

Table 5.2: Mean HR and HRV data for heart failure patients and healthy controls. The heart failure patients experienced a significant reduction in LF/HF ratio during tVNS ($p = 0.05$). The healthy volunteers experienced a small but significant decrease in mean HR during tVNS ($p = 0.005$).

	Patients (n = 8)			Controls (n = 8)		
	Baseline	**Stimulation**	***p***	**Baseline**	**Stimulation**	***p***
Mean heart rate (bpm)	68.29 ± 3.24	67.57 ± 3.25	0.141	**65.15 ± 3.64***	**63.25 ± 3.95***	**0.005**
Total power (ms^2)	264.97 ± 65.24	289.63 ± 84.27	0.401	841.98 ± 155.46	775.31 ± 187.43	0.526
VLF power (ms^2)	64.19 ± 17.27	63.59 ± 16.83	0.889	236.49 ± 64.09	262.18 ± 112.11	1.0
LF power (ms^2)	148.77 ± 43.73	136.01 ± 41.76	0.479	510.48 ± 209.01	408.97 ± 149.99	0.263
HF power (ms^2)	51.90 ± 11.63	88.78 ± 32.15	0.093	414.88 ± 225.40	371.10 ± 164.84	1.0
LF/HF ratio	**3.09 ± 0.62***	**2.08 ± 0.61***	**0.05**	1.99 ± 0.52	1.32 ± 0.15	0.121

Table 5.2: Mean HR and HRV data for heart failure patients and healthy controls

	Patients (n = 8)			Controls (n = 7)		
	Baseline	**Stimulation**	***p***	**Baseline**	**Stimulation**	***p***
Mean Systolic BP (mmHg)	98.34 ± 8.09	97.68 ± 8.60	0.836	120.21 ± 3.44	118.13 ± 4.59	0.266
Mean Diastolic BP (mmHg)	**48.11 ± 5.19***	**45.19 ± 5.20***	**0.026**	65.92 ± 1.94	65.56 ± 3.00	0.789
Mean BP (mmHg)	64.85 ± 5.81	62.69 ± 5.98	0.228	84.02 ± 2.21	83.34 ± 3.23	0.640
Mean BRS (ms/mmHg)	**5.36 ± 1.18***	**7.33 ± 1.57***	**0.017**	9.34 ± 1.17	10.63 ± 1.94	0.494

Table 5.3: Blood pressure and BRS data for heart failure patients and healthy controls. The heart failure patients had a significant reduction from baseline in mean diastolic BP (p = 0.026) and an increase in mean BRS (0.017) during tVNS. No significant changes in BP or BRS were detected in the healthy controls during tVNS.

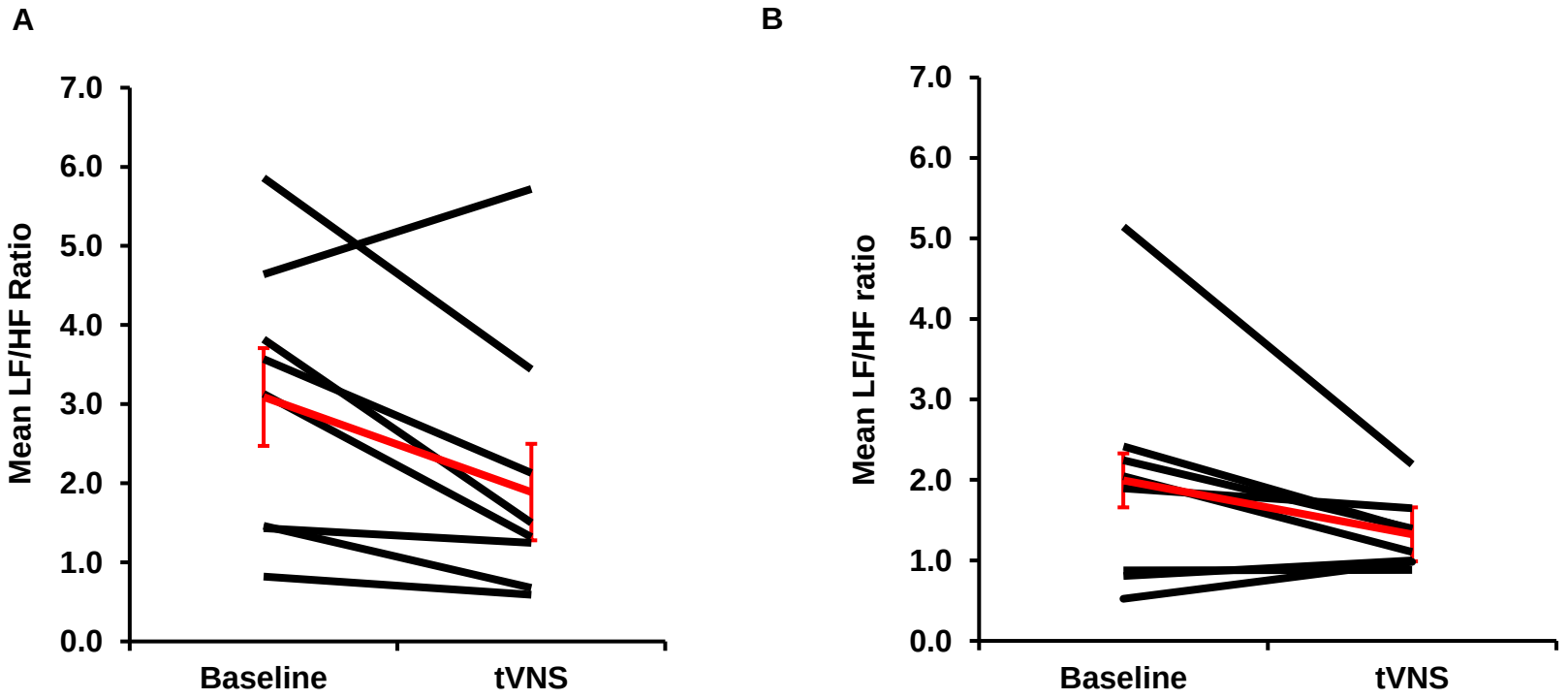


Figure 5.1: The effects of tVNS on LF/HF ratio in individual heart failure patients and healthy controls.

A significant decrease in LF/HF ratio occurred during tVNS only in the heart failure patients (A; n = 8; p = 0.05). No significant change in LF/HF ratio was observed in the age-matched and sex-matched healthy controls (B; n = 8; p = 0.121).

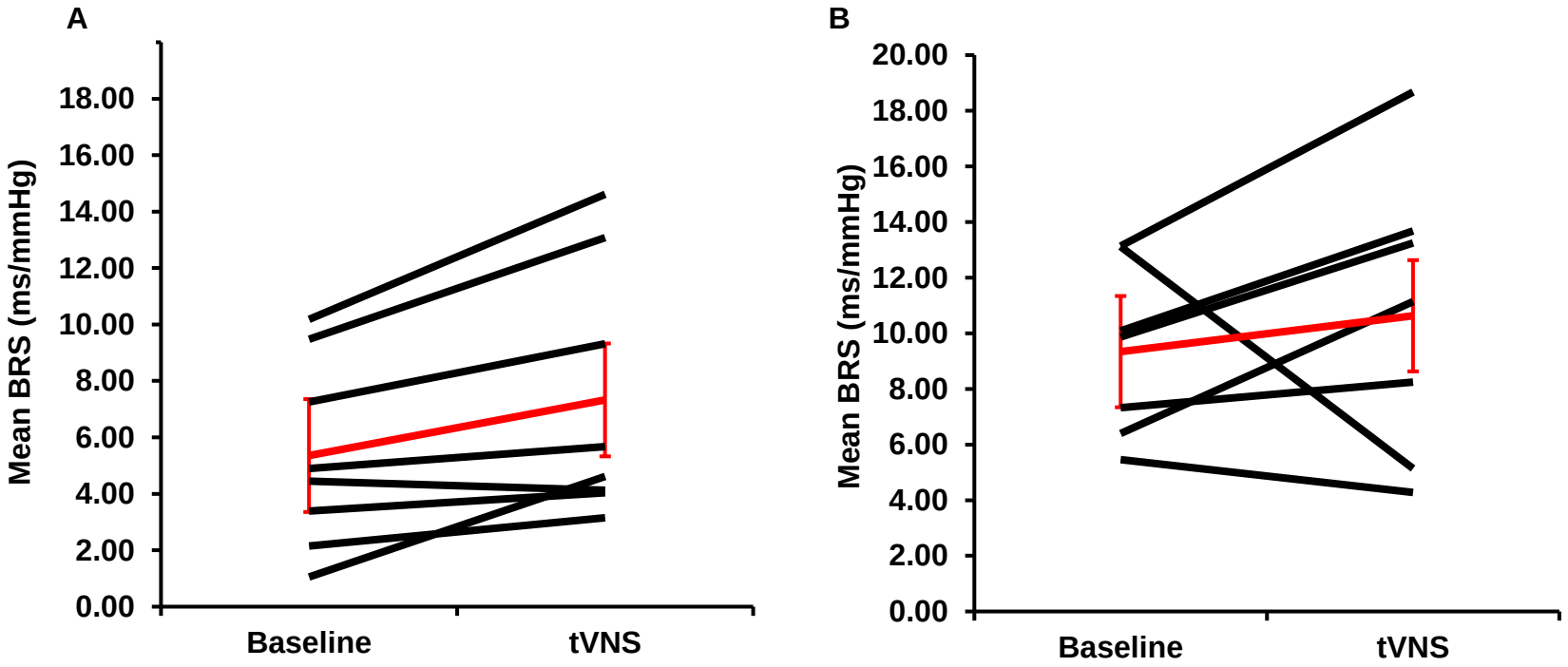


Figure 5.2: The effects of tVNS on spontaneous BRS in individual heart failure patients and healthy controls. A significant increase in BRS occurred during tVNS only in the heart failure patients ($p = 0.017$). No significant change in LF/HF ratio was observed in the healthy controls ($p = 0.494$).

5.6.3 Effects of tVNS on HR and BP

There was no difference between patients and healthy controls in terms of baseline mean heart rate (p=0.382). A slight but significant decrease in mean heart rate from baseline was observed in the healthy controls during tVNS (p = 0.005; Table 5.2, but not in the heart failure patients (p=0.141). Systolic blood pressure was significantly lower in the patient group at baseline and these effects may have been the result of CHF medication such as beta-blockers (p = 0.033, see Table 5.3 for BP values). This was further reflected in decreased values for diastolic BP (p = 0.011) and mean BP (p = 0.013) compared to the control group.

5.6.4 Tolerability questionnaire results

All heart failure patients enrolled in the study were asked to complete a tolerability questionnaire which asked if they experienced any discomfort or other sensations during tVNS. The patients were also asked if they experienced any dizziness, anxiety or chest palpitations during stimulation. In terms of the tolerability questionnaire, none of the patients reported any side effects or discomfort caused by tVNS and tolerated the procedure well (Table 5.4).

Question	Mean Score
Did you find the ear stimulation uncomfortable	0
Did you experience any pins and needles during the ear stimulation?	0.63 ± 0.39
Did you experience any warmth sensation at the stimulation site	0.13 ± 0.12
Did you feel any dizziness during the experiment?	0.25 ± 0.23
Did you feel anxious during the experiment?	0
Did you experience any palpitations during the experiment?	0
Did you find lying on the couch uncomfortable	0.13 ± 0.12

Table 5.4: Results of the tVNS tolerability questionnaire. Questions were rated from 0 – 5 with 0 = not at all, 3 = somewhat and 5 = extremely.

5.7 Discussion

This case-control study demonstrates that tVNS can decrease LF/HF ratio in heart failure patients, indicating a shift in cardiac autonomic control towards parasympathetic predominance. This alteration was also shown to coincide with an increase in baroreflex sensitivity in the patient group. Given that all of the patients were receiving optimal medical therapy (including beta-blockers) at the time of participation in the study, the results suggest that tVNS may be able to influence cardiac autonomic nervous system activity when it is already to an extent impaired. Some evidence of this impairment may be in the reduced total power, LF power, HF power and reduced BRS exhibited by the patients compared to the healthy controls free from cardiovascular disease. Decreased parasympathetic activity, indicated by reduced HRV and BRS, is known to be associated with an increased risk of mortality after myocardial infarction and in patients with heart failure (La Rovere and Schwartz, 1997, La Rovere et al., 1998, La Rovere et al., 2011).

In the clinical trials with heart failure patients, cervical VNS was shown to elicit alterations in autonomic activity as indicated by time-domain HRV measures (Premchand et al., 2014, Zannad et al., 2015, Gold et al., 2016). However, it should be noted that the majority of patients enrolled in these trials were NYHA class III, indicating at least moderate symptoms such as breathlessness following mild physical exertion e.g. walking. This was exemplified by INOVATE-HF, where all 707 heart failure patients enrolled were NYHA class III (Gold et al., 2016). In the present study, the majority of the included patients were NYHA class I or II (n=6), with only one NYHA class III patient and one NYHA class IV patient. The severity of heart failure symptoms may thus have an influence on the efficacy of tVNS and a larger sample size is needed to identify if there is any correlation.

In the present study, the heart failure patients had a greater baseline mean LF/HF ratio compared to the aged healthy control group, although because of the small sample size and wide variation in baseline LF/HF ratios this was not found to be significantly different ($p = 0.414$). In Chapter 4, linear regression analysis revealed a significant relationship between baseline LF/HF ratio and response to tVNS in healthy participants aged 60 and older who were free from

cardiovascular disease. A greater decrease in LF/HF ratio was observed in healthy participants who had an elevated baseline LF/HF ratio. This trend was not observed in the heart failure patients, again potentially due to a small sample size. A larger controlled study of the effects of tVNS in heart failure should be carried out to clarify differences in response to tVNS between patients and healthy controls. In addition, further studies with heart failure patients should be controlled for the potential confounding effects of time of day and also caffeine and nicotine intake. This was not feasible with the present cohort of patients due to time constraints.

As a result of the time constraints with some patients, it was not possible to evaluate the presence of any immediate residual effects following short-term stimulation i.e. within 15 minutes of the stimulus being switched off. Clancy et al. (2014) reported a sustained reduction in LF/HF ratio and single-unit MSNA in healthy younger participants following tVNS (Clancy et al., 2014), although Chapter 4 of this thesis found only a sustained reduction in single-unit MSNA in healthy older participants, with the LF/HF ratio returning to baseline level 15 minutes after stimulation had ceased. The cardiovascular benefits of repeated daily exposure to tVNS in coronary artery disease patients has been previously demonstrated (Zamotrinsky et al., 1997, Zamotrinsky et al., 2001) and as such there may be potential for similar residual effects to be observed in heart failure.

The primary advantage for tVNS is its ease of application, followed by a lack of side-effects and complications from using the technique (Ben-Menachem et al., 2015). The present study found acute tVNS to be well-tolerated in the heart failure patients and no side-effects such as chest palpitations were reported as a direct result of the stimulation. For these patients, the most commonly reported sensation was paraesthesia or a ‘tingling’ sensation before the electrical current was titrated to a comfortable level. In addition, none of the patients asked for tVNS to be stopped as a result of these sensations and did not report any further stimulus-associated discomfort. This is in contrast to VNS, which although safe and also generally well-tolerated is known to cause adverse effects in some patients as a direct result of stimulation (Beekwilder and Beems, 2010, Ben-Menachem et al., 2015). VNS requires a surgical procedure under general anaesthesia to implant the electrodes and a subcutaneous generator in the chest wall, increasing the risk of post-operative

side-effects such as dysphonia and infection (Elliott et al., 2011). In the longer term, hardware failure may require revision surgery to replace faulty electrodes, leaving patients at risk of experiencing a relapse in their symptoms (Spuck et al., 2010, Dlouhy et al., 2012). Furthermore, the invasiveness of VNS poses an issue in particular for very ill or elderly patients, for whom surgery may not be advisable. This severely constrains the potential clinical utility of VNS.

The case for trialling tVNS in cardiovascular disease states rather than VNS may be furthered by the ineffective outcomes of the recent clinical trials investigating the efficacy of right-sided VNS in heart failure (Zannad et al., 2015, Gold et al., 2016). In particular, INOVATE-HF was stopped due to statistical futility when patients in the treatment arm of the trial did not see a significant improvement in all-cause mortality or unplanned HF-related hospitalisations (Gold et al., 2016). The lack of efficacy reported in these trials may be due in part to the complex mixed fibre composition of the human cervical vagus nerve along with the potential activation of sympathetic nerve fibres transmitted within the cervical vagus, which may have a confounding effect (Seki et al., 2014, Verlinden et al., 2016). Indeed, when the cervical vagus nerve was stimulated in anesthetized dogs, activity of neurones in the stellate ganglion increased; this increased sympathetic nerve activity would clearly be detrimental to patients suffering from cardiovascular disease (Rhee et al., 2015). Given the drawbacks inherent to cervical VNS, a simpler, more practical alternative without the need for a surgical procedure may be offered by tVNS.

5.7.1 tVNS as a potential anti-arrhythmic agent

The present study excluded 14 out of the 28 enrolled heart failure patients (50%) due to frequent ventricular extrasystoles or premature ventricular complexes (PVCs), which if uncorrected can cause spectral abnormalities in the low-frequency and high frequency power bandwidths for frequency-domain HRV analysis (Wen and He, 2011). PVCs are arrhythmic events which are commonly asymptomatic, occurring in around 6% of 45 - 65 year olds (Sapoznikov et al., 1992). The frequency of these PVCs is known to increase with age, although they are perceived to be harmless in individuals with no obvious structural heart disease. However, a longitudinal study of elderly

people aged 65 and over (n = 1139) with normal left ventricular ejection fraction (LVEF) at baseline and no history of congestive heart failure showed that an elevated frequency of PVCs may be associated with a reduction in LVEF, an increased risk of developing heart failure and increased mortality (Dukes et al., 2015). Previous longitudinal studies have also found that men with frequent PVCs have an increased risk of developing coronary artery disease (Hirose et al., 2010, Bikkina et al., 1992) and PVC frequency may be an independent risk factor for ischaemic stroke (Agarwal et al., 2015). Moreover, there is an association between frequent PVCs and the development of atrial fibrillation in adults aged ≥50 years old (Watanabe et al., 2006), indicating that these frequent disruptions in normal cardiac rhythm may have a deleterious effect on the cardiac conduction system.

A study with CAD patients by Popov et al. demonstrated that tVNS is able to influence the frequency of PVCs, as measured by 24 hour ECG recording (Popov et al., 2013). Ten days of tVNS led to a significant reduction in PVC frequency one month after the course had been completed. A number of other studies have found tVNS to have a pronounced anti-arrhythmic effect. Yu and colleagues (2013) used tVNS of the right tragus to inhibit the induction of atrial fibrillation from three hours of rapid atrial pacing in anaesthetised dogs (Yu et al., 2013). Further tVNS studies associated the tVNS-induced suppression of atrial fibrillation (AF) with reduced loss of connexin 40 and connexin 43 in the atrial myocardium (Chen et al., 2015c, Chen et al., 2015a) and improved cardiac function and reduced ventricular remodelling (Wang et al., 2015b, Wang et al., 2014). The results observed in animal models informed a study where one hour of tVNS under general anaesthesia was trialled in 20 patients with paroxysmal atrial fibrillation and the effects compared to a control group of AF patients who received sham tVNS (n = 20). Active tVNS significantly decreased the duration of atrial fibrillation and also reduced serum levels of pro-inflammatory cytokines such as tumour necrosis factor, C-reactive protein and interleukin-6, suggesting that tVNS is able to modulate immune system activity (Stavrakis et al., 2015). These studies suggest that tVNS may be able to benefit patients with a range of cardiac arrhythmias by reducing the frequency of arrhythmic events through modulation of the cardiac conduction system, myocardial remodelling and a dampening of the immune response.

5.8 Conclusion

These preliminary results suggest that tVNS may be able to influence autonomic activity in conditions such as heart failure which are associated with autonomic dysfunction. Moreover, tVNS is an inexpensive and non-invasive method of activating similar neural pathways to cervical VNS that may be utilised by a much greater number of patients. Further investigation is needed in both clinical and animal studies to elucidate the efficacy of tVNS as an adjunct for the treatment of heart failure.

Chapter 6

The autonomic effects of supraorbital trigeminal nerve stimulation in healthy human research participants

6.1 Introduction

The anti-epileptic effects of VNS led to the investigation of trigeminal nerve stimulation (TNS) as a potential therapeutic and minimally-invasive alternative form of neuromodulation (Penry and Dean, 1990, DeGiorgio et al., 2006). Subsequent trials sought to establish a standardised method of non-invasively applying TNS to the supraorbital and supratrochlear nerves of the ophthalmic division (V_1) of the trigeminal nerve using adhesive surface electrode positioned on the forehead (DeGiorgio et al., 2006; see Figure 2.2 in Chapter 2). These trials have shown TNS to be safe and well-tolerated, with no significant effect on blood pressure although there is some evidence that short-term (60 minutes) exposure to TNS can exert a mild (4%) decrease in heart rate (Cook et al., 2013, Pop et al., 2011, Schrader et al., 2011). However, heart rate and blood pressure are crude measures of autonomic function and it is unclear what effect TNS may have on other measures such as heart rate variability and baroreflex sensitivity. Acute stimulation of trigeminal afferents can induce cardiovascular effects such as bradycardia, a phenomenon variously reported in the literature as the trigeminal depressor response or the trigeminocardiac reflex (Kumada et al., 1975, Shelly and Church, 1988, Lang et al., 1991). This has been reported to occur during surgical procedures where blunt contact between surgical instruments and a trigeminal nerve branch can cause bradycardia and a decrease in mean arterial pressure by as much as 20% (Schaller et al., 2009). A related trigeminal reflex is the mammalian diving reflex, where exposure of facial skin to cold water also leads to increased vagal tone but in this instance there is a simultaneous increase in sympathetic nervous system activity (Alboni et al., 2011). However microneurographic recordings of MSNA and SSNA in humans during exposure to cold water showed that this sympathetic activation was primarily directed towards the skeletal muscle vasculature rather than the cutaneous vascular beds (Fagius and Sundlof, 1986). A later study in young healthy men found that stimulation of trigeminal afferents via facial cooling led to significantly increased MSNA and mean arterial pressure (Fisher et al., 2015).

6.1.1 Knowledge gap

There have been few studies which have specifically investigated the cardiovascular autonomic effects of electrical TNS. In anaesthetised rabbits, non-invasive electrical stimulation (10Hz, 0.5 ms, 5V) of the nasal mucosa generated an increase in cerebral blood flow as assessed by laser Doppler flowmetry (Gurelik et al., 2004). The nasal mucosa are densely innervated by trigeminal afferents from the nasociliary nerve (V_1) and this study failed to detect any changes in mean heart rate or mean arterial blood pressure as a result of stimulation. A later study by Hanamoto et al. investigated the autonomic effects of lingual nerve (V_3) stimulation in cats (n = 38) sedated using controlled doses of pentobarbital sodium, a widely-used anaesthetic for animal studies (Hanamoto et al., 2012). A light dose of anaesthetic caused an increase in HR and BP when 10 seconds of TNS (10Hz, 5ms, 2Ma) was applied. This may have been due to sympathetic activation, whereas a moderate dose with TNS produced a depressor reflex response, suggesting that the reflex may be dependent on the depth of anaesthesia and the type of anaesthetic agent used (Hanamoto et al., 2012). It is less clear how stimulation of trigeminal afferents using TENS, such as the TNS protocols employed in clinical trials, may alter autonomic nervous system activity in awake humans. A recent study in healthy volunteers (n = 16) by Waki et al. found that electroacupuncture delivered at 100Hz (compared to 120Hz for the TNS in epilepsy patient trials) for 5 minutes to the supraorbital region elicited a significant decrease in heart rate along with an increase in HF power during stimulation (Waki et al., 2017). A bilateral increase in cerebral blood flow to the prefrontal cortices was also detected using near-infrared spectroscopy compared to a control group who did not receive stimulation and rested quietly (Waki et al., 2017).

Anatomical tracing studies have demonstrated that the trigeminal nerve projects to similar brainstem structures at the ABVN such as the nucleus tractus solitarius and spinal trigeminal nucleus (Jacquin et al., 1983, Nomura and Mizuno, 1984, Chien et al., 1996), suggesting that transcutaneous electrical nerve stimulation applied to a site such as the tragus may stimulate the trigeminal nerve as well as the ABVN. Chapter 3 of this thesis provides some indication that auriculotemporal nerve may contribute to the cardiovascular

autonomic effects seen with tVNS applied to the tragus, as participants in the tragus responder subgroup (n = 12; participants who experienced a decrease in LF/HF ratio during tragus stimulation) experienced a decrease in LF/HF ratio in response to helix (auriculotemporal nerve) stimulation. It should be noted that the auriculotemporal branch of the trigeminal nerve which innervates the external ear and overlaps with the cutaneous innervation of the ABVN is derived from the mandibular V_3 division as opposed to the ophthalmic V_1 or maxillary V_2 divisions, which have been previously targeted in the TNS clinical trials with refractory epilepsy patients (DeGiorgio et al., 2006). However, if the trigeminal nerve is involved in the alteration in cardiovascular autonomic function observed during tragus stimulation, then stimulation at an alternative site to the tragus e.g. the supraorbital region may elicit a similar autonomic effect if the same stimulation parameters are used (30Hz, 200µs, continuous current for 15 minutes). There may also be a frequency-dependent effect whereby stimulation at a higher frequency e.g. 120Hz as per the stimulation protocols used by DeGiorgio et al. might alter cardiovascular autonomic function in a different way to stimulation at a lower frequency (DeGiorgio et al., 2006). As none of the TNS for treatment-resistant epilepsy trials have assessed the effects of TNS on autonomic measures such as HRV or BRS, this data could be useful for the optimisation of future trials with this treatment-resistant population.

6.2 Hypothesis

Transcutaneous electrical nerve stimulation applied to the supraorbital branches of the trigeminal nerve (TNS) in healthy research participants will alter cardiovascular autonomic function.

6.3 Aims and objectives

The present study investigated the effects of TNS on cardiovascular autonomic activity in healthy participants by non-invasive measurements of heart rate and finger arterial blood pressure. Two different stimulation parameters (low or high frequency TNS) were employed to assess if there were any frequency-dependent effects, as well as a sham TNS protocol.

6.4 Materials and methods

6.4.1 General protocol

University of Leeds ethical approval was secured (Ethics Reference: BIOSCI 13-025) and the all experiments conformed to the standards outlined in the Declaration of Helsinki. Informed written consent was obtained voluntarily by all research participants and their data were anonymised and stored securely according to the Data Protection Act (1998). All experiments were carried out in a dedicated human physiology study room at University of Leeds. These experiments occurred between the hours of 8am and 10am in order to minimise the impact of circadian rhythm variations on the autonomic nervous system. The ambient temperature of the study room was maintained at 21 ± 2°C. Inclusion criteria were male or female volunteers aged ≥ 18 years old. Exclusion criteria were a prior medical history of hypertension, cardiac disease, diabetes mellitus or epilepsy. Female participants were asked to indicate if they were receiving hormonal replacement therapy (HRT) for treatment of menopausal symptoms, as HRT has been shown to independently alter cardiac autonomic activity (Yildirir et al., 2001). All participants were required to abstain from caffeine, alcohol, nicotine and strenuous exercise for a minimum of 12 hours prior to their visit. They were further required to consume a light breakfast and use the toilet prior to attending for the experiment. Participants were asked to attend University of Leeds to receive low-frequency TNS (L-TNS, 30Hz) or high-frequency TNS (H-TNS, 120Hz) at separate visits. The protocols for delivering these stimulation parameters are outlined in Chapter 2. Ten participants were selected at random from the group to return for an additional visit where sham TNS was performed and this visit was included into the randomisation order.

6.4.2 Cardiovascular autonomic measurements and data acquisition

Recordings of heart rate, respiration, blood pressure and MSNA were obtained as described in Chapter 2. The data acquisition protocol and analyses of HRV and BRS were performed as described in Chapter 2. In some participants it was not possible to obtain reliable finger arterial blood pressure recordings or to

detect BRS sequences and the sample sizes for these analyses are included in Table 6.3 for BP and Table 6.4 for BRS.

6.4.3 Statistical Analysis

All statistical analyses for this study were performed using SPSS (version 24). Shapiro-Wilk tests were performed on each variable to assess normality. Participant subgroup characteristics were compared using independent t-tests or Mann Whitney U-tests. One-way repeated measures ANOVA was used to analyse effect of time (baseline, stimulation and recovery) for each visit (L-TNS, H-TNS or sham TNS) and Bonferroni post-hoc correction applied. Non-parametric Friedman test with Bonferroni post-hoc test for pairwise comparisons was used where data were not normally distributed. All data are presented as group mean ± standard error of the mean (S.E.M).

6.5 Results

6.5.1 Baseline characteristics

30 participants with no previous medical history of cardiovascular disease, diabetes or epilepsy were enrolled at the study and written informed consent was obtained at the first visit. Two participants (n = 2 male) were excluded from the study due to elevated blood pressure (> 140 mmHg systolic BP) at rest as measured using a brachial cuff and digital blood pressure monitor. One volunteer (female, age = 21 years) was excluded due to frequent ectopic beats. One volunteer (female, age = 38 years) was excluded due to discomfort unrelated to the experiments. The baseline characteristics of the remaining 26 participants (n = 11 female and n = 15 male) included in this arm of the study are presented in Table 6.1. No significant differences in baseline characteristics such as age, BMI, mean heart rate or mean blood pressure were identified between male and female volunteers (male n = 11; female n = 15).

	N	Age (years)	BMI (kg/m^2)	Mean Blood Pressure	Heart Rate (bpm)	Baseline LF/HF
All participants	26	29.69 ± 2.13	23.0 ± 0.59	76.31 ± 2.12	61.59 ± 1.24	1.15 ± 0.13
Male participants	11	26.09 ± 2.27	23.65 ± 0.97	77.01 ± 3.87	59.74 ± 1.17	1.32 ± 0.13
Female participants	15	32.33 ± 3.18	22.53 ± 0.75	75.80 ± 2.40	62.94 ± 1.94	1.03 ± 0.20

Table 6.1: Baseline characteristics of participants enrolled in the TNS study. No significant differences were identified between male and female participants.

6.5.2 Cardiovascular autonomic measurements

There was no significant difference in baseline mean LF/HF ratio values between the L-TNS and H-TNS visits for the entire group ($p = 0.118$, Table 6.2). When the 26 included participants were compared at baseline between the stimulation parameters, no significant differences were detected between parameters at each time point (baseline, stimulation and recovery) for any measure of HRV (Friedman test, $p > 0.05$). This may have been due to the relatively small sample size and the skewed non-normal distribution of data. There were no significant differences between participants who received L-TNS first due to the order of randomisation compared to those who received H-TNS first for any measure.

No significant changes were observed in respiration rate, BRS or blood pressure for any of the stimulation parameters (Table 6.5 for BP data and Table 6.6 for BRS data). Mean HR was observed to decrease from baseline during L-TNS (Figure 6.1; repeated measures ANOVA; $p = 0.02$) and in the recovery period ($p < 0.001$). Mean HR decreased only in the recovery period for H-TNS

(Figure 6.1; repeated measures ANOVA; p = 0.017) and no significant changes were observed for sham TNS (repeated measures ANOVA; p = 0.157).

Within-groups HRV analysis of the entire sample (n = 26) failed to detect any changes in HRV as a result of L-TNS, H-TNS or sham TNS. However, several differing sex-specific effects were identified when the sample was divided into male (n = 11; Table 6.3) and female (n = 15; Table 6.4) participants. While no significant differences from baseline were detected for L-TNS or sham TNS for either sex, H-TNS reduced HF power in females during stimulation (Friedman test; HF power change from baseline p = 0.001). Both HF power and LF power were found to have decreased from baseline in females following H-TNS in the recovery period (HF power recovery period p = 0.005; LF power recovery period p = 0.006). However no significant change was observed in LF/HF ratio for the female participants as a result of H-TNS (Friedman test; p = 0.420). In addition, neither normalised LF power or normalised HF power were found to be significantly reduced for the female participants as a result of H-TNS. This disparity between the normalised and absolute measures may be due to the relatively small sample size. The observed changes in the female participants for measures of frequency-domain HRV were not seen with the male participants. Both male and female participants experienced a significant decrease in mean heart rate following L-TNS in the recovery period (repeated measures ANOVA; female mean HR p = 0.001; male mean HR p = 0.005). A significant decrease in mean HR was also observed in the recovery period following H-TNS (repeated measures ANOVA; p = 0.035), but no significant change in mean heart rate was detected in the male participants as a result of H-TNS (repeated measures ANOVA; p = 0.253).

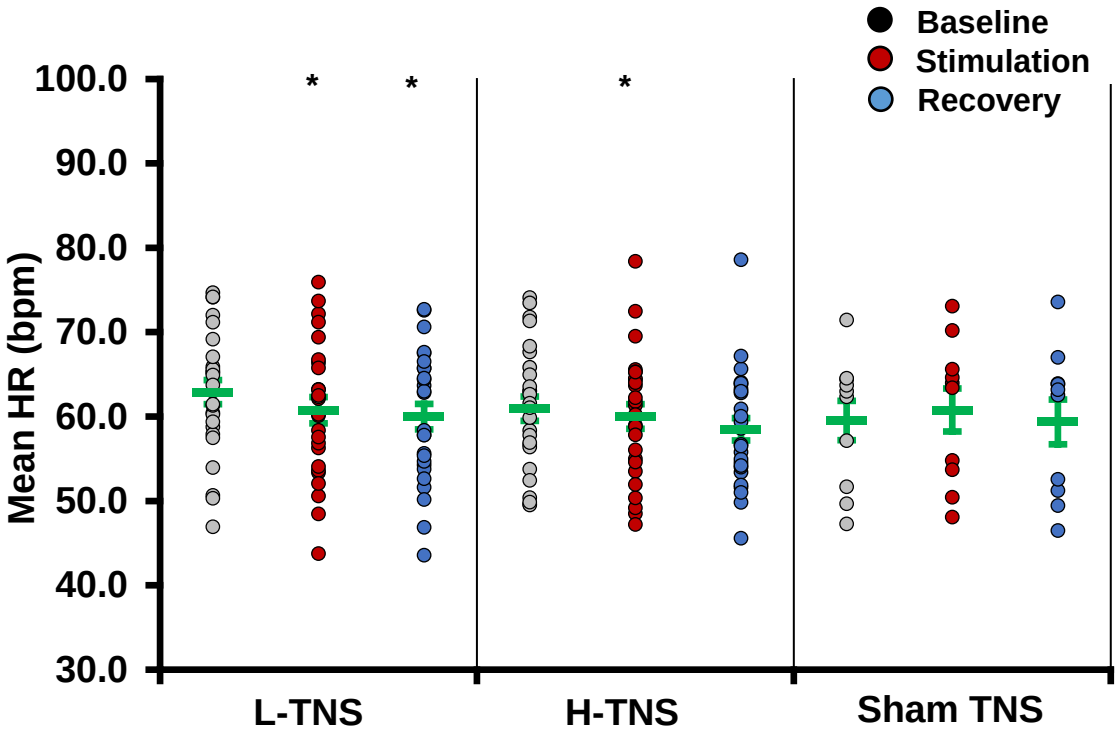


Figure 6.1: Mean HR data for all participants (n = 26). A significant reduction in mean HR from baseline was observed during L-TNS (Repeated measures ANOVA; $p = 0.02$) and in the recovery period ($p < 0.001$). A significant decrease in mean HR from baseline was only observed in in the recovery period for H-TNS (Repeated measures ANOVA; $p = 0.017$). No significant changes in mean HR were observed for sham TNS (Repeated measures ANOVA; $p = 0.157$).

Table 6.2: HRV data for all participants (n = 26). No significant differences were observed as a result of any stimulation parameter (L-TNS, H-TNS or sham TNS) when HRV data from all participants was analysed.

Table 6.2: HRV data for all participants (n = 26)

	L-TNS			H-TNS			Sham TNS		
	Baseline	**Stimulation**	**Recovery**	**Baseline**	**Stimulation**	**Recovery**	**Baseline**	**Stimulation**	**Recovery**
Total Power (ms^2)	6508.19 ± 1678.64	6205.75 ± 1651.53	4678.31 ± 1093.27	5619.14 ± 1519.89	4882.91 ± 1219.30	3712.59 ± 719.95	6568.30 ± 1485.87	6182.53 ± 1324.43	5986.90 ± 1479.20
LF Power (ms^2)	2575.18 ± 590.69	2729.97 ± 780.38	2094.72 ± 548.45	2105.69 ± 489.00	1977.43 ± 514.19	1428.37 ± 266.73	3030.20 ± 926.23	2427.15 ± 622.95	2350.34 ± 667.37
HF Power (ms^2)	3313.81 ± 1029.02	2677.17 ± 707.48	2108.05 ± 577.30	2896.32 ± 952.40	1250.51 ± 329.08	1130.46 ± 264.51	2743.99 ± 698.31	2710.92 ± 738.01	2639.78 ± 645.63
Normalised LF Power (n.u.)	0.48 ± 0.03	0.50 ± 0.03	0.51 ± 0.03	0.49 ± 0.03	0.49 ± 0.03	0.49 ± 0.03	0.51 ± 0.05	0.53 ± 0.05	0.51 ± 0.04
Normalised HF power (n.u.)	0.52 ± 0.03	0.50 ± 0.03	0.50 ± 0.03	0.51 ± 0.03	0.51 ± 0.03	0.51 ± 0.03	0.49 ± 0.05	0.47 ± 0.05	0.49 ± 0.04
LF/HF	1.13 ± 0.15	1.15 ± 0.13	1.19 ± 0.14	1.12 ± 0.13	1.12 ± 0.11	1.16 ± 0.13	1.25 ± 0.23	1.25 ± 0.19	1.14 ± 0.14

Table 6.3: HRV data for male participants (n = 11). No significant differences were detected.

	L-TNS			H-TNS		
	Baseline	**Stimulation**	**Recovery**	**Baseline**	**Stimulation**	**Recovery**
Total Power (ms^2)	4133.03 ± 1047.85	3898.93 ± 1244.02	4636.62 ± 1436.37	2756.51 ± 534.16	3631.18 ± 1071.62	3426.83 ± 841.25
LF Power (ms^2)	2136.88 ± 607.09	1923.53 ± 613.75	2686.13 ± 1105.96	1341.74 ± 271.54	1508.10 ± 381.10	1684.66 ± 376.46
HF Power (ms^2)	1610.37 ± 451.79	1377.90 ± 375.71	1613.38 ± 330.21	902.46 ± 220.74	1170.39 ± 608.67	1046.53 ± 406.32
Normalised LF Power	0.56 ± 0.03	0.57 ± 0.03	0.57 ± 0.04	0.59 ± 0.03	0.59 ± 0.03	0.62 ± 0.03
Normalised HF power	0.44 ± 0.03	0.43 ± 0.03	0.44 ± 0.03	0.41 ± 0.03	0.41 ± 0.03	0.38 ± 0.03
LF/HF	1.46 ± 0.24	1.48 ± 0.24	1.51 ± 0.27	1.57 ± 0.17	1.57 ± 0.17	1.71 ± 0.18

Table 6.4: HRV data for female participants (n = 15). HF power decreased from baseline during H-TNS (p = 0.001). Both HF power and LF power were found to have decreased from baseline in females following H-TNS in the recovery period (HF power recovery period p = 0.005; LF power recovery period p = 0.006).

	L-TNS			H-TNS		
	Baseline	**Stimulation**	**Recovery**	**Baseline**	**Stimulation**	**Recovery**
Total Power (ms^2)	8249.97 ± 2763.07	7897.42 ± 2673.68	4708.87 ± 1618.23	7874.49 ± 2478.99	5984.66 ± 1967.65	4099.90 ± 1115.78
LF Power (ms^2)	2896.60 ± 932.45	3321.36 ± 1276.18	1661.01 ± 508.51	**2748.42 ± 795.08***	2408.05 ± 847.06	**1333.34 ± 386.70†**
HF Power (ms^2)	4563.00 ± 1705.03	3629.98 ± 1149.80	2470.81 ± 975.82	**4407.19 ± 1548.87*†**	**1367.79 ± 413.23***	**1247.46 ± 374.84†**
Normalised LF Power	0.43 ± 0.04	0.45 ± 0.04	0.46 ± 0.04	0.42 ± 0.03	0.44 ± 0.03	0.41 ± 0.04
Normalised HF power	0.57 ± 0.04	0.55 ± 0.04	0.54 ± 0.04	0.58 ± 0.03	0.56 ± 0.03	0.59 ± 0.04
LF/HF	0.89 ± 0.16	0.91 ± 0.10	0.96 ± 0.13	0.85 ± 0.17	0.86 ± 0.11	0.80 ± 0.13

	L-TNS			H-TNS			Sham TNS		
	Baseline	**Stimulation**	**Recovery**	**Baseline**	**Stimulation**	**Recovery**	**Baseline**	**Stimulation**	**Recovery**
Systolic BP (mmHg)	111.69 ± 2.85	111.96 ± 3.41	113.09 ± 3.49	108.31 ± 3.01	110.18 ± 3.90	111.65 ± 3.13	108.45 ± 2.66	115.72 ± 2.17	112.63 ± 4.91
Diastolic BP (mmHg)	66.10 ± 1.98	65.96 ± 2.67	67.95 ± 2.60	66.93 ± 3.05	67.16 ± 3.58	67.87 ± 2.42	63.22 ± 2.49	65.29 ± 1.90	66.84 ± 2.59
Mean BP (mmHg)	81.30 ± 2.06	81.29 ± 2.68	82.99 ± 2.68	80.72 ± 2.53	81.50 ± 3.56	82.46 ± 2.40	78.29 ± 1.93	82.10 ± 1.73	82.10 ± 3.09

Table 6.5: Blood pressure data for all participants included in the study (n = 24). It was not possible to obtain reliable BP recordings in two participants. No significant differences in BP were observed for systolic BP, diastolic BP or mean BP as a result of L-TNS, H-TNS or sham TNS.

	N	Baseline	Stimulation	Recovery
L-TNS	18	20.29 ± 1.78	19.29 ± 1.74	21.61 ± 2.29
H-TNS	18	19.35 ± 1.90	21.56 ± 1.85	21.73 ± 2.44
Sham TNS	7	19.41 ± 2.94	21.10 ± 2.95	21.91 ± 2.90

Table 6.6: Baroreflex sensitivity data for all participants with detectable BRS sequences. No significant changes in BRS were observed as a result of L-TNS, H-TNS or sham TNS. Units = ms/mmHg.

6.5.3 Reported sensations due to trigeminal nerve stimulation

L-TNS and H-TNS were well-tolerated by all subjects and all participants reported a tingling sensation on their forehead and scalp as a result of the stimulus. Some participants reported an additional mildly uncomfortable "wiggling" or "worm-like" sensation under the skin of the forehead (n = 7) during the electrical current titration stage, but this sensation diminished once a more comfortable stimulus intensity was achieved. During this initial stage four participants also reported piloerection of the anterior scalp hair, which disappeared in all subjects once the stimulus had been titrated down to a comfortable level.

6.6 Discussion

The present study is the first reported investigation of the effects of non-invasive TNS using TENS on heart rate variability and baroreflex sensitivity in healthy humans. It provides evidence that transcutaneous electrical nerve stimulation applied to the supraorbital branches of the trigeminal nerve has a minimal effect on cardiovascular autonomic function in healthy human adults.

While no significant differences in blood pressure were observed for the trigeminal stimulation parameters, a small but significant decrease in mean HR was observed as a result of both L-TNS and H-TNS. A safety study by Pop et al. (2011) reported a small but significant ($p = 0.01$) 4% decrease in heart rate as a result of 60 minutes of stimulation and this decrease was only observed to occur following at least 15 minutes of stimulation (Pop et al., 2011). Interestingly, the present study observed a 4% decrease in mean HR 10 – 15 minutes after stimulation had been switched off (25-30 minutes after the onset of stimulation), but no change was observed during the 15 minutes in which H-TNS was applied. This may suggest a residual effect of H-TNS on mean HR and a slightly longer on-time for stimulation e.g. 20 minutes H-TNS may produce an observable effect on mean HR during H-TNS. A small reduction in mean HR (4.61%) was observed during L-TNS, suggesting that the mild bradycardic effects on heart rate may be frequency-dependent in the short-term. Further investigation is needed to clarify if a reduction in mean HR can still be observed for both L-TNS and H-TNS over the course of one hour of stimulation, as per the protocol used by Pop et al. to assess the cardiac safety of the H-TNS parameters (Pop et al., 2011).

The observed effects on mean HR in the present study may have been the result of a mild form of the oculocardiac reflex, a variant of the trigeminocardiac reflex involving the branches of V_1 such as the supraorbital nerves as the afferent limb (Schaller et al., 2009, Kim et al., 2012). The oculocardiac reflex has been reported as bradycardia of 10 – 20% during ophthalmic or neurosurgical procedures where surgical instruments come into accidental peri-operative physical contact with branches of V_1, as well as in rare cases of orbital fractures where damage to V1 branches has occurred

(Kim et al., 2012, Kasi et al., 2014, Meuwly et al., 2015). This bradycardic effect is thought to be elicited by increased vagal activation and thus the trigeminocardiac reflex can also be referred to as the trigeminovagal reflex (Meuwly et al., 2015). However, the present study failed to detect any changes in HRV or BRS which might indicate some level of vagal activation at the same time as the observed reductions in mean HR as a result of L-TNS and H-TNS.

The participants in the present study were slightly younger than those in Chapter 3 (29.69 ± 2.13 years and 33.09 ± 2.18 years respectively) and this age difference may have had an influence on baseline HRV. The high baseline HRV of the subjects in this study may have impacted on the response to TNS, as high HRV suggests that cardiovascular autonomic function in these subjects was already well-regulated and as such may not be readily modulated by an external stimulus. However, profound autonomic dysfunction affecting both the parasympathetic and sympathetic nervous systems has been associated with different types of epilepsy during seizures (known as the ictal period), in the period immediately following a seizure (postictal period) and also in the latent period between seizure attacks (interictal period) (Devinsky, 2004). A common observation during seizures is an increase in sympathetic nervous system activity which manifests in symptoms such as sinus tachycardia (Leutmezer et al., 2003). Given this sympathetic activation associated with seizures, future clinical trials of TNS for intractable epilepsy should include methods of assessing sympathetic nervous system activity such as microneurography to determine if there is any change in MSNA. Measures of HRV and BRS could also be included to provide non-invasive although less specific measurements of the effects of TNS on cardiovascular autonomic function over time.

In the majority of clinical studies of TNS, stimulation was delivered for a relatively long duration e.g. ≥ 8 hours per night for ≥ 3 months, meaning that it is difficult for the outcomes of the present study with healthy participants to be generalised to a clinical context. Shorter durations of TNS have been investigated as part of these trials in patients with epilepsy (stimulus on-time

= 60 minutes) and migraine (20 minutes) (Pop et al., 2011, Cook et al., 2013, DeGiorgio et al., 2013). However with the exception of Pop et al., where a small decrease in heart rate was observed on an acute basis, these trials have generally failed to detect significant changes in heart rate or blood pressure as a result of stimulation. In a randomised crossover study with healthy volunteers (n = 16), Waki et al. found that 5 minutes of electroacupuncture applied to the supraorbital region at 100Hz in resting volunteers did not alter the LF/HF ratio compared to controls, although an increase in HF power was observed during stimulation (Waki et al., 2017). This study however did not specify the proportion of male and female participants included in the sample.

In the present study, a reduction in total power, LF power and HF power was observed as a result of H-TNS in female participants, suggesting that H-TNS may be able to evoke an acute reduction in vagal activation which is sex-specific in character. However this should be interpreted with caution as no significant changes in normalised LF power or normalised HF power were detected at the same time-points for H-TNS. This may be due to the small sample size and normalised measures of LF and HF power are known to minimise the effect of changes in total power on LF and HF power (Malik, 1996). Pre-menopausal women are known to have a predominance of parasympathetic nervous system activity over sympathetic activity and this relatively increased vagal tone, reflected in higher HRV, may protect them from the onset of cardiovascular disease and sudden cardiac death (Kuo et al., 1999, Behbahani et al., 2016). There is limited information on the influence of sex on HRV in patients with epilepsy, although Behbahani et al. found that middle-aged female patients with epilepsy had lower values for HF power compared to men (Behbahani et al., 2016). This would suggest that female epilepsy patients may have reduced vagal tone compared to men and this may be an important consideration for future trials of TNS using the H-TNS parameters

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6.7 Conclusion

In conclusion, the results of the present study suggest that short-term application of non-invasive TNS delivered at either a lower frequency (30Hz) or a higher frequency (120Hz) has a limited effect on cardiovascular autonomic function in young healthy volunteers. Future clinical trials of TNS for disorders such as treatment-resistant epilepsy should include assessments of autonomic function such as HRV, BRS and microneurography to further elucidate the impact of TNS in conditions where autonomic dysfunction may be present.

Chapter 7

General Discussion

7.1 Summary of findings

7.1.1 TENS applied to different sites on the external ear can elicit variable cardiovascular autonomic responses in healthy adults

This thesis found that transcutaneous electrical nerve stimulation (TENS) applied to the tragus could induce a reduction in LF/HF ratio and an increase in BRS in a subgroup of participants (n = 12; 54.5%) who were termed tragus responders. This reduction in LF/HF ratio also occurred during TENS applied to the spine of the helix, which is known to be innervated by the auriculotemporal nerve, but no change in BRS was observed as a result of stimulation at this site. In addition, no significant changes in cardiovascular autonomic function were observed during stimulation of the earlobe (innervated by cervical spinal afferents) or the cymba concha (innervated by ABVN). This would suggest that stimulation of the auriculotemporal nerve may have an influence on autonomic activity, as this nerve can also be found at the tragus, but *in vivo* investigation is needed to investigate the exact mechanisms of tragus stimulation before this can be verified.

7.1.2 tVNS applied to the tragus of the external ear can alter cardiovascular autonomic function towards parasympathetic predominance in healthy aged adults and heart failure patients

This thesis provides physiological evidence that tVNS applied to the tragus can induce acute changes in cardiovascular autonomic function in healthy adults aged 60 years and older with no prior medical history of cardiovascular disease. A significant decrease in LF/HF ratio occurred alongside an increase in spontaneous BRS during active tVNS but not sham tVNS. Microneurographic recording of MSNA in a subset of participants showed a decrease in vasoconstrictor sympathetic nerve activity, suggesting that the change in cardiovascular autonomic function was a shift towards parasympathetic predominance. In addition, tVNS applied to the tragus appears to have a similar modulatory effect on cardiovascular autonomic

function in a small sample of patients with chronic heart failure who were already receiving optimal medical therapy, with a reduction in LF/HF ratio and an increase in BRS during stimulation. However, it was not possible to perform microneurography in these patients to confirm changes in sympathetic nervous system activity. No adverse effects were identified in these patients during stimulation and tVNS was well-tolerated.

7.1.3 Supraorbital TNS has a minimal effect on cardiovascular autonomic function in healthy humans

This thesis demonstrates that non-invasive trigeminal nerve stimulation delivered to the supraorbital region of the forehead has a minimal acute effect on cardiovascular autonomic function in healthy human volunteers. This is regardless of whether stimulation was delivered at a lower frequency (L-TNS; 30Hz) or a higher frequency (H-TNS, 120Hz). Both induced small reductions in mean heart rate but no significant changes were observed in measures of HRV or BRS. However, future studies in both healthy and clinical populations should include microneurography to assess changes in sympathetic outflow, as well as assessment of measures of HRV and BRS over longer durations of stimulation.

7.2 Potential mechanisms behind the cardiovascular autonomic effects of tVNS applied to the tragus

While the exact mechanisms behind the autonomic effects of tVNS are unclear, there is evidence that the activation of vagal afferents projecting to the NTS may be responsible. The NTS is a key relay point for the processing of cardiovascular reflexes and the control of autonomic outflow, with a number of animal studies reporting that the ABVN also has projections to the NTS. ABVN afferents have been found to project to the NTS in rats (He et al., 2013), while application of HRP to the ABVN in cats showed significant neuronal labelling not only in the NTS but also in the spinal trigeminal nucleus, principal sensory trigeminal nucleus and cuneate nucleus (Nomura

and Mizuno, 1984). In humans, BOLD fMRI have detected brainstem activation as a result of tVNS applied to the tragus (Kraus et al., 2007, Kraus et al., 2013). A further human fMRI study showed that electrical stimulation of the left cymba concha could activate the ipsilateral NTS and could also induce significant bilateral activation of the spinal trigeminal nuclei, locus coeruleus and dorsal raphe nuclei (Frangos et al., 2015). In a rat model of transient focal ischaemia, Ay et al. showed that electroacupuncture applied to the ABVN dermatome caused a 28% reduction in infarct volume (Ay et al., 2015a). This study also identified bilateral c-fos staining in the NTS, confirming activation of the NTS as a result of tVNS. In this way, the ABVN projections to the NTS may be significant with regards to the potential for tVNS to influence cardiovascular activity.

However, the tragus responder subgroup of participants in Chapter 3 (n = 12) observed a decrease in LF/HF ratio as a result of stimulation to both the tragus and the helix using the same stimulation parameters. Consequently, this may implicate afferent fibres in the auriculotemporal nerve rather than the ABVN as being responsible for the change in cardiovascular autonomic function seen with tragus stimulation. A potential explanation for this may be auriculotemporal afferent fibre projections to the NTS, as a previous neuroanatomical tracing study by Jacquin et al. showed some evidence of auriculotemporal nerve projections to the NTS in rats, although a later study by Takemura et al. did not report any projections to this site (Jacquin et al., 1983, Takemura et al., 1987). In addition, Chien et al. reported some HRP-labelled nerve terminals in the canine NTS from the rostral internal auricular nerve, the canine equivalent of the auriculotemporal nerve (Chien et al., 1996). The study also observed HRP-labelling from the middle and caudal internal auricular nerves in the NTS and the majority of neurons were found to have their cell bodies in the jugular ganglion of the vagus nerve, consistent with the ABVN (Chien et al., 1996). Evidence of NTS activation has also been found in an fMRI study where electrical stimulation was applied to the inner surface of the tragus in human volunteers (Kraus et al., 2013). In this way, activation of the NTS by afferent fibres from one or both of the ABVN or the auriculotemporal nerve could be responsible for the autonomic effects of

tragus stimulation. Activation of the NTS could then also activate the nucleus ambiguus and dorsal motor nucleus of the vagus to elicit an increase in parasympathetic outflow via the vagus nerve (Izzo et al., 1993). This is consistent with the observation that the tragus responder subgroup of participants exhibited a significant increase in normalised HF power during both tragus and helix stimulation, with an additional increase in BRS observed during tragus stimulation. However, as it is not possible to perform direct recordings of vagus nerve activity in humans, further preclinical in vivo experiments are necessary to clarify the effects of tragus stimulation on parasympathetic activity. Nonetheless, this thesis provides evidence that tVNS can increase BRS and HRV in both aged healthy volunteers and heart failure patients, suggesting an increase in vagal tone.

Of particular relevance to this thesis is the finding that tVNS significantly reduced the concentration of plasma norepinephrine in conscious dogs with healed myocardial infarction following bilateral stimulation of the tragus, adding further evidence to the ability of tVNS to modulate sympathetic nerve activity (Wang et al., 2015a). A more recent study in anaesthetised dogs by the same research group showed that the acceleration in heart rate produced in response to stimulation of the right stellate ganglion was attenuated following tVNS applied to the tragus (Zhou et al., 2016). This was further accompanied by a reduction in LF/HF ratio (Zhou et al., 2016). tVNS was also found to reduce c-fos expression in the right stellate ganglion, suggesting a reduction in cardiac sympathetic activation. The precise mechanisms underlying this sympathoinhibitory pathway are uncertain, but excitation of ABVN (or auriculotemporal) afferent projections to the NTS may inhibit activity in the rostral ventrolateral medulla (RVLM) via activation of the caudal ventrolateral medulla. The RVLM regulates the basal level of central sympathetic outflow and tVNS may therefore be able to reduce sympathetic nerve activity through RVLM inhibition (Figure 7.1; Guyenet, 2006). In this way, tVNS applied to the tragus may be a potential therapeutic adjunct for conditions associated with chronic sympathetic activation such as in heart disease and hypertension, which have an increased prevalence with age.

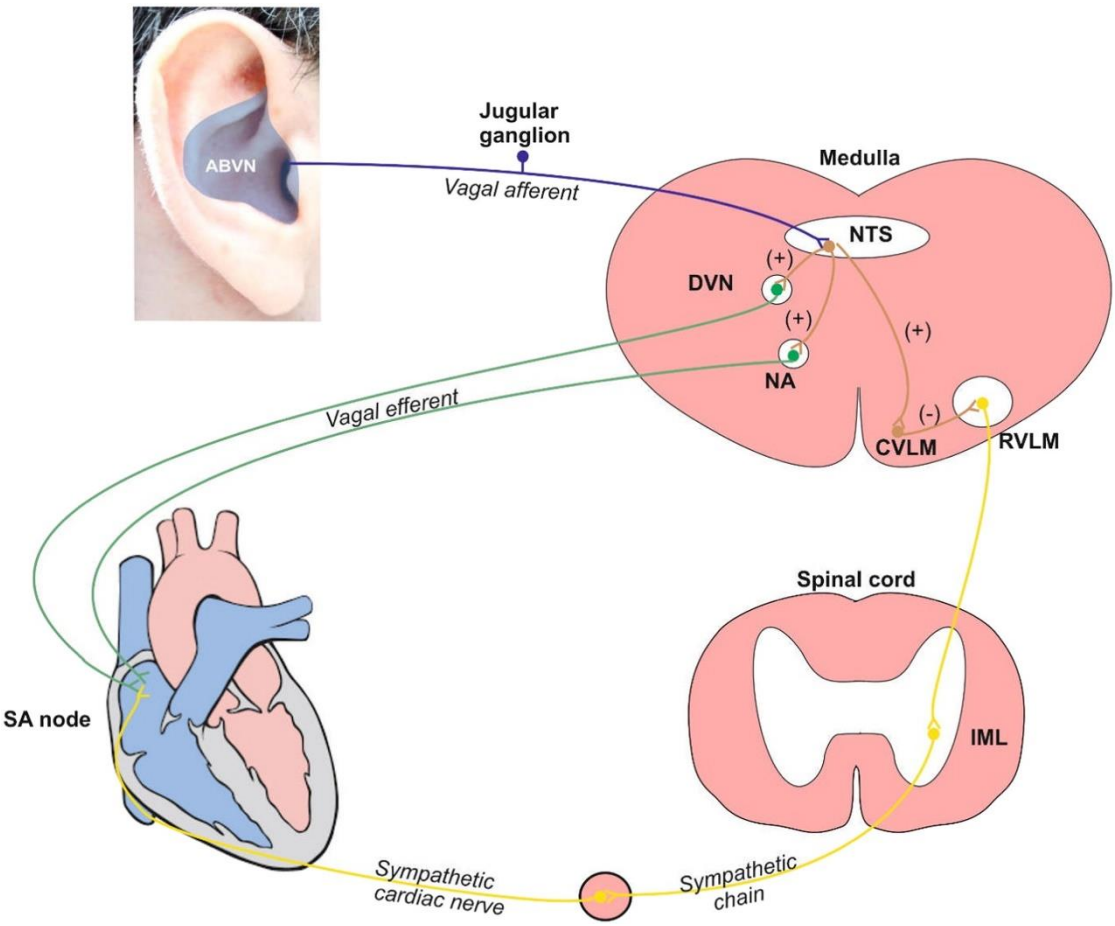

Figure 7.1: Outline of potential neural pathways through which stimulation of the auricular branch of the vagus nerve (ABVN) could influence cardiac activity. Electrical stimulation applied to skin innervated by ABVN afferents increases input to the nucleus tractus solitarius (NTS) in the dorsal medulla. This in turn leads to increased activation of NTS neurones projecting to the dorsal vagal nucleus (DVN) and nucleus ambiguus (NA), which contain vagal preganglionic efferent neurones. These vagal efferent neurones increase activation at the sinoatrial (SA) node, inducing a bradycardiac response. Stimulation of ABVN afferents may also excite neurones in the NTS with excitatory projections to the caudal ventrolateral medulla (CVLM). The CVLM has inhibitory projections to the rostroventrolateral medulla (RVLM), the principal source of excitatory drive to sympathetic preganglionic neurones in the intermediolateral cell column (IML) of the spinal cord. This inhibition would lead to a reduction in sympathetic nervous system activity. Image sourced from Murray et al. (2016).

7.3 Potential clinical applications of tVNS applied to the tragus

7.3.1 tVNS for treatment-resistant hypertension

Hypertension is the foremost risk factor for premature mortality and heart disease, with the global prevalence predicted to increase to 1.56 billion people by 2025 (Kearney et al., 2005). The prevalence of hypertension in England has been determined to be around 30% of the population (Joffres et al., 2013). Up to half of hypertensive patients are treatment-resistant, defined as persistently high blood pressure that remains elevated despite the use of at least three antihypertensive pharmacological agents including a diuretic (Calhoun et al., 2008, Esler et al., 2010). As such, there has been substantial interest in the use of neuromodulatory therapies in treatment-resistant hypertensive patients.

One such technique is renal nerve denervation, where afferent and efferent nerve fibres surrounding the renal artery are ablated using radiofrequency or ultrasound energy in an attempt to reduce sympathetic activation (Esler et al., 2010). This is achieved using specialised catheter-based electrodes inserted percutaneously into the femoral artery and fed superiorly into the renal artery to allow for large-scale ablation of nerve fibres in the arterial adventitia (Patel et al., 2015). The SYMPLICITY-HTN1 trial of renal nerve denervation in treatment-resistant hypertension patients (n = 45) showed therapeutic promise at 12 months after denervation, with ambulatory BP recordings detecting a mean reduction in systolic blood pressure of 27 mmHg and diastolic blood pressure of 17 mmHg from a mean baseline BP of 177/110 mmHg (Krum et al., 2009). By three years, 93% of patients had experienced a reduction in systolic blood pressure of at least 10 mmHg (Krum et al., 2014). The SYMPLICITY-HTN2 trial, where patients (n = 106) were randomised to renal nerve denervation or optimum medical therapy, showed a reduction in office BP measured in clinic of -32/12 mmHg and a reduction in ambulatory BP of -11/7mmHg, no significant changes in the control group (Esler et al., 2010). However the SYMPLICITY-HTN3 randomised sham-

controlled trial (n = 500) failed to detect a significant reduction in systolic BP at 6 months between patients who received renal nerve denervation and patients who received a sham procedure (Bhatt et al., 2014).

Another invasive neuromodulatory technique which has been trialled for treatment-resistant hypertension has been baroreflex activation therapy (BAT). Given that an increase in BP will trigger activation of carotid sinus baroreceptors to initiate a subsequent reduction in BP via the arterial baroreflex, electrical stimulation applied to the carotid sinus could be used to improve BP control in treatment-resistant hypertension. In this way, a baroreflex-mediated decrease in sympathetic nervous system activity and blood pressure could also be achieved in these patients. Baroreflex activation therapy was initially tested using the Rheos™ carotid sinus stimulation system, which involved the surgical implantation of bilateral bipolar electrodes around the carotid sinus (Tordoir et al., 2007, Scheffers et al., 2010). A significant decrease in BP was observed following 12 months of BAT in 45 patients with treatment-resistant hypertension (Scheffers et al., 2010). Reductions in MSNA have also been reported in hypertensive patients as a result of BAT (Heusser et al., 2010). A subsequent randomised sham-controlled trial in 265 treatment-resistant hypertension patients involved patients receiving either active BAT one month after implantation (treatment group) or six months after implantation (sham group) (Bisognano et al., 2011). While the trial did not observe a significant difference between groups in its primary efficacy endpoint (decrease of ≥ 10mmHg systolic BP after 6 months), 42% of participants in the active BAT group achieved a decrease in systolic BP to < 140mmHg (Bisognano et al., 2011). In addition, the reductions in systolic BP were observed at long-term follow-up in this cohort of patients (Bakris et al., 2012). A second-generation system has since been developed called Barostim neo™, which uses a smaller electrode to perform unilateral carotid sinus stimulation as opposed to bilateral stimulation (Hoppe et al., 2012, Gassler and Bisognano, 2014). Preliminary testing in 30 patients with resistant hypertension showed a mean systolic BP reduction of 26 mmHg at 6 months, comparable to the effects observed with the early Rheos system (Hoppe et al., 2012). However, as with renal nerve

denervation, both of these methods of BAT requires an invasive surgical procedure, which ultimately limits their clinical potential.

The results of this thesis have shown that tVNS can reduce MSNA and increase BRS in humans with no medical history of cardiovascular disease, so there is potential for tVNS to have a similar effect in patients with hypertension. To date there has not been much investigation into the antihypertensive effects of VNS or tVNS in humans, but as tVNS is a non-invasive technique it may offer the greater therapeutic potential.

7.3.3 Obstructive sleep apnoea

Obstructive sleep apnoea (OSA) is a common sleep disorder which may affect up to around 100 million individuals worldwide (Senaratna et al., 2017). OSA is characterised by recurrent episodes of apnoea (obstruction of the pharyngeal section of the airway) leading to reduced airflow and consequently hypoxia which can then disrupt normal sleep (Gaspar et al., 2017). Moreover, ageing is known to significantly increase the risk of developing OSA, with around 24 – 62% (Young et al., 2002). Patients with OSA have been found to have increased sympathetic activation while awake, as assessed by measures such as plasma norepinephrine and MSNA (Somers et al., 1995, Narkiewicz et al., 1998b). Reduced heart rate variability has also been demonstrated in OSA patients compared to controls (Narkiewicz et al., 1998a). Patients with severe OSA have also been found to have evidence of impaired BRS, but crucially this impairment in baroreceptor reflex function was restricted to severe cases of OSA and no baroreflex dysfunction was observable in patients with mild OSA (Blomster et al., 2015, Sforza et al., 2016). A common treatment strategy for OSA is continuous positive airway pressure (CPAP) and this has been found to reduce MSNA (Narkiewicz et al., 1999, Henderson et al., 2016). However, a major limitation of CPAP is the need to wear a mask for the device while sleeping, which in itself can lead to sleep disturbances as well as poor compliance (Hussain et al., 2014). A potential therapeutic alternative may be

tVNS, which might be better tolerated but could also reduce sympathetic activation. Moreover, tVNS might also increase BRS in patients with severe OSA.

7.3.3 Rheumatoid Arthritis

Rheumatoid arthritis (RA), a chronic inflammatory disorder which affects around 1% of the Western world (Firestein, 2003). It typically manifests in mid-life as musculoskeletal joint problems such as swelling and pain accompanied by persistent fatigue (Stack et al., 2013). However the symptom remission rate for RA patients is low despite the existence of a number of pharmacological therapies which target the inflammatory mechanisms of RA (Smolen et al., 2017). In addition, RA patients have an increased risk of cardiovascular morbidity and mortality (Solomon et al., 2003, Kapetanovic et al., 2011), which could be in part due to chronic cardiovascular autonomic dysfunction. RA patients have been found to have impaired BRS (Aydemir et al., 2010, Adlan et al., 2017) as well as increased sympathetic activation measured through plasma norepinephrine (Vlcek et al., 2008) and microneurographic recordings of multi-unit MSNA (Adlan et al., 2016). Heart rate variability has also been shown to be reduced in RA patients. Evrengul et al. described a sample of RA patients (n = 48) who had a greater LF/HF ratio and a reduced HF power compared to a group of healthy controls (n = 50), suggesting reduced vagal tone (Evrengul et al., 2004).

Implanted VNS has been trialled in a 17 RA patients who had > 6 months of symptoms and increased CRP levels (≥ 7mg/L) at baseline (Koopman et al., 2014, Koopman et al., 2016). VNS initiated two weeks after implantation for 60 seconds per day showed improvements in RA severity at day 42 as determined by reduced values for the Disease Activity Score-28 for Rheumatoid Arthritis with CRP (DAS28-CRP) composite scoring tool (Koopman et al., 2016). VNS was then stopped for 14 days and DAS28-CRP was found to have increased, indicating a worsening of RA severity

(Koopman et al., 2016). However this trial did not assess any changes in autonomic function and a number of adverse events were reported during the trial including dysphonia, dyspnoea and paraesthesia of the skin of the neck (Koopman et al., 2014; Koopman et al., 2016). Although VNS appears to be effective in reducing RA severity, the invasiveness of the implantation procedure and the side-effects associated with VNS limit its therapeutic potential. Thus, tVNS applied to the tragus to stimulate the ABVN could be a potential non-invasive alternative to VNS which may have a similar efficacy in RA patients. The effects of tVNS on cardiovascular autonomic function described in this thesis for healthy volunteers and heart failure patients may also be observed in patients with RA.

7.4 Limitations

A significant limitation of this thesis is the small sample size of CHF patients recruited to the study in Chapter 5. There was a high incidence of cardiac arrhythmias in CHF outpatients who were screened at the outpatient clinic, which had a detrimental effect on recruitment. Moreover, many patients who were recruited had no prior diagnosis of arrhythmia but were found to have frequent ectopic beats during the experimental recordings. This meant that it was not possible to analyse HRV in these patients due to the data loss caused by the ectopic beats. In addition, the early phase of the study involved recruitment and testing of patients during the outpatient clinic hours, which severely restricted the time available for each experiment. This meant that it was not possible to obtain post-stimulation recovery recordings for all patients to clarify the short-term residual effects of tVNS in CHF. Later experiments conducted in the Cardiovascular Clinical Research Facility at Leeds General Infirmary were able to offer patients the opportunity to return at a convenient day and time for their experiment, although due to frequent ectopic beats only two patients from this phase were eligible for inclusion to the study. In addition, patient recruitment for the CHF study was stopped prematurely due to a shortage of clinical care staff available to screen the CHF patients during outpatient clinics. In future, this could be prevented by

obtaining funding for a part-time research assistant or research nurse who would be required to screen patients.

All of the studies included in this thesis relied analyses of HRV and BRS, two non-invasive estimates of cardiovascular autonomic function as opposed to direct measurements. However, the lack of significant change in sham tVNS and sham TNS in Chapters 4 and 6, where no stimulus was applied to either the tragus or the supraorbital region, suggested that the effects observed with these non-invasive measures during active stimulation were valid. Sham tVNS was not performed in the heart failure patients in Chapter 5, or in the study with healthy participants in Chapter 3. However, Chapter 3 included earlobe stimulation, which has been previously used in some tVNS studies as a control stimulation site (Frangos et al., 2015; Yakunina et al., 2016). No significant differences in measures such as BRS or the LF/HF ratio were identified for earlobe stimulation, suggesting that stimulation of great auricular nerve afferents may not significantly influence autonomic activity in humans. However, due to experimental time constraints it was not possible to perform microneurography for the study in Chapter 3 to directly assess changes in sympathetic nervous system activity.

Microneurography was however performed in healthy participants in Chapter 4, which showed a reduction in MSNA as a result of active tVNS. Nevertheless, a limitation of this technique is that changes in MSNA represent changes in sympathetic outflow at a systemic rather than regional level and it was not possible to measure regional changes in sympathetic nervous system activity as part of this study. In future, this could be investigated in healthy volunteers or heart failure patients using cardiac norepinephrine spill-over measurements obtained using a catheter inserted into the coronary sinus.

7.5 Future directions

7.5.1 Effects of chronic tVNS in healthy aged volunteers

The elderly are at an increased risk of developing chronic cardiovascular disease (North and Sinclair, 2012). The preliminary data presented in Chapter 4 indicates that tVNS applied to the tragus of the external ear can alter cardiovascular autonomic function in healthy older adults with no previous history of cardiovascular disease in a potentially beneficial way. Specifically, this alteration was a reduction in sympathetic nervous system activity assessed through microneurographic recording of MSNA and an increase in parasympathetic predominance observed through a decrease in LF/HF ratio and an increase in spontaneous BRS. In this way, tVNS may represent a method of preventing or attenuating the onset of cardiovascular disease in aged individuals. However, the long-term autonomic effects of tVNS in older people are at present unknown. The changes in HRV and BRS observed in Chapter 4 were short-lived, with values for LF/HF ratio and BRS returning to near-baseline levels within 15 minutes of the end of stimulation (recovery period). Daily application of tVNS for 15 minutes or more over a number of weeks or months could, however, have a residual cumulative effect on cardiovascular autonomic control.

To investigate the autonomic effects of chronic tVNS, an initial pilot study design would be to recruit a cohort of elderly volunteers who would receive 15 minutes of tVNS twice daily (morning and evening) over the course of one month. An initial baseline visit would assess the short-term response to 15 minutes tVNS using non-invasive measures of autonomic function such as HRV and BRS, as well as microneurography to record MSNA in participants who consent to this more invasive technique. At the end of this baseline visit, participants would receive their own auricular electrode clips and a TENS machine so that they could self-apply tVNS in their own home. The TENS machine would have a built-in compliance meter to monitor use and participants would be further required to keep a diary to record use and any

self-reported effects of tVNS. Participants would be asked to return for two additional visits at day 14 and day 28 to assess if the residual effects of chronic tVNS. Following completion of 28 days of tVNS, a follow-up period of 28 days could be undertaken where the TENS machines are taken away from the participants, who are then asked to return for additional visits at days 42 and 56 to assess if there have been any persistent post-tVNS changes in HRV, BRS and MSNA. If positive results are detected at the end of this period e.g. reductions in MSNA, increased BRS and HRV, then the TENS machines could be returned to the participants for daily use and an extended protocol of six months to one year of chronic tVNS performed with assessment visits every three months. In addition, quality of life questionnaires could be used to assess any changes in mood as a result of chronic tVNS.

7.5.2. Effects of long-term application of tVNS in chronic heart failure

Following on from the work in Chapter 4 with the healthy aged volunteers, a group of chronic heart failure (CHF) patients were recruited in an effort to investigate the short-term autonomic effects of tVNS in individuals with a pre-existing cardiac condition. As with the older volunteers in Chapter 4, the CHF patients saw a decrease in LF/HF ratio and an increase in BRS during tVNS. This change was not reflected in a sample of age-matched and sex-matched healthy controls (n = 8) who were compared to the patients, but this lack of an effect may have been due to the small sample size. It was not possible to obtain post-stimulation (recovery period) recordings for the majority of patients due to time constraints in the experiments. Moreover, the time constraints in the clinical setting meant it was not possible to perform microneurography.

However, the promising results observed for HRV and BRS during 15 minutes of tVNS supports further investigation of the long-term effects of tVNS in patients with CHF. A similar study design could be used as with the proposal for chronic tVNS in elderly participants (section 7.5.2) and the CHF

patients could self-apply tVNS twice daily for one month in the first instance. In addition to measures of autonomic function such as HRV and BRS, clinical measurements could be incorporated into the assessment visits including quality of life questionnaires to identify improvements in mood, 6-minute walk tests to assess the effects of tVNS on exercise tolerance and echocardiography to detect any changes in measures of cardiac function such as LVEF. Depending on the outcome of an initial one month pilot study, further investigation could be carried out to identify the autonomic and clinical effects of tVNS over the course of six months to one year. In this way, the clinical efficacy of long-term exposure to daily tVNS in patients with CHF could be compared and contrasted with the outcomes of the VNS for CHF trials (DeFerrari et al., 2011, Premchand et al., 2014, Zannad et al., 2015, Gold et al., 2016).

7.5.3 Distribution of the ABVN

Chapter 3 attempted to compare the autonomic effects of stimulation at different sites on the ear, with the sites selected based on the auricular innervation patterns described in 7 human cadavers (Peuker and Filler, 2002). However, the physiological effects observed in Chapter 3, in particular the lack of any significant change in HRV or BRS as a result of cymba concha stimulation, suggests that the reported distribution of nerves such as the ABVN may not be completely accurate. The cymba concha was reported by Peuker and Filler to be innervated exclusively by the ABVN, yet the response observed during cymba concha stimulation was similar to that observed for the earlobe, which is innervated by the great auricular nerve (GAN) (Peuker and Filler, 2002). A larger-scale cadaveric dissection study is needed to determine the extent of inter-individual anatomical variation in the ABVN dermatome, as this information could be used to optimise the electrode placement of tVNS.

7.5.4 Determining the central mechanisms of tVNS

A potential method of investigating the central mechanisms of tVNS is through the working heart brainstem preparation (WHBP), an arterially perfused *in situ* decerebrate rodent preparation which does not require the use of anaesthetic agents (Paton, 1996). The WHBP allows for intracellular recordings of the neurons associated with medullary autonomic neuronal circuits, as these can be preserved using WHBP. The baroreceptor reflex can be activated in the WBHP through maximally increasing the arterial perfusion pressure, thereby eliciting a reduction in heart rate (Lall et al., 2012). Modified versions of the electrode clips used in this thesis could be used to investigate the autonomic effects of stimulation at different sites on the rodent ear in a similar way to the study design of Chapter 3. Another approach would be to use a carbocyanine lipophilic neuronal tracer such as DiI in fixed human cadaveric tissue, as DiI can travel through fixed neuronal tissue in both an anterograde and retrograde direction (Lanciego and Wouterlood, 2011). DiI applied to the cut central end of nerves such as the ABVN or auriculotemporal nerve could be used to visualise the central terminations of afferents from these nerves using an epifluorescence microscope.

7.6 Conclusion

This thesis has demonstrated that it is possible to use transcutaneous electrical nerve stimulation applied to the tragus (tVNS) to elicit a modulatory effect on autonomic function in older healthy humans and also in heart failure patients. This thesis also shows that supraorbital TNS appears to have a minimal influence on autonomic function in healthy humans. Further clinical studies are needed to determine if tVNS applied to the tragus could be an effective adjunctive therapy for disorders where autonomic dysregulation is present. In addition, the precise mechanisms behind the autonomic effects of tVNS should be further investigated in animal studies in order to optimise the technique and facilitate future translational work.

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Appendix

Ethics Approvals

Performance, Governance and Operations
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UNIVERSITY OF LEEDS

Biological Sciences Faculty Research Ethics Committee
University of Leeds

Mr Aaron Murray
Faculty of Biological Sciences
University of Leeds
LS2 9JT

20 February 2014

Dear Aaron

Title of study: **The effects of transcutaneous vagus nerve stimulation and trigeminal nerve stimulation on autonomic nervous control of the heart.**
Ethics reference: **BIOSCI 13-025**

I am pleased to inform you that the above research application has been reviewed by the Faculty of Biological Sciences Research Ethics Committee and following receipt of your response to the Committee's initial comments, I can confirm a favourable ethical opinion as of the date of this letter. The following documentation was considered:

Document	*Version*	*Date*
BIOSCI 13-025_Corrections to Ethics Review Application (Aaron Murray).docx	2	06/02/14
BIOSCI 13-025_Project Poster (Aaron Murray).pptx	2	06/02/14
BIOSCI 13-025_Participant Information Sheet (Aaron Murray).docx	2	06/02/14

Please notify the committee if you intend to make any amendments to the original research as submitted at date of this approval, including changes to recruitment methodology. All changes must receive ethical approval prior to implementation. The amendment form is available at http://ris.leeds.ac.uk/EthicsAmendment.

Please note: You are expected to keep a record of all your approved documentation, as well as documents such as sample consent forms, and other documents relating to the study. This should be kept in your study file, which should be readily available for audit purposes. You will be given a two week notice period if your project is to be audited. There is a checklist listing examples of documents to be kept which is available at http://ris.leeds.ac.uk/EthicsAudits.

We welcome feedback on your experience of the ethical review process and suggestions for improvement. Please email any comments to ResearchEthics@leeds.ac.uk.

Yours sincerely
Karen Clinton, Administrative Support Officer, Research & Innovation Service
On behalf of Karen Birch, Chair, BIOSCI Faculty Research Ethics Committee

CC: Student's supervisor

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UNIVERSITY OF LEEDS

Biological Sciences Faculty Research Ethics Committee
University of Leeds

Aaron Murray
Faculty of Biological Sciences
University of Leeds
LS2 9JT

20 December 2016

Dear Aaron

Title of study: The effect on autonomic nervous system activity of electrical nerve stimulation at different sites on the ear in healthy human research subjects
Ethics reference: BIOSCI 16-009

I am pleased to inform you that the above research application has been reviewed by the Faculty of Biological Sciences Research Ethics Committee and following receipt of your response to the Committee's initial comments, I can confirm a favourable ethical opinion as of the date of this letter. The following documentation was considered:

Document	*Version*	*Date*
BIOSCI 16-009 Ear stimulation sites study consent form version 2 with amendments.docx	2	16/12/16
BIOSCI 16-009 Ear stimulation sites study ethics application form (Aaron Murray) version 2 with amendments.doc	2	16/12/16
BIOSCI 16-009 Ear stimulation sites study health questionnaire version 2 with amendments.docx	2	16/12/16
BIOSCI 16-009 Ear stimulation sites study information document version 2 with amendments.docx	2	16/12/16

Please notify the committee if you intend to make any amendments to the information in your ethics application as submitted at date of this approval as all changes must receive ethical approval prior to implementation. The amendment form is available at http://ris.leeds.ac.uk/EthicsAmendment.

Please note: You are expected to keep a record of all your approved documentation, as well as documents such as sample consent forms, and other documents relating to the study. This should be kept in your study file, which should be readily available for audit purposes. You will be given a two week notice period if your project is to be audited. There is a checklist listing examples of documents to be kept which is available at http://ris.leeds.ac.uk/EthicsAudits.

We welcome feedback on your experience of the ethical review process and suggestions for improvement. Please email any comments to ResearchEthics@leeds.ac.uk.

Yours sincerely
Victoria Butterworth

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10 April 2013

John Greenwood
Academic Unit of Cardiovascular Medicine
G Floor
Jubilee Wing
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Leeds
LS1 3EX

Dear Mr Greenwood

Study title:	**Non invasive transcutaneous vagus nerve stimulation in heart failure: a pilot study**
REC reference:	**12/YH/0354**
Amendment number:	**V1.1**
Amendment date:	**22 March 2013**
IRAS project ID:	**109128**

The above amendment was by the Sub-Committee in correspondence.

Ethical opinion
The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Protocol	1.1	22 March 2013
Notice of Substantial Amendment (non-CTIMPs)	3.5	26 March 2013

Membership of the Committee
The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval
All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance
The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

12/YH/0354:	Please quote this number on all correspondence

Yours sincerely

pp

Janet Holt
Vice Chair

E-mail: hayley.jeffries@nhs.net

Enclosures: *List of names and professions of members who took part in the review*

Copy to: Ms Anne Gowing, R&D Department, Leeds Teaching Hospitals NHS Trust

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Health Research Authority

NRES Committee Yorkshire & The Humber - Leeds East
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24 June 2014

Professor John P Greenwood
Professor of Cardiology, Honorary Consultant Cardiologist
University of Leeds
Department of Cardiology
Sunshine Corridor
Leeds General Infirmary
LS1 3EX

Dear Professor Greenwood

Study title:	**Non invasive transcutaneous vagus nerve stimulation in heart failure**
REC reference:	**14/YH/0178**
IRAS project ID:	**154178**

Thank you for your letter of 23 June 2014, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair together with Professor Kenneth Brodlie.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact the REC Manager, Miss Sarah Grimshaw, nrescommittee.yorkandhumber-leedseast@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publicly accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact Catherine Blewett (catherineblewett@nhs.net), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering letter on headed paper	P. Bijsterveld	09 May 2014
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only)	Henderson	19 September 2014
GP/consultant information sheets or letters	1.0	18 June 2014
Non-validated questionnaire [Tolerability Questionnaire]	1.1	18 June 2014
Participant consent form	1.1	18 June 2014
Participant information sheet (PIS)	1.1	18 June 2014
REC Application Form	154178/604349/1/429	02 May 2014
Research protocol or project proposal	1.1	18 June 2014
Response to Request for Further Information	Petra Bijsterveld	23 June 2014
Summary CV for Key Investigator	A. Murray	

Summary CV for Key Investigator	J. Deuchars	27 June 2012
Summary CV for Chief Investigator (CI)	J. Greenwood	

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website: http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

14/YH/0178	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project.

Yours sincerely

S. Grimshaw

pp
Dr C E Chu
Chair

Email:nrescommittee.yorkandhumber-leedseast@nhs.net

Enclosures: "After ethical review – guidance for researchers" *SL-AR2*

Copy to: *Ms Clare Skinner, University of Leeds*
Ms Anne Gowing, Leeds Teaching Hospitals NHS Trust
Ms Petra Bijsterveld, University of Leeds