The effects of non-invasive neuromodulation on autonomic nervous system function in humans

Jennifer Ann Clancy

Submitted in accordance with the requirements for the degree of
Doctor of Philosophy

The University of Leeds
School of Biomedical Sciences

July 2013

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

Acknowledgements

I would firstly like to thank Jim and Sue Deuchars who have been excellent supervisors, allowing me to develop and improve my research skills. The Deuchars lab is a wonderful place to work and is testament to the support, encouragement and enthusiasm of Jim and Sue. Indeed, I am very grateful to all the members of the Deuchars lab for making my PhD a surprisingly fun and enjoyable experience. In particular I would like to thank Varinder, for always listening and keeping me going, and Ian for his limitless patience and always making time to help.

I am indebted to many people for their help and expertise including David Mary who taught me most of the techniques used in this thesis, Petra Bijsterveld, John Greenwood and Klaus Witte who helped me gain access to the heart failure clinic and Mark Mon-Williams and Robyn Johnson for the use of the tDCS device.

I would also like to thank my Mum and Dad who have always supported me in everything I have done. Your love and encouragement are the reason I undertook a PhD but don't worry, I won't make you read the rest of this book!

Poor Stuart, thank you for being the first guinea pig for everything I tried. Not only that, but for putting up with my ranting and raving. Your relentless positivity and support forced me to believe I could do this.

Lastly, I would be nowhere without the generosity of all the volunteers who gave up their time to take part in this research. It has been wonderful meeting so many friendly, interesting and helpful people.

Abstract

Neuromodulation, the alteration of nerve activity through the use of targeted electrical stimulation or pharmacology, is a rapidly advancing field with applications in a plethora of conditions e.g. epilepsy. Many of these techniques are invasive and expensive such as vagus nerve stimulation limiting their applicability to patient populations. Autonomic nervous system imbalance is a characteristic of many conditions including heart failure. The ability to favourable alter autonomic function non-invasively would, therefore, offer potential therapeutic benefit to a large number of patients. This thesis investigated the effects of two non-invasive neuromodulatory techniques on cardiovascular autonomic function in humans – transcutaneous vagus nerve stimulation (tVNS) and transcranial direct current stimulation (tDCS).

tVNS was performed using surface electrodes placed on the ear to stimulate the auricular branch of the vagus nerve (ABVN). High pulse width and frequency tVNS was found to alter heart rate variability (HRV) towards parasympathetic predominance in healthy participants (n = 34) and heart failure patients (n = 8). Furthermore, microneurography recordings performed in healthy participants (n = 10) revealed that this effect may have been mediated, at least in part, by a reduction in muscle sympathetic nerve activity (MSNA).

tDCS was performed by placing electrodes over the motor cortex and the contralateral supraorbital region of healthy participants (n = 22). Anodal stimulation (positive electrode over the motor cortex) altered HRV towards sympathetic predominance and increased MSNA whereas cathodal and sham tDCS had no effect.

tVNS and anodal tDCS over the motor cortex had opposite effects on cardiovascular autonomic function. These techniques may be tailored to the needs of individual patients to shift cardiovascular autonomic function towards either parasympathetic (tVNS) or sympathetic (tDCS) predominance. tVNS and tDCS are simple, non-invasive and inexpensive allowing a wide cohort of patients to access these potential therapies.

Publications

Papers

Clancy, JA., Johnson, R., Raw, R., Deuchars, SA., Deuchars, J. (2013) Anodal transcranial direct current stimulation over the motor cortex increases sympathetic nerve activity. *Brain Stimulation* (Epub 18th Sept).

Clancy, JA., Deuchars, SA., Deuchars, J. (2013) The wonders of the Wanderer. *Experimental Physiology* **98 (1)** pp.38-45.

Clancy, JA., Deuchars SA. (2012) Highlights in basic autonomic neurosciences: Exploring novel and atypical circuits. *Autonomic Neuroscience* **171 (1-2)** pp.1-3.

Clancy, JA., Lall, VK., Deuchars, SA. (2011). Highlights in basic autonomic neurosciences: new avenues for the study of autonomic function in health and disease. *Autonomic neuroscience* **162 (1-2)** pp.49-52.

Abstracts

Clancy JA., Deuchars, SA., Deuchars, J (2013) Non-invasive vagus nerve stimulation enhances parasympathetic influence on autonomic output in healthy humans and heart failure patients. Proc 37th IUPS, PCC077

Clancy JA., Johnson R., Wilkie R., Mon-Williams M., Deuchars SA., Deuchars J. (2012) The influence of transcranial direct current stimulation on cardiac autonomic function in healthy human subjects. *FASEB ,* 26:891.5.

Clancy JA., Deuchars SA., Deuchars J. (2012) The influence of transcutaneous vagus nerve stimulation on cardiac autonomic control in healthy human subjects. *Proc Physiol Soc* 27.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ABVN – auricular branch of the vagus nerve

ACE – angiotensin converting enzyme

ACh – acetylcholine

AF – atrial fibrillation

ANOVA – analysis of variance

ANS – autonomic nervous system

BMI – body mass inde

BOLD – blood oxygenation level dependent

BP – blood pressure

bpm – beats per minute

BRS – baroreflex sensitivity

CN – cranial nerve

CNS – central nervous system

CVLM – caudal ventrolateral medulla

DMO - dextromethorphan

DVN – dorsal vagal nucleus

DR – dextran-tetramethylrhodamine

ECT – electroconvulsive therapy

FFT – fast Fourier transform

FG – fluorogold

fMRI – functional magnetic resonance imaging

GABA - gamma-aminobutyric acid

HF – high frequency component of heart rate variability

HRP – horse radish peroxidase

HRR – heart rate recovery

HRT – heart rate turbulance

HRV – heart rate variability

H-tVNS – high pulse width and frequency transcutaneous vagus nerve stimulation

IHG – isometric handgrip

LC – locus coeruleus

LF – low frequency component of heart rate variability

LF/HF – low frequency to high frequency ratio of heart rate variability

L-tVNS – low pulse width and frequency transcutaneous vagus nerve stimulation

LVEF – left ventricular ejection fraction

MDD – major depressive disorder

MEPs – muscle evoked potentials

MI – myocardial infarction

mPFC – medial prefrontal cortex

MSNA – muscle sympathetic nerve activity

MVC – maximal voluntary contraction

NA – nucleus ambiguus

NMDA – N-methyl-D-aspartate

N-N – normal to normal heart beat interval

NTS – nucleus tractus solitaries

NYHA – New York Heart Association

OSA – obstructive sleep apnoea

PAG – periaqueductal grey

PCOS – polycystic ovary syndrome

pNN50 – number of pairs of adjacent N-N intervals differing by > 50 ms

PVN – paraventricular nucleus

rCBF – regional cerebral blood flow

RHR – resting heart rate

RMSSD – square root of the sum of squares of differences between adjacent normal – normal intervals

R-R – interbeat interval

RSA – respiratory sinus arrhythmia

RVLM – rostral ventrolateral medulla

SDANN – standard deviation of the averages of normal – normal intervals in 5 minute segments of an entire recording

SDNN – standard deviation of normal – normal intervals

sMSNA – single unit muscle sympathetic nerve activity

SSNA – skin sympathetic nerve activity

tDCS – transcranial direct current stimulation

TENS – transcutaneous electrical nerve stimulation

TNF-alpha – tumour necrosis factor alpha

tVNS – transcutaneous vagus nerve stimulation

VF – ventricular fibrillation

VLF – very low frequency component of heart rate variability

VNS – vagus nerve stimulation

VSEP – vagus somatosensory evoked potential

WGA-HRP – wheat germ agglutinin-horse radish peroxidase

WHBP – working heart brainstem preparation

Chapter 1
General Introduction: the autonomic nervous system in health and disease

1.1 The autonomic nervous system

The autonomic (Greek auto = self, nomos = rule) nervous system (ANS) is appropriately named as it generally operates without conscious input to regulate visceral function and maintain homeostasis. The ANS has three divisions; the sympathetic, parasympathetic and enteric divisions. The enteric division is situated in the gastrointestinal tract and controls digestive reflexes. The sympathetic and parasympathetic divisions innervate cardiac muscle, smooth muscle and exocrine glands. Many organs receive dual input from both the sympathetic and parasympathetic divisions and these divisions are often considered to be functionally antagonistic. For example, the sympathetic division is regarded as responsible for the 'fight or flight' response e.g. increasing heart rate and the parasympathetic division as active during 'rest and digest' conditions e.g. decreasing heart rate. In reality the ANS is more sophisticated; both sympathetic and parasympathetic divisions are tonically active providing integrated and co-ordinated responses that allow fine-tuned control of visceral function and homeostasis. Indeed, heart rate is under tonic inhibition at rest through parasympathetic input to the sino-atrial node (Jose and Collison, 1970). The initial increase in heart rate during exercise is due to parasympathetic withdrawal that is followed by increased sympathetic activity to further increase heart rate (Fagraeus and Linnarsson, 1976). The ability to rapidly alter sympathetic and parasympathetic activity in response to changing physiological demands is essential to healthy function. This can be impaired in numerous conditions such as heart failure with detrimental results.

1.1.1 The sympathetic nervous system

The sympathetic nervous system is widely distributed throughout the body and innervates many organs including lungs, heart, blood vessels, sweat glands, erector pili muscles and many abdominal and pelvic organs. The cell bodies of sympathetic preganglionic neurones are located in the lateral horns of the spinal cord from the level of the first thoracic segment to the second lumbar segment (T1-L2). The small–diameter, myelinated axons of these neurones exit the spinal cord in the ventral nerve roots and enter the

ganglia of the sympathetic chain through the white rami communicantes (Gilbey and Spyer, 1993). The axons of preganglionic sympathetic neurones may travel rostrally or caudally in the sympathetic chain before synapsing with postganglionic sympathetic neurones (Gilbey and Spyer, 1993). Non-myelinated postganglionic sympathetic neurones exit the sympathetic chain through the grey rami communicantes and are distributed to their respective organs in branches of the spinal nerves or along the carotid arteries. Some preganglionic neurones pass through the sympathetic chain without synapsing and exit as the sympathetic splanchnic nerves. These project to the prevertebral ganglia including the coeliac, superior mesenteric and inferior mesenteric ganglia and then synapse with postganglionic neurones. Some of the preganglionic fibres in the greater splanchnic nerve project directly to the medulla of the adrenal gland (Strack et al., 1989). The cells of the adrenal medulla are modified postganglionic sympathetic neurones that secrete adrenaline and noradrenaline.

The neurotransmitters used in the sympathetic nervous system are acetylcholine and noradrenaline. Sympathetic preganglionic neurones release acetylcholine (ACh) which activates postganglionic sympathetic neurones. Postganglionic sympathetic neurones primarily use noradrenaline which acts on alpha or beta receptors of the effector organ. The exception is sympathetic innervation of sweat glands through cholinergic postganglionic sympathetic axons (Dale and Feldberg, 1934) which act on muscarinic receptors (Patton, 1948).

1.1.2 The parasympathetic nervous system

The parasympathetic nervous system is described as having a craniosacral outflow as preganglionic parasympathetic neurones are located in brainstem nuclei and sacral spinal cord segments (S2-4). The parasympathetic brainstem cranial nerve (CN) nuclei include the Erdinger-Westphal nucleus (CN III – oculomotor nerve), the superior and inferior salivatory nuclei (CN VII – facial nerve and CN IX – glossopharyngeal nerve), the dorsal vagal nucleus (DVN) and nucleus ambiguus (NA; both CN X – vagus nerve). The majority of cardiac preganglionic neurones are located in the NA as revealed

by retrograde labelling following injection of cholera-toxin conjugated to horse radish peroxidase into rat myocardium (Izzo et al., 1993). Extracellular recordings of cardiac preganglionic neurones in the NA of cats revealed that they fire in synchrony with the cardiac cycle (McAllen and Spyer, 1978). Simultaneously recording afferent baroreceptor activity from the carotid sinus nerve confirmed that this is due to baroreceptor input and synchrony could be abolished by compressing the carotid arteries and sectioning the aortic nerve (McAllen and Spyer, 1978). In contrast to the sympathetic nervous system, the axons of parasympathetic preganglionic neurones are much longer as parasympathetic ganglia are located close to the target organ, however, they also use ACh as a neurotransmitter. Postganglionic parasympathetic neurones are relatively short and use ACh as a neurotransmitter which acts on muscarinic receptors of the target tissue.

1.1.3 The enteric nervous system

The enteric nervous system is relatively independent, however, it does receive some sympathetic and parasympathetic input. It is composed of two nerve plexuses that extend the length of the gastrointestinal tract. The myenteric (Auerbach's) plexus between the circular and longitudinal muscle layers is responsible for co-ordinating muscle contraction. The submucosal (Meissner's) plexus lies between the circular muscle and mucosa and regulates secretion. The enteric nervous system is vital in regulating gastrointestinal function, however, as this is not the focus of this thesis it will not be described in detail.

1.1.4 Central control of cardiovascular autonomic function

Precise and dynamic regulation of the autonomic control of the cardiovascular system is essential for healthy function and to respond to changing physiological demands. To achieve this, afferent information from the cardiovascular system is integrated into central pathways which control autonomic outflow. The nucleus tractus solitarius (NTS), located in the dorsal medulla, is essential in processing cardiovascular reflexes and

maintaining cardiovascular homeostasis e.g. arterial baoreceptor reflex controlling blood pressure. The NTS is the principal recipient of cardiovascular receptor afferents conveying information from baroreceptors and chemoreceptors through the glossopharyngeal and vagus nerves. This was evidenced by retrograde neuronal tracing of the carotid sinus nerve and aortic depressor nerve in cats (Ciriello et al., 1981). The NTS also receives many other visceral and somatic inputs that influence cardiovascular function including afferents from skeletal muscles (Kalia et al., 1981) and kidneys (Weiss and Chowdhury, 1998).

The NTS also receives afferents from other autonomic centres both medullary (e.g. ventrolateral medulla and medullary raphe nuclei) and supramedullary (e.g. paraventricular nucleus [PVN], lateral hypothalamic nucleus, central nucleus of the amygdala and insular cortex) as demonstrated by neuronal tracing studies in rats (van der Kooy et al., 1984) (Ross et al., 1981) and rabbits (Schwaber et al., 1982; Gieroba et al., 1991). Furthermore, the NTS also projects to parts of the brain and spinal cord important for cardiovascular control including the lateral horn of the spinal cord, rostral and caudal ventrolateral medulla, NA, PVN and central nucleus of the amygdala and receives reciprocal projections from these areas too as demonstrated by anterograde and retrograde neuronal tracing in cats (Loewy and Burton, 1978) and rats (Ricardo and Koh, 1978; Ross et al., 1985). These numerous and reciprocal projections to the NTS allow the NTS to integrate and influence reflex autonomic control at the level of the brainstem and also allows higher centres to influence autonomic function.

In addition to the NTS, other brainstem areas important for autonomic control include the DVN, NA, caudal ventrolateral medulla (CVLM) and rostral ventrolateral medulla (RVLM). As mentioned in Section 1.1.2, the DVN and NA contain preganglionic parasympathetic neurones. The DVN lies ventral to the NTS and the NA lies dorsolateral to the inferior olivary nucleus. There are also sparse preganglionic parasympathetic neurones located in the reticular formation which lies between the DVN and NA (Izzo et al., 1993). The majority of cardiac preganglionic neurones are located in the NA which also contains motor neurones innervating the muscles of the pharynx and larynx. It is possible to differentiate between these two groups of

neurones electrophysiologically as cardiac neurones show pulse synchronous activity that is abolished by baroreceptor denervation (McAllen and Spyer, 1978).

The RVLM is vital in regulating blood pressure (Dampney, 1994) and has projections to the lateral horn of the spinal cord demonstrated by microinjecting the retrograde neuronal tracer horseradish peroxidase into the RVLM (Ross et al., 1984b). It was later shown that the RVLM projects directly to sympathetic preganglionic neurones in the spinal cord by combining anterograde tracing of RVLM projections with retrograde tracing of preganglionic neurones innervating the adrenal medulla and visualising the results using electron microscopy (Zagon and Smith, 1993). Electrical stimulation of the RVLM in anesthetised rats caused increased blood pressure and heart rate plus increased plasma noradrenaline levels. (Ross et al., 1984a). Microinjection of excitatory amino acids (L-glutamate and kainic acid) into the RVLM also increased blood pressure and heart rate whereas the inhibitory amino acid GABA decreased blood pressure and heart rate (Ross et al., 1984a). The RVLM is pivotal in maintaining blood pressure and lesioning of this area caused a decrease in resting blood pressure (Dampney and Moon, 1980) indicating that the RVLM has a tonic influence on vasoconstriction. The importance of different cell types in the RVLM is being investigated using optogenetics. By introducing a virus into the RVLM that expresses channel rhodopsin under the control of a specific promoter it is possible to selectively stimulate neurones using light. This elegant method has recently revealed that stimulating only catecholaminergic neurons within the NTS increased blood pressure and sympathetic nerve activity providing direct evidence that catecholaminergic neurones in the RVLM are sympathoexcitatory (Abbott et al., 2009).

The CVLM projects to the RVLM and is essential in regulating neuronal activity of the RVLM. Direct projections from the CVLM to sympathetic premotor neurones of the RVLM were demonstrated by combining injection of an anterograde neuronal tracer into the CVLM and a trans-ganglionic retrograde tracer into the adrenal medulla which revealed double labelled neurones in the RVLM (Li et al., 1992). Neurones in the CVLM have an inhibitory effect on the RVLM that can be elicited by

microinjecting glutamate into the CVLM causing a decrease in blood pressure (Blessing, 1988). This effect was abolished by injecting bicuculline (GABA antagonist) into the RVLM, indicating that the CVLM acts on the RVLM through a GABAergic pathway (Blessing, 1988). These robust findings could be enhanced through the use of optogenetics to directly stimulate CVLM neurones, however, this has not yet been performed.

The raphe nuclei, located in the midline of the medulla, project to both supramedullary areas of autonomic control (PVN) (Standish et al., 1995) and the lateral horn of the spinal cord (Allen and Cechetto, 1994; Standish et al., 1995) as revealed by retrograde neuronal tracing. The raphe nuclei also project to the ventral horn of the spinal cord and injections of neuronal tracer into lateral and ventral horns revealed double labelled neurones in the raphe nuclei (Allen and Cechetto, 1994). This suggests that the raphe nuclei may integrate autonomic and somatic motor activity. Stimulation of the raphe nuclei in cats caused both increases and decreases in blood pressure providing direct evidence of their involvement in autonomic regulation (McCall, 1984).

Supramedullary areas also play an important role in autonomic regulation. The hypothalamus is key in integrating neuroendocrine and autonomic functions whereas the insular cortex and the central nucleus of the amygdala co-ordinate emotional responses. The PVN of the hypothalamus has projections to the RVLM and preganglionic sympathetic neurones in the lateral horn of the spinal cord as demonstrated by injecting fluorescent retrograde tracers into these areas (Pyner and Coote, 2000). The PVN also projects to the DVN and NA (Portillo et al., 1998; Palkovits, 1999) and thereby influences parasympathetic activity. In addition to these diverse projections, the PVN is also neurochemically diverse containing GABAergic, glutamatergic, dopaminergic, oxytocinergic and vasopressinergic neurones (Pyner, 2009). This variety explains the conflicting results of stimulation studies of the PVN which caused either increases or decreases in blood pressure (Yang and Coote, 1998).

1.1.5 Reflex control of cardiovascular autonomic function

Reflex control of cardiovascular function permits tight regulation of the cardiovascular system and rapid responses to physiological challenges such as postural changes. The arterial baroreceptor reflex is a good example of reflex autonomic regulation to maintain blood pressure and prevent excessive perturbations. Baroreceptors are specialised stretch receptors located in the carotid sinus and aortic arch which project to the NTS through the vagus (aortic) and glossopharyngeal (carotid) nerves (Ciriello et al., 1981). An increase in blood pressure causes stretching of carotid and aortic arterial walls and increases baroreceptor firing. Through a glutamatergic projection from the NTS, this causes activation of the CVLM which inhibits the RVLM (Agarwal et al., 1990). This, in turn, decreases sympathetic outflow resulting in vasodilation to decrease blood pressure. The NTS also activates the NA to increase cardiac parasympathetic activity and decrease heart rate (Neff et al., 1998). Conversely, a decrease in blood pressure disinhibits the RVLM leading to an increase in sympathetic activity and vasoconstriction to preserve blood pressure (Minson et al., 1994).

In addition to the arterial baroreceptor reflex, cardiopulmonary reflexes mediated by low pressure stretch receptors in the heart, great vessels and lung vasculature modulate central sympathetic outflow (Mitchell and Victor, 1996; Minisi, 1998). Application of lower body negative pressure < -20 mmHg unloads cardiopulmonary baroreceptors by decreasing central venous pressure without altering arterial blood pressure, allowing the effects of cardiopulmonary reflexes to be studied (Jacobsen et al., 1993). Recording muscle sympathetic nerve activity (MSNA) during lower body negative pressure in healthy humans revealed increased burst frequency, incidence and amplitude (Vissing et al., 1989; Jacobsen et al., 1993). Activation of cardiopulmonary baroreceptors has also been investigated in humans using lower body *positive* pressure. Pressures of 10-20 mmHg caused an increase in atrial dimensions measured using echocardiography and this was accompanied by a decrease in MSNA (Fu et al., 1998). Vagal afferents are involved in this reflex as blocking the cervical vagus nerves in dogs by cooling to 0 – 2 ºC attenuated cardiopulmonary reflex responses (Bishop and Peterson, 1978). The cardiopulmonary reflex may influence the arterial

baroreflex particularly during exercise when the arterial baroreflex is reset. Using manoeuvres to increase central blood volume and hence load cardiopulmonary baroreceptors during exercise e.g. cycling in a supine position as opposed to upright, attenuated the increase in blood pressure and resetting of the baroreflex sensitivity curve (Ogoh et al., 2007).

The chemoreflex is also an important component in maintaining homeostasis and influences sympathetic activation. The peripheral chemoreceptors are located in the carotid bodies and respond most sensitively to hypoxia whereas central chemoreceptors in the brainstem respond to hypercapnia (Guyenet, 2000). Peripheral chemoreceptor afferents project to the NTS and activation of the chemoreflex causes increased blood pressure, bradycardia and tachypnoea. The increase in blood pressure, through increased sympathetic vasoconstrictor activity, is mediated by the RVLM and is abolished by bilateral microinjection of the glutamate receptor antagonist kynurenic acid into the RVLM (Koshiya et al., 1993).

Similarly, central chemoreceptor activation causes sympathoexcitation, increased blood pressure and tachypnoea, however, the mechanisms are unclear (Somers et al., 1989). Sympathetic nerve activity varies with respiration, first described in anaesthetised rabbits and anaesthetised or decerebrate cats (Adrian et al., 1932). Respiratory modulation of muscle sympathetic nerve activity has also been demonstrated in humans using microneurography, with a peak in activity at end-expiration and minimum activity at end-inspiration (Eckberg et al., 1985). The source of this respiration dependent modulation is unclear however, simultaneous extracellular recordings of barosensitive GABAergic CVLM neurones and phrenic nerve activity in the rat revealed a central respiratory drive of neuronal activity in the CVLM (Mandell and Schreihofer, 2006). GABAergic CVLM neurones inhibit RVLM neurones which also exhibit central respiratory drive activity that mirrors that of CVLM neurones and this could explain respiration dependent modulation of sympathetic activity (Mandell and Schreihofer, 2006). Independent of central respiratory drive, CNS PCO_2 also influences sympathetic activity (Guyenet et al., 2010). It is postulated that this could be mediated by neurones of the retrotrapezoid

nucleus that are sensitive to PCO_2 and project to the CVLM and RVLM (Rosin et al., 2006) in rats, however, there is currently no direct evidence of this pathway.

Metabolically sensitive muscle afferents (group III and IV afferents) activated by metabolites (e.g. lactate, ATP, pH) also influence cardiovascular autonomic control through the metaboreflex (Guyenet, 2006). This was first described in man by Alam and Smirk (1937) who reported that placing inflatable cuffs around exercising limbs to prevent blood flow and induce ischaemia increased blood pressure to a greater degree than exercise with unimpeded circulation. Furthermore, leaving the cuffs in place to preserve ischaemia after exercise had ceased maintained the increase in blood pressure. Recording MSNA during post-exercise ischaemia, induced by inflating arm cuffs after isometric handgrip exercise, revealed that in addition to a maintained increase in blood pressure, there was also a maintained increase in MSNA burst frequency and amplitude (Mark et al., 1985). Interestingly, heart rate was only elevated during exercise and decreased during post exercise ischaemia (Mark et al., 1985). There has been some debate as to the effects of the metaboreflex on the autonomic control of heart rate. Recently, the effects of metaboreflex activation on heart rate have been elucidated through the use of parasympathetic (glycopyrrolate) and beta-adrenergic (metoprolol or propanolol) blocking agents administered to healthy human volunteers who performed leg cycling and isometric handgrip exercises. Parasympathetic blockade augmented the elevation in heart rate during post exercise ischaemia induced after isometric handgrip exercise performed at 25% of maximal voluntary contraction but had no effect when this exercise was performed at 40% MVC (Fisher et al., 2010; Fisher et al., 2013). Parasympathetic blockade also had no effect on heart rate during post exercise ischaemia following leg cycling exercise (Fisher et al., 2013). Beta blockade abolished heart rate elevation following isometric handgrip exercise at 25% MVC and attenuated heart rate elevation following 40% MVC handgrip exercise and had no effect on heart rate elevation following leg cycling exercise. The increase in heart rate following greater effort (40% MVC isometric handgrip) or larger muscle mass (leg cycling exercise) appears to be mediated by increased cardiac sympathetic

activity with cardiac parasympathetic withdrawal whereas exercise at lower intensity (25% MVC isometric handgrip) causes a smaller elevation in heart rate which may be due to parasympathetic reactivation. It therefore requires larger exercising muscle mass or greater effort for the metaboreflex to overcome the reflex decrease in heart rate and parasympathetic activation that occurs after cessation of exercise.

1.2 Measuring cardiovascular autonomic function

The majority of techniques to measure autonomic function in humans are indirect due to the difficulty and invasiveness of direct recordings. This approach provides estimates of autonomic balance rather than directly quantifying sympathetic and parasympathetic activity.

1.2.1 Non-invasive estimates of autonomic function

1.2.1.1 Resting heart rate

Resting heart rate (RHR) is one of the simplest measures of sympathovagal balance and is usually 60-80 beats per minute (bpm). RHR can be much lower in trained athletes at 30 bpm and, conversely, can be as high as 100 bpm in sedentary individuals (Cook et al., 2006). High RHR (implying increased sympathetic activity and/or decreased parasympathetic activity) in healthy population studies was associated with an increased risk of mortality (Shaper et al., 1993; Jouven et al., 2005). A long term study of 5713 asymptomatic working men aged 42 to 53 years (Jouven et al., 2005) found that a RHR > 75 bpm resulted in a four-fold risk of sudden death compared to a RHR < 60 bpm. RHR is an indicator of the net contribution of the parasympathetic and sympathetic nervous systems to cardiac control.

1.2.1.2 Heart rate recovery

The decline in heart rate after exercise is another indication of autonomic cardiovascular regulation. The immediate heart rate recovery (HRR) after

exercise is mainly due to vagal activity as vagal blockade with atropine slows HRR in the 2 minute post exercise period (Imai, 1994). Studies investigating HRR in various populations have found that a low rate of recovery can be a strong predictor of mortality (Cole et al., 1999; Shetler et al., 2001; Jouven et al., 2005). A long term study of HRR in 2428 patients who had been referred for first time coronary angiography found that, after adjustments for age, sex, medication and resting heart rate etc, a HRR of ≤12 bpm in the first minute after exercise was a strong predictor of mortality (adjusted relative risk 2.0; $p < 0.001$) (Cole et al., 1999). This finding was confirmed by Shetler *et al*. (2001) who also examined HRR in 2193 patients referred for coronary angiography and reported that a HRR <22 bpm was predictive of a higher risk of mortality. This value differs from that given by Cole et al., however, Shetler et al. used a longer recovery time of 2 minutes after exercise. Of particular interest is a long term study of HRR in 5713 asymptomatic men (42-53 years) (Jouven et al., 2005) which reported that HRR < 25 bpm was a strong predictor of sudden death from myocardial infarction (MI) (relative risk, 2.2).

1.2.1.3 Baroreflex sensitivity

Baroreflex sensitivity (BRS) can be investigated through the change in heart rate in response to a change in blood pressure (BP). There are a number of techniques to measure BRS in the laboratory, however, some are invasive e.g. using drugs (phenylephrine/nitroprusside) to produce transient changes in blood pressure (Parati et al., 2000; La Rovere et al., 2008). Traditionally, measurements of BRS utilised intra-arterial monitoring of blood pressure, however, a strong correlation was found between measurements using this technique and non-invasive BP measurement using photoplethysmography (Pinna et al., 2000). This offers an easily implemented technique to measure BRS.

A non-pharmacological alternative to induce changes in BP is to use the Valsalva manoeuvre. This requires forced expiration (straining) either against a closed glottis or resistance such as blowing into a tube connected to a manometer (approximately 40 mmHg). The changes in blood pressure

and heart rate during the Valsalva manoeuvre can be divided into 4 phases (Freeman, 2006). In phase I, at the onset of straining, there is a transient increase in blood pressure and a fall in heart rate. This is a result of the increase in intra-thoracic pressure compressing the aorta and forcing blood into the peripheral circulation. In phase II (during straining) there is a decrease in blood pressure due to impaired venous return. This results in sympathetic activation with tachycardia and vasoconstriction (Parati et al., 2000; La Rovere et al., 2008). At the end of straining (phase III) there is a decrease in blood pressure and an increase in heart rate due to the release of intra-thoracic pressure. This is followed by an increase in blood pressure above baseline values (overshoot, phase IV) due to unimpaired venous return and residual vasoconstriction. This leads to parasympathetic activation to reduce heart rate and thereby decrease blood pressure (Parati et al., 2000; La Rovere et al., 2008). The Valsalva manoeuvre can be considered a natural challenge to the baroreceptors compared to pharmacological methods, however, it can be difficult to perform. The neck chamber technique, which can apply positive or negative pressure to the neck leading to deactivation or activation of the baroreceptors, is better tolerated although the equipment is expensive (Parati et al., 2000; La Rovere et al., 2008). Alternatively, spontaneous changes in blood pressure can be used to assess BRS and are arguably more physiological requiring no interventions.

There are two main approaches to measuring spontaneous BRS – the sequence method and spectral methods. The sequence method identifies sequences of 3 or more heart beats where increases or decreases in systolic blood pressure are followed by lengthening or shortening of inter-beat intervals measured between the R peaks of the ECG (R-R intervals) (Parati et al., 2000; La Rovere et al., 2008). Spectral methods use a transfer function between oscillations in systolic blood pressure and R-R interval in the same frequency band to estimate BRS (Parati et al., 2000; La Rovere et al., 2008). The benefits of both these techniques are that they are simple, non-invasive and inexpensive, however they provide estimates of BRS rather than direct measurements.

Impaired BRS (<3 ms/mmHg) has been shown by the ATRAMI (Autonomic Tone and Reflexes After Myocardial Infarction) study to be an independent predictor of mortality (La Rovere et al., 1998). This was demonstrated by measuring BRS using the phenylephrine technique in almost 1300 patients after a recent MI (< 28 days). Impaired BRS, using non-invasive methods of BRS measurement, has also been shown to be a significant risk factor (Pinna et al., 2005), however, this technique is limited as it cannot be applied if ectopic beats (e.g. premature ventricular complexes) occur.

1.2.1.4 Heart rate turbulence

Heart rate turbulence (HRT) takes advantage of the oscillations in heart rate that occur during an ectopic heart beat and may provide an alternative to BRS in subjects prone to ectopic heart beats. There is an initial acceleration in heart rate preceding an ectopic beat followed by a deceleration. The percentage difference between heart rate deceleration and acceleration is measured as the turbulence onset (TO). There is also a fall in systolic blood pressure following an ectopic beat due, in part, to the short diastolic filling time (Bauer et al., 2010). Through the baroreflex, this leads to vagal withdrawal and sympathetic activation which can be measured as changes in the intervals between heart beats (R-R intervals) plotted as a linear regression (turbulence slope - TS) (Bauer et al., 2010). Impaired HRT has been shown to be an independent risk factor post MI, however, 17-19% of patients were excluded from this study due to atrial fibrillation or absence of ectopic beats (Schmidt et al., 1999).

1.2.1.5 Heart rate variability

Normal heart rate varies beat to beat and is a reflection of both parasympathetic and sympathetic input to the sinoatrial node in response to a variety of factors e.g. baroreceptors, circadian rhythm, renin-angiotensin system, respiration and exercise. There are two main methods to analyse heart rate variability (HRV) either using time domain measures or frequency domain measures.

Time domain measures are perhaps the simplest as they comprise simple statistical and geometric analyses of R-R intervals (also termed normal-normal [N-N] intervals). Time domain measures are recommended for long term (24 hour) ECG recordings and include SDNN (the standard deviation of all N-N intervals) which is a measure of overall HRV; SDANN (the standard deviation of the averages of N-N intervals in all 5 min segments of the entire recording) which is a measure of long-term components of HRV and RMSSD (the square root of the mean of the sum of the squares of differences between adjacent N-N intervals) and pNN50 (number of pairs of adjacent N-N intervals differing by > 50 ms) which are measures of short term components of HRV such that a higher value indicates increased vagal modulation (Task Force of the European Society of Cardiology and the North American Society of Pacing Electrophysiology, 1996).

Frequency domain measures use power spectral analysis to describe how variance (power) distributes as a function of frequency. There are a number of algorithms that can be used in frequency analysis but the Fast Fourier Transform (FFT) is most commonly used. The components of HRV spectral analysis are: very low frequency (< 0.04 Hz; VLF); low frequency (0.04-0.15 Hz; LF) and high frequency at (0.15-0.4 Hz; HF). Studies have shown that the HF component of HRV is related to rapid variations in heart rate associated with respiratory sinus arrhythmia mediated by vagal activity (Malliani, 2005). Akselrod et al. (1981) investigated the effect of parasympathetic and sympathetic blockade (using glycopyrrolate and propanolol respectively) on HRV in conscious dogs. Parasympathetic blockade abolished the HF component of HRV whereas sympathetic blockade had little effect confirming that HF is a reflection of parasympathetic activity. Interpretation of the LF component has been subject to debate. Some claim the LF component is a reflection of sympathetic activity only (Malliani, 2005) whereas others believe that both sympathetic and parasympathetic activity contribute to the LF component (Akselrod et al., 1981). Studies have shown that sympathetic blockade with propanolol significantly decreased the LF component in rats but did not completely abolish it (Aubert et al., 1999). Parasympathetic blockade with

atropine, as well as completely abolishing the HF component, also reduced LF power indicating that both sympathetic and parasympathetic activity contribute to the LF component (Aubert et al., 1999; Lahiri et al., 2008). It is agreed that the relative ratio of LF/HF is a reflection of sympathovagal balance (Task Force of the European Society of Cardiology and the North American Society of Pacing Electrophysiology, 1996). This means that HRV analysis provides information about the degree of autonomic modulation rather than the level of autonomic tone. Kleiger et al. (1987) first demonstrated that reduced HRV, indicating reduced parasympathetic influence on heart rate, was a risk factor by investigating the standard deviation of R-R intervals of patients post MI. This revealed a 5.3 times greater risk of mortality in patients with SD of R-R intervals (SDNN) < 50 ms compared to those with a SDNN >100 ms.

1.2.2 Invasive measures of autonomic function

Indirect measures of autonomic function such as those described above are useful, however, they are an estimate of autonomic function. There is no method to directly measure parasympathetic activity in humans, however, it is possible to directly assess sympathetic activity. The development of radiotracer-derived measurements of noradrenaline spill-over to plasma and microneurography revolutionised investigations of sympathetic activity (Engleman et al., 1968; Hagbarth and Vallbo, 1968).

1.2.2.1 Plasma noradrenaline levels

Noradrenaline is the neurotransmitter released by sympathetic adrenergic nerve endings. Most noradrenaline is removed by reuptake mechanisms, however, approximately 20% diffuses into plasma (Esler et al., 1985). Although only a little of the noradrenaline released enters the circulation, perturbations that increase sympathetic nerve activity such as exercise cause a prompt increase in plasma noradrenaline indicating that it may be used as a measure of sympathetic activity (Cohn et al., 1984). The first approach to measure systemic noradrenaline levels as an insight to

sympathetic function analysed urinary levels of excreted noradrenaline. (Chidsey et al., 1965). Urinary noradrenaline originates from the filtration of plasma noradrenaline at the glomerulus and noradrenaline released by renal sympathetic nerves within the kidney. This technique may therefore be biased by renal sympathetic activation. Noradrenaline levels in plasma were also measured from blood samples as an indicator of systemic sympathetic activity (Cohn et al., 1984). Both urinary and plasma measures of noradrenaline levels are crude, depending on the rate of noradrenaline reuptake and clearance from circulation. Measuring noradrenaline spill-over using a radiotracer derivative is a more accurate measurement of sympathetic activity. This requires infusion of radiolabelled noradrenaline into a peripheral vein until a steady state is achieved (approx. 90 mins). Blood samples are then taken from another vein and the proportion of endogenous noradrenaline to infused radiolabelled noradrenaline is used as a measure of noradrenaline spill-over into plasma (Esler et al., 1985). This technique has been further refined by placing catheters into the venous drainage of specific organs to measure regional differences in noradrenaline spill-over and thereby sympathetic activity. This is a powerful but highly invasive technique e.g. cardiac noradrenaline spill-over is measured by placing the catheter into the cardiac sinus. A less invasive method to assess sympathetic activity is microneurography.

1.2.2.2 Microneurography

Microneurography was pioneered in the late 1960s and involves inserting a tungsten microelectrode into a superficial nerve, commonly the peroneal (fibular) nerve as it courses round the neck of the fibula (Hagbarth and Vallbo, 1968). Microneurography allows the direct recording of sympathetic nerve activity to intramuscular blood vessels (Hilz and Dutsch, 2006; Charkoudian and Rabbitts, 2009). Initially, this technique was used to record bursts of muscle sympathetic nerve activity (MSNA), however, this was subsequently refined to permit the recording of individual vasoconstrictor neurones (single units) (Macefield et al., 1994).

Muscle sympathetic nerve activity is closely related to blood pressure and the arterial baroreflex. Negative feedback through the baroreflex results in bursts in MSNA that are pulse synchronous. Indeed, bilateral baroreceptor deactivation through injection of lidocaine around the vagus and glossopharyngeal nerves in the neck abolishes the rhythmicity of MSNA in humans (Fagius et al., 1985). This technique is rather crude as anaesthetic was applied to these nerves as they exited the skull thereby affecting other afferent information e.g. from cardiopulmonary receptors, however, animal studies confirm that complete baroreceptor denervation abolishes pulse synchronous muscle sympathetic nerve activity (Gebber, 1980). Further confirmation of the importance of the baroreflex in the regulation of sympathetic nerve activity comes from stimulation of the carotid sinus nerve which led to a decrease in MSNA activity (Wallin et al., 1975). Microneurography has contributed much to the study of sympathetic activity, however, its application is restricted to superficial peripheral nerves and, unlike regional noradrenaline spill-over studies, cannot provide information on regional variations in sympathetic activity. Combining both invasive and non-invasive measures of autonomic function has led to the appreciation of the role of sympathetic activation in many conditions including heart failure.

1.2.3 Sympathoexcitation and heart failure

Heart failure is a complex syndrome arising from an abnormality in heart structure or function at rest that prevents the heart supplying adequate levels of oxygen to body tissues (Members et al., 2012). Heart failure is characterised by neurohumoral activation involving activation of the sympathetic nervous system and renin angiotensin system. Initially, this is compensatory for the initial abnormality, however, chronic sympathetic activation is cardiotoxic and leads to the progression of heart failure. The symptoms of heart failure are assessed using the New York Heart Association (NYHA) classification (I-IV). Patients in class I are asymptomatic, however, as heart failure progresses symptoms worsen. Patients in class II and III experience fatigue and dyspnoea that limits physical activity and patients in class IV experience dyspnoea even at rest.

Heart failure is a leading cause of mortality and it is estimated that 45-60% of patients die within 5 years of diagnosis (Bui et al., 2011).

The first evidence of sympathetic activation in heart failure came from analysis of catecholamine excretion in urine from heart failure patients (n = 110) compared to normal subjects (n = 13) (Chidsey et al., 1965). Noradrenaline excretion was significantly higher in NYHA class III and IV heart failure patients (46.4 and 58.1µg/day respectively) compared to normal subjects and combined class I and II heart failure patients (22.5 and 22.4 µg/day respectively). This finding may reflect the renal sympathetic activation that occurs in heart failure patients (Petersson et al., 2005).

Increased sympathetic activity in heart failure was confirmed by measuring plasma noradrenaline levels which were significantly elevated compared to control subjects and correlated with the severity of heart failure (Thomas and Marks, 1978). The more refined method of regional noradrenaline spill-over revealed not only regional differences in the degree of sympathetic activation in heart failure but also an impairment in noradrenaline reuptake. This may lead to overestimation of sympathoexcitation, however, cardiac noradrenaline spill-over was markedly higher compared to plasma and renal (Hasking et al., 1986). Importantly, the degree of sympthoexcitation in heart failure, measured using plasma noradrenaline, is correlated with an increased risk of mortality (Cohn et al., 1984; Kaye et al., 1995)

Further direct evidence of sympathoexcitation in heart failure comes from microneurography studies. The frequency of both MSNA bursts and single units was elevated in heart failure patients (n = 8) (Macefield et al., 1999). Additionally, high levels of MSNA (> 49 bursts/ min) were associated with a significantly lower survival rate in heart failure patients followed up for 1 year (n = 122) (Barretto et al., 2009).

Heart rate variability is impaired in heart failure and indicates a shift in autonomic balance towards sympathetic predominance. The UK Heart Failure Evaluation and Assessment of Risk Trial (UK-HEART) (Nolan et al., 1998) was the first large prospective study of HRV in heart failure and its potential in identifying patients with an increased risk of death. 433

outpatients with chronic heart failure were recruited and HRV was analysed using time domain measures. Patients with a standard deviation of all N-N intervals (SDNN) of < 50 ms had a mortality rate of 51.4% compared to 5.5% of patients with SDNN > 100 ms. This is similar to findings of studies investigating predictors of post myocardial infarction prognosis (Kleiger et al., 1987; La Rovere et al., 1998).

Baroreflex sensitivity is another index of impaired autonomic function in heart failure. Both phenylephrine and non-invasive techniques have shown that BRS is an indicator of prognosis in heart failure. The phenylephrine method of BRS determination revealed that BRS of < 3 ms/mmHg was an independent predictor of sudden death or cardiac transplantation in heart failure patients (n = 282) (Mortara et al., 1997). Impaired BRS using the spectral method (<3.1 ms/mmHg) was also associated with a significantly higher risk of cardiac death (n = 228) (Pinna et al., 2005).

Overall, these disparate methods of assessing autonomic function illustrate that increased sympathetic activity is associated with poor prognosis in heart failure. To combat this, pharmaceutical interventions have targeted the sympathoexcitation underlying the pathophysiology of heart failure in order to alleviate symptoms and slow disease progression.

1.2.4 Treatment of heart failure

Current drug treatments for heart failure include beta blockers to combat the effects of sympathoexcitation. Lampert *et al*. (Lampert et al., 2003) investigated the effect of the beta blocker propanolol on HRV indices in 88 patients hospitalised with acute MI. After 6 weeks, patients treated with propanolol had a significant decrease in LF/HF ratio compared to patients given a placebo (n= 96) indicating an improvement in sympathovagal balance toward parasympathetic predominance. There was also an improvement in outcome with a decreased incidence of death, MI or congestive heart failure in propanolol treated patients (9% propanolol vs 23% placebo; p = 0.02). Carvedilol, another beta blocker, increased BRS and the HF frequency component of HRV and reduced cardiac

noradrenaline spill-over in heart failure patients treated for 4 months (n = 10). This indicates that beta blocker therapy may also augment vagal activity through the withdrawal of the inhibitory effects of noradrenaline (Kubo et al., 2005).

Despite the availability of treatments such as beta blockers, ambulant outpatients with heart failure have an annual mortality rate of 10% (Nolan et al., 1998) therefore the development of new therapies is essential. Recently, increasing parasympathetic activity has become a target in the treatment of heart failure.

1.2.3 The parasympathetic nervous system and heart failure

Heart failure is not only characterised by sympathoexcitation but also parasympathetic/vagal withdrawal (Triposkiadis et al., 2009; Sabbah et al., 2011a). Eckberg et al. (1971) first demonstrated impaired parasympathetic activity in heart failure patients compared to normal subjects as an impaired increase in heart rate in response to parasympathetic blockade using atropine. Heart rate increased by 55% in healthy subjects compared to 23% in heart failure patients. Vagally mediated heart rate recovery after exercise is attenuated in heart failure patients (Imai, 1994) and the magnitude of the increase in heart rate to parasympathetic blockade was attenuated in a canine model of heart failure compared to controls (Dunlap et al., 2003). Modulation of the parasympathetic nervous system e.g. vagus nerve stimulation therefore presents a new approach to altering the underlying autonomic imbalance in heart failure.

1.3 Neuroanatomy of the vagus nerve

The vagus nerves are the tenth and longest pair of cranial nerves and originate from the medulla. The vagus nerve is appropriately named as it derives from the Latin for ‘wanderer’. The vagus nerves exit the skull through the jugular foramina of the skull then pass through the neck and thorax to reach the abdomen. The vagus nerve is normally regarded as the main

parasympathetic output of the autonomic nervous system, however, it has many diverse functions. Indeed, counts of afferent and efferent fibres in silver preparations of feline cervical vagus nerve sections (n = 11) revealed that it is composed of 80% afferent fibres and only 20% efferent fibres (DuBois and Foley, 1936).

The afferent component of the vagus nerve consists of general visceral, special visceral and general somatic afferent fibres. The general visceral afferent fibres carry sensory information from thoracic and abdominal internal organs including the larynx, trachea, lungs, heart and gastrointestinal tract up to the splenic flexure of the colon (Figure 1.1). There is also some evidence of vagal afferents from the uterus (Berthoud and Neuhuber, 2000). The vagus nerve also carries baroreceptor and chemoreceptor information from the aortic arch. Special visceral afferent vagal fibres carry taste information from taste buds on the epiglottis. The cell bodies of visceral afferent sensory neurones reside in the nodose ganglion of the vagus nerve and project to the NTS through which they play an important part in the reflex control of respiration, blood pressure, heart rate, swallowing and digestion (Ruffoli et al., 2011). Unlike visceral afferent neurones, the cell bodies of the somatic afferent neurones are located in the jugular ganglion and project to the spinal trigeminal nucleus. The vagus nerve carries somatic sensory information from the lower pharynx, larynx, trachea, oesophagus, posterior dura mater and parts of the external ear (Figure 1.1). Interestingly, there are reports of activation of visceral reflexes in response to stimulation of the ear e.g. ear syringing can evoke cough or even bradycardia responses in a small percentage of the population (Prasad, 1984; Boghossian et al., 2010) indicating that the neuroanatomy of the vagus nerve is not fully understood and requires further investigation.

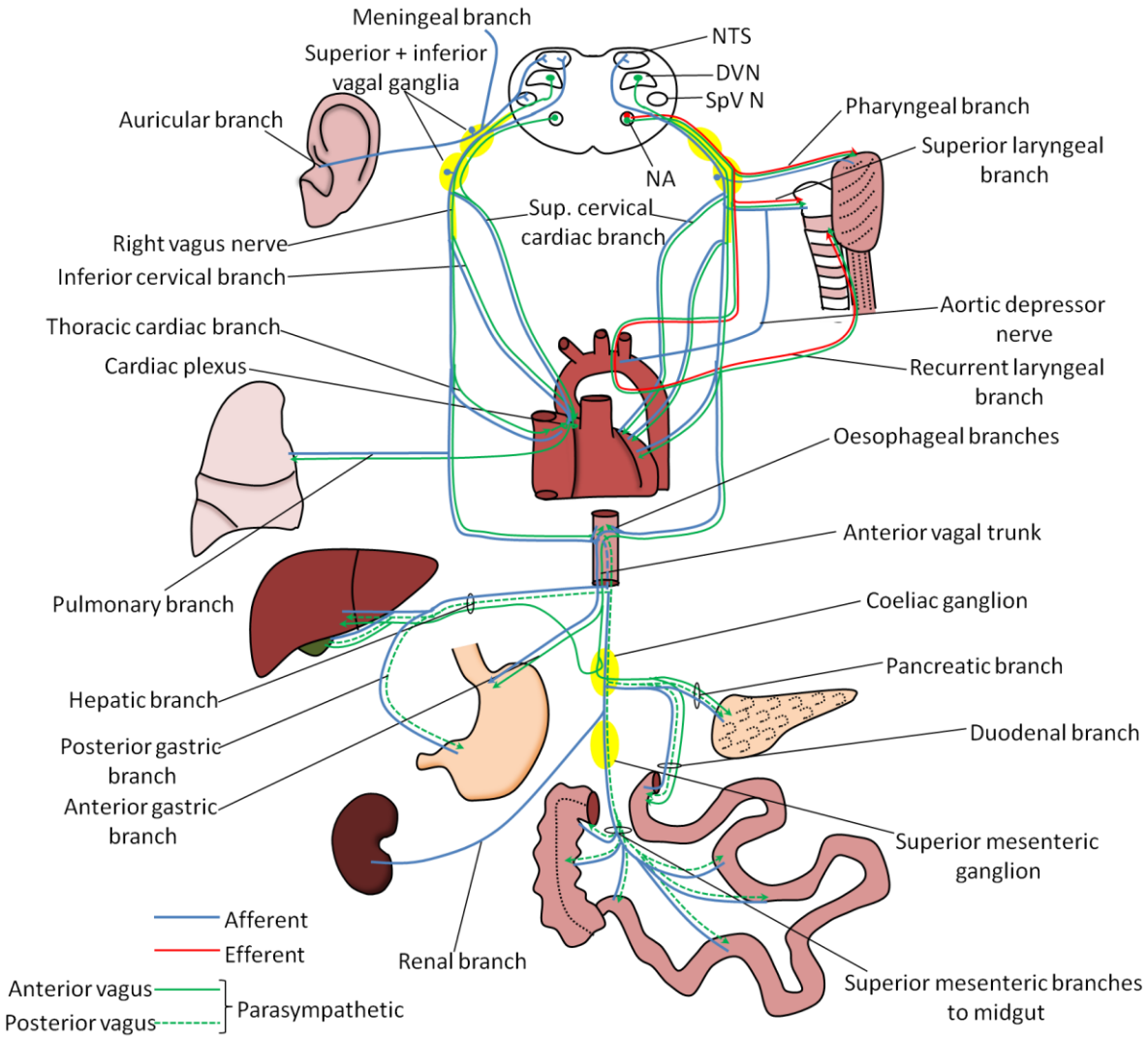


Figure 1.1 Representation of the distribution and central connections of the vagus nerve. All cervical and thoracic branches are bilateral and may have been omitted for clarity. The right recurrent laryngeal nerve (not shown) passes round the right subclavian artery instead of the arch of the aorta. Left and right vagus nerves form the oesophageal plexus and then become anterior and posterior trunks as they pass through the diaphragm to supply the abdominal viscera up to 2/3 along the transverse colon. NTS- nucleus tractus solitarius; DVN- dorsal vagal nucleus; SpV N- spinal trigeminal nucleus; NA- nucleus ambiguus.

The efferent component of the vagus nerve may be smaller but is no less vital. It consists of general and special visceral efferent components. The general visceral efferent component consists of preganglionic parasympathetic neurones that originate primarily in the DVN. These synapse in parasympathetic ganglia close to or in the walls of the organs they supply which are the organs of the thoracic and abdominal cavities including the gastrointestinal tract up to the splenic flexure of the colon. There is an additional contribution of preganglionic fibres from the NA supplying the heart. The vagus nerve has a special visceral efferent component that supplies the muscles of the 4th branchial pouch in the embryo. Neuronal tracing studies found that these motor neurones originated in the NA and supplied the striate muscle of the palate, larynx, pharynx (except stylopharyngeus) and the upper oesophagus (Bieger and Hopkins, 1987; Kitamura et al., 1991).

The importance of the vagus nerve in relaying sensory information that influences the regulation of internal organs and its extensive distribution throughout the body means it is little wonder that modulation of vagal activity is being investigated as a possible therapy for a wide range of conditions.

1.3.1 Vagus nerve stimulation

Stimulation of the vagus nerve was first pioneered as a therapy for epilepsy over 100 years ago and is now an approved therapy for treatment resistant epilepsy. Vagus nerve stimulation (VNS) is also an approved therapy for treatment resistant depression in the USA and is being investigated in a range of other conditions including heart failure, tinnitus, obesity and inflammatory conditions (Clancy et al., 2013).

1.3.1.1 Vagus nerve stimulation for epilepsy

Epilepsy is one of the most common neurological disorders and is estimated to affect 70 million people worldwide. Epilepsy encompasses a wide number of syndromes characterised by recurrent seizures. The mechanisms of epileptogenesis are not fully understood, however, an underlying feature is

hyperexcitability of the cortex (Badawy et al., 2009). Approximately 30% of epilepsy patients have refractory epilepsy or unacceptable side effects from anti-epileptic drug treatment (Connor et al., 2012). Resective surgery is an alternative approach for treating patients with partial epilepsy where the epileptogenic focus can be located and removed (Connor et al., 2012). Refractory epilepsy patients who are not candidates for resection or whose epilepsy is still uncontrolled after surgery rely on palliative interventions including corpus callostomy, ketogenic diet and vagus nerve stimulation (Beleza, 2009).

Electrical stimulation of the vagus nerve to treat epilepsy was first proposed in 1880s by Corning, an American neurologist. It was believed that cerebral hyperaemia was responsible for epilepsy, therefore, Corning attempted to reduce blood flow to the brain by compressing the carotid arteries and reducing cardiac output through transcutaneous electrical stimulation of the cervical vagus nerves (Corning, 1884). However, direct electrical stimulation of the central end of the cervical vagus nerve was later shown to reduce strychnine induced seizures in cats (Schweitzer and Wright, 1937). Further, electrical vagal stimulation desynchronised EEG activity in spinally transected (C1), aortic baroreceptor-denervated anaesthetised cats and reduced strychnine induced epileptic like activity in the cortex (Zanchetti et al., 1952). This demonstrated that VNS could modulate cortical activity independent of cardiovascular alterations and was dependent on vagal afferent stimulation.

The first human studies of cervical VNS in epilepsy were conducted in the late 1980s (Penry and Dean, 1990; Terry et al., 1990). An electrode was wrapped around the left cervical vagus and connected by a lead to a pulse generator embedded subcutaneously below the clavicle. The generator can subsequently be programmed non-invasively to alter stimulation parameters. VNS is generally well tolerated and side effects (hoarseness, coughing, pain etc.) tend to be mild and transient. VNS has been used to treat over 50,000 patients with refractory partial epilepsy and long term trials have shown VNS to be effective in reducing seizure frequency by 50% or more in approximately 40-50% of patients (Shahwan et al., 2009).

The mechanism by which VNS alleviates epilepsy symptoms is poorly understood. The original hypothesis was that stimulation of vagal afferents could alter the excitability of the cortex through relays in the NTS. The vagus nerve is composed of myelinated A- and B-fibres and unmyelinated C-fibres which have different activation thresholds. A-fibres have the lowest threshold (0.02-0.2mA), followed by B-fibres (0.4-0.6mA) and C-fibres require the highest stimulation parameters for activation (>2mA) (Erlanger and Gasser, 1930). Therapeutic VNS parameters vary but are below levels required to activate C-fibres suggesting the anti-epileptic effects of VNS are mediated through A- and B-fibres (Ruffoli et al., 2011). Indeed, destruction of C-fibres through administration of capsaicin in rats did not affect VNS reduction of pentylenetetrazol (PTZ) induced seizures further supporting that C-fibres are not involved in the anti-epileptic mechanism of VNS (Krahl et al., 2001). This study was carried out using anaesthetised rats and may not apply to conscious animals. In addition, there is no selective method to destroy A- and B-fibres therefore the importance of different fibre types in VNS therapeutic effects requires further investigation.

It has been suggested that VNS may alter neurotransmitter levels or processing in the brain. GABA is the major inhibitory neurotransmitter of the central nervous system and could play an important part in seizure reduction. Marrosu et al. (2003) investigated the possible role of GABA receptors in the anti-epileptic effects of VNS using single photon emission computed tomography (SPECT) to image $GABA_A$ receptor density before VNS and after 1 year of VNS therapy in 10 patients with refractory partial epilepsy. A significant correlation between reduced seizure frequency and increased $GABA_A$ receptor density suggested that increased GABA mediated inhibition is an important component of the VNS mechanism.

Noradrenergic and serotonergic signalling are also altered by VNS and may influence epileptic activity (Dorr and Debonnel, 2006). The effects of VNS on locus coeruleus (LC) noradrenergic neurones and dorsal raphe serotonergic neurones basal firing rates were recorded extracellularly *in vivo* (rats). Long term (2 weeks to 3 months) VNS increased firing rates of both LC noradrenergic neurones and dorsal raphe serotonergic neurones (Dorr and Debonnel, 2006) which in turn influence the function of many higher

CNS regions (Fornai et al., 2011). Both chronic and acute LC lesions significantly reduced the anti-seizure effects of VNS in rats (Krahl et al., 1998). Another hypothesis is that VNS acts to reduce inflammation associated with epilepsy (De Herdt et al., 2009). It therefore appears likely that the anti-epileptic effects of VNS are polymodal and much more work is required before the mechanisms involved are fully understood.

1.3.1.2 Vagus nerve stimulation as a treatment for depression

Depression is a disabling condition and it is estimated that 30-45% of major depressive disorder (MDD) patients are treatment resistant (Schosser et al., 2012). Vagus nerve stimulation is also approved by the US Food for Drug Adminstration (FDA) for MDD treatment. Anti-depressive effects of VNS were first observed in patients with refractory epilepsy. Improvement in mood was observed even in those patients who experienced little or no change in seizure frequency (Harden et al., 2000). VNS for treatment resistant depression has been somewhat controversial due to the lack of controlled studies of its efficacy in depression (Martin and Martín-Sánchez, 2012). In one randomised controlled trial of VNS in patients with depression, VNS performed no better than placebo (Rush et al., 2005a). However, this trial only lasted 10 weeks and VNS effects displayed increasing response rates at 3, 6, 9 and 12 months suggesting that it may be a long term adjunctive therapy rather than an acute treatment (Rush et al., 2005b).

1.3.1.3 Vagus nerve stimulation as an anti-inflammatory therapy

The vagus nerve is a critical component of the inflammatory reflex. This reflex has received much attention recently and parts of the pathway involved have been elucidated. The afferent arm of the reflex is the vagus nerve which is activated by pro-inflammatory cytokines (Tracey, 2009). The central pathway is unknown, however, as the vagus nerve projects to the NTS which in turn projects to the hypothalamus this is thought to be key in mediating the efferent response which is also through the vagus nerve. The efferent arm of this reflex is debated. Stimulation of the vagus nerve increases acetylcholine (ACh) release from ACh synthesising T cells in the

spleen (Rosas-Ballina et al., 2011). This in turn acts on the α7 nicotinic acetylcholine receptor expressed by macrophages to reduce cytokine release. Vagus nerve stimulation can reduce pro-inflammatory cytokine release and tissue injury in models of inflammatory diseases (Borovikova et al., 2000). This led to the suggestion that efferent vagal fibres may synapse with splenic nerves in the coeliac ganglion and thereby influence acetylcholine production in the spleen (Rosas-Ballina et al., 2011). However, electrophysiological experiments in the rat revealed that stimulation of vagal efferents had no effect on the splenic nerve activity (Bratton et al., 2012). Furthermore, combining retrograde tracing of splenic nerve fibres and anterograde tracing of vagal efferents revealed no putative synaptic contacts using confocal analysis (Bratton et al., 2012). This provides strong evidence that the anti-inflammatory effects of VNS are not mediated through the splenic nerve and require further investigation.

Whilst the mechanisms underlying the anti-inflammatory reflex are unknown, this reflex is a potential therapeutic target in many conditions in which excessive inflammation plays a role in tissue damage, including heart failure. Indeed, VNS attenuated the increase in plasma C reactive protein (a marker of systemic inflammation) compared to the control group in a canine model of heart failure (Zhang et al., 2009b).

1.3.1.4 Vagus nerve stimulation to reverse pathological remodelling in tinnitus

The potential neuroplastic effects of VNS through alterations in CNS neurotransmitter levels and/or processing have led to considering VNS as a potential therapy for tinnitus. Tinnitus is the perception of ‘ringing in the ear’ despite the absence of an external sound. Persistent tinnitus affects approximately 10-15% of the population over their lifetime and can impair the ability to concentrate, follow conversations or it can even disturb sleep (Baigi, 2011; Schnupp, 2011). Tinnitus is usually caused by excessive noise exposure that leads to pathological plasticity of the auditory cortex. Engineer et al. (2011) investigated a potential method to reverse this process in a rat model of tinnitus by playing tones (outside the putative tinnitus frequencies)

and simultaneously stimulating the left cervical vagus nerve to induce and target plasticity. After 10 days of VNS paired with tones, noise exposed rats significantly improved in detecting a gap in presented background noise compared to sham treated rats. This improvement persisted for the 3 weeks tested post-treatment. A neurophysiological correlate was determined since tinnitus-induced increases in auditory cortical responses to tone presentation were almost reversed by VNS. These results have led to a clinical pilot study of VNS paired with tones in patients with tinnitus (n = 12) (Vanneste et al., 2012). 4 weeks of treatment resulted in improved symptoms indicating that this may be an effective therapy for tinnitus. The potential for VNS to induce cortical plasticity is very exciting and could be exploited in the treatment of many conditions. Indeed, pairing VNS with movement increased motor cortical representation of the movements (Porter et al., 2011). This could potentially be used to treat movement disorders including the rehabilitation of stroke patients.

1.3.1.5 Obesity and vagus nerve stimulation

Weight loss has been observed in patients receiving VNS therapy for epilepsy and may be a welcome side effect for some patients (Burneo et al., 2002). This observation was also made in a group of patients receiving VNS for refractory depression. Furthermore, the degree of weight loss correlated with baseline BMI (Pardo et al., 2007). This has led to the investigation of VNS as a potential strategy to tackle obesity. The vagus nerve plays an important role in satiety, signalling gastric distension and the release of gastrointestinal hormones such as cholecystokinin and leptin (Berthoud, 2008). The vagus nerve therefore offers a potential target to modify appetite, however, results are conflicting. Another study investigating the effects of VNS on weight loss in epilepsy patients found no significant effect (Koren and Holmes, 2006). These patients were followed up for 2 years after VNS therapy commenced, similar to the other studies, however baseline BMI information is not provided. Since the degree of weight loss is dependent on BMI (Pardo et al., 2007) then it may be that the participants recruited in this study were already under the threshold for a significant effect of VNS.

There has been no dedicated study of the effects of VNS on weight loss in obese humans, however, animal studies report positive results. Stimulation of the left subdiaphragmatic vagus nerve reduced food intake and weight gain in rats fed a high fat diet (Gil et al., 2011), however, this is not a suitable model for weight loss in obesity. To combat this, Val-Laillet et al. (2010) utilised obese mini-pigs (n = 8) to study the effects of bilateral stimulation of the thoracic vagus nerves. VNS did not cause weight loss, however, weight gain was significantly lower in VNS animals compared to sham from 5 weeks post operatively until the end of the study (14 weeks). In addition, VNS animals consumed significantly less compared to sham animals. Intriguingly, VNS also altered the food preferences of treated animals with a diminished preference for sweet food (Val-Laillet et al., 2010). These results are encouraging, however, the sample numbers used in the animal studies are small. Larger studies are required to confirm these findings before VNS can be investigated in obese humans.

1.3.1.5 Vagus nerve stimulation as a potential heart failure therapy

Early support of VNS in heart failure came from animal studies. In a canine model of heart failure (healed MI) there was a significant reduction in the number of dogs with ventricular fibrillation during exercise and coronary artery occlusion in the group that received VNS compared to the control group (10% vs. 92%) (Vanoli et al., 1991). This striking result indicates that vagal stimulation is protective against ventricular fibrillation. Indeed, when exercise was repeated in the VNS group with the stimulators switched off, 89% developed ventricular fibrillation. VNS also improved survival in a rat model of chronic heart failure. Compared to the control heart failure group, the vagal stimulation heart failure group had improved left ventricular function, decreased biventricular weight and a 73% reduction in relative risk ratio of death (Li et al., 2004). Interestingly, the VNS treated heart failure rats also had significantly reduced plasma noradrenaline levels indicating attenuated sympathetic activation compared to the control heart failure group. The autonomic effects of VNS were also investigated in a canine ventricular pacing heart failure model (Zhang et al., 2009a). LF/HF ratio was

significantly lower in VNS treated heart failure dogs with significantly reduced LF power and increased HF power indicating a shift towards parasympathetic predominance. BRS was also significantly increased in the VNS group and plasma noradrenaline and C reactive protein levels were reduced. These results corroborate evidence that VNS may reduce sympathetic activation and may also increase parasympathetic activity in heart failure. In addition, these results suggest that VNS has an anti-inflammatory effect that may be beneficial in heart failure. These numerous beneficial effects are encouraging, however, this study also reported side effects including coughing, retching, vomiting and reduced food intake.

Dedicated research into the cardiovascular autonomic effects of VNS in humans is sparse, however Kamath et al. (1992) studied the effect of VNS of the left cervical vagus nerve on HRV in 8 epilepsy patients. Patients in the low stimulation group (n = 4; 2 Hz, 130 ms pulse) showed no significant change in LF/HF ratio after two weeks of stimulation whereas patients in the high stimulation group (30 Hz, 500 ms pulse) showed a significant decrease in LF/HF ratio and a significant increase in HF power indicative of increased vagal tone.

The first clinical study of VNS in heart failure patients was conducted by Schwartz et al. (2008), in which 8 patients with advanced heart failure reported improved quality of life scores and had improved left ventricular function at 1, 3 and 6 months. Extension into a multicentre trial with an additional 24 heart failure patients revealed that chronic right sided VNS could improve NYHA classification, quality of life scores and left ventricular function at 3 months and these changes were maintained or accentuated at 1 year follow up (De Ferrari et al., 2011). These results are encouraging, suggesting VNS may be a powerful adjunctive therapy to improve the symptoms and quality of life of heart failure patients, however, VNS is an invasive and expensive procedure that is associated with complications and side effects. This includes technical and surgical complications such as wound infection, cardiac arrhythmia under test stimulation and electrode malfunction (Spuck, 2010). In addition, side effects include hoarseness, dysphagia, cough and pain (Elliott et al., 2011). A non-invasive method of VNS with reduced side effects is desirable. The vagus nerve has a small

cutaneous distribution to the external ear through the auricular branch of the vagus nerve (ABVN). This offers an easily accessible point for non- invasive VNS.

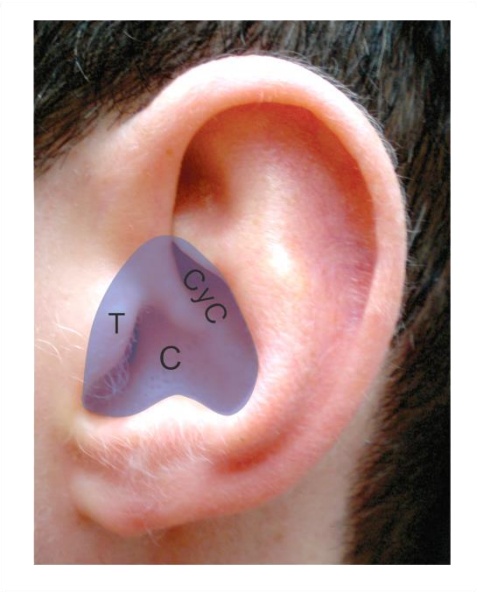


Figure 1.2 The peripheral distribution of the auricular branch of the vagus nerve. The shaded area represents the distribution of the ABVN (extrapolated from (Peuker and Filler, 2002)). T – tragus, C – concha, CyC – cymba concha.

1.3.2 The auricular branch of the vagus nerve

The auricular branch of the vagus nerve (ABVN) originates from the superior (jugular) ganglion and traverses the temporal bone to reach the external ear. Information regarding the terminal distribution of the ABVN is sparse. Peuker & Filler (2002) dissected 14 ears in 7 human cadavers and demonstrated that the ABVN was the sole innervation of the cymba conchae (100%). They also reported that the ABVN contributed to innervation of the antihelix (73%),

tragus (45%) and cavity of concha (45%; Figure 1.2). The variability of the distribution of the ABVN is unknown, therefore, further investigation of the terminal distribution of the ABVN is required to confirm this finding.

1.3.2.1 Central projections of the auricular branch of the vagus nerve

Little is known about the central projections of the ABVN. Chien et al. (1996) traced the central projections of auricular nerves in the dog. The rostral, middle or caudal auricular nerves were isolated in the pinna and treated with cholera toxin subunit B conjugated with horse radish peroxidise (CTB-HRP) but this included the trigeminal, facial and vagus nerves. The rostral auricular nerve of the dog is regarded as the auriculotemporal branch of the trigeminal nerve. Chien et al. (Chien et al., 1996) found that the majority of fibres of the middle and caudal auricular nerves were vagal (88% and 79% respectively) with some fibres from the facial nerve. Labelling from the middle and caudal auricular nerves was found in the spinal trigeminal nucleus, the cuneate nucleus, DVN, and NTS. Afferents from the ear projecting to the NTS could be involved in autonomic control (Chien et al., 1996).

Nomura & Mizuno (1984) provide the only study of the central projections of the ABVN alone. Using cats, they isolated the ABVN in the mastoid canaliculus of the temporal bone before it reached the facial canal and used HRP to trace its central connections in the brainstem. The results showed that fibres of the ABVN project to the principle sensory trigeminal nucleus, spinal trigeminal nucleus, cuneate nucleus and NTS, similar to the findings of Chien et al. (1996). The principle sensory trigeminal nucleus and spinal trigeminal nucleus are both responsible for somatic sensation. It is interesting that some of the fibres from the ABVN terminated in the NTS of the brainstem as this is where general visceral afferents terminate. Moreover, these terminations were located in the dorsomedial part of the caudal NTS which corresponds with the subdivision of the NTS that receives baroreceptor afferents (Ciriello et al., 1981). This indicates that the ABVN may be capable of influencing cardiovascular and visceral autonomic control through the NTS.

The ABVN has been reported to mediate several somatovisceral reflexes the most common being the ear-cough reflex (Arnold's reflex) (Gupta et al., 1986; Tekdemir et al., 1998). This reflex can be elicited by impacted cerumen, foreign bodies or syringing the ear. The incidence of this reflex is estimated to be 2.3% by Tekdemir et al. (1998) who examined 514 patients (admitted to the ear, nose and throat department) using a blunt probe to palpate the external auditory meatus. Gupta et al. (1986) used the same method to study 500 randomly selected out-patients and found a 4.2% incidence. Gupta et al. (1986) also observed the auriculo-palatal reflex (gag reflex) in 1.8%, the auriculo-lacrimal reflex in 2% and the auriculo-cardiac reflex (auricular syncope) in 0.6%. Another interesting phenomenon involving the ABVN is pain referred to the external ear from viscera supplied by the vagus nerve in conditions such as lung cancer (Bindoff and Heseltine, 1988; Abraham et al., 2003; Palmieri, 2006), gastroeosophageal reflux (Blau, 1989) and myocardial infarction (Rothwell, 1993; Amirhaeri, 2010). Furthermore, cervical vagus nerve stimulation has also been reported to cause ear pain (Myers, 2008; Schwartz et al., 2008). The ABVN may therefore provide a non-invasive alternative to cervical VNS, that could be accessible to many more patients than surgical implantation of an electrode round the cervical vagus nerve.

1.3.3 Transcutaneous vagus nerve stimulation

Stimulation of the peripheral distribution of the ABVN to the external ear – transcutaneous vagus nerve stimulation (tVNS) – is receiving increasing interest as a non-invasive method of VNS. Electroencephalography (EEG) revealed that tVNS of the tragus elicits vagus sensory evoked potential (VSEPs; n = 6) (Fallgatter et al., 2003). These had a similar latency to cochlear nerve evoked potentials (2-5 ms) and were only produced by stimulation of the tragus. Stimulation of other parts of the ear not supplied by the ABVN (ear lobe, scapha, superior crus of the antihelix and the top of the helix) did not produce a VSEP. Functional magnetic resonance imaging (fMRI) during tVNS of the tragus revealed similar activation patterns to conventional VNS (Kraus et al., 2007). Recently, these results have been

confirmed and strengthened with the inclusion of a sham stimulation comparison (Kraus et al., 2013). Furthermore, this study found changes in brainstem signals of the NTS and locus coeruleus during tVNS of the anterior auditory canal (i.e. inner tragus) but not the posterior auditory canal. These results indicate that the tragus may be an acceptable site for tVNS.

Few studies have investigated the cardiovascular effects of tVNS (Table 1.1 and Table 2.2). Electroacupuncture and manual acupuncture of the concha caused a significant decrease in heart rate and blood pressure in anaesthetised rats (n = 18) (Gao et al., 2008). The mechanisms behind these effects were investigated through extracellular recordings of cardiac-related NTS neurones during manual acupuncture of the concha (n = 58 rats) (Gao et al., 2011). Neurones were classified as cardiac-related if they displayed spontaneous activity synchronised with the R peak of the ECG (n = 34). The firing rate of these neurones increased during stimulation while blood pressure and heart rate decreased. Intravenous administration of atropine attenuated the decrease in heart rate and blood pressure in response to stimulation and also attenuated the increased firing rate of cardiac-related neurones in the NTS. These results indicate that the NTS plays a key role in mediating the cardiovascular effects of tVNS. tVNS of the right tragus using surface electrodes has also been found to suppress atrial fibrillation in a canine rapid atrial pacing model (n = 10) (Yu et al., 2013). Crucially, this effect was abolished by sectioning left and right vagus nerves indicating that these must also be intact.

Table 1.1 Animal studies of VNS and tVNS

Paper	**Animal**	**VNS location**	**VNS Parameters**	**Results**
Gao et al. (2008)	Rats – healthy (n = 18)	A1-apex helix A2-middle of helix A3-tail of helix A4-inferior concha A5- middle antihelix	Electroacupuncture for 30 s at: 4 Hz; 0.4 mA; 4 Hz; 1.0 mA; 100 Hz; 0.4 mA; 100 Hz; 1.0 mA Manual acupuncture for 30s	Low frequency electroacupuncture (EA) had no significant effects. High frequency EA evoked a significant decrease in BP at all 5 areas and heart rate at A3, A4 and A5. Manual acupuncture reduced heart rate only in A4 and BP in A1, A3 and A4.
Gao et al (2011)	Rats – healthy (n = 58)	Inferior concha	Manual acupuncture	Significant decrease in HR and BP and increase in firing rate of cardiac related neurons in NTS. Effect eliminated by intravenous atropine.
Li et al. (2003)	Rats – 1 month after MI (VNS = 11; control = 13)	Right cervical vagus nerve	0.2 ms; 20 Hz; 0.1-0.13 mA for 6 weeks	Significantly lower heart rate 40bpm and improved left ventricular haemodynamics. Significantly lower mortality (14% vs 50% p = 0.008). No effect on BP.
Tsutsumi et al. (2008)	Mice – 28 days after MI (VNS = 6; control = 6)	Right cervical vagus	1 ms; 500 mV; 10 Hz for 15mins.	Decrease in heart rate 10% below baseline and a significant reduction in cardiac norepinephrine level
Vanoli et al. (1991)	Dogs – 1 month after MI (VNS = 30; control = 24)	Right cervical vagus nerve	3 ms; 3-8 Hz; 1.0-3.0 mA during exercise and ischaemia test	VF significantly ($p < 0.001$) lower in VNS group compared to control group (11.5% vs. 92%)
Yu et al., (2013)	Dogs – 6 hours rapid atrial pacing (n = 10)	Right tragus	1 ms; 20 Hz; 80% below voltage required to slow sinus rate	Increased effective refractory period and suppressed atrial fibrillation. Eliminated by sectioning left and right vagi.
Zhang et al. (2009)	Dogs – 8 weeks into high rate ventricular pacing (VNS = 8; control = 7)	Right cervical vagus	1.5 mA ± 0.6 mA; 20Hz; for 8 weeks	No effect on BP. Left ventricular function improved in VNS group. After 4 weeks VNS group had significant increase in HF power and lower LF/HF ratio; improved BRS; lower plasma levels of CRP and NA.

Table 1.2 Studies of tVNS in healthy humans

Paper	Sample Group	Study Design	Electrode positions	Stimulation parameters	Study outcomes
Fallgatter et al. (2003)	Healthy participants (n = 6)	Pilot study	Right tragus, ear lobe, helix, anti-helix, scapha	0.1 ms pulses of 8 mA at 2 s intervals.	Vagus Sensory Evoked Potentials (VSEP) only elicited with stimulation of the tragus.
Haker et al. (2000)	Healthy participants (n = 12)	Pilot study	Left concha	Manual acupuncture 25 mins with manual stimulation every 5th min.	Significant increase in HF during stimulation and post-stimulation period of 60mins. No significant change in heart rate or BP
Kraus et al. (2007)	Healthy participants (n = 8)	Pilot study	Left tragus	20 µs; 8 Hz; 30.7-33.1 V for 4x30 s at 1 min intervals.	No effects on heart rate or BP. fMRI shows similar activation pattern to invasive VNS.
Kraus et al., 2013	Healthy participants (n = 16)	Sham controlled crossover study	Left auditory canal: anterior wall or posterior wall	20 µs; 8 Hz; 14 – 57 V for 4x30s at 1 min intervals.	fMRI during tVNS of anterior auditory wall shows similar activation pattern to invasive VNS plus changes in brainstem areas.
La Marca et al. (2010)	Healthy participants (n = 14)	Sham controlled cross-over study	Left concha	Manual + electro-acupuncture 30 minutes 2.65 V; 0.5 s; 10 Hz	Manual acupuncture no effect, electroacupunture increased respiratory sinus arrhythmia.
Polak et al.(2009)	Healthy participants (n = 20)	Pilot study	Left and right tragus	5-10 mA; 0.1 ms at 2 s intervals	Optimal intensity 8mA for VSEP detection without pain. No effect of side or gender on VSEP amplitude.

Only two studies have specifically investigated the autonomic effects of tVNS in humans. Manual acupuncture of the left concha in healthy volunteers caused no change in heart rate or blood pressure, however, HRV analysis revealed a significant increase in the HF component, representing vagal modulation of heart rate, during stimulation (n = 12) (Haker et al., 2000). Electroacupuncture of the same site (left concha) was also found to increase respiratory sinus arrhythmia mediated by the vagus nerve (n = 14) (La Marca et al., 2010). These results suggest that tVNS may be capable of modulating cardiovascular autonomic control and merits further investigation. Indeed, electroacupuncture of the concha has been reported to alleviate symptoms in coronary artery disease patients and reduce the use of vasodilator medication (Zamotrinsky et al., 1997; Zamotrinsky et al., 2001).

Vagus nerve stimulation is not the only neuromodulatory technique currently receiving interest. Transcranial direct current stimulation (tDCS) is a non-invasive neuromodulatory technique used to influence cortical excitability. This is under investigation in a range of conditions including depression (Brunoni et al., 2013a), pain (Borckardt et al., 2011), Parkinson's disease (Pereira et al., 2013) and stroke rehabilitation (Schulz et al., 2013).

1.4 Transcranial direct current stimulation

Neuromodulation, the alteration of nerve activity through the use of targeted electrical stimulation or pharmacology, is a rapidly advancing field with applications in a plethora of conditions that have previously proven difficult to treat. In addition to vagus nerve stimulation (Section 1.3.1) other neuromodulation techniques, such as renal nerve ablation and carotid sinus stimulation, are being investigated as potential therapies for treatment resistant hypertension (Esler et al., 2010; Jordan et al., 2012). Another neuromodulatory technique of interest is transcranial direct current stimulation (tDCS).

The use of cranial electric therapy dates back to the Roman physician Scribonius Largus (AD 46) who applied live electric torpedo fish to the scalp

to treat headache (Kellaway, 1946). Pliny the Elder and the Greek physician Galen also described similar findings and the Muslim physician Ibn-Sidah (11th century) recommended the use of live electric catfish applied to the forehead to treat epilepsy (Kellaway, 1946). Scientific investigations of the effects of electrical cranial stimulation were not carried out until much later when Galvani and Volta (18th century) introduced the field of electrophysiology (Priori, 2003). Indeed Galvani's nephew, Aldini, reported the successful use of transcranial galvanic/direct current stimulation in the treatment of melancholia in 1804 (Priori, 2003). The results of subsequent tDCS studies were variable and, with the introduction of electroconvulsive therapy (ECT) by Bini and Cerlutti in the 1930s (Endler, 1988), interest in tDCS diminished (Stagg and Nitsche, 2011). ECT is still currently used in therapy resistant depression (Kellner et al., 2012), however, the principles between this and tDCS differ significantly. ECT is performed using much higher currents (200 – 900 mA) (d'Elia G, 1983) with the aim of inducing seizure activity whereas tDCS is performed at 1 mA and modulates spontaneous neuronal activity (Nitsche et al., 2003b). tDCS was revived in recent years due to improved understanding of the mechanisms involved.

1.4.1 Mechanisms of transcranial direct current stimulation

tDCS has been shown to influence the spontaneous activity of cortical neurones. *In vivo* studies, applying direct current directly to the cortex in cats and rodents, have shown a sub-threshold depolarisation of the resting membrane potential of neurones underlying the anode (positive electrode) (Creutzfeldt et al., 1962; Bindman et al., 1964; Purpura and McMurtry, 1965; Nitsche and Paulus, 2000). Anodal tDCS does not directly elicit action potentials, rather the sub-threshold depolarisation enables other inputs to reach threshold. This was demonstrated by measuring motor evoked potentials (MEPs) elicited using transcranial magnetic stimulation before and during anodal tDCS. The amplitude of MEPs was significantly higher during anodal tDCS (1 mA) compared to baseline (Nitsche and Paulus, 2000). Conversely, beneath the cathode (negative electrode) there is hyperpolarisation causing a decrease in spontaneous neuronal activity

(Creutzfeldt et al., 1962; Bindman et al., 1964; Purpura and McMurtry, 1965; Nitsche and Paulus, 2000).

Remarkably the polarity specific alteration in cortical activity persists after stimulation has ceased. *In vivo*, direct current stimulation of 5-10 minutes resulted in effects that lasted 1-5 hours (Bindman et al., 1964). The effects of tDCS in humans are similar with effects lasting up to 90 minutes after 13 minutes of stimulation (Nitsche and Paulus, 2000, 2001). The long-term effects of tDCS depend on both the duration and strength of current application (Bindman et al., 1964; Purpura and McMurtry, 1965) and are thought to be mediated by N-methyl-D-aspartate (NMDA) receptors (Stagg and Nitsche, 2011). Indeed, the residual effects of both anodal and cathodal tDCS in healthy humans (n = 11) were blocked using dextromethorphan (DMO), an NMDA receptor antagonist (Liebetanz et al., 2002). This suggests that synaptic plasticity, such as long term potentiation or long term depression, mediates the effects of tDCS (Lang et al., 2005; Stagg and Nitsche, 2011).

Positron emission tomography of regional cerebral blood flow (rCBF) has shown that the effects of tDCS are not limited to the area of cortex underlying the electrode. Both anodal and cathodal tDCS caused widespread changes in rCBF not only in other areas of the cortex but also in subcortical structures (Lang et al., 2005). Modelling studies also predict widespread distribution of the electric field used in tDCS suggesting that it may even induce an electric field in the brainstem (Im et al., 2012). While widespread activation of the cortex may facilitate plasticity, there is potential that this may have unintentional effects on brain function. For example, this may affect central regulation of autonomic function, not only through the possible spread of the electrical field to the brainstem but also through cortical projections from the areas under stimulation that may influence autonomic control. The potential effects of tDCS on autonomic function have received little attention and the results are conflicting (Table 1.3) (Accornero et al., 2007; Vandermeeren et al., 2010; Montenegro et al., 2011; Raimundo et al., 2012). This may be due to the different electrode positions and stimulation parameters used in these studies. As tDCS is being investigated

as potential therapy in patient groups that often exhibit autonomic dysfunction e.g. stroke patients, this merits further consideration.

1.4.2 tDCS in stroke rehabilitation therapy

The ability of tDCS to modulate cortical excitability and perhaps induce synaptic plasticity led to investigation of tDCS as a therapy to enhance stroke motor rehabilitation. Approximately 56% of stroke patients suffer marked hemiparesis up to 5 years post stroke (Bolognini et al., 2009). This limits the ability of patients to carry out activities of daily life, reducing independence and quality of life scores. Anodal tDCS over the motor cortex improved motor learning (Boggio et al., 2006; Reis et al., 2009) in healthy subjects and improved motor function in stroke patients compared to sham stimulation (Fregni et al., 2005b; Boggio et al., 2007; Stagg et al., 2012). To date, tDCS studies involving stroke patients are few with small sample numbers, however, a recent meta-analysis of tDCS in motor rehabilitation found significant improvement compared to sham (Butler et al., 2013).

Table 1.3 Studies of the effects of tDCS on autonomic variables

Paper	Sample Group	Electrode Positions	Stimulation Parameters	Study Outcomes
Accornero et al. (2007)	Healthy participants (n = 20)	Occipital region and neck base	1 mA for 3 or 10 mins	No change in heart rate, blood pressure or body temperature during anodal or cathodal tDCS.
Montenegro et al. (2011)	Healthy participants; athletes (n = 10) and non–athletes (n = 10)	Left temporal lobe (T3) and contralateral supraorbital region	2 mA for 20 mins	Anodal tDCS increased HF component of HRV and reduced LF/HF in athletes. No effect in non-athletes.
Raimundo et al. (2012)	Healthy participants (n = 50)	Left motor cortex (C3) and contralateral supraorbital region	1mA for 20 mins	No change in heart rate, blood pressure, body temperature or respiratory rate.
Vandermeeren et al. (2010)	Healthy participants (n = 30)	Midline frontal cortex (Fz) and right tibia	1mA for 20 mins	Compared to sham stimulation there was no effect on heart rate, blood pressure, respiratory rate or HRV.

1.4.3 tDCS in the treatment of depression

A greater understanding of the mechanisms underlying tDCS has led to consideration of this technique in the treatment of major depressive disorder (Fregni et al., 2005), however, the results are conflicting. Anodal tDCS over the left dorsolateral prefrontal cortex has been reported to cause a significant improvement in mood in MDD patients compared to sham tDCS (Fregni et al., 2006a; Loo et al., 2012). Conversely, this technique has been reported to perform no better than sham tDCS (Palm et al., 2012). A meta-analysis of randomised control trials of tDCS in MDD patients found no difference between active and sham conditions (Berlim et al., 2013). Subsequently, the efficacy of tDCS compared to a selective serotonin reuptake inhibitor (sertraline) has been investigated in MDD patients (n = 120). There was a significant improve in depression scores in the active tDCS group compared to sham. Furthermore, combined tDCS and sertraline treatment resulted in a significant improvement compared to either treatment on its own (Brunoni et al., 2013b). Further investigation into the anti-depressive effects of tDCS is warranted, particularly as tDCS may offer an alternative to ECT in treatment resistant depression.

1.4.4 tDCS and cognition

Alterations in cortical excitability are observed in cognitive processes which has led to investigation of tDCS as a potential method to improve cognition (Kuo and Nitsche, 2012). Anodal tDCS over the posterior parietal cortex improved attention in healthy humans during a visual exploration task (Bolognini et al., 2010). Working memory was also improved by anodal tDCS over the left dorsolateral prefrontal cortex in healthy humans who were asked to recall specific letters (Ohn et al., 2008). Interestingly, studies of tDCS in patients with depression also found improvements in working memory and attention (Fregni et al., 2006a; Loo et al., 2012). The effects of tDCS on cognition may contribute to the improvement in symptoms reported in depression patients.

tDCS has also been investigated in learning and long term memory studies. Anodal tDCS of the left motor cortex in healthy humans over three

sessions improved learning of a visually guided pinch task (Schambra et al., 2011) supporting the application of tDCS in stroke rehabilitation. In addition, anodal tDCS has also been reported to improve verbal learning (de Vries et al., 2009) and arithmetic abilities (Cohen Kadosh et al., 2010) in healthy volunteers by targeting different areas of the cortex (Broca's area and right parietal lobe respectively). There are few studies of the cognitive effects of tDCS, however, the potential ability of tDCS to improve learning may have ethical implications in the future.

1.4.5 tDCS and chronic pain therapy

The prevalence of chronic pain in the adult European population is estimated to be around 19% and can severely impact quality of life (Luedtke et al., 2012). Chronic pain is often unresponsive to pharmacological therapy therefore surgical interventions, including brain stimulation, are being investigated. A multi-centre trial of deep brain stimulation of the periaqueductal grey matter or the thalamus showed modest improvements in short term pain perception in patients (3 and 6 months) but was not effective long term (Coffey, 2001). Stimulation of the motor cortex has also been investigated in chronic pain patients and reduced pain perception (Lima and Fregni, 2008), however, this is an invasive technique requiring surgery. tDCS over the motor cortex has been proposed as a non-invasive alternative therapy in chronic pain. Preliminary results of clinical studies indicate that anodal tDCS over the motor cortex may reduce pain in a range of neuropathic pain conditions including spinal cord injury (Fregni et al., 2006b; Soler et al., 2010), fibromyalgia (Fregni et al., 2006c) and chronic pelvic pain (Fenton et al., 2009). These results suggest that tDCS may be a non-invasive method to alleviate pain symptoms, however, the studies are based on small sample groups and larger control trials are required.

1.5 General Hypothesis

It is possible to non-invasively alter autonomic function using neuromodulatory techniques. Transcutaneous vagus nerve stimulation and transcranial direct current stimulation will alter cardiovascular autonomic function.

1.6 Aims and Objectives

The aim of this thesis was to investigate the effects of 2 non-invasive neuromodulatory techniques on cardiovascular autonomic function. These techniques were transcutaneous vagus nerve stimulation (tVNS) and transcranial direct current stimulation (tDCS). The objectives for the tVNS study were to:

1. investigate the effects of tVNS on non-invasive estimates of cardiovascular autonomic function (HRV and BRS) in healthy human volunteers
2. determine the optimal stimulation parameters and electrode placement for tVNS to increase parasympathetic predominance
3. investigate the mechanisms of tVNS effects on cardiovascular autonomic control through direct recordings of sympathetic nerve activity (microneurography) and neuronal tracing of the ABVN in fixed human tissue
4. investigate the effects of tVNS on HRV and BRS in heart failure patients
5. investigate the tolerability of tVNS in HF patients

The objectives for the tDCS study were to:

1. investigate the effects of anodal and cathodal tDCS on non-invasive estimates of cardiovascular autonomic function (HRV and BRS) in healthy human volunteers
2. investigate the mechanisms of tVNS effects on cardiovascular autonomic control through direct recordings of sympathetic nerve activity (microneurography).

Chapter 2
The influence of transcutaneous vagus nerve stimulation on autonomic function

2.1 Introduction

Electrical stimulation of the cervical vagus nerve has been approved for treatment resistant epilepsy in Europe and the USA for over 15 years (Shahwan et al., 2009). VNS is also an approved therapy for treatment resistant depression in the USA (Milby et al., 2008) and has been investigated as a potential therapy for a wide range of conditions including inflammation (De Herdt et al., 2009), Alzheimer's disease (Merrill et al., 2006), obesity (Val-Laillet et al., 2010), chronic pain (Kirchner A. et al., 2000) and tinnitus (Engineer et al., 2011). Indeed, VNS has proven effective in pilot studies for the treatment of heart failure (Schwartz et al., 2008) and in a subsequent multi-centre trial that is on-going (ClinicalTrials.gov Identifier: NCT01303718)(De Ferrari et al., 2011). However, despite positive indications from pilot studies, large scale trials are rare.

One factor that may hinder larger trials is the invasive nature of VNS. VNS requires surgical implantation of a bipolar electrode around the cervical vagus nerve and implantation of a generator subcutaneously in the thoracic wall. This is associated with technical and surgical complications including wound infection, cardiac arrhythmia under test stimulation and electrode malfunction (Spuck, 2010). In addition, side effects include hoarseness, dysphagia, cough and pain (Elliott et al., 2011).

A potential non-invasive route for VNS is electrical stimulation of the auricular branch of the vagus nerve (ABVN), which is distributed to the external ear (Peuker and Filler, 2002). This stimulation can be performed transcutaneously by applying surface electrodes or acupuncture needles to the external ear (tVNS). tVNS has been piloted in some patient groups as an alternative to implanted VNS e.g. tinnitus (Lehtimäki et al., 2012). Short term tVNS (7 treatments lasting 45-60 mins over 10 days) paired with sound therapy in tinnitus patients decreased perception of tinnitus and improved mood and symptoms assessed using questionnaires (n = 10) (Lehtimäki et al., 2012). Furthermore, magnetoencephalography revealed a decrease in amplitude of evoked auditory cortical responses during tVNS (Lehtimäki et al., 2012). Conversely, tVNS has been reported to have no effect on tinnitus

(n = 24) (Kreuzer et al., 2012), however, this study did not use simultaneous sound therapy.

As VNS is currently used in refractory epilepsy, tVNS has been piloted as a non-invasive alternative. tVNS was investigated in 7 patients with refractory epilepsy (Stefan et al., 2012). Patients were treated for 9 months (1 hour 3 times daily) and kept logs of seizures. Patients also underwent EEG at baseline and every 3 months during the study, however, as 4 of the 7 patients showed no clinical seizures in the baseline EEG, comparisons were limited. 2 of the patients exhibited a significant reduction in seizure number (29% and 50% decrease) during EEG at 9 month follow-up whereas 1 patient had a significant increase in seizure number (175%) (Stefan et al., 2012). From the patients' logs, 5 experienced a decrease in seizure frequency whereas 2 reported an increase. These mixed preliminary results make it unclear if tVNS is beneficial in refractory epilepsy. A larger sample group, with the addition of a suitable control such as sham tVNS, is required.

tVNS is also being investigated as a possible non-invasive therapy in depression. 2 weeks of tVNS (15 minutes 1-2 times daily 5 days a week) significantly decreased depression rating scores using the Beck Depression Inventory compared to sham tVNS, however, there was no difference in scores assessed using the Hamilton Depression Rating Scale (Hein et al., 2013). Interestingly, the Beck Depression Inventory is utilised by patients whereas the Hamilton Depression Rating Scale is based on clinicians' ratings of symptoms, therefore, there was an improvement in patient rated scores but not in clinician scores. This may suggest a placebo effect or it may reflect the limitations of depression rating scales. Alternatively, the conflicting results may also be due to the short treatment time; evidence from invasive VNS for depression suggests that long term stimulation is required for therapeutic effects (Rush et al., 2005b).

tVNS may also be a therapeutic approach to reduce pain perception. tVNS in healthy participants (n = 48) reduced pain sensitivity in response to tonic heat and increased mechanical and pressure pain thresholds when compared to sham tVNS (Busch et al., 2013). tVNS has also been

investigated in patients with chronic pain (endometriosis; n = 15) - responses to evoked mechanical pain stimuli were decreased compared to sham stimulation (Napadow et al., 2012).

Electroacupuncture of the ear has also been reported to improve symptoms in coronary artery disease patients (Zamotrinsky et al., 1997; Zamotrinsky et al., 2001). 15 minutes of stimulation per day for 10 days abolished angina symptoms at rest and reduced the use of vasodilator medication. Furthermore, patients subsequently underwent coronary artery bypass surgery and had improved postoperative outcomes compared to the control group.

These recent studies of tVNS in the clinical setting have provided some positive initial results, however, the outcomes investigated are varied and mostly subjective. In addition, the stimulation parameters used differ widely (Table 2.1), therefore, little is known about the optimal parameters for tVNS. There is also a dearth of information regarding the potential effects of tVNS on central autonomic control. Neuronal tracing studies in cats and dogs revealed projections of the ABVN to the NTS (Nomura and Mizuno, 1984; Chien et al., 1996). The NTS receives visceral vagal afferents and plays a key role in integrating autonomic control through projections to the DVN, NA, CVLM and RVLM (see General Introduction 1.1.4), therefore, stimulation of the ABVN may increase efferent vagal parasympathetic activity and/or decrease sympathetic outflow. Indeed, acupuncture of the concha of the ear in healthy volunteers has been reported to significantly increase the HF component of HRV indicating an increase in parasympathetic modulation of the heart (Haker et al., 2000). Many of the clinical pilot studies recorded ECG data to ensure that tVNS was safe to use and reported no effect on heart rate (Kreuzer et al., 2012; Lehtimäki et al., 2012; Napadow et al., 2012; Busch et al., 2013). However, heart rate is a crude measure of autonomic function and the sample sizes were small. If tVNS can be shown to influence autonomic control towards parasympathetic predominance it could provide a method to correct autonomic imbalance in conditions with parasympathetic withdrawal such as heart failure.

2.2 Hypothesis

Transcutaneous stimulation of the auricular branch of the vagus nerve will alter cardiovascular autonomic control.

2.3 Aims and Objectives

The aim of this study was to investigate the effects of tVNS on cardiovascular autonomic function in healthy human volunteers. The objectives were to:

1. determine an optimal tVNS protocol by investigating the effects of different electrode positions and stimulation parameters (pulse width and frequency) on cardiovascular autonomic function (measured by HRV and BRS analyses).
2. determine the effects of the optimal tVNS protocol on sympathetic nerve activity measured by microneurography.

Table 2.1 Studies of tVNS in patient populations.

Paper	Sample Group	Study Design	Electrode positions	Stimulation parameters	Study outcomes
Hein et al. (2013)	Major depression patients (tVNS = 17; sham = 20)	Sham controlled	Left and right ears	1.5 Hz; 0-600 µA	Decreased depression rating scores using the Beck Depression Inventory; no difference in scores using the Hamilton Depression Rating Scale.
Kreuzer et al. (2012)	Tinnitus patients (n = 24)	Pilot study (no control)	Exact site unclear	25 Hz; 0.1 – 10 mA; 24 weeks	No effect on tinnitus symptoms. No effect on heart rate. Reduction in QRS complex duration.
Lehtimäki et al. (2013)	Tinnitus patients (n = 10)	Pilot study (no control)	Left tragus	25 Hz; approx. 0.8 mA and sound therapy; 10 days	Improved mood and tinnitus severity. Reduced amplitude of evoked auditory cortical responses. No effect on heart rate.
Napadow et al. (2012)	Endometriosis patients (n = 15)	Sham controlled cross-over	Left cymba concha and anti helix	450 µs; 30 Hz; during exhalation	Reduced pain responses to evoked mechanical pain stimuli and reduced anxiety. No effect on HRV (n = 10).
Stefan et al (2012)	Epilepsy patients (n = 7)	Pilot study (no control)	Left ear. Exact site unclear	300 µs; 10 Hz; 25V; 9 months	Reduced seizure frequency.
Zamotrinsky et al. (1997)	Coronary artery disease patients (VNS = 10; control = 10)	Randomised control trial	Left and right ears Exact site unclear	Electro-acupuncture 0.2-1.5mA; 3Hz;1.5ms 15mins/day for 10 days	After day 4 angina did not develop at rest. After day 7 patients had increased tolerance to exercise. Patients no longer needed vasodilators (glycerol trinitrate). Effects lasted 2-3 weeks after tVNS treatment.
Zamotrinsky et al. (2001)	Coronary artery disease patients (VNS = 8; control = 10)	Randomised control trial	Left and right ears Exact site unclear	Electro-acupuncture 0.2-1.5mA; 3Hz;1.5ms 15mins/day for 10 days	tVNS caused a transient decrease in heart rate and BP during first 4-5 days. After 4-5 days heart rate and BP chronically lower compared to baseline. Reduction in angina episodes and reduced need for medication. Improved postoperative outcome compared to control group.

2.4 Methods

2.4.1 General Protocol

The study was approved by the University of Leeds Ethics Committee (Appendix - BIOSCI 09-021) and was conducted in accordance with the Declaration of Helsinki. Informed written consent was obtained from all participants. 150 healthy participants were recruited for the study. Inclusion criteria for healthy participants were male or female over the age of 18 years. Exclusion criteria were a history of cardiovascular disease, diabetes or hypertension. To avoid possible confounding factors, all experiments were carried out in a similar manner between 8-10 am to limit the effect of circadian variation on autonomic function. The study was conducted in a dedicated study room at 21 ± 2°C. All participants were asked to avoid alcohol and intense exercise 12 hours prior to attendance. They were also asked to avoid caffeine and nicotine on the morning of the study. Each participant was asked to have a light breakfast and to void their bladder before the study commenced. Demographic data including age, weight, height, medication and activity levels were recorded. Participants were asked to lie on a couch with a memory foam mattress topper in a semi-supine position while heart rate, blood pressure and respiration were monitored continuously. Following experimental set up, participants rested for 10-20 minutes while heart rate and blood pressure were monitored until a steady state was reached. Data were recorded at baseline, during tVNS and during recovery for 15 minutes each. Between each recording, participants rested until heart rate and blood pressure returned to baseline levels. Participants were asked to remain still and refrain from talking during recordings.

2.4.2 Transcutaneous Vagus Nerve Stimulation (tVNS)

tVNS was performed using a Transcutaneous Electrical Nerve Stimulation (TENS) device (V-TENS Plus, Body Clock Health Care Ltd, UK) with modified surface electrodes (auricular clips, Body Clock Health Ltd, UK). tVNS was applied for 15 minutes. The effects of varying several stimulation

parameters were investigated; electrode position, single vs. bilateral stimulation and the pulse with and pulse frequency of stimulation. In all experiments, amplitude was adjusted to the level of sensory threshold (10-50 mA).

2.4.2.1 Electrode positioning for tVNS

Three different electrode configurations were investigated in healthy participants (n = 63); tragus (n = 34), tragus + cymba (n = 15) or concha (n = 14; Figure 1.2). tVNS was performed with a pulse width of 20 μs at 15Hz for 15 minutes.

2.4.2.2 The effects of tVNS of the right ear vs. bilateral tVNS

In the same group of participants (n = 63) in which electrode positioning was investigated, the efficacy of tVNS of either the right ear only (n = 21) or both ears simultaneously (n = 42) was also investigated. tVNS was performed with a pulse width of 20 μs at 15Hz for 15 minutes.

2.4.2.3 Stimulation parameters for tVNS

The effects of different pulse width and frequency stimulation was investigated. As no significant difference was found between different electrode positioning and sides on HRV, this group were termed the low pulse width and pulse frequency group (L-tVNS; 20 μs pulse width at 15 Hz; n = 63). A further 34 participants were recruited for the high pulse width and frequency group (H-tVNS).

2.4.2.4 Sham tVNS

Sham tVNS (n = 14) was performed by placing the electrodes on the tragus and increasing amplitude until the participant reported feeling sensation. Participants were then told that the amplitude would be reduced slightly to prevent discomfort but the electrode leads were disconnected from the TENS machine without the participants' knowledge.

2.4.3 Heart Rate Variability (HRV)

A three lead ECG was used to monitor and record heart rate. Electrodes (Ambu, UK) were placed on left and right clavicles and costal margins enabling electrode polarities to be changed to select the lead that detected the most prominent R peak for subsequent HRV analysis (normally lead II). HRV was analysed offline using LabVIEW (National Instruments, USA) software. A threshold was set to detect R peaks from an 8 minute ECG recording and R-R intervals used to produce a tachogram. The ECG was inspected to ensure all R peaks were detected and there were no abnormalities in the ECG such as ectopic beats (e.g. premature ventricular complexes). Ectopic beats could be corrected using a linear spline to average the R-R interval prior to and following the ectopic. If more than 2 ectopic beats were detected the recording was excluded. The resulting tachogram was resampled at 5 Hz and underwent 512 point Fast Fourier Transform (78% overlap) with a Hanning window to calculate the power spectrum of HRV with the low frequency (LF) component at 0.04 - 0.15 Hz and the high frequency (HF) component at 0.15 - 0.40 Hz (Figure 2.1). LF and HF powers were also converted to normalised units as a percentage of the total power minus very low frequency power (VLF; 0.00 – 0.04 Hz) to determine the LF/HF ratio. The HF component reflects parasympathetic modulation of heart rate (Chapleau and Sabharwal, 2011) and the LF component reflects both sympathetic and parasympathetic modulation of heart rate (Akselrod et al., 1981). The ratio of low frequency and high frequency oscillations of heart rate variability can be used as an index of cardiac autonomic balance such that a decrease in LF/HF ratio indicates a shift in cardiac autonomic balance towards parasympathetic predominance (Task Force of the European Society of Cardiology and the North American Society of Pacing Electrophysiology, 1996; Chapleau and Sabharwal, 2011).

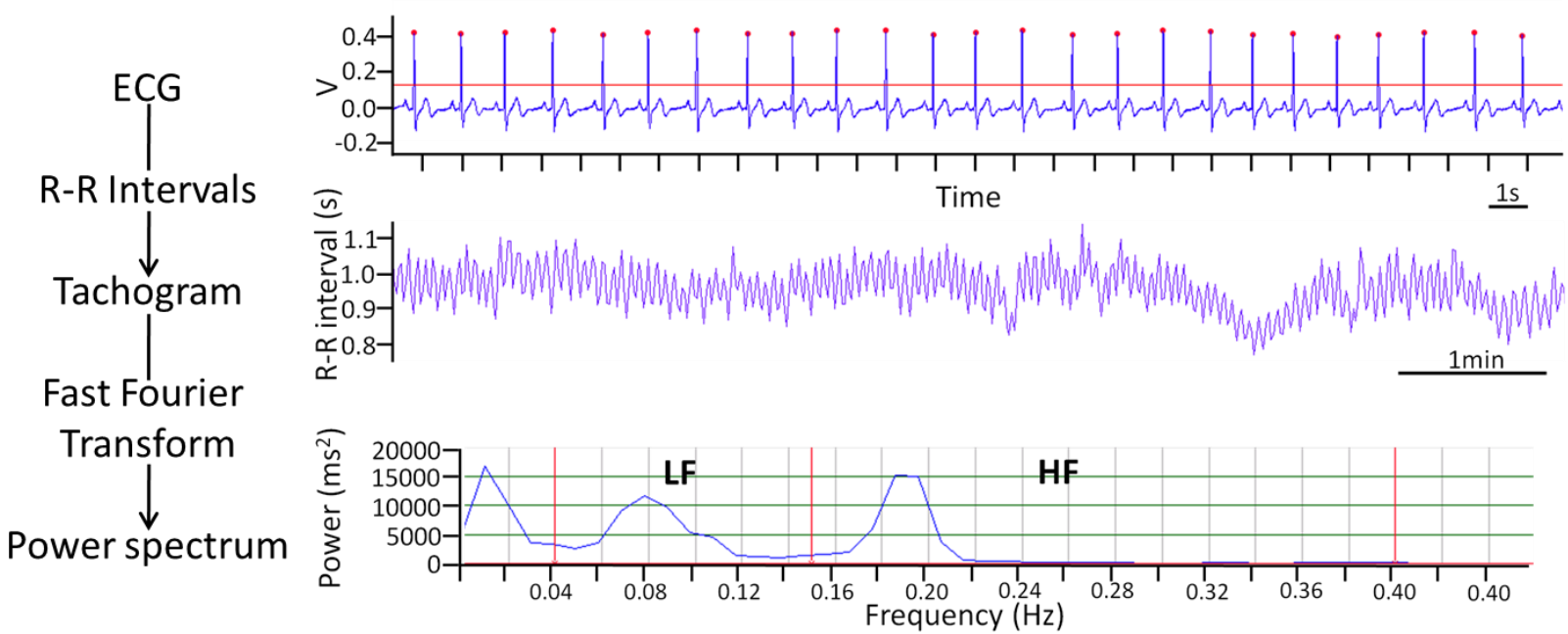


Figure 2.1 Example of heart rate variability analysis. The high frequency (HF) component of HRV represents parasympathetic/vagal modulation of heart rate whereas the LF component reflects both sympathetic and parasympathetic influences. The ratio of LF/HF can be used as an estimate of cardiac sympathovagal balance.

2.4.4 Respiration

A piezo-electric transducer (Pneumotrace, UFI, USA) was placed round the upper thorax to monitor and record respiration rate. A respiration rate <10 breaths/min was unacceptable for HRV analysis as the HF component is respiration dependent. At slow respiration rates the HF peak of the HRV spectrum can merge with the LF peak (Malliani, 2005). In this case participants were asked to use a breathing metronome set at 16 breaths/min (n = 3).

2.4.5 Blood pressure

A Finometer device (Finometer Medical Systems B.V., Arnhem, Netherlands) was used to monitor and record blood pressure continuously including pulse-to-pulse changes. This device utilises the volume clamp method proposed by Peňáz in 1973 (Parati et al., 2003). An inflatable cuff was placed round the middle phalanx of the middle finger on the left hand. The finger cuff has an infrared light source and sensors that detect changes in blood volume (photoplethysmography). The finger cuff was connected to the front-end unit that was strapped to the subject's wrist. This receives plethysmography data and adjusts pressure in the finger cuff to keep the diameter of the digital arteries constant (clamped). The front-end unit on the wrist was connected to the main unit of the Finometer. The main unit consists of a pump used to inflate the finger cuff and the PhysioCal software that calculates the set point at which the artery wall is unloaded i.e. when the intra-arterial pressure of the digital artery matches air pressure in the finger cuff. PhysioCal was switched off during recordings as it interferes with data collection but it was switched on again between recordings to ensure accurate measurements. The Finometer output screen displayed the arterial waveform and also the numerical values for heart rate, systolic, diastolic, and mean blood pressure.

The device also has a hydrostatic height correction system to compensate for hand position with respect to the level of the heart. One sensor was placed at the level of the heart and another on the finger cuff. An agreement within 10 mmHg for systolic and diastolic values obtained by the

Finometer device and blood pressure readings from the arm using an automatic blood pressure machine was sought. If there was a discrepancy then a variety of adjustments were tried including:

1. reapplying the finger cuff
2. using a different sized finger cuff
3. warming the hands
4. using a different finger

Although precise values of systolic and diastolic blood pressure cannot be obtained using the Finometer device, this does provide accurate measurement of beat-to-beat changes in blood pressure that are required for baroreflex sensitivity measurement (Parati et al., 2003). The alternative method is the use of an intra-arterial catheter to measure the precise values for systolic and diastolic blood pressure which is highly invasive and was not feasible. La Rovere et al. (1997) simultaneously measured intra-arterial blood pressure and finger cuff blood pressure during BRS assessment in 620 patients and found a high correlation between the two methods ($r = 0.92$, $p = 0.001$).

2.4.6 Baroreflex sensitivity (BRS)

Spontaneous BRS can be used as an index of cardiovagal activity (La Rovere et al., 2008). Systolic blood pressure variability was calculated using a similar method to HRV except the threshold was set to detect peak systolic pressure. Cross spectral analysis of oscillations in systolic blood pressure and R-R interval was performed. The alpha index was used as an estimate of BRS and was calculated as the square root of the ratio of HRV LF power over systolic blood pressure LF power (Robbe et al., 1987). Coherence between oscillations in systolic blood pressure and heart rate exceeded 0.5 for BRS analysis to be accepted.

2.4.7 Microneurography

Muscle sympathetic nerve activity (MSNA) was recorded as previously described (Macefield et al., 1994; Greenwood et al., 1999) in 10 healthy volunteers in the H-tVNS group (8 male, 2 female; 29-59 years). The right leg was flexed at the knee (approximately 70º) and supported with foam cushions. The peroneal nerve was identified by palpation as it coursed round the neck of the fibula (Figure 2.2) and the skin was cleaned using alcohol wipes. Microneurography was not performed if there were skin lesions present in the area of electrode insertion. Two tungsten microelectrodes (FHC Inc., USA) were inserted percutaneously below the knee. The electrodes were 35 mm long with a diameter of 200 µm tapering to a tip. The recording electrode was epoxy insulated with an impedance of 0.3 ± 0.6 MΩ. An electrode with high impedance was used as this limits the area over which neural activity is picked up and is necessary for single unit MSNA recordings (Macefield et al., 2002). This was inserted into the peroneal nerve and the second (reference) electrode was inserted into subcutaneous tissue 1-2 cm away. The electrodes were connected to a headstage (Neurolog NL100AK, UK) which was connected to an AC pre-amplifier (x50k amplification; Neurolog NL104A, UK). The signal was passed through a Humbug (Quest Scientific, Canada) to filter out mains noise at 50 Hz and a bandpass filter (0.7-2.0 kHz; Neurolog NL125/6, UK). The signal was sampled at 16 kHz and digitised (Power 1401, CED, UK). The signal was then passed to a PC (Dell laptop) where it was displayed in real time and recorded using Spike2 software (version 7; CED, UK). The time base could be expanded to allow inspection of individual action potentials during the experiment. The recording microelectrode was manipulated until a prominent single action potential could be visualised. Manipulations were small and limited to 45 minutes in accordance with guidelines (Mano et al., 2006).

To confirm that action potentials were recorded from a sympathetic vasoconstrictor fibre the following criteria were used;

1) the action potential occurred in diastole
2) there was an inverse relationship between single unit activity and blood pressure as expected of a sympathetic vasoconstrictor fibre

3) single unit activity increased in response to cold pressor test or isometric handgrip exercise
4) there was an absence of paraesthesia and no change in single unit activity in response to cutaneous stimuli (stroking the skin of the lateral leg and the dorsum of the foot).

Further confirmation was obtained during off-line analysis by superimposing all putative MSNA single units to ensure the amplitude and shape remained constant indicating that the same unit was recorded throughout the recording period. MSNA frequency (per min) was calculated by counting all single units that occurred in the first 2 minutes of each recording. MSNA incidence (per 100 heart beats) was also calculated to limit the effect of any changes in heart rate. Baseline data were normalised to 1 and stimulation and recovery data were normalised to baseline due to a high degree of inter-individual variation. Raw values of MSNA frequency and incidence are presented in Table 2.7.

2.4.7.1 Cold pressor test

Control data were recorded for 1 minute then the participant was asked to submerge the entire left hand and wrist into ice water (approximately 4ºC). The hand was submerged for 1 minute unless the discomfort level was too high. The hand was removed from the water and placed in a towel while a further 1 minute of recovery data was obtained. The cold pressor test is useful in discriminating between MSNA and skin sympathetic nerve activity (SSNA). The cold pressor test evokes an increase in blood pressure that correlates well with increasing MSNA (Fagius et al., 1989; Kregel et al., 1992). Crucially, there is a delay in the increase of MSNA of approximately 30 s. The degree of increased MSNA is related to the perceived level of discomfort and this may account for the variation in onset (Victor et al., 1987; Kregel et al., 1992) whereas there is no consistent change in skin sympathetic activity (Kregel et al., 1992). The response to the cold pressor test was therefore derived from the last 30 s of immersion compared to the control period.

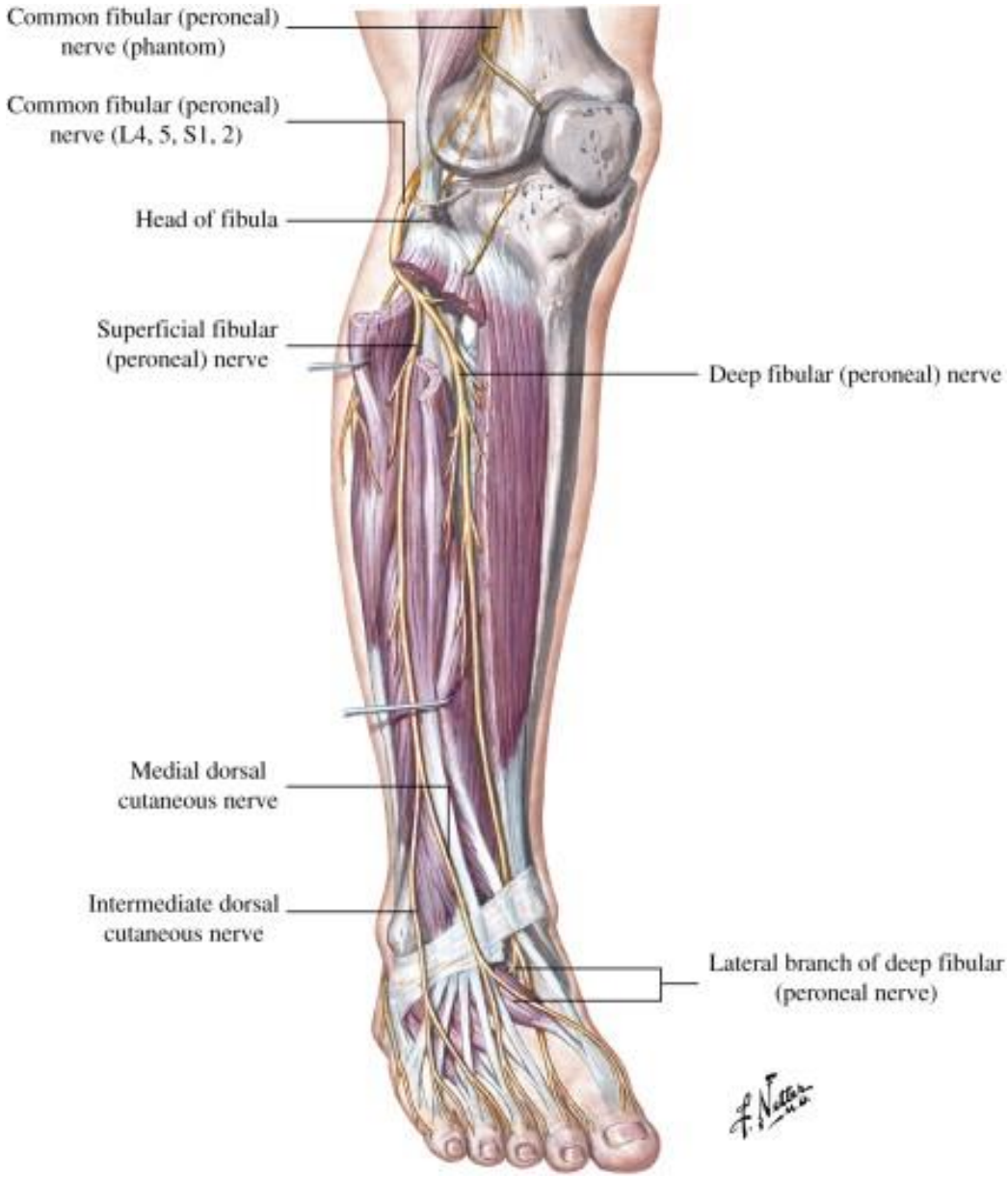


Figure 2.2 The course and distribution of the common, deep and superficial peroneal (fibular) nerves in the leg (Flanigan and DiGiovanni, 2011)

2.4.7.2 Isometric handgrip exercise (IHG)

Maximal voluntary contraction (MVC) was determined during the experimental set up by asking the participant to squeeze a handgrip as hard as possible. The handgrip was connected to a dynamometer (MIE Medical Research Ltd, UK) which provided a numerical display. In addition, the signal from the dynamometer was digitised (Coulbourn Lab Sinc V, Coulbourn Ltd, USA), passed to a PC and displayed as a dial on the monitor to aid participants during the exercise. Control data were recorded for 1 minute then the participant was asked to squeeze the handgrip until the pointer was in the green area of the dial, representing 30-40% of MVC, and to maintain this for 2 minutes. Participants were asked to try and keep all other muscles as relaxed as possible and to avoid breath holding during the handgrip. After 2 minutes participants were asked to stop the handgrip and a further 1 minute of recovery data was recorded. IHG causes an immediate and continuous increase in blood pressure mediated in the initial stage by tachycardia and subsequently by increased 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 s and then gradually increases throughout the exercise (Seals et al., 1988; Saito et al., 1990) (Figure 2.3). MSNA from the second minute of IHG was therefore compared to the control period.

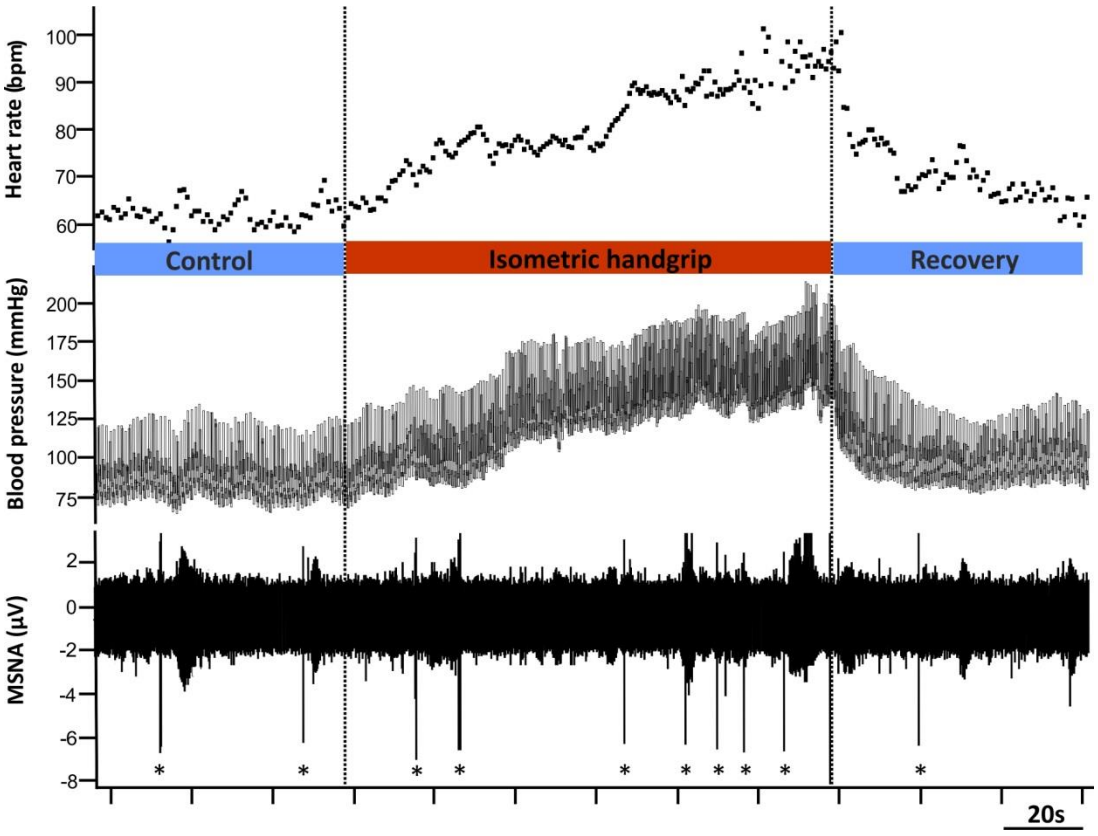


Figure 2.3 Example isometric handgrip exercise recording. There are 2 putative MSNA units during the 1 minute control period and during the first minute of exercise. The firing of this unit increases during the second minute of exercise to 5 units indicating that this is a sympathetic vasoconstrictor unit (* = single unit).

2.4.8 Data acquisition

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

2.4.9 Statistical analysis

All statistical analyses were carried out using SPSS (version 18). Independent t-tests or Mann-Whitney U tests were used to compare group characteristics. Repeated measures ANOVA was used to analyse time effect (baseline, stimulation, recovery) in each group alone with post hoc Bonferroni correction. To analyse the effects of different methods of stimulation, mixed mode ANOVA with group (electrode site, side or stimulation parameters) and time was used. For groups that differed at baseline, ANCOVA was used with baseline values held constant as covariates. Where interactions were revealed, post hoc analyses were undertaken using repeated measures ANOVA on each group separately. The Greenhouse – Geisser correction was used where data did not meet sphericity. Linear regression was used to explore the relationship between variables. Data are presented as group mean ± standard error of the mean (S.E.M.) unless stated otherwise. A 2-tailed probability value < 0.05 was considered statistically significant.

2.4.10 Study Failures

There were 132 volunteers for the first part of the study investigating the effects of different stimulation protocols on cardiovascular autonomic control, however, 21 records were excluded for the following reasons:

- 6 had multiple ectopic beats
- 5 had pre-existing conditions (hypertension, type 1 diabetes, persistent cough, back pain and stroke)
- 3 had cardiac arrhythmia
- 2 were excluded due to moving and talking during recordings
- 1 file was corrupted
- 1 had poor signal to noise ratio in ECG
- 1 had vasomotor symptoms of menopause
- 1 needed to urinate
- 1 was excluded due to noise from on-going building works

There were 18 volunteers for the second part of the study utilising microneurography however, 8 were excluded for the following reasons:

- 6 were excluded due to failure to locate an acceptable MSNA unit
- 1 had a poor signal to noise ratio
- 1 had a lesion present at the electrode insertion site.

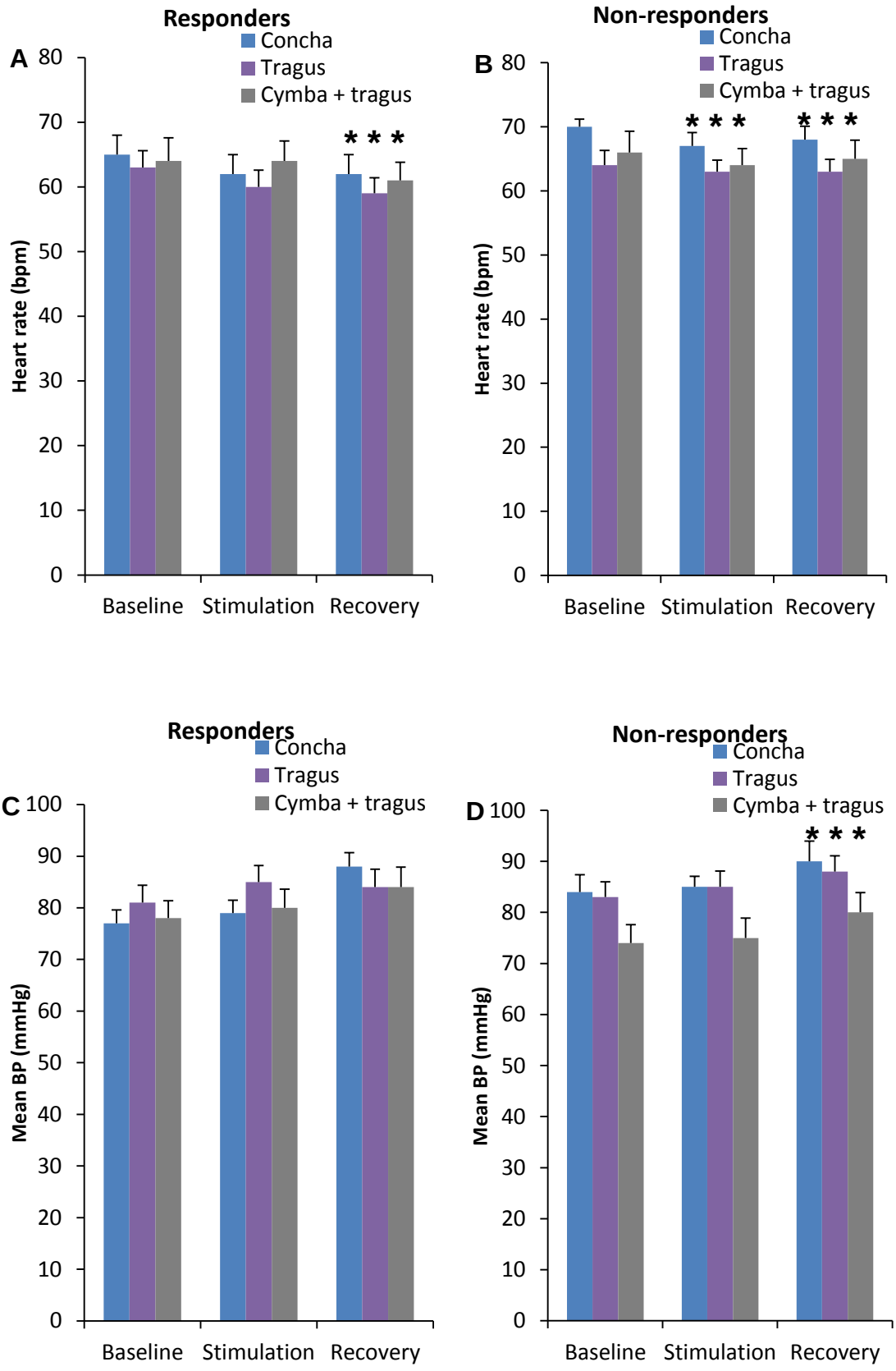
2.5 Results

2.5.1 Electrode positioning for tVNS

The auricular branch of the vagus nerve is distributed to parts of the external ear including the tragus, concha and cymba concha (Peuker and Filler, 2002) (Figure 1.2). The effect of stimulating these three areas by specific electrode placement at the ear was investigated in 63 healthy volunteers (34 female, 29 male; 20-66 years old). L-tVNS was performed for 15 minutes using a TENS machine (pulse width 20 μs; pulse frequency 15 Hz) and modified surface electrodes. Repeated measures ANOVA revealed there was no significant change in HRV or BRS during tVNS (Figure 2.5 A; Table 2.2– L-tVNS group). To test if this was due to the inter-individual variation in resting cardiac autonomic control, HRV was analysed for responders (those who had a decrease in LF/HF ratio during tVNS; n = 31) and non-responders (n = 32; Table 2.3). This decision was validated by the fact that there was a significant difference in baseline LF/HF ratios between responders and non-responders suggesting higher vagal tone in the non-responder group (1.32 and 1.04 respectively; $p = 0.038$; Table 2.3). There was no significant difference between responder and non-responder groups in other baseline characteristics such as body mass index (BMI), age, resting heart rate and blood pressure (Mann-Whitney U test, Table 2.4– L-tVNS group). Mixed mode ANOVA confirmed there was no significant change in LF/HF ratio (time effect, $p > 0.05$). There was also no impact of group (responder or non-responder main effect, $p > 0.05$), however, there was a significant interaction between group and time ($p < 0.0005$). Further analysis of the interaction

using a repeated measures ANOVA of LF/HF ratio in the responder group alone revealed a significant decrease in LF/HF ratio (Figure 2.5; time effect, $p < 0.0005$) during L-tVNS indicating a shift in autonomic balance towards parasympathetic predominance. Conversely, there was a significant increase in LF/HF ratio in the non-responder group (time effect, $p < 0.0005$) indicating that the interaction is due to an increase in LF/HF ratio in the non-responder group compared to a decrease in the responder group. There was no significant change in LF or HF power. There was no impact of electrode site on LF/HF in the responder group alone (tragus n = 34; concha n = 14; tragus + cymba n = 15; Figure 2.4; main effect for site, $p > 0.05$; Figure 2.5). In addition, there was no interaction between electrode site and time. There was a significant decrease in heart rate (time effect $p < 0.0005$) in the responder group alone (Figure 2.4). There was a significant decrease in heart rate (time effect, $p > 0.0005$) and an increase in mean BP in the non-responder group (time effect, $p = 0.001$). There was no impact of electrode site on heart rate (main effect for side, $p > 0.05$) or mean BP (main effect for side $p > 0.05$) and there was no interaction between electrode site and time.

Figure 2.4 Cardiovascular variables of responder and non-responder groups during L-tVNS of different electrode sites. In the responder group alone, there is a significant decrease in heart rate (time effect, $p < 0.0005$) and no change in mean BP (time effect, $p > 0.05$). There is a significant decrease in heart rate (time effect, $p > 0.0005$) and an increase in BP in the non-responder group (time effect, $p = 0.001$). Electrode site had no effect on heart rate (main effect for site, $p > 0.05$) or mean BP (main effect for side, $p > 0.05$) and there was no interaction between site and time (* = significantly different from baseline).


A
Responders
Concha
Tragus
Cymba + tragus
Heart rate (bpm)
0
10
20
30
40
50
60
70
80
* * *
Baseline
Stimulation
Recovery
B
Non-responders
Concha
Tragus
Cymba + tragus
Heart rate (bpm)
0
10
20
30
40
50
60
70
80
* * *
* * *
Baseline
Stimulation
Recovery
C
Responders
Concha
Tragus
Cymba + tragus
Mean BP (mmHg)
0
10
20
30
40
50
60
70
80
90
100
Baseline
Stimulation
Recovery
D
Non-responders
Concha
Tragus
Cymba + tragus
Mean BP (mmHg)
0
10
20
30
40
50
60
70
80
90
100
* * *
Baseline
Stimulation
Recovery

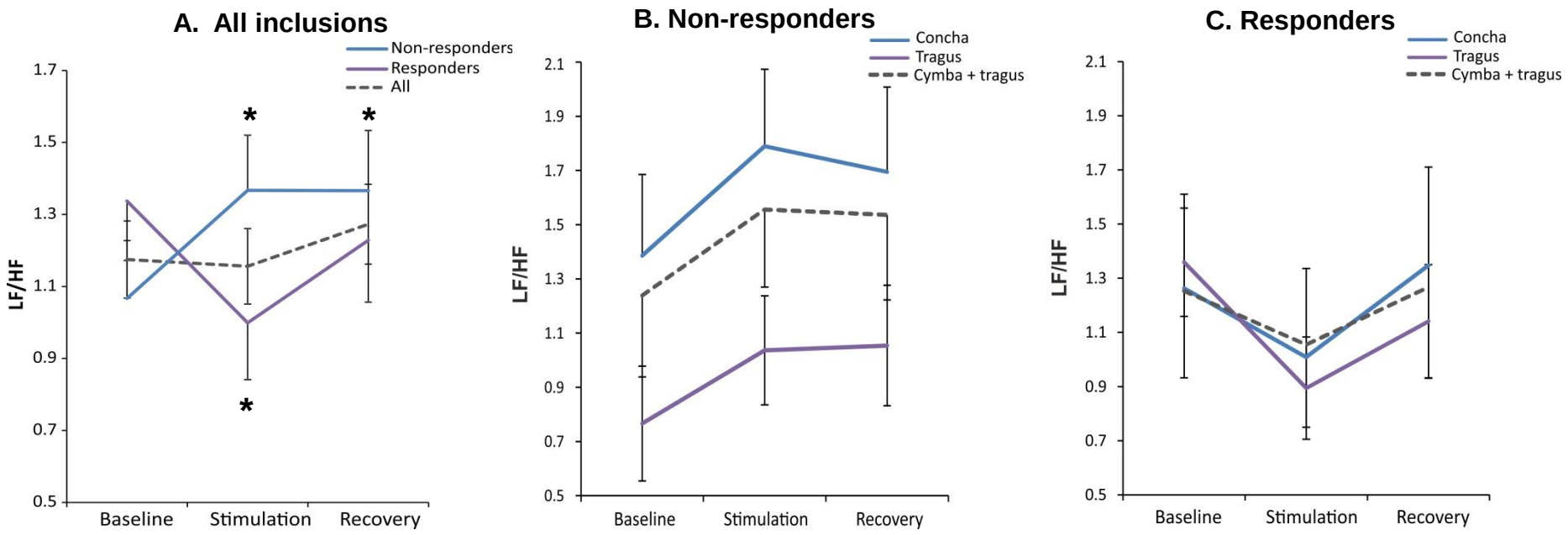


Figure 2.5 Effects of different L-tVNS electrode positions on HRV in responder and non-responder groups. There was no significant change in LF/HF (A; time effect $p > 0.05$) and group (responder or non-responder) had no impact (main effect for group ($p > 0.05$), however there was a significant interaction between group and time ($p < 0.0005$). In the non-responder group alone, there was a significant increase in LF/HF (time effect, $p < 0.0005$; $n = 32$). There was a significant decrease in LF/HF ratio during L-tVNS in the responder group ($p < 0.0005$; $n = 31$). There was no impact of electrode site on the concha ($n = 14$), tragus ($n = 34$) or tragus + cymba concha ($n = 15$; main effect for site, $p > 0.05$; C) and no interaction between electrode site and side (* = significantly different from baseline).

2.5.2 L-tVNS of the right ear vs. bilateral tVNS

In the same group of participants in which electrode positioning was investigated, the effect of stimulating just the right ear (n = 21) versus left and right ears simultaneously was investigated (n = 42; Figure 2.6). Mixed mode ANOVA revealed no significant change in LF/HF (time effect, $p > 0.05$) and no impact of group (responder or non-responder), however there was a significant interaction between group and time. Further analysis of the interaction using a repeated measures ANOVA of the responder group alone revealed a significant increase in LF/HF ratio (time effect, $p < 0.0005$). There was a significant increase in LF/HF ratio in the non-responder group ($p > 0.0005$). There was no impact of side on LF/HF (main effect for side, $p > 0.05$) and there was no interaction between side and time. There was a significant decrease in heart rate (time effect, $p < 0.0005$) and increase in mean BP (time effect, $p < 0.0005$) in the responder group alone (Figure 2.6). There was also a decrease in heart rate (time effect, $p < 0.0005$) and an increase in mean BP in the non-responder group (time effect, $p < 0.0005$). There was no impact of electrode site on heart rate (main effect for side, $p > 0.05$) or mean BP (main effect for side $p > 0.05$) and there was no interaction between electrode site and time (Figure 2.7). Based on these results, tVNS of the tragus of both left and right ears was used for all subsequent experiments due to ease of application.

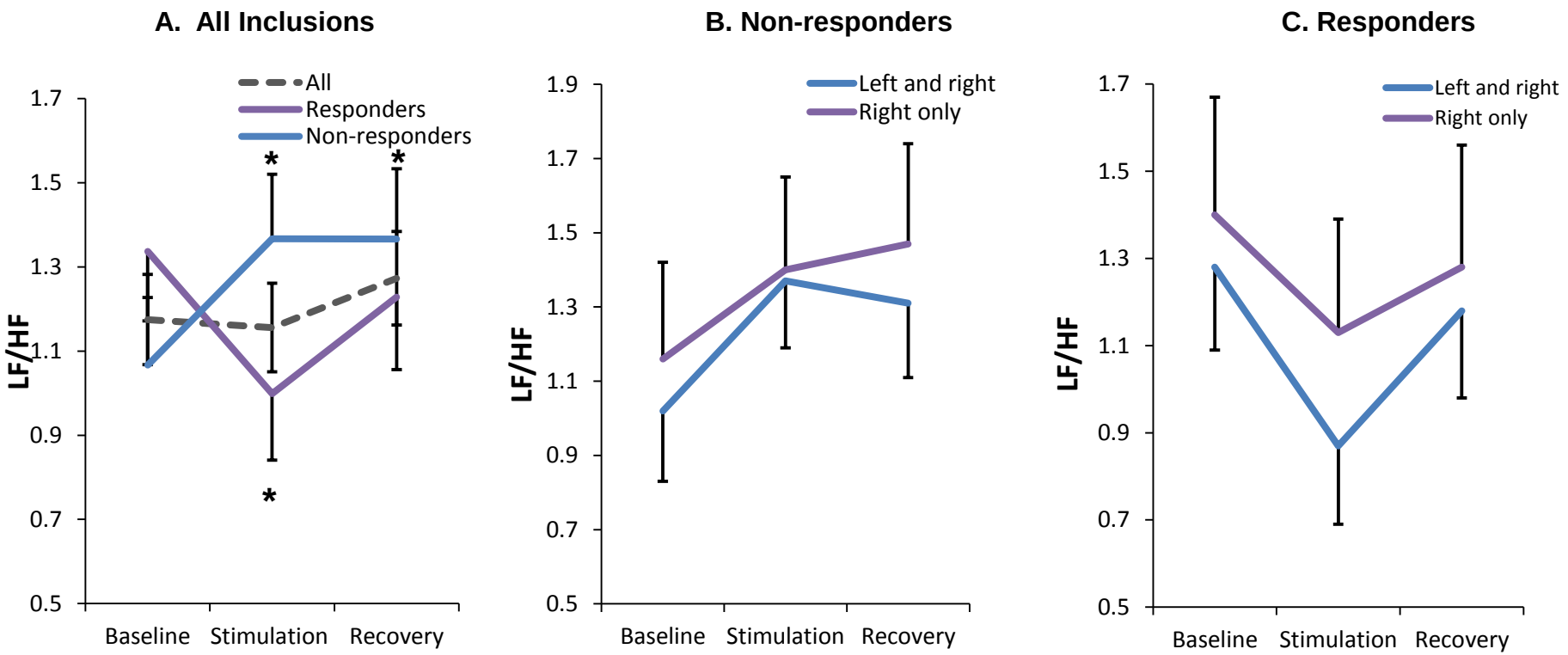
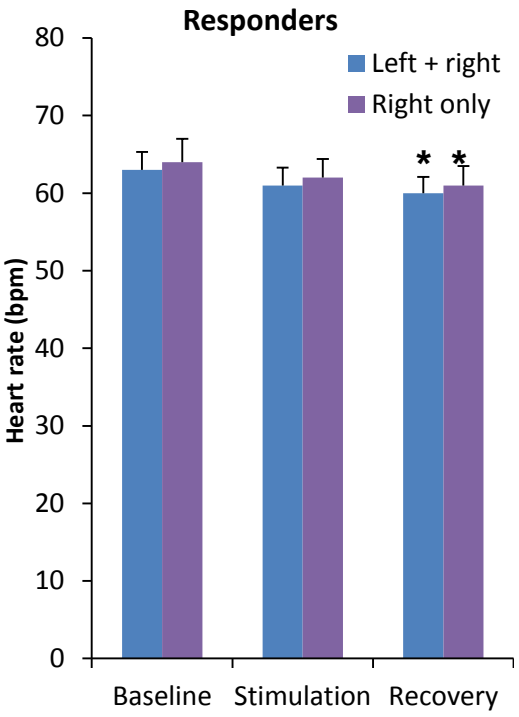
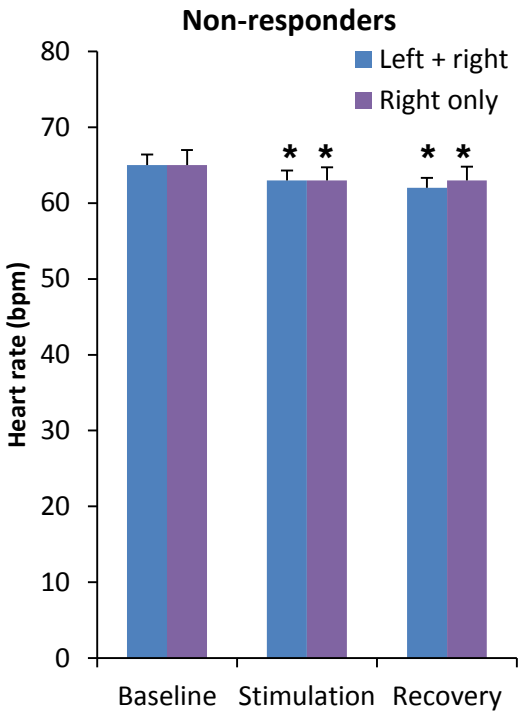
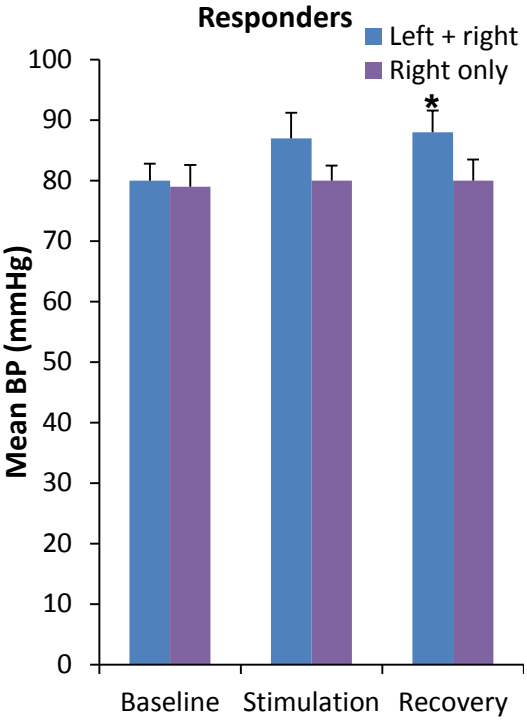


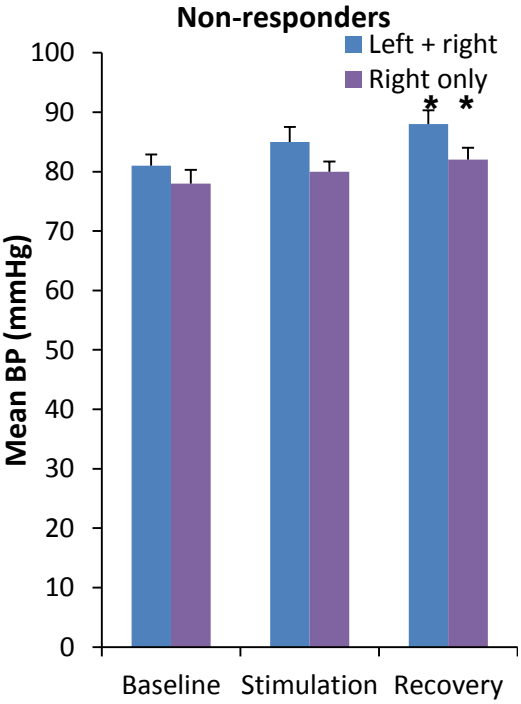
Figure 2.6 Effects of L-tVNS of the right ear only compared to left and right ears simultaneously. There was no significant change in LF/HF (A; time effect $p > 0.05$) and group (responder or non-responder) had no impact (main effect for group, $p > 0.05$), however there was a significant interaction between group and time ($p < 0.0005$). In the non-responder group alone, there was a significant increase in LF/HF (time effect, $p < 0.0005$; $n = 32$). There was a significant decrease in LF/HF ratio during L-tVNS in the responder group alone (time effect, $p < 0.0005$; $n = 31$). There was no impact of side (main effect for side, $p > 0.05$; C) and no interaction between side and side (* = significantly different from baseline).

Figure 2.7 Cardiovascular variables of responder and non-responder groups during L-tVNS of right vs. both ears simultaneously. In the responder group alone, there is a significant decrease in heart rate (time effect, $p < 0.0005$) and an increase in mean BP (time effect, $p < 0.0005$). There is also a decrease in heart rate (time effect, $p < 0.0005$) and an increase in mean BP in the non-responder group (time effect, $p < 0.0005$). Electrode site had no effect on heart rate (main effect for site, $p > 0.05$) or mean BP (main effect for site, $p > 0.05$) and there was no interaction between site and time (* = significantly different from baseline).


Responders
Left + right
Right only
Heart rate (bpm)
80
70
60
50
40
30
20
10
0
Baseline
Stimulation
Recovery
*
*


Non-responders
Left + right
Right only
Heart rate (bpm)
80
70
60
50
40
30
20
10
0
Baseline
Stimulation
Recovery
*
*
*
*


Responders
Left + right
Right only
Mean BP (mmHg)
100
90
80
70
60
50
40
30
20
10
0
Baseline
Stimulation
Recovery
*


Non-responders
Left + right
Right only
Mean BP (mmHg)
100
90
80
70
60
50
40
30
20
10
0
Baseline
Stimulation
Recovery
*
*

2.5.2 Stimulation parameters for tVNS

The cardiovascular autonomic effects of different stimulation parameters were investigated in 111 healthy volunteers (58 female, 53 male; 20-66 years old). tVNS was performed with low pulse width and pulse frequency (L-tVNS = 20 μs at 15 Hz; n = 63), high pulse width and pulse frequency (H-tVNS = 200 μs at 30 Hz; n = 34) or sham stimulation (electrodes placed on the ear without electrical stimulation; n = 14) and HRV analysed (including responders and non-responders). The L-tVNS group included stimulation using different electrode positions but as no significant difference was found between positions all were included for investigation of stimulation parameters. Mixed mode ANOVA revealed a significant decrease in LF/HF during tVNS (time effect, $p = 0.040$). Stimulation parameters (L-tVNS or H-tVNS group) had no impact on LF/HF ratio (main effect of stimulation parameters, $p > 0.05$), however, there was a significant interaction between stimulation parameters and time ($p = 0.048$; Figure 2.8). Further analysis of the interaction using a repeated measures ANOVA of the H-tVNS group alone revealed a significant decrease in LF/HF ratio (time effect, $p = 0.026$; Figure 2.8). There was no significant change in LF/HF ratio in L-tVNS and sham groups. Splitting the H-tVNS group into responders to tVNS and non-responders again revealed a significant difference in baseline LF/HF ratios between responders and non-responders (1.54 and 0.64 respectively; $p = 0.001$; Table 2.3). Repeated measures ANOVA revealed a significant decrease in LF power during tVNS in the H-tVNS responder group (time effect, $p = 0.001$; Table 2.3) whereas total power and HF power did not change significantly. There was no significant change in total power, LF power or HF power in the non-responder group during H-tVNS.

Table 2.2 Heart rate variability values for Sham tVNS, L-tVNS and H-tVNS groups including responders and non-responders. There was a significant decrease in LF/HF during tVNS (time effect, $p = 0.040$). Stimulation parameters (L-tVNS or H-tVNS group) had no impact on LF/HF ratio (main effect of stimulation parameters, $p > 0.05$), however, there was a significant interaction between stimulation parameters and time ($p = 0.048$). There was a significant decrease in LF/HF ratio during tVNS in the H-tVNS group alone (time effect, $p = 0.026$). There was no significant difference in baseline total power, LF power, HF power or LF/HF ratio between these groups.

	Sham (n = 14)			L-tVNS (n = 63)			H-tVNS (n = 34)		
	Base	**Stim**	**p**	**Base**	**Stim**	**p**	**Base**	**Stim**	**p**
Total power (ms^2)	2463.75 ±732.50	2789.22 ±843.02	ns	2072.56 ±291.50	2033.08 ±343.28	ns	2735.31 ±422.23	3212.17 ±497.45	ns
LF Power (ms^2)	615.52 ±168.65	664.63 ±160.85	ns	578.83 ±92.29	588.49 ±81.16	ns	906.39 ±133.74	821.49 ±177.62	ns
HF Power (ms^2)	1109.41 ±395.90	1286.40 ±469.05	ns	823.99 ±143.75	779.12 ±123.28	ns	972.67 ±208.32	1043.02 ±178.65	ns
LF/HF	1.16 ±0.30	1.19 ±0.32	ns	1.18 ±0.11	1.16 ±0.10	ns	1.26 ±0.15	1.04 ±0.14	**0.026**
BRS (ms/mmHg)	10.47 ±4.01	11.46 ±4.81	ns	11.40 ±0.82	11.28 ±.82	ns	11.51 ±1.46	12.55 ±1.75	ns

Table 2.3 Heart rate variability values for H-tVNS and L-tVNS responder and non-responder groups. There is a significant decrease in LF/HF ratio during stimulation (time effect, p = 0.040). Stimulation parameters had no impact on LF/HF (main effect of stimulation parameters, p >0.05). There is an interaction between stimulation parameters and time (p = 0.048). There is a significant decrease in LF/HF ratio in the H-tVNS group alone (n=34; time effect, p=0.026). There is no change in LF/HF in the L-tVNS (n=63) or Sham (n = 14) groups. There is a significant difference in baseline LF power between H-tVNS responders and non-responders (*p = 0.041) and in baseline LF/HF ratio between H-tVNS (**p = 0.001) and L-tVNS groups (†p < 0.038). There is a significant decrease in LF power during stimulation in the H-tVNS responder group (time effect, p = 0.001). Both L-tVNS and H-tVNS responder groups show a significant decrease in LF/HF ratio during stimulation (time effect, p < 0.0005). There is a significant increase in LF/HF ratio in the L-tVNS non-responder group (time effect, p < 0.0005).

	L-tVNS						H-tVNS					
	Responder			Non-responder			Responder			Non-responder		
	Base	**Stim**	**p**	**Base**	**Stim**	**p**	**Base**	**Stim**	**p**	**Base**	**Stim**	**p**
Total Power (ms^2)	1953.7 ±345.9	2041.1 ±298.3	ns	2187.7 ±340.4	2025.3 ±293.6	ns	2957.5 ±590.4	3183.1 ±836.1	ns	1895.5 ±853.7	2799.8 ±1208.9	ns
LF Power (ms^2)	623.0 ±94.8	567.4 ±93.4	ns	536.0± 93.3	608.9 ±91.9	ns	**1041.9*** ±202.4	779.9 ±172.1	0.001	**476.0*** ±292.6	783.6 ±248.8	ns
HF Power (ms^2)	596.4 ±210.3	761.2 ±163.3	ns	1044.5 ±206.9	796.5 ±160.7	ns	934.3 ±210.9	964.2 ±226.6	ns	868.6 ±305.1	1031.4 ±327.7	ns
LF/HF	**1.32†** ±0.15	0.95 ±0.15	<0.0005	**1.04†** ±0.15	1.35 ±0.14	<0.0005	**1.54**** ±0.14	1.09 ±0.11	<0.005	**0.64**** ±0.20	0.88 ±0.17	ns

Table 2.4 Baseline characteristics of H-tVNS, L-tVNS and sham groups. There was a significant difference in baseline LF/HF ratio between H-tVNS responders and non-responders (**p = 0.001) and L-tVNS responders and non-responders (*p = 0.038). There was also a significant difference in baseline LF/HF ratio between H-tVNS and L-tVNS non-responder groups (†p = 0.041).

	Sham tVNS	**H-tVNS**	**H-tVNS responder**	**H-tVNS non-responder**	**L-tVNS**	**L-tVNS responder**	**L-tVNS non-responder**
Number	14 (6 ♀; 8 ♂)	34 (18 ♀; 16 ♂)	23 (10 ♀; 13 ♂)	11 (8 ♀; 3 ♂)	63 (34 ♀; 29 ♂)	31 (16 ♀; 15 ♂)	32 (18 ♀; 14 ♂)
Age (years)	38±3.48	34±2.3	37±3.1	35±3.3	38±1.66	38±2.39	37±2.35
BMI (kg/m^2)	23.9±0.67	24.9±0.71	25.27±0.95	24.34±0.75	24.5±0.57	24.2±0.54	24.8±1.00
Heart rate (bpm)	64±2.49	64±1.31	62±1.73	66±1.71	65±1.16	63±1.79	66±1.48
Mean BP (mmHg)	79±3.59	83±1.99	84±2.62	80±2.57	80±1.46	80±2.17	81±2.00
LF/HF	1.15±0.28	1.25±0.14	**1.54±0.17****	**0.64±0.10**,†	1.18±0.11	**1.32±0.15***	**1.04±0.15***,†

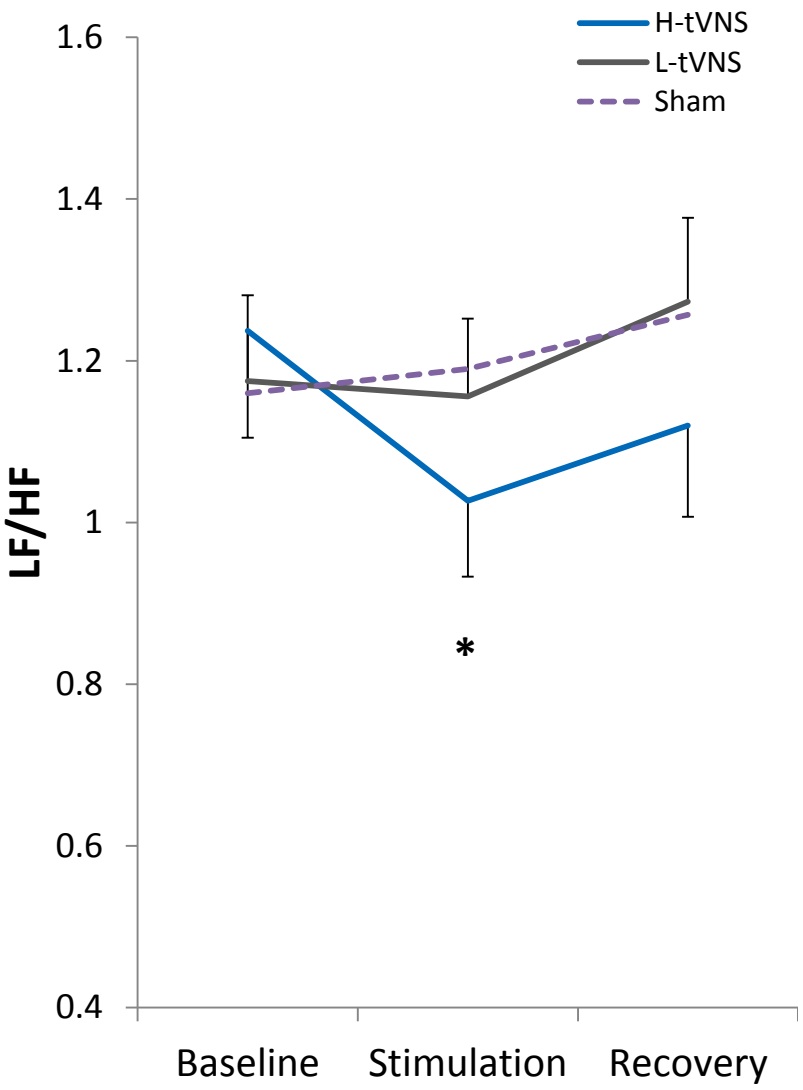


Figure 2.8 Comparison of high pulse width and frequency tVNS and low pulse width and frequency tVNS on HRV. There is a significant decrease in LF/HF ratio during stimulation (time effect, p = 0.040). Stimulation parameters had no impact on LF/HF (main effect of stimulation parameters, p >0.05). There is an interaction between stimulation parameters and time (p = 0.048). There is a significant decrease in LF/HF ratio in the H-tVNS group alone (n=34; time effect, p=0.026). There is no change in LF/HF in the L-tVNS (n=63) or Sham (n = 14) groups (error bars omitted from the sham group to aid clarity; * = significantly different from baseline).

Linear regression revealed a relationship between baseline LF/HF ratio and the change in LF/HF ratio during H-tVNS such that a higher baseline LF/HF ratio predicts a greater response to H-tVNS ($R^2 = 0.58$; $p < 0.0005$; Figure 2.9). Aging is associated with changes in cardiovascular autonomic function with increasing sympathetic activity and decreasing parasympathetic activity (Umetani et al., 1998). Indeed, a relationship between increasing age and higher baseline LF/HF values was also observed in this study ($R^2 = 0.19$; $p = 0.013$; Figure 2.9) although this relationship was weaker.

There was no change in BRS during tVNS, however, there was a modest but significant decrease in heart rate (time effect, $p < 0.0005$; Figure 2.10), although this was not affected by stimulation parameters (L-tVNS, H-tVNS or sham groups; main effect of stimulation parameters, $p = 0.05$) and there was no interaction between stimulation parameters and time. There was also a significant increase in mean BP (time effect, $p < 0.0005$; Figure 2.10) during stimulation that did not recover. Similar to heart rate, there was no impact of stimulation parameters on mean BP (main effect of stimulation parameters, $p > 0.05$) and no interaction between stimulation parameters and time. The increase in BP may be due to the method of BP measurement (Finometer) which was in place throughout the experiment. The Finometer calibration system was used during each experiment, however it was temporarily switched off during recordings for 15 minutes. It is possible that this affected accurate BP monitoring. It has also been reported that long term Finometer measurements may cause local oedema affecting BP detection (Ristuccia et al., 1997). To investigate this, 12 subjects also had their BP measured from the arm using an automatic BP machine. Three arm readings of BP were taken at baseline, during stimulation and during recovery and the average of the readings compared to the Finometer measurements. A mixed mode ANOVA revealed that there was a significant increase in systolic blood pressure during stimulation and recovery (time effect, $p = 0.006$; Figure 2.10) and the method of measurement had a significant effect (main effect method of measurement, $p = 0.024$). Furthermore there was an interaction between method of measurement and time ($p = 0.002$). Further analysis of this interaction using repeated

measures ANOVA revealed a significant increase in systolic BP in the Finometer group (time effect, $p = 0.005$). There was no change in systolic BP measure from the arm. A mixed mode ANOVA also revealed a significant increase in mean BP during stimulation and recovery (time effect, $p = 0.016$; Figure 2.10). Method of measurement had no impact on mean BP, however, there was an interaction between method of measurement and time ($p = 0.009$). Further analyses of these interactions using repeated measures ANOVA of BP measurements taken using the arm method alone showed no significant change. There was a significant increase in mean BP (time effect, $p = 0.016$) measured using the Finometer alone. As there was no significant change in BP measurements taken using an arm sphygmomanometer whereas the increase in BP measured using the Finometer persisted into the recovery period, this suggests that the increase may be due to the method of measurement – perhaps constriction and oedema caused by the finger cuff. This may also have affected the analysis of BRS from Finometer records.

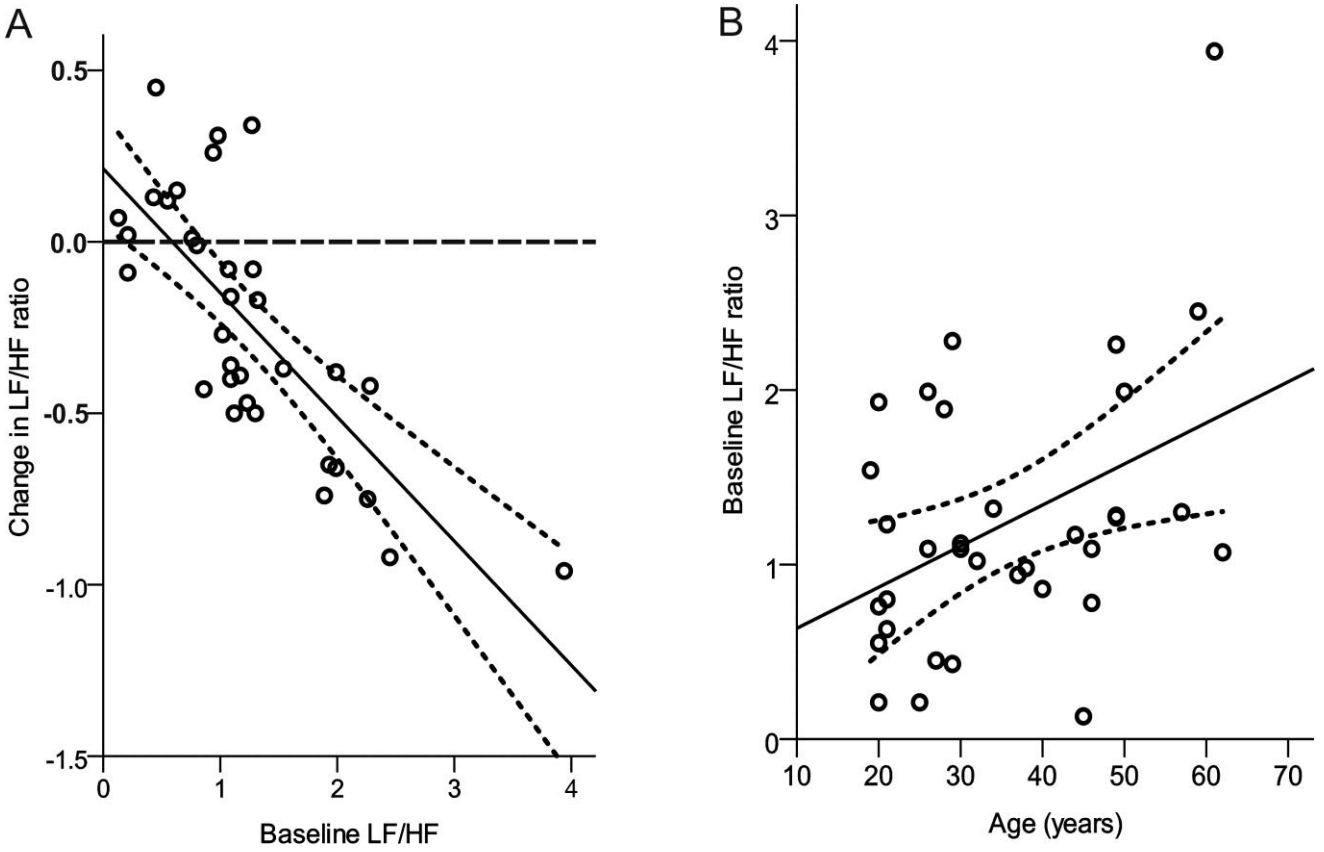
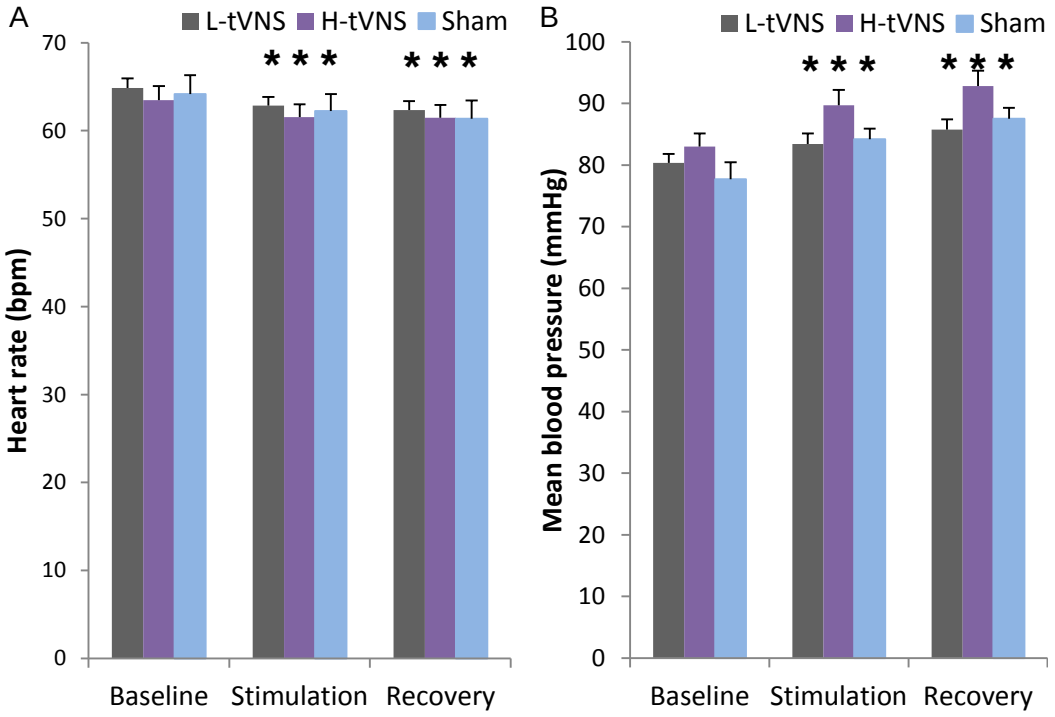
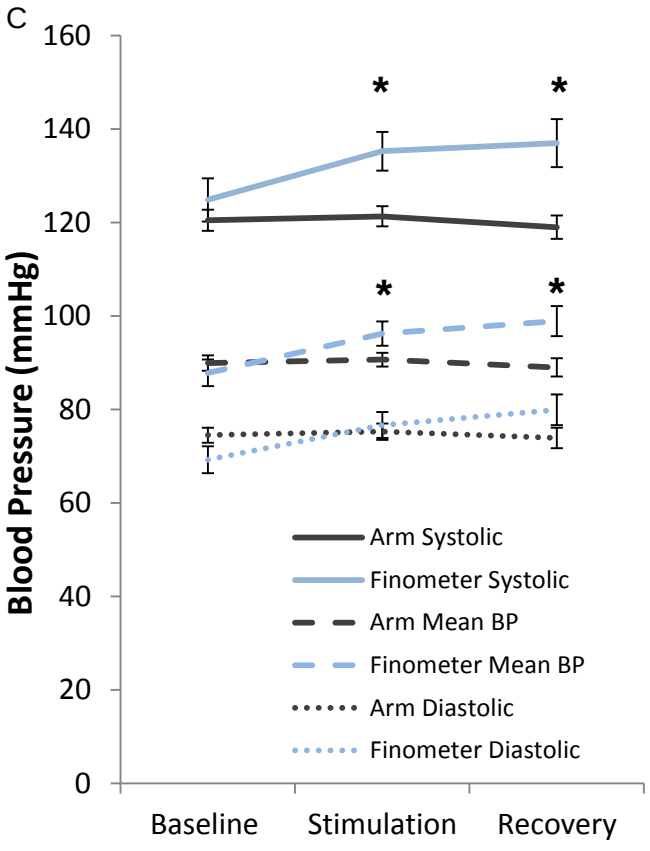


Figure 2.9 The relationship between baseline LF/HF and response to H-tVNS. There is a relationship between baseline LF/HF ratio and change in LF/HF ratio during H-tVNS indicating that higher baseline LF/HF ratios predict a greater decrease in LF/HF during H-tVNS (R^2=0.58; p<0.0005; A). There is a relationship between age and baseline LF/HF ratio (R^2=0.19; p=0.013; B).

Figure 2.10 There is a significant reduction in heart rate and an increase in BP during tVNS and recovery. There is a small but significant decrease in heart rate (time effect, $p < 0.0005$; A). There is also a modest but significant increase in BP (time effect, $p < 0.0005$; B) during stimulation and the recovery period. There is no impact of stimulation parameters on heart rate (main effect of stimulation parameters, $p > 0.05$) or mean BP (main effect of stimulation parameters, $p > 0.05$) and no interaction between stimulation parameters and time.

The effects of different methods of measuring BP (arm vs. Finometer) was investigated ($n = 12$; C). There was a significant increase in systolic blood pressure during stimulation and recovery (time effect, $p = 0.006$) and the method of measurement had a significant effect (main effect method of measurement, $p = 0.024$). Furthermore there was an interaction between method of measurement and time ($p = 0.002$). Further analysis of this interaction revealed a siginificant increase in systolic BP measured using the Finometer (time effect, $p = 0.005$). There was no change in systolic BP measured from the arm. There was also a significant increase in mean BP during stimulation and recovery (time effect, $p = 0.016$). Method of measurement had no impact on mean BP, however, there was an interaction between method of measurement and time ($p = 0.009$). Further analysis of this interaction revealed a significant increase in mean BP (time effect, $p = 0.016$) measured using the Finometer alone. There is no change in mean BP measured from the arm (* = significantly different from baseline).


A
L-tVNS
H-tVNS
Sham
Heart rate (bpm)
70
60
50
40
30
20
10
0
* * *
* * *
Baseline
Stimulation
Recovery
B
L-tVNS
H-tVNS
Sham
Mean blood pressure (mmHg)
100
90
80
70
60
50
40
30
20
10
0
* * *
* * *
Baseline
Stimulation
Recovery


C
Blood Pressure (mmHg)
160
140
120
100
80
60
40
20
0
*
*
*
*
Arm Systolic
Finometer Systolic
Arm Mean BP
Finometer Mean BP
Arm Diastolic
Finometer Diastolic
Baseline
Stimulation
Recovery

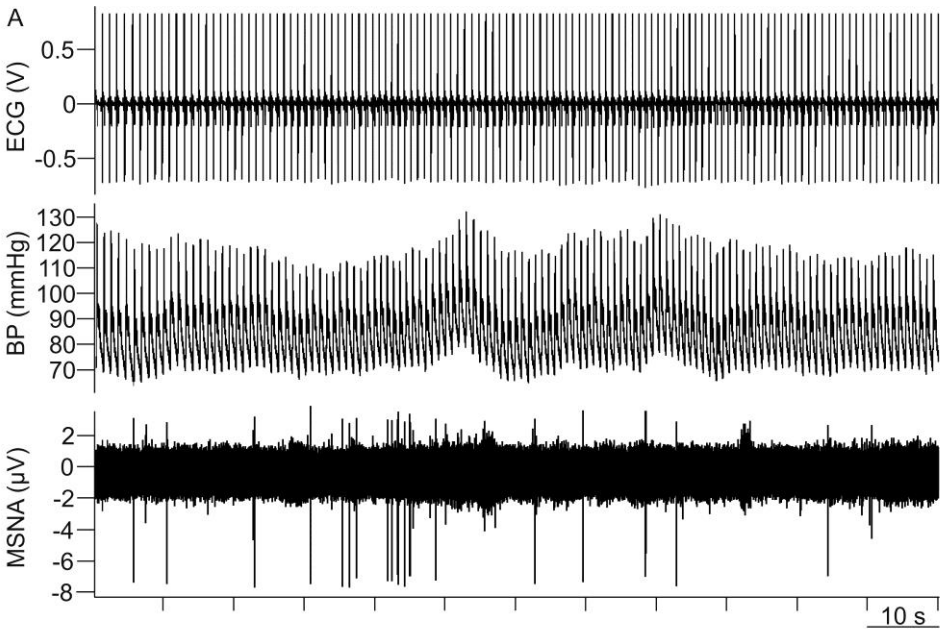
2.5.3 Contribution of the sympathetic nervous system to cardiovascular autonomic changes during H-tVNS

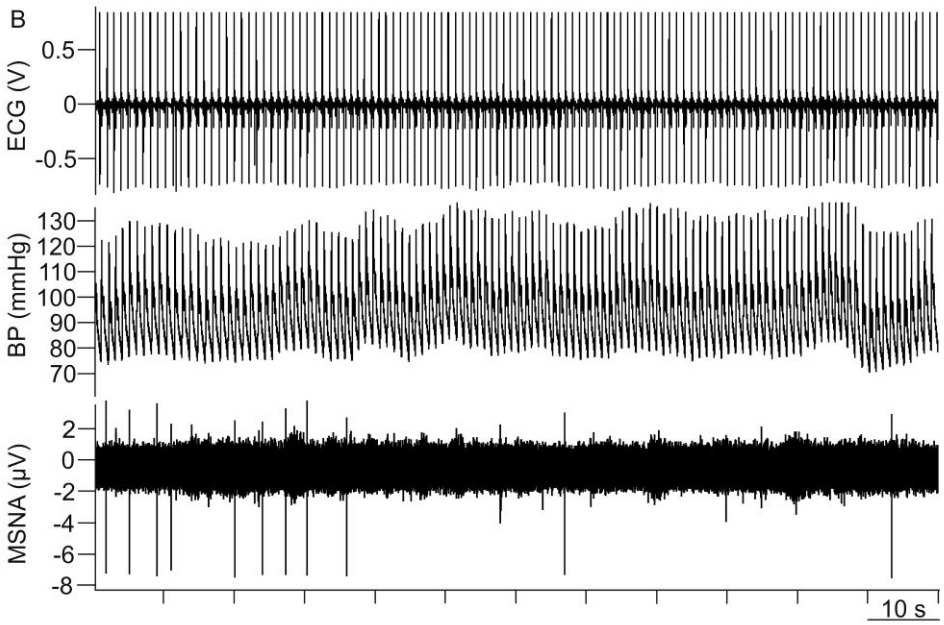
H-tVNS altered cardiac autonomic control towards vagal predominance, however, the lack of increase in HF power suggests this is not due to an increase in vagal tone. Alternatively, the shift in HRV towards parasympathetic predominance may be due to reduced sympathetic activity. While cardiac sympathetic activity can be investigated using cardiac noradrenaline spill-over, the invasiveness of this technique was not in keeping with the ethos of this study. Instead, vascular vasoconstrictor activity was recorded using microneurography. While there are regional differences in sympathetic outflow, there is a correlation between HRV and MSNA (Pagani et al., 1997). Sympathetic vasoconstrictor single unit activity was recorded in 10 healthy volunteers during H-tVNS (Figure 2.11; Table 2.5). Cardiovascular and MSNA data during the cold pressor test and isometric handgrip test confirmed the identification of sympathetic vasoconstrictor units (Table 2.6). There was a significant decrease in MSNA frequency (time effect, $p = 0.001$) and incidence (time effect, $p = 0.002$; Figure 2.12) during H-tVNS. Similar to the H-tVNS group as a whole, there was a modest but significant decrease in heart rate (time effect, $p < 0.0005$; Table 2.7) and mean BP (time effect, $p < 0.0005$) during H-tVNS and recovery. There was a reduction in LF/HF ratio, however, this did not reach significance (time effect, $p > 0.05$).

Table 2.5 Raw microneurography values. Results are presented as mean ± S.E.M.

	Baseline	**Stimulation**	**Recovery**
MSNA frequency (units/min)	1.20 ± 0.29	0.55 ± 0.28	0.90 ± 0.29
MSNA incidence (units/100 heart beats)	2.00 ± 0.49	0.91 ± 0.46	1.54 ± 0.48

Figure 2.11 Example microneurography recordings from one individual before and during H-tVNS. Raw ECG, BP and MSNA activity at baseline (A) and during H-tVNS (B). Action potentials from a single MSNA unit (C) and overlaid to show consistent morphology (D).


A
ECG (V)
0.5
0
-0.5
BP (mmHg)
130
120
110
100
90
80
70
MSNA (μV)
2
0
-2
-4
-6
-8
10 s


B
ECG (V)
0.5
0
-0.5
BP (mmHg)
130
120
110
100
90
80
70
MSNA (μV)
2
0
-2
-4
-6
-8
10 s

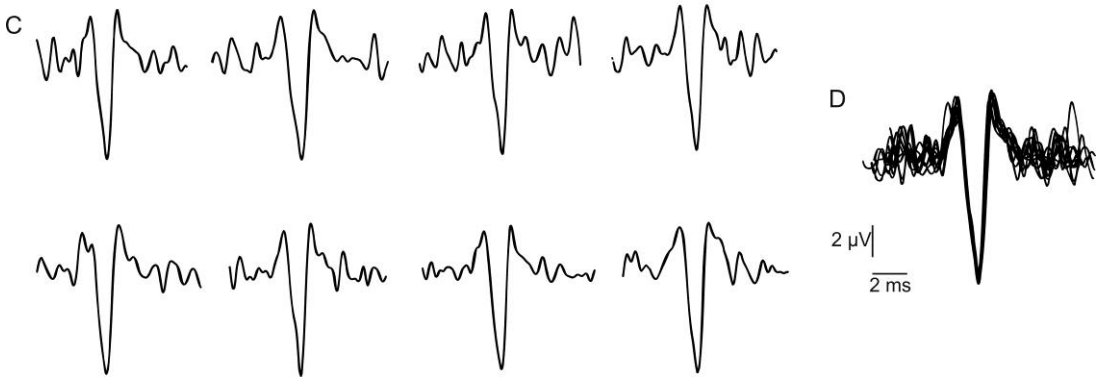

C
D
2 μV
2 ms

Table 2.6 Cardiovascular and MSNA data during cold pressor and isometric handgrip tests.

	Baseline	CPT	Recovery	Baseline	IHG	Recovery
Heart rate (bpm)	61 ± 2.7	66 ± 2.8	60 ± 2.8	62 ± 2.8	81 ± 2.9	61 ± 2.7
Mean BP (mmHg)	87 ± 3.1	108 ± 3.4	89 ± 3.2	86 ± 3.0	123 ± 3.3	90 ± 3.1
MSNA frequency (units/min)	1.24 ± 0.30	2.78 ± 0.32	1.03 ± 0.29	1.30 ± 0.28	3.84 ± 0.28	1.46 ± 0.29
MSNA incidence (units/100 heart beats)	2.03 ± 0.48	4.21 ± 0.46	1.72 ± 0.46	1.66 ± 0.49	4.74 ± 0.48	2.39 ± 0.49

Table 2.7 Effects of H-tVNS on cardiovascular and HRV variables of participants who underwent microneurography (n = 10). There is a modest but significant decrease in heart rate (time effect, $p < 0.0005$; Table 2.7) and mean BP (time effect, $p < 0.0005$; * = significantly different from baseline).

	Baseline	Stimulation	Recovery
Total Power (ms^2)	2822 ± 1469	3603 ± 2127	2783 ± 1290
LF Power	912 ± 471	711 ± 337	505 ± 140
HF Power	861 ± 465	906 ± 457	603 ± 170
LF/HF	1.28 ± 0.19	0.99 ± 0.15	1.13 ± 0.22
Heart rate (bpm)	60 ± 2.7	**59 ± 2.7***	**58 ± 2.6***
Mean BP (mmHg)	86 ± 3.0	**94 ± 3.3***	**96 ± 3.0***
BRS (ms/mmHg)	13.15 ± 4.08	14.71 ± 4.96	13.03 ± 2.93

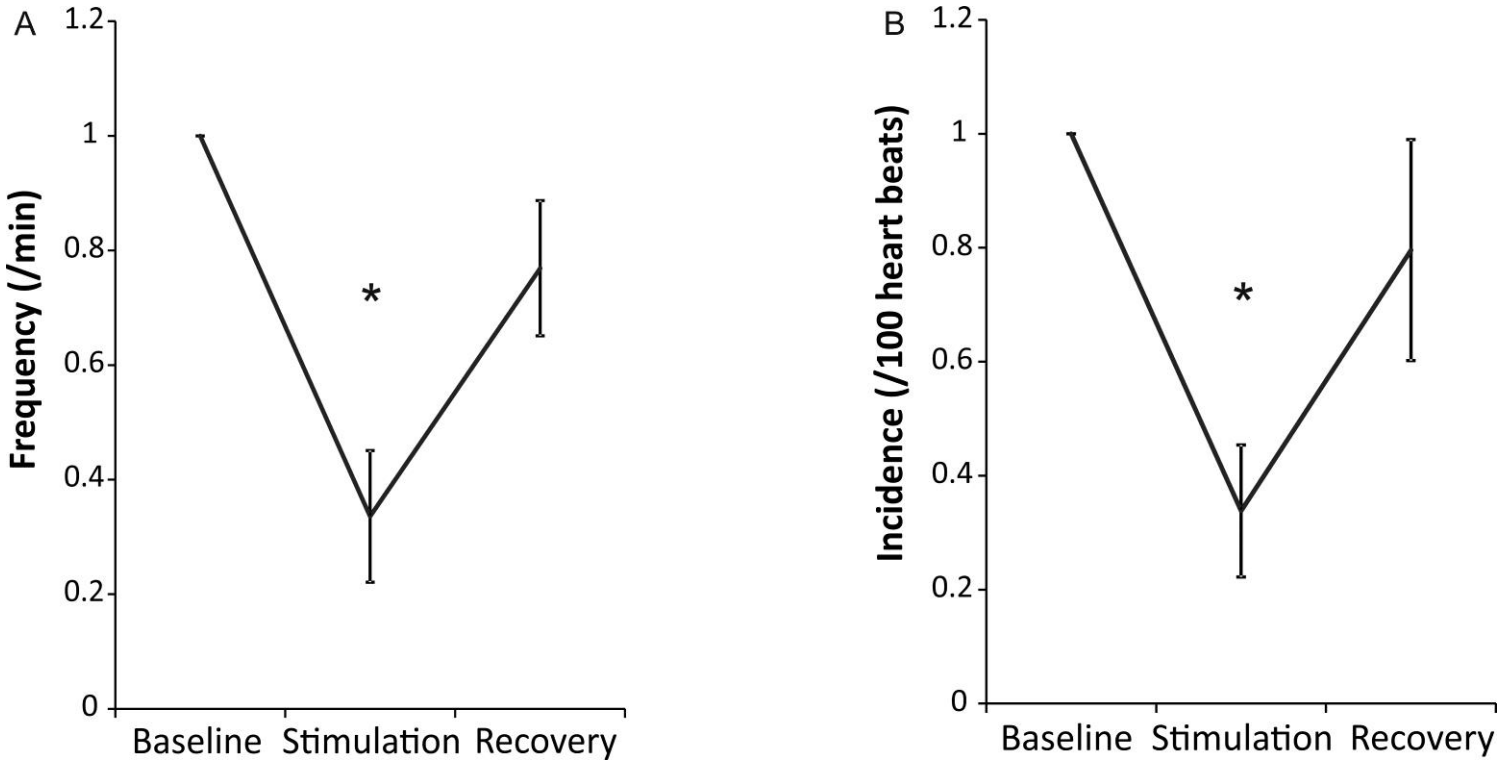


Figure 2.12 H-tVNS significantly reduces single unit MSNA frequency (A; p = 0.001) and incidence (B; p = 0.002; normalised data; n = 10; * = significantly different from baseline).

2.6 Discussion

This study shows for the first time that transcutaneous vagus nerve stimulation can alter cardiovascular autonomic control in healthy participants. The results also highlight the role of the sympathetic nervous system in mediating tVNS effects. High pulse width and high pulse frequency tVNS significantly decreased LF/HF ratio, indicating improved heart rate variability with a shift in cardiac autonomic balance towards parasympathetic/vagal dominance. A significant decrease in MSNA during H-tVNS indicated that this protocol may activate central autonomic centres to elicit an indirect decrease in sympathetic nerve activity.

2.6.1 tVNS effects on cardiovascular autonomic function

Increased sympathetic activity and/or reduced parasympathetic activity as indicated by HRV is not only a powerful and independent predictor of poor prognosis in patients with cardiovascular disease (Kleiger et al., 1987; Nolan et al., 1998), but is also a risk factor in healthy populations (Nunan et al., 2010). Reduced heart rate variability in a healthy population (n = 2501) was associated with a significantly increased risk of subsequent cardiac events (Tsuji et al., 1996). Increased MSNA is associated with poor prognosis in heart failure and is also elevated in hypertension, obstructive sleep apnoea and obesity (Charkoudian and Rabbitts, 2009). The ability to favourably alter HRV and MSNA through H-tVNS in a healthy population is significant and could be applied to many populations where cardiovascular autonomic balance is shifted toward sympathetic predominance e.g. older or sedentary populations (Mischel and Mueller, 2011) or in other conditions with sympathoexcitation such as hypertension (Charkoudian and Rabbitts, 2009). Indeed, the significant correlation between baseline LF/HF ratio and the change in LF/HF ratio during stimulation implies that H-tVNS may be even more effective in these populations.

The stimulation parameters used for tVNS are critical. The percentage of those who responded to tVNS with a decrease in LF/HF ratio, indicating increased parasympathetic predominance, was 67 % in the H-

tVNS group compared to 49 % in the L-tVNS group. These results suggest that H-tVNS parameters are effective in altering cardiac autonomic control towards vagal predominance in a healthy population sample. Indeed, the baseline LF/HF ratio of H-tVNS non-responders was significantly lower than L-tVNS non-responders (0.64 and 1.04 respectively). This was consistent with a higher proportion of respondents to H-tVNS, suggesting that H-tVNS was more effective than L-tVNS in altering the LF/HF ratio towards parasympathetic predominance even in those who have relatively high baseline vagal tone. Furthermore, there was a significant decrease in LF power in the responder sub-group during tVNS whereas there was no significant change in HF power. This was unexpected; tVNS may be predicted to increase HF power as this component of HRV represents vagal modulation of heart rate. However, as the participants in this study were relatively young and healthy and were recorded at rest vagal tone would already be high and it may not be possible to increase it further. The decrease in LF power is difficult to interpret due to the ambiguity of the origin of this component of HRV.

Of particular interest in this study is the finding that the LF/HF ratio remains lower than baseline levels during the recovery period after H-tVNS has ceased. The stimulation and recovery periods lasted 15 minutes therefore the long term effects of H-tVNS on cardiovascular autonomic control require further investigation, however, auricular acupuncture increased HF power (indicating increased parasympathetic activity) for at least an hour after stimulation had ceased (Haker et al., 2000). The increase in HF power reported by Haker et al. (2000) is contrary to our findings. This may be due to the smaller sample size used (n = 12) or the use of a different stimulation technique - manual auricular acupuncture without any electrical stimulation. In addition, these conflicting results may also be due to the limitation of HRV analysis as an indirect measure of cardiac autonomic activity. La Marca et al. (2010) demonstrated that auricular electroacupuncture increased respiratory sinus arrhythmia (RSA, mediated by the vagus nerve) in healthy participants (n = 14) suggesting increased vagal activity, however, this is also an indirect measure of parasympathetic activity. One of the limitations of these studies, including the study presented

here, is that they have all used healthy participants therefore the extent of the effects that might be observed in patient populations, which characteristically have reduced parasympathetic activity, e.g. heart failure seems likely to be underestimated. Another limitation of this study was the omission of HRV time domain analysis. Time domain analysis of HRV was originally recommended for long term (24 hour) recordings (Task Force of the European Society of Cardiology and the North American Society of Pacing Electrophysiology, 1996), however, analysis of short term measures of HRV such as pNN50 may provide more information regarding tVNS effects on parasympathetic modulation of heart rate in the future. Furthermore, while respiration rate was monitored throughout the study and remained unaltered formal analysis of the effects of tVNS should be conducted. Another observation is that the MSNA frequencies recorded in this study are low in comparison to other studies. This may due to the dearth of microneurography studies involving healthy individuals which makes it difficult to define a ‘normal’ range, particularly in the presence of high inter-individual variability.

Recently, tVNS has gained interest as a possible non-invasive therapy in a number of conditions. The first clinical study of tVNS found that electroacupuncture of both ears was beneficial for patients with coronary artery disease (Zamotrinsky et al., 1997; Zamotrinsky et al., 2001). Patients (n = 10) received auricular electroacupuncture for 15mins/day for 10 consecutive days. After 4 treatments, angina symptoms at rest were abolished and patients no longer required vasodilators. After 7 treatments, patients had improved exercise tolerance and were able to climb 5-7 flights of stairs without developing angina symptoms. These studies also reported that the improvement in angina symptoms persisted after the cessation of tVNS treatment for up to 3 weeks. Recently, tVNS using surface electrodes has been investigated as a possible analgesic (Napadow et al., 2012; Busch et al., 2013) and has been trialled as an alternative to invasive cervical VNS in patients with refractory epilepsy (Stefan et al., 2012). These studies reported no significant changes in heart rate during tVNS. Napadow et al. (2012) also analysed ECG data for HRV and found tVNS had no significant effect (n = 10). These findings are contrary to the results of the present

study, however this seems likely to be due to the smaller sample sizes and the different stimulation parameters used.

2.6.2 Potential pathways of tVNS cardiovascular autonomic effects

The neurocircuitry underlying tVNS autonomic effects requires further elucidation. The auricular branch of the vagus nerve has previously received little attention and hence there is a dearth of information regarding its central projections and its peripheral distribution (see General Introduction 1.3.2.1). The ABVN has been reported to project to the NTS which plays an integral role in relaying vagal afferent visceral information (Nomura and Mizuno, 1984; Chien et al., 1996). Based on the results of this study, the proposed pathway of tVNS autonomic effects could involve activation of the NTS by ABVN afferents. This could activate the caudal ventrolateral medulla to inhibit the rostral ventrolateral medulla and thus reduce sympathetic output (Guyenet, 2006). In addition, the NTS could also activate the dorsal motor nucleus of the vagus and the nucleus ambiguus to increase parasympathetic activity (Izzo et al., 1993). However, the effects of tVNS on parasympathetic activity are unclear. If H-tVNS increased cardiac parasympathetic modulation a significant increase in HF power of HRV would be expected. This was not observed in the present study, however, this may be due to the healthy cohort of volunteers used who were tested under resting conditions when parasympathetic activity is already high. It may be that in populations with reduced parasympathetic activity e.g. heart failure patients, H-tVNS would increase HF power and this requires further investigation. In addition, there is no direct measure of vagal/parasympathetic nerve activity in humans, therefore, *in vivo* experiments to record vagus nerve activity directly may be required to clarify the effects of H-tVNS on the parasympathetic activity.

2.6.3 Scope of tVNS therapy for cardiovascular diseases

VNS is already being trialled as a potential heart failure therapy and has resulted in positive clinical outcomes (De Ferrari et al., 2011). The findings presented here support the use of tVNS as a non-invasive method of VNS for cardiovascular diseases. Of particular interest is the finding that H-tVNS reduces sympathetic outflow. Sympathoexcitation is the hallmark of many conditions including heart failure, hypertension and obstructive sleep apnoea (Charkoudian and Rabbitts, 2009). The tVNS approach described here may therefore offer a simple, non-invasive and economical alternative that could make vagus nerve stimulation a widely available therapy and potentially improve quality of life for patients with heart failure and other conditions characterised by sympathoexcitation.

Chapter 3
The influence of transcutaneous vagus nerve stimulation on autonomic function in heart failure

3.1 Introduction

The first pilot study of VNS in heart failure was reported in 2008 (Schwartz et al., 2008). An implantable neurostimulator that delivered pulses synchronous with heart beat was placed on the right cervical vagus nerve of 8 male patients. During follow-up at 1, 3 and 6 months there was a modest but significant reduction in resting heart rate and a marked improvement in quality of life score and symptoms as assessed by the Minnesota quality of life questionnaire and New York Heart Association (NYHA) classification. There was also improved left ventricular function indicating that VNS treatment is beneficial in heart failure. The reason that the changes were small may be that the targeted stimulation parameters of either 5.5 mA or a reduction in heart rate of 5-10 bpm were not reached due to patient discomfort. Interestingly, patients complained of ear pain which may be referred pain caused by VNS to the area of skin supplied by the ABVN. This, combined with the small sample group used who all had advanced heart failure, may account for the relatively small changes reported. This study has now been expanded to a multi-centre trial with data from an additional 24 patients added to the original pilot data (De Ferrari et al., 2011). The results are similar with an improvement in NYHA class and quality of life scores accompanied by a significant reduction in heart rate and a significant increase in left ventricular ejection fraction (LVEF). There was also a significant improvement in HRV (time domain measures - RMSSD and pNN50) at 3 months and 6 months follow-up. Most patients (n = 23) were also followed up at one year and these encouraging data demonstrated that the beneficial effects of VNS were maintained or even further improved, most notably LVEF continued to improve to 34% at 1 year follow-up compared to 21% at baseline.

If VNS is to become a treatment to restore sympathovagal balance in heart failure, a less invasive and better tolerated method is desirable. The possibility of using oesophageal vagal afferents has been explored (Bajwa et al., 1997). VNS was performed for 2-5 minutes in 13 healthy male subjects using a manometer with an electrode inserted into the oesophagus. HRV analysis showed an increase in the HF component during stimulation

compared to baseline levels indicating increased parasympathetic influence on heart rate. While this technique is not surgical it is still invasive and not ideal for regular use.

tVNS utilising the auricular branch of the vagus nerve may provide a 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. Based on results of H-tVNS in a healthy population (Chapter 2), showing improved HRV and reduced MSNA, permission was obtained to pilot H-tVNS in heart failure patients.

3.2 Hypothesis

Transcutaneous vagus nerve stimulation will alter cardiovascular autonomic function in heart failure patients. Based on previous results in healthy participants, H-tVNS will decrease LF/HF ratio indicating a shift towards parasympathetic predominance.

3.3 Aims and Objectives

The aim of this study was to investigate the effects of H-tVNS on cardiovascular autonomic function in heart failure patients. The objectives were to:

1. determine the effects of H-tVNS, using the optimal parameters defined in Chapter 2, on cardiovascular autonomic function in heart failure patients.
2. investigate the tolerability of H-tVNS in heart failure patients.

3.4 Methods

3.4.1 General Protocol

The study was approved by the National Research Ethics Service (Appendix - 12/YH/0354) and was conducted in accordance with the Declaration of Helsinki. Informed written consent was obtained from all participants. Heart failure patients were recruited from the outpatients heart failure clinic in Leeds General Infirmary. Inclusion criteria for heart failure patients were male or female over the age of 18 years with a diagnosis of heart failure. Heart failure patients also had to be in sinus rhythm for inclusion. Heart failure patients were excluded if they were unable to transfer safely to a couch for the duration of the procedures or had known bradycardia or postural hypotension. Heart failure patients were also excluded if they had cardiac arrhythmia including atrial fibrillation. All heart failure patients were on optimal medical therapy including angiotensin converting enzyme inhibitors, angiotensin receptor blockers, beta-blockers and loop diuretics (Table 3.1). 4 heart failure patients also had cardiac resynchronisation therapy using a pacing device, however, none were atrially paced. None of the heart failure patients had diabetes. As patients were recruited directly from the clinic, the time at which experiments took place was not controlled. In addition, it was not possible to control for medication, caffeine, nicotine or physical activity prior to attendance. Experiments were carried out in a quiet room in Leeds General Infirmary. Patients were asked to lie semi-supine on a couch while heart rate, blood pressure and respiration were recorded as described in Chapter 2.

3.4.2 Transcutaneous vagus nerve stimulation

Stimulation was performed using the H-tVNS protocol described in Chapter 2. Briefly, auricular clips were applied to left and right tragi and connected to a TENS machine. Stimulation was delivered as pulses of 200 μs duration at 30 Hz for 15 minutes. Amplitude was adjusted to the level of sensory threshold for each individual (10 – 50 mA).

Table 3.1 Heart failure medication for each participant.

	Beta-blocker	ACE Inhibitor	Diuretic
Participant 1	Bisoprolol 10 mg	Ramipril 10 mg	Furosemide 80 mg
Participant 2	Bisoprolol 2.5 mg	Ramipirl 10 mg	Furosemide 40 mg
Participant 3	Bisoprolol 2.5 mg	Ramipril 10 mg	NA
Participant 4	Bisoprolol 5 mg	Candesartan 4 mg	Furosemide 80 mg
Participant 5	Bisoprolol 1.25 mg	Perindopril 8 mg	Furosemide 40 mg
Participant 6	Metoprolol 25 mg	Ramipril 5 mg	Furosemide 40 mg
Participant 7	Bisoprolol 10 mg	Ramipril 10 mg	NA
Participant 8	Carvedilol 25 mg	Candesartan 32mg	Furosemide 80mg

3.4.3 Heart rate variability

HRV was analysed offline using Spike 2 software (version 7.1; CED, UK). The ECG was inspected to ensure all R peaks were detected and there were no abnormalities in the ECG e.g. premature ventricular complexes. Ectopic beats could be corrected by averaging the R-R interval prior to and following the ectopic. A threshold was set to detect R peaks from a 5 minute ECG recording and R-R intervals used to produce a tachogram. This was saved as a memory channel and used to produce a virtual channel on which power spectral analysis could be carried out. Data were resampled at 5 Hz and DC removal process applied to remove any bias in digitisation and to set the channel offset to zero. Fast Fourier Transform was then applied (512 point; 50% overlap) with a Hanning window to calculate the power spectrum of HRV. The power spectrum was divided into VLF, LF and HF (Chapter 2) components. This data was exported to Excel (2010) and normalised LF and HF calculated to determine LF/HF ratio.

3.4.4 Baroreflex sensitivity

Systolic blood pressure variability was calculated using a similar method to HRV except the threshold was set to detect peak systolic pressure. The

coherence between systolic blood pressure and R-R interval was calculated using a Spike2 script and the alpha index calculated as described in Chapter 2.

3.4.5 Tolerability questionnaire

All heart failure patients were asked to complete a tolerability questionnaire at the end of the experiment to assess any possible discomfort caused by H-tVNS. Participants were asked to rate any discomfort, pins and needles or warmth sensations experienced during H-tVNS using a Likert type scale accompanied by a visual analogue scale to aid interpretation. The scale ranged from 0 to 5 with 0 representing ‘not at all’ and 5 representing ‘extremely’. Heart failure patients were also asked if they experienced any palpitations or feelings of anxiety during the experiment and if they found the couch uncomfortable. In addition, there was space for additional comments or suggestions they wished to make.

3.4.6 Statistical analysis

Wilcoxon signed ranks test was used to analyse the effect of H-tVNS on HRV compared to baseline. The Mann-Whitney U test was used to compare baseline characteristic between heart failure patients and healthy participants recruited for the study in Chapter 2.

3.4.7 Study Failures

24 heart failure patients were recruited for the study, however, 16 patients were excluded for the following reasons:

- 10 had ectopic beats or cardiac arrhythmias
- 2 had persistent coughing
- 2 had pre-existing pain conditions (arthritis of back and shoulder) that made lying on the couch painful
- 1 had pain in left arm

3.5 Results

3.5.1 H-tVNS significantly improved HRV in heart failure patients

There was a significant decrease in LF/HF ratio ($p = 0.035$; $n = 8$; Figure 3.1; Table 3.2) during H-tVNS. BRS was not calculated for many of the heart failure patients due to poor coherence between oscillations in heart rate and blood pressure. There was no significant change in heart rate or blood pressure.

The heart failure patients were significantly older (66 ± 4.7 vs. 38 ± 2.3; $p < 0.005$) than the healthy participants who received H-tVNS. The heart failure patients also had a significantly higher LF/HF ratio at baseline (4.23 ± 1.21 vs. 1.20 ± 0.08; $p = 0.009$; Table 3.3). There was no significant relationship between baseline LF/HF ratio and the change in LF/HF ratio during H-tVNS and there was also no significant relationship between age and baseline LF/HF ratio. This may be due to the small sample size in this study.

Table 3.2 Heart rate variability values for heart failure patients. There is a significant decrease in LF/HF ratio during H-tVNS ($p = 0.035$).

	Baseline	**Stimulation**	**p**
Total power (ms^2)	475.75 ± 172.69	434.36 ± 168.98	ns
LF Power (ms^2)	225.30 ± 54.30	174.71 ± 48.29	ns
HF Power (ms^2)	135.70 ± 88.38	180.98 ± 114.95	ns
LF/HF	4.23 ± 1.21	3.11 ± 1.14	**0.035**

Table 3.3 Baseline characteristics of heart failure patients.

Number	8 (2 female, 6 male)
Age (years)	66 ± 4.70
LVEF (%)	31.88 ± 3.65
NYHA class	3 class I; 3 class II; 1 class III; 1 class IV
Duration of heart failure (months)	36.57 ± 5.29
Baseline LF/HF	4.23 ± 1.21
Heart rate (bpm)	68 ± 3.42
Mean BP (mmHg)	68 ± 7.01

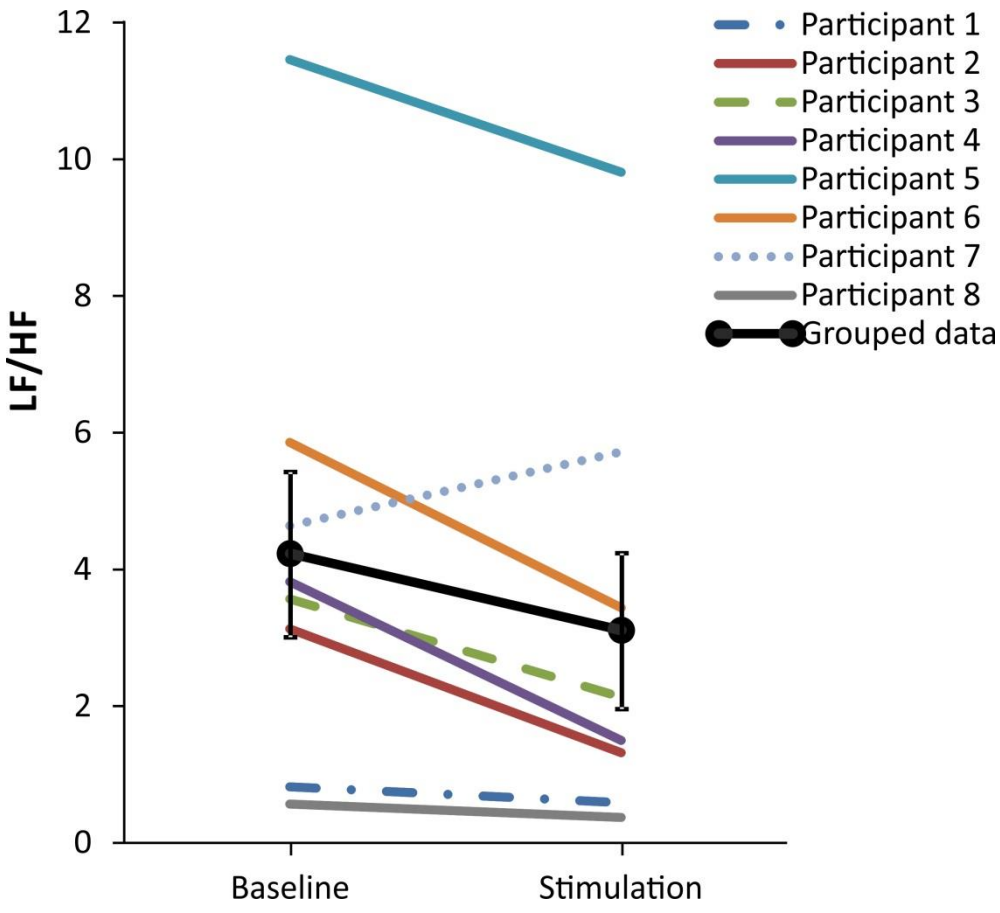


Figure 3.1 The effects of H-tVNS on HRV in heart failure patients. There is a significant decrease in LF/HF ratio during H-tVNS ($p = 0.035$). In the one patient (participant 7) where an increase was observed, the patient reported that they found lying semi-supine uncomfortable which may have caused the increase in HRV.

3.5.2 Patient tolerability of H-tVNS

Patients were asked to complete a tolerability questionnaire which included specific questions about discomfort or unpleasant sensations caused by H-tVNS. Patients were also asked if they experienced any dizziness, anxiousness or palpitations during the procedure. In terms of the tolerability questionnaire, none of the patients reported any side effects or discomfort caused by H-tVNS and tolerated the procedure well. However, participant 7 rated the couch as 'somewhat uncomfortable' (Table 3.4). This may explain the increase in LF/HF ratio observed in this patient as the discomfort may have caused some sympathoexcitation.

Table 3.4 Results of tolerability questionnaire. Questions were rated from 0-5 with 0 = not at all, 3 = somewhat and 5 = extremely**.**

Question	Average Score (S.E.M.)
Did you find the ear stimulation uncomfortable?	0.13 ± 0.04
Did you experience any pins and needles during the stimulation?	0.75 ± 0.25
Did you experience any warmth sensation during the stimulation?	0.13 ± 0.04
Did you feel any dizziness during the visit?	0.25 ± 0.08
Did you feel anxious during the visit?	0
Did you experience any palpitations?	0
Did you find lying on the couch uncomfortable?	0.5 ± 0.17

3.6 Discussion

For the first time, this study shows that H-tVNS can decrease LF/HF ratio in heart failure patients indicating a shift in cardiac autonomic control towards parasympathetic predominance. This is despite maximal medical therapy including beta blockers. Decreased parasympathetic activity, as determined by impaired HRV and BRS, is associated with an increased risk of mortality in heart failure patients and post myocardial infarction (La Rovere et al., 1997; La Rovere et al., 1998), therefore, the ability to increase parasympathetic activity in this population is highly desirable.

This first indication that H-tVNS may alter cardiac autonomic control towards parasympathetic predominance in heart failure is important. Cervical VNS is reserved for moderate to severe heart failure patients who are unsuitable for other therapies e.g. cardiac resynchronisation therapy. This is due to the invasive nature, cost and side effects of implantable VNS. In contrast, H-tVNS is a simple, inexpensive and non-invasive therapy that could potentially be used by a wider cohort of patients. This study also shows that H-tVNS is well tolerated by heart failure patients. Paraesthesia (pins and needles) was the most commonly reported sensation during tVNS (n = 3; maximum score = 3), however, none of the participants requested the procedure to be stopped indicating that this sensation was not sufficient to cause marked discomfort.

The heart failure patients in this study were significantly older ($p < 0.0005$) and also had a significantly higher baseline LF/HF ratio ($p = 0.009$), indicating accentuated sympathetic influence on heart rate compared to healthy participants (Chapter 2). In healthy participants, there was a significant relationship between baseline LF/HF ratio and the magnitude of the response to H-tVNS such that higher baseline LF/HF ratios were associated with a greater decrease during tVNS. This was not found in the heart failure group, however, this may be due to the small sample size. This may also explain why there was no relationship between age and baseline LF/HF in this group unlike the findings reported in Chapter 2. Age matched controls without cardiovascular conditions may help determine the degree of elevation in baseline LF/HF that is due to heart failure. In addition, future

studies should be controlled for time of day and provide participants with information prior to attendance to control caffeine, nicotine and food intake.

The mechanisms behind the effects of H-tVNS in heart failure patients require investigation. There is a decrease in LF power during H-tVNS that is similar to the findings in healthy participants, however, this does not reach significance. There is also an increase in HF power that does not reach significance. The lack of significance may be due to the small sample size in this study. Further work to increase patient numbers is required to determine the mechanism of H-tVNS effects on HRV in heart failure. The effects of H-tVNS on sympathetic nerve activity in heart failure patients also need to be determined e.g. by recording MSNA using microneurography. Due to the sympathoexcitation that occurs in heart failure, the incidence of MSNA bursts can be 100% i.e. a burst occurring during every heart beat (Macefield et al., 1999). Single units do not fire during every burst, therefore, the recording and analysis of single unit MSNA offers vital information that MSNA bursts cannot provide. This would determine if the effects of H-tVNS on sympathetic nerve activity in heart failure patients are similar to healthy participants without cardiovascular disease (Chapter 2).

The residual effect of H-tVNS on HRV, as observed in healthy participants in Chapter 2, was not investigated due to time constraints in the clinic. A residual effect may make H-tVNS a more practical and patient friendly therapy if only intermittent application is required rather than continuous, therefore, this merits investigation in the future. Auricular electroacupuncture in coronary artery disease patients was reported to have no effect on symptoms after 1 treatment, however after 4 treatments, angina no longer occurred at rest and after 7-8 treatments, patients had no symptoms during moderate exercise (Zamotrinsky et al., 1997; Zamotrinsky et al., 2001). Moreover, tVNS also improved post-surgical (coronary artery bypass) outcomes with improved haemodynamics and sinus rhythm compared to the sham treated group. This is remarkable as surgery took place 2-3 weeks after tVNS ceased and symptoms of angina remained reduced during this time (Zamotrinsky et al., 1997; Zamotrinsky et al., 2001). This indicates that chronic tVNS may produce a prolonged residual effect that precludes the need for constant stimulation.

3.6.1 Manifold beneficial effects of H-tVNS in heart failure

The potential beneficial effects of H-tVNS in heart failure may not be restricted to improved cardiac autonomic function. H-tVNS may reduce sympathetic activity in heart failure and thereby prevent cardiac remodelling as high levels of noradrenaline are cardiotoxic causing apoptosis. Applying noradrenaline to cultured isolated rat cardiomyocytes caused apoptosis that was eliminated by propranolol indicating that this process is mediated by beta-receptors (Communal et al., 1998). Reducing cardiac sympathetic activity would reduce catecholamine release and may attenuate pathological remodelling of the heart.

Inflammatory markers (tumour necrosis factor alpha – TNF-alpha and interleukin 6 – IL6) are elevated in heart failure and the degree of activation is correlated to NYHA classification (Torre-Amione et al., 1996). The importance of TNF-alpha in the development of heart failure was demonstrated in transgenic mice that overexpressed TNF-alpha in the myocardium. All mice developed heart failure and the severity and progression of left ventricular impairment were dependent of the degree of overexpression (Franco et al., 1999). The vagus nerve is an essential component of the inflammatory reflex (Rosas-Ballina et al., 2011) and stimulation reduced levels of pro-inflammatory cytokines in a rat model of endotoxaemia (Borovikova et al., 2000). Indeed, cervical VNS in a canine model of heart failure also reduced plasma levels of inflammatory markers (Zhang et al., 2009b). Using a rat model of endotoxaemia, tVNS also reduced plasma levels of pro-inflammatory cytokines, moreover, this effect was abolished by cervical vagotomy (Zhao et al., 2012). This indicates that tVNS may be capable of reducing inflammation in heart failure and thereby slow the progression of heart failure.

Approximately one third of heart failure patients die of sudden cardiac death (Narang et al., 1996), most commonly due to ventricular tachycardia (84%) (de Luna et al., 1989). In a ground-breaking study, cervical VNS reduced the incidence of ventricular tachycardia and improved survival in a canine model of heart failure (Vanoli et al., 1991). The mechanisms behind the protective effect of VNS against ventricular arrhythmias are under

investigation. VNS affects electrical restitution in the heart (the relationship between action potential duration and diastolic interval) and this is thought to contribute to the anti-fibrillatory effect of VNS. A steep restitution curve results in electrical instability and is related to the occurrence of ventricular fibrillation (VF) (Riccio et al., 1999). Flattening of the restitution curve with drugs such as verapamil (a calcium channel blocker) prevented the induction of VF in dogs (Riccio et al., 1999). The effects of VNS on restitution have been studied using an isolated rabbit heart preparation in which the autonomic innervation is intact (Ng et al., 2001). VNS flattened the restitution curve and increased the threshold at which VF could be induced (Ng et al., 2007). This occurred in the absence of sympathetic nerve activity suggesting that the anti-fibrillatory effect of VNS is direct and does not require antagonistic sympathetic activity. Zamotrinsky et al. (1997) reported that 10 days of tVNS treatment reduced the occurrence of post-operative arrhythmias in patients undergoing coronary artery bypass surgery, however, there has been no specific investigation of the effects of tVNS on arrhythmias.

VNS is approved for treatment resistant depression in the US and this is pertinent in light of recent developments in psychogenic heart disease – the association between mental stress or psychiatric illness and the development of cardiovascular disease (Esler, 2010). Major depressive disorder significantly increases mortality risk (Faller et al., 2007; Sherwood et al., 2007; Lesman-Leegte et al., 2009) and hospitalisation (Sherwood et al., 2007) in heart failure patients. It is also associated with reduced quality of life scores (Bekelman et al., 2007) compared to heart failure patients without depression. Depression, like heart failure, is associated with sympathetic activation. Plasma noradrenaline levels were elevated in patients with major depression compared to controls (Veith et al., 1994). Pilot data have indicated that tVNS reduced depression symptoms measured using the Beck Depression Inventory in MDD patients (Hein et al., 2013) and results presented in Chapter 2 demonstrated that H-tVNS reduced MSNA in healthy volunteers. These results support the potential of H-tVNS to reduce comorbidity of depression in heart failure and may improve prognosis and quality of life in this patient population.

Cervical VNS has led to improved quality of life scores in heart failure patients (De Ferrari et al., 2011). Depression was not assessed in this study and was not mentioned in the inclusion/exclusion criteria, therefore, it is difficult to speculate the impact this may have had on quality of life scores. Improved quality of life may be related to improved 6 minute walking test results and heart failure symptoms which would lead to increased ability to carry out activities of daily life. tVNS in patients with coronary artery disease improved exercise tolerance during a cycling test with 90% tolerating 50 W after 10 days of treatment compared to 0% at baseline (Zamotrinsky et al., 1997). These patients also reported an improved ability to carry out activities of daily life after treatment. These preliminary results require validation, however, tVNS may facilitate independence of heart failure patients and thereby improve quality of life.

3.6.2 Is VNS suitable for all heart failure patients?

Atrial fibrillation (AF) occurs in approximately one third of heart failure patients and is associated with an increased risk of mortality (McManus et al., 2013). The genesis of atrial fibrillation is complex involving both sympathetic and parasympathetic activation. Acetylcholine perfused into the sinus node artery in dogs consistently induced AF (n = 20) (Sharifov et al., 2004). Subsequent perfusion of isoproterenol (beta-receptor agonist) facilitated AF induction and reduced the concentration of ACh required (Sharifov et al., 2004). Sympathetic activation increased calcium entry and spontaneous calcium release from the sarcoplasmic reticulum whereas vagal activation reduced the effective refractory period (Shen et al., 2012). Combined intracellular calcium mishandling and shortened action potentials contribute to the generation and maintenance of AF (Shen et al., 2012). Indeed, cervical VNS is commonly used to induce AF in animal models (Katsouras et al., 2009; Zhang and Mazgalev, 2011). These findings suggest that VNS is not suitable for patients with AF and may risk promoting the development of AF in previously unaffected patients, however, AF has not been reported as a side effect despite the large numbers of refractory epilepsy patients treated with VNS (Zhang and Mazgalev, 2011).

Furthermore, VNS may be beneficial in reducing AF in heart failure. Using a stimulation protocol similar to that used in patients, left VNS reduced the frequency of AF in a canine AF model (atrial pacing) compared to sham (Shen et al., 2011). Extracellular recordings from the left stellate ganglion showed reduced neuronal discharge during VNS compared to baseline indicating a reduction in sympathetic nerve activity. Immunohistochemistry of the left stellate ganglion after 1 week of VNS revealed a significant reduction in the number of tyrosine hydroxylase positive neurones compared to control dogs suggesting that VNS may cause neuronal remodelling in the stellate ganglion (Shen et al., 2011). The stimulation parameters for VNS used to induce experimental AF are much higher and cause a significant decrease in heart rate whereas therapeutic levels of VNS are lower and produce little change in heart rate (Zhang and Mazgalev, 2011). This may be crucial to the differential effects of VNS on the atria. Transcutaneous VNS (at 80% below the voltage required to change heart rate) has recently been investigated in a canine model of atrial fibrillation and was capable of supressing AF and reversing acute atrial remodelling (i.e. shortened effective refractory period) induced by rapid atrial pacing. Furthermore, this effect was abolished by transection of left and right thoracic vagus nerves (Yu et al., 2013) indicating that H-tVNS may be an alternative approach to cervical VNS. This is encouraging, however, a limited number of studies have investigated the effects of low level VNS in AF and further evidence of safety is warranted before trialling in patients with AF.

3.6.3 Conclusion

Studies of tVNS in clinical populations or animal models are preliminary in nature and require validation, however, initial results are encouraging and merit further investigation. tVNS may have manifold effects that could be beneficial, not only in heart failure, but a variety of conditions characterised by autonomic imbalance such as hypertension (Charkoudian and Rabbitts, 2009). tVNS is a simple, inexpensive and, crucially, non-invasive technique that may be utilised by a wider cohort of patients than cervical VNS.

Chapter 4
Central projections of the auricular branch of the vagus nerve in humans

4.1 Introduction

The results presented in Chapters 2 and 3 of this thesis indicate that H-tVNS can alter cardiovascular autonomic function in healthy participants and heart failure patients towards parasympathetic predominance, however, the mechanisms underlying these effects require investigation. There is a lack of detailed information regarding the precise innervation of the external ear, particularly in humans. The external ear is supplied by the great auricular nerve and the lesser occipital nerve, which originate from the cervical plexus (C2 and C3 spinal nerve roots), the auriculotemporal nerve (a branch of the trigeminal nerve), and the auricular branch of the vagus nerve (ABVN) (Peuker and Filler, 2002).

The ABVN (also called Arnold's nerve or the Alderman's nerve) has received little attention and hence there is a dearth of information regarding its anatomy. The ABVN arises from the superior (jugular) ganglion of the vagus nerve (Nomura and Mizuno, 1984; Gupta et al., 1986; Folan-Curran et al., 1994; Tekdemir et al., 1998; Folan-Curran and Cooke, 2001). It then passes posterior to the internal jugular vein to enter the mastoid canaliculus in the lateral wall of the jugular fossa (Gupta et al., 1986; Tekdemir et al., 1998). The mastoid canaliculus runs through the temporal bone to the tympanomastoid fissure (Tekdemir et al., 1998). Before reaching the tympanomastoid fissure, the mastoid canaliculus crosses the facial canal about 4.5 mm above the stylomastoid foramen at which point the ABVN crosses the facial canal and then emerges through the tympanomastoid fissure (Gupta et al., 1986; Tekdemir et al., 1998). The ABVN is principally distributed to the antihelix, conch, cymba concha and tragus of the ear (Peuker and Filler, 2002).

The central projections of the ABVN have been investigated in cats and dogs by applying the neuronal tracer horse radish peroxidase (HRP) to the nerve (Nomura and Mizuno, 1984; Chien et al., 1996). The main central projection of the ABVN was to the spinal trigeminal nucleus which processes touch, pain and temperature sensations from the face (Chien et al., 1996). There was also a smaller projection to the nucleus tractus solitarius, a nucleus which is integral to central autonomic control. Interestingly, the

ABVN projected to the dorsomedial part of the caudal NTS which is the area that receives baroreceptor afferents (Ciriello et al., 1981). The central projections of the ABVN in humans have never been explored. This could be achieved by applying a neuronal tracer to the ABVN in human cadavers, however, the majority of neuronal tracers e.g. neurobiotin rely on active metabolism for axonal transport and therefore are inappropriate for fixed tissue (Lanciego and Wouterlood, 2011). This can be overcome by using a strongly lipophilic carbocyanine tracer e.g. DiI (Lanciego and Wouterlood, 2011). DiI diffuses along the plasma membrane and was first used as a neuronal tracer in fixed tissue by Godement et al. (1987) to investigate the development of the optic nerve in embryonic mice. DiI was reported to travel retrogradely and anterogradely and estimated to travel 6 mm/day in living tissue compared to 2 mm/week in fixed tissue (Molnár et al., 2006). Clarifying the central projections of the ABVN using neuronal tracing could aid interpretation of the effects of H-tVNS on cardiovascular autonomic control and the mechanisms involved.

4.2 Hypothesis

The auricular branch of the vagus nerve projects to the NTS and/or areas of the brainstem involved in autonomic regulation in humans.

4.3 Aim

The aim of this study was to investigate the central projections of the ABVN in human cadavers using a neuronal tracer.

4.4 Methods

4.4.1 Obtaining human tissue for research purposes

Ethics approval was obtained from the University of Leeds (Appendix - BIOSCI 11-001). Donors that had provided prior written consent for tissue to be used for research purposes and for images to be taken were identified in the University of Leeds Anatomy Department. 4 heads that met these conditions, had intact auricles and brainstems and were no longer needed for teaching purposes were selected. A Tissue Transfer Agreement was completed and signed to transfer the tissue from the Anatomy Human Tissue license to the Research licence. The donors included 2 females and 2 males and were > 65 years old at death. Tissue had been embalmed 3-5 years previously using embalming fluid containing 75% methanol, 1.6% formaldehyde, phenol, glycerol and water.

4.4.2 Dissection of the ABVN

Dissections were made posterior to the ear to locate the ABVN as it emerged from the tympanomastoid fissure. The mastoid process was palpated and a longitudinal skin incision made 1-2 cm posterior to this structure. A horizontal incision was made superior to the external ear and extended to the first incision. The skin overlying the temporal bone and the posterior surface of the ear was then reflected. The underlying parotid fascia was removed and the auricularis posterior muscle reflected. A magnifier with a mounted light was used to carefully remove deep fascia and to identify auricular nerves. A nerve was accepted as being the ABVN if it:

1) emerged from the tympanomastoid fissure
2) continued straight over the posterior surface of the auricle
3) pierced the auricular cartilage to reach the anterior surface of the ear.

The ABVN could not be located in one head therefore 3 were used for the study (2 right and 1 left ABVN; Figure 4.1). In one case, 2 nerves on the same side met the criteria and both were utilised for tracer application (Figure 4.1 **Error! Reference source not found.**B).

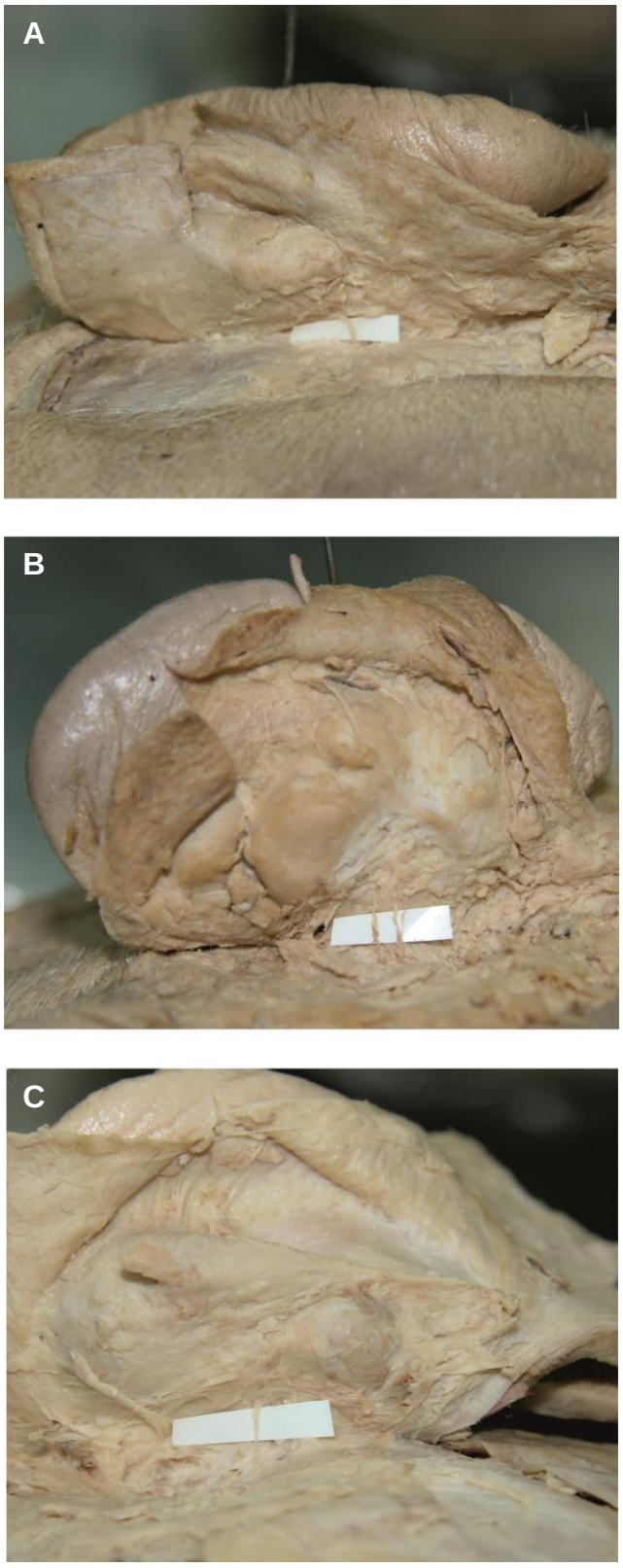


Figure 4.1 Dissections of the right ABVN. Posterior view of dissected ears showing the ABVN overlying white card (A – left ABVN; B and C – right ABVN). One case presented with a double right ABVN (B).

4.4.3 Application of neuronal tracer

The tissue used in this study was fixed using formaldehyde, therefore, a carbocyanine neuronal tracer was selected as these diffuse along the plasma membrane even in fixed tissue. This can be a long process as standard DiI ($DiIC_{18}$ or 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) travels along axons at approximately 0.4 mm/day (Godement et al., 1987). Dilinoleyl DiI (also called FAST DiI or 1,1'-dilinoleyl-3,3,3',3'-tetramethylindocarbocynanine perchlorate; Biotium Inc.,USA) is an analogue of DiI in which the 2 hydrocarbons chains are unsaturated (in contrast to saturated) allowing faster diffusion (Freidland et al., 1996). Dilinoleyl DiI was selected as the most appropriate tracer for this study due to the distance of diffusion required (approximately 5 cm). Once the ABVN was identified, it was sectioned and DiI oil pasted onto the proximal end using forceps. Care was taken to ensure DiI was not applied to any other tissue. The proximal end of the ABVN was then enclosed in a small, blind-ending plastic tube which was fixed in place using superglue to isolate the DiI coated end and ensure it did not touch the surrounding tissue. The tissue was then left at room temperature in the mortuary for 7 months.

4.4.4 Tissue Preparation

After 7 months, the ABVN was further dissected into the tympanomastoid fissure and removed using scissors. To remove the brainstem, firstly, the cervical musculature and the occipital belly of the occipitofrontalis muscle were removed using a large scalpel to expose the cervical vertebrae and the occipital bone. The pedicles of the upper cervical vertebrae were cut using a bone saw and the neural arch of the vertebrae removed to expose the spinal cord and caudal medulla. The occipital bone and posterior parts of the parietal and temporal bones were removed using a bone saw to expose the posterior surface of the occipital lobe and cerebellum. A large scalpel was inserted into the fissure between the occipital lobe and cerebellum, inferior to the tentorium cerebelli, to section the midbrain. The midbrain, pons, medulla, cerebellum and rostral cervical spinal cord were then carefully extracted. The superior, middle and inferior cerebellar peduncles were sectioned to

detach the cerebellum and the remaining brainstem was divided at the pontomedullary junction. Ventral and dorsal rootlets of the first cervical spinal root were identified and the spinal cord inferior to this point was removed. The meninges covering the medulla were removed using forceps. The medulla was then mounted on a vibrating microtome (Leica VT1000M, UK) and transverse sections of 50 µm were cut. Sections were mounted on glass slides with Vectashield (Vector Laboratories, USA).

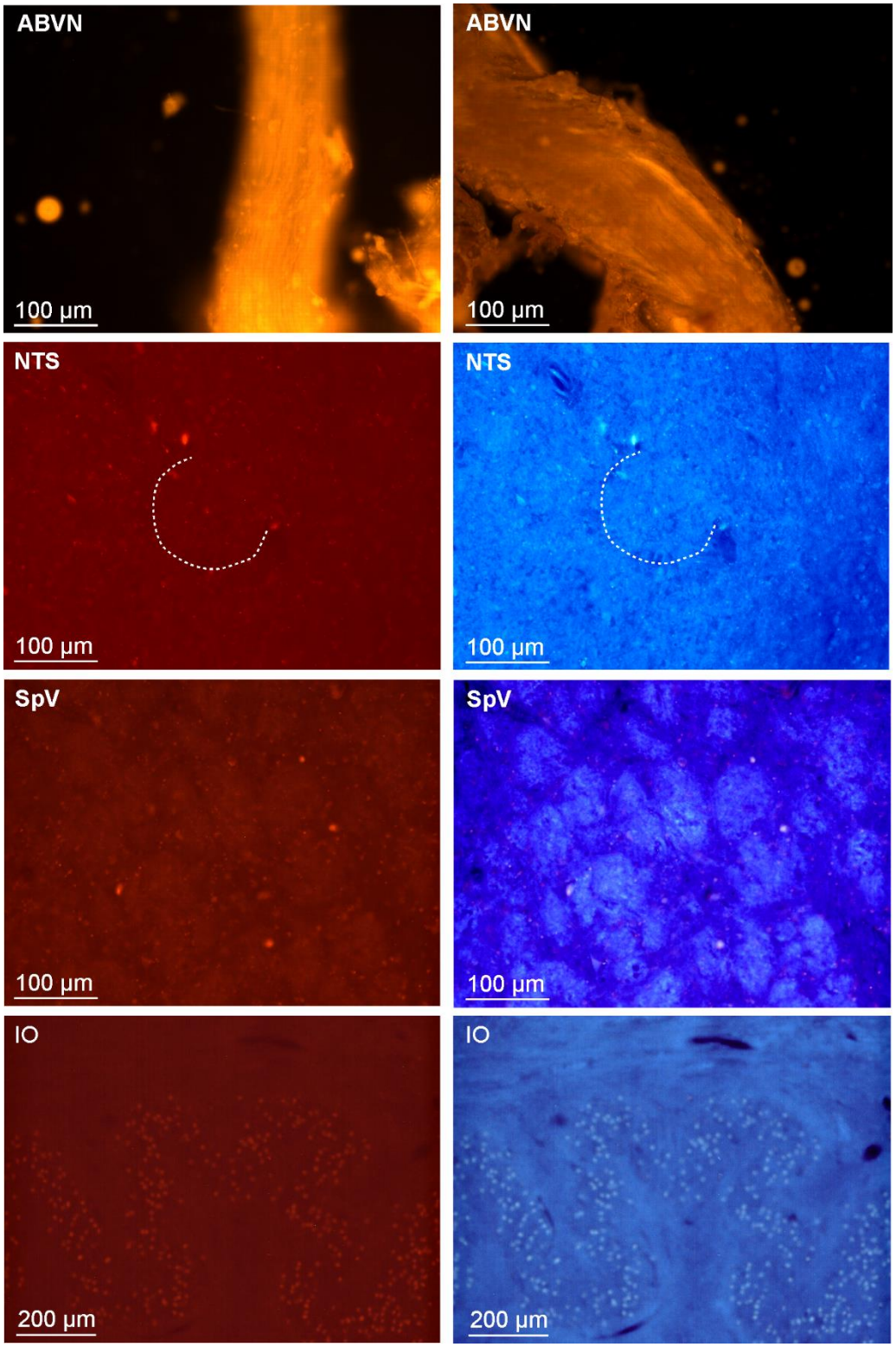
4.4.5 Microscopy

Slides were viewed under a microscope equipped with epi-fluorescence (Nikon Eclipse E600) and images were obtained using an integrated CCD camera attached to an Aquis image capture system (Synoptics Ltd. UK).

4.5 Results

DiI was observed in the ABVN proximal to the site of application as the nerve entered the tympanomastoid fissure indicating diffusion of the tracer along the nerve, however, DiI was not observed in any of the sections of the medulla (Figure 4.2). There was a high level of autofluorescence in medulla sections which could be observed in both the red and blue fluorescence channels and this may have obscured any discrete DiI labelling. Autofluorescence was observed in all sections and was present throughout the medulla including the NTS and was very pronounced in the inferior olivatory nucleus. There was no evidence of any axonal-like labelling, indicating that DiI had not entered the medulla through projections of the ABVN. There was also no evidence of DiI labelled projections to the spinal trigeminal nucleus which receives the majority of ABVN projections.

Figure 4.2 Evidence of DiI tracing in the ABVN from two donors and autofluorescence in the NTS, spinal trigeminal nucleus (SpV) and inferior olive (IO). DiI can be seen diffusing superiorly along fibres of the ABVN away from the application site (inferior portion). Intensity is greatest at the application site and diminishes with distance. Areas of fluoresence in the same section of NTS (demarcated by dotted line), SpV and IO are visible in the red channel and blue channel indicating that this is autofluoresence and not DiI labelling.


ABVN
100 µm
ABVN
100 µm
NTS
100 µm
NTS
100 µm
SpV
100 µm
SpV
100 µm
IO
200 µm
IO
200 µm

4.6 Discussion

In this unique study an attempt was made to trace the central projections of the ABVN in humans using DiI, however, no evidence of DiI reaching the medulla was found. This may be due to the extensive autofluorescence that was observed and may have obscured DiI labelling or it may be that 7 months was not sufficient to allow diffusion of DiI from the ABVN at the tympanomastoid fissure to the medulla.

DiI is a lipophilic neuronal tracer that diffuses along the plasma membrane, therefore, with enough time DiI should travel along the plasma membrane of axons in the ABVN to the medulla. It was estimated that the distance from the ABVN as it exits the skull at the tympanomastoid fissure to the median of the medulla was 50 mm and that seven months would be sufficient time to allow DiI to diffuse this distance. This would require a diffusion rate of 1.8 mm per week. There is no published diffusion rate data for ‘fast’ DiI (used in this study), however, reports using standard DiI state that two weeks were sufficient to obtain staining of axons in fixed mouse tissue for a distance of 5 mm (Godement et al., 1987). It has been shown that the diffusion rate of standard DiI slows with time and distance from the application site. Indeed, this same study found that, by 6 weeks, standard DiI tracing had reached a distance of 12 mm (Godement et al., 1987). This indicates that the average diffusion rate had slowed from 2.5 mm/week to 2.0 mm/week. Despite this it was predicted that, as ‘fast’ DiI should have a more rapid diffusion rate due to the unsaturated hydrocarbon chains, 7 months should be sufficient. Detailed investigations of the dynamics of ‘fast’ DiI diffusion along peripheral nerves are required to establish the time required for long distance neuronal tracing.

In the future, modifications to the protocol to optimise the performance of the neuronal tracer may limit the time needed for diffusion. For example, incubating the tissue at 37ºC significantly increased standard DiI diffusion time in fixed rat spinal cord compared to tissue kept at room temperature (Chen et al., 2006). Applying a direct current to fixed human tissue has also been found to increase the diffusion time of DiI along the median and ulnar nerves. Applying a DC electric field of 40 V/cm increased the diffusion rate

100 times compared to control tissue (Swift et al., 2005). Another option may be to dissect the ABVN within the mastoid canaliculus or the jugular foramen of the temporal bone in order to reduce the diffusion distance to the medulla.

4.6.1 Sources of autofluorescence

A high degree of autofluorescence was present in the medulla which may have masked DiI labelling. Autofluorescence refers to the presence of endogenous fluorescence in the tissue or fluorescence induced by the tissue fixation process. A source of endogenous fluorescence is lipofuscin, the breakdown product of red blood cells (Billinton and Knight, 2001). Lipofuscin is prominent in neurones and levels of lipofuscin increase with normal ageing (Katz and Robison Jr, 2002; Ohtaki et al., 2012). The donors in this study were elderly, therefore, high levels of lipofuscin may account for the prominent autofluorescence present in this tissue. Indeed, the inferior olive is reported to accumulate more lipofuscin with ageing than any other area of the brain (Rogers et al., 1980) which is in keeping with the observations of this study.

Aldehyde fixation can also result in autofluorescence. Aldehydes react with amine groups in tissue to form fluorescent compounds (Clancy and Cauller, 1998). Furthermore, aldehyde induced fluorescence increases with the duration of fixation. The tissue in this study was previously used in anatomy teaching and was fixed 3-5 years ago. The prolonged fixation time may have contributed to the intense autofluorescence observed in this study.

In future studies it would be advisable to utilise tissue from younger donors to limit levels of lipofuscin in the tissue, however, it is unlikely that tissue would be obtained from younger donors as all the donations to the anatomy department are from elderly individuals. An alternative solution may be to obtain tissue from post-mortems, however, acquiring consent prior to death from younger individuals may be rare. It is possible to alter the fixation process to reduce autofluorescence. Reducing the concentration of formaldehyde (soft-embalming) would reduce the level of autofluorescence. One such technique utilises glycol, salts and boric acid to preserve the tissue and reduces the concentration of formaldehyde required (Eisma et al.,

2013). This is called the Thiel method and results in flexible, life-like fixation that is considered ideal for surgical training (Eisma et al., 2013). The degree of autofluorescence induced by this method of fixation is unknown, however, it would be predicted that the lower concentration of formaldehyde used would reduce the level of autofluorescence generated. Furthermore, decreasing the levels of formaldehyde used would also reduce cross-linking of proteins, facilitating the diffusion of DiI and other lipophilic neuronal tracers.

4.6.2 Conclusions

The results of this study are inconclusive, however, elucidating the central projections of the ABVN in humans is vital in understanding the mechanisms of H-tVNS and its effects on cardiovascular autonomic function. There are a number of possibilities to enhance the neuronal tracing protocol and reduce autofluorescence that warrant investigation. This would clarify if the central projections of the ABVN in humans are similar to those reported in studies of cats and dogs (Nomura and Mizuno, 1984; Chien et al., 1996). Results from animal studies indicate that the ABVN is likely to project to the NTS, therefore, stimulating this pathway could influence central control of autonomic function.

Chapter 5
The influence of transcranial direct current stimulation on autonomic function

5.1 Introduction

Transcranial direct current stimulation (tDCS) is a non-invasive neuromodulatory technique that has been used to influence cortical excitability in a range of conditions, including depression (Brunoni et al., 2013a) and pain (Borckardt et al., 2011), and has also been investigated in stroke rehabilitation (Schulz et al., 2013). Different stimulation parameters and electrode montages have been used in tDCS research however the most common arrangement consists of one surface electrode placed over the motor cortex and the other placed on the contralateral supraorbital region (Stagg and Nitsche, 2011; Im et al., 2012). A small direct current, typically 1-2 mA, is then applied which influences the spontaneous activity of cortical neurones (Nitsche and Paulus, 2000).

tDCS alters cortical excitability through a polarity-specific shift in resting membrane potential resulting in changes in spontaneous neuronal firing rates. The effects of tDCS on motor cortex excitability can be assessed by measuring the amplitude of muscle evoked potentials (MEPs) elicited by transcranial magnetic stimulation of the motor cortex. Transcranial magnetic stimulation produces a magnetic pulse that, in turn, produces an electric current in the underlying brain tissue (Fregni and Pascual-Leone, 2007). Anodal tDCS causes a sub-threshold depolarisation and increases the amplitude of MEPs indicating increased cortical excitability. This is mediated by voltage dependent calcium and sodium channels, demonstrated by orally administering carbamazepine (CBZ; sodium channel blocker), flunarizine (FLZ; calcium channel blocker) or placebo drugs to healthy participants prior to tDCS. CBZ and FLZ reduced the effects of anodal tDCS and had no effect on cathodal tDCS (Nitsche et al., 2003b).

The effects of anodal tDCS over the motor cortex on MEP amplitude persist for up to 90 minutes post stimulation suggesting that tDCS may also induce synaptic plasticity through long term potentiation and long term depression like mechanisms. This is supported by the finding that dextromethorphan (DMO; NMDA receptor antagonist), administered to healthy participants prior to tDCS, abolished the residual effects of both anodal and cathodal tDCS (Liebetanz et al., 2002). This indicates that

changes in glutamatergic signalling through NMDA receptors plays an important role in the residual effect of tDCS. Magnetic resonance spectroscopy has also revealed a polarity dependent change in neurotransmitter levels following tDCS (Stagg et al., 2009). There was a decrease in GABA concentration in the motor cortex of healthy human volunteers (n = 11) following anodal tDCS and a decrease in glutamate concentration following cathodal tDCS (n = 7). This suggests that a reduction in GABAergic inhibition contributes to the residual effects of anodal tDCS and the inhibitory effects of cathodal stimulation are mediated by a reduction in glutamatergic signalling.

Anodal tDCS has previously been reported to cause respiratory depression in a healthy volunteer during frontal tDCS with an extra-cephalic electrode (Lippold and Redfearn, 1964; Redfearn et al., 1964). This suggests that brainstem respiratory centres may be affected by tDCS. Furthermore, this implies that tDCS may also affect autonomic control through modulation of neuronal activity in the brainstem, however, only a handful of studies have investigated the potential autonomic effects of tDCS with conflicting results (see General Introduction Table 1.3) (Accornero et al., 2007; Vandermeeren et al., 2010; Montenegro et al., 2011; Raimundo et al., 2012). These studies utilised a variety of tDCS montages and autonomic measures making it difficult to draw any conclusions. Indeed, many of the autonomic measures used were crude estimates such as heart rate, blood pressure and respiratory frequency which are not sufficiently accurate to detect potential changes in autonomic function. Clarifying any potential influence of tDCS on autonomic function is essential as this may be an unknown and underappreciated side effect of tDCS treatment.

5.2 Hypothesis

Transcranial direct current stimulation over the motor cortex will influence central control of cardiovascular autonomic function.

5.3 Aims and Objectives

The aim of this study was to investigate the effects of tDCS on cardiovascular autonomic function in healthy human volunteers. The objectives were to determine:

1. the effects of anodal tDCS over the motor cortex on cardiovascular autonomic function.
2. the effects of cathodal tDCS over the motor cortex on cardiovascular autonomic function.
3. the effects of tDCS on sympathetic nerve activity measured by microneurography.

5.4 Methods

5.4.1 General Protocol

The study was approved by the University of Leeds Ethics Committee (Appendix - BIOSCI 11-019) and conducted in accordance with the Declaration of Helsinki. Informed written consent was obtained from all participants. 22 healthy participants were recruited for the study. Male and female participants over 18 years were included. Exclusion criteria were a history of cardiovascular disease, diabetes, hypertension, migraine or epilepsy. Participants were also excluded if they had any metal implants, were taking any psychotropic drugs (e.g. anti-depressants) or were pregnant. The study was conducted in the same room and similar environment as described in Chapter 2 to avoid confounding factors affecting autonomic control. Data were recorded at baseline, during tDCS and during recovery. The study used a double-blind, sham controlled design. 17 participants visited the laboratory twice (at least 7 days apart) and received active or sham stimulation. The order of the stimulation was random so that half received sham stimulation on the first visit and half received active first. An additional 5 participants visited the laboratory once for microneurography recordings and received anodal tDCS.

5.4.2 Transcranial direct current stimulation

Different parameters and electrode montages have been used in tDCS research however the most common arrangement consists of one surface electrode placed over the motor cortex and the other placed on the contralateral supraorbital region (Stagg and Nitsche, 2011; Im et al., 2012). tDCS was delivered by a specially developed constant current stimulator (Eldith DC stimulator, Magstim, UK) connected to rubber surface electrodes (5 cm by 7 cm, area = 35 cm^2) housed in saline (0.9%) soaked sponges. For anodal stimulation over the non-dominant primary motor cortex (M1), the anodal electrode was placed over C3/4 (using the International 10-20 EEG system) and the cathodal electrode was placed over the contralateral supraorbital area. Electrodes were held in place by elastic straps placed round the head. For cathodal tDCS, the electrode positions were reversed.

On the first visit, after experimental setup but before baseline recordings, participants experienced 10 s of 1 mA active tDCS to familiarise them with the procedure. This was performed in order to attenuate anxiety during subsequent monitoring and familiarise participants with any sensations they might experience during the stimulation (e.g. itching). The aim was to reassure participants thereby minimising changes in heart rate, blood pressure and respiration linked to anxiety.

During active stimulation, a constant current of 1 mA was applied for 15 minutes, taking 30 seconds to ramp up at the start of stimulation and 30 seconds to ramp down at the end of stimulation. Current density was 0.029 mA/cm^2 in accordance with safety criteria (Nitsche et al., 2003a). 14 participants (7 male, 7 female; 21-48 years) received anodal stimulation and 8 (4 male, 4 female; 21-45 years) received cathodal stimulation. For sham stimulation, electrodes were placed in the same positions as for active stimulation. There was a 30 s ramping up period at the start of sham stimulation then the current was immediately ramped down again. This mimicked the cutaneous sensations experienced during active stimulation. In all conditions, recording of autonomic variables commenced after the initial 30s when the current reached maximal test parameters (Figure 5.1).

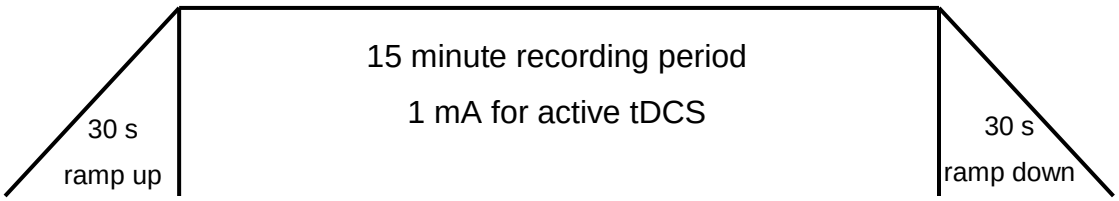


Figure 5.1 Stimulation protocol for active tDCS. Current was applied for 15 minutes and was either anodal (anode placed over the motor cortex) or cathodal (cathode placed over the motor cortex).

5.4.3 Blinding procedure

The participants and the investigator performing data analyses were blinded as to whether tDCS was active or sham. The tDCS device remained out of the participants' and investigator's sight at all times. Another un-blinded investigator, not involved in data analysis, administered tDCS. Participants were asked after the experiments whether they were able to determine which experimental session was "real" (active) stimulation and which one was "not real" (sham) stimulation. Half of the participants subsequently guessed correctly and as this was no better than chance, this was accepted as a suitable sham condition.

5.4.4 Tolerability Questionnaire

After each visit, participants (n = 9) were asked to provide feedback about their experience in the form of a questionnaire. Participants were asked to score experiences on a scale of 0-5 with 0 equal to none at all and 5 equal to extreme. This was also presented as a visual analogue scale to aid interpretation (Likert scale). There was no significant difference between scores for active and sham visits (Table 5.1). The most commonly reported experiences were itching or tingling during tDCS.

Table 5.1 Summary of tDCS tolerability scores. There was no significant difference in scores between active and sham tDCS. tDCS was well tolerated and no participants withdrew from the study.

	Active tDCS	**Sham tDCS**
Discomfort	1.38 ± 0.32	1.13 ± 0.35
Tingling	2.44 ± 0.24	2.22 ± 0.32
Itching	2.78 ± 0.36	2.00 ± 0.29
Warmth	0.89 ± 0.26	0.78 ± 0.22
Burning	0.78 ± 0.32	0.56 ± 0.24

In addition, space was provided for free-text comments and suggestions. Four comments were left in total;

- “tingling and itching only in first minute or so”
- “stinging”
- “initial burning sensation surprising”
- “hat strap unpleasant”

Participants were asked to notify the investigator if there were any symptoms e.g. headache after tDCS but none were reported. No participants withdrew from the study and all returned for the second visit.

5.4.5 Cardiovascular autonomic control and data acquisition

Heart rate, blood pressure, respiration and MSNA were recorded as described in Chapter 2. Muscle sympathetic nerve activity (MSNA) was recorded in 5 volunteers during anodal tDCS (2 male, 3 female; 21-46 years). Microneurography was only performed for anodal tDCS after data analyses revealed that anodal tDCS affected HRV. It was considered unethical to perform microneurography in cathodal and sham tDCS groups as this in an invasive procedure and analyses revealed cathodal tDCS had

no effect on HRV. Data acquisition and analyses of HRV, BRS and MSNA were conducted as in Chapter 2.

5.4.6 Statistical analysis

All statistical analyses were carried out using SPSS (version 18). Independent t-test or Mann-Whitney U test was used to compare group characteristics. Freidman's test with post hoc Bonferroni correction was used to analyse within subject effects of tDCS on HRV. To analyse the effects of different modes of stimulation (anodal or cathodal) on heart rate and blood pressure, mixed mode ANOVAs were used. Data are presented as group mean ± standard error of the mean (S.E.M.) unless stated otherwise. P-values < 0.05 were considered significant.

5.5 Results

5.5.1 Effect of transcranial direct current stimulation on heart rate variability

There was an increase in LF/HF ratio during anodal tDCS which continued into the post-stimulation phase and reached significance (n = 14;Freidman's test, p = 0.017) whereas there was no significant change in cathodal (n = 8) and sham (n = 17) tDCS groups (Figure 5.2; Table 5.2). There was also a significant increase in LF power during anodal tDCS (Freidman's test, p = 0.011) whereas HF power did not change significantly (Figure 5.3; Table 5.3). There was no significant difference between those who received active tDCS on the first visit compared to those who received sham first. There was no significant change in BRS. Mixed mode ANOVAs revealed a significant decrease in heart rate (time effect, p = 0.010) and increase in mean BP (time effect, p <0.0005). Mode of stimulation (anodal or cathodal) had no impact on heart (main effect for mode of stimulation, p > 0.05) or mean BP (main effect form mode of stimulation > 0.05). Furthermore, there was no interaction between mode of stimulation and time (Figure 5.4).

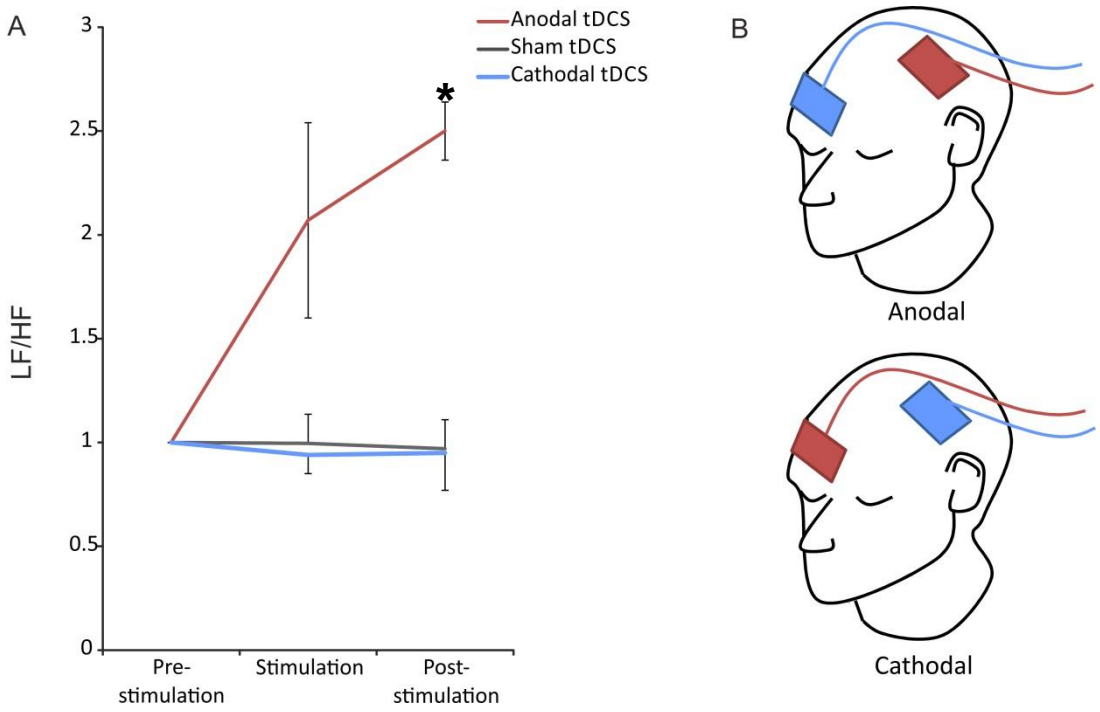


Figure 5.2 The effects of anodal, cathodal and sham tDCS on heart rate variability. (A) There was an increase in LF/HF ratio during anodal tDCS which continued into the recovery phase and reached significance (n = 14; time effect, p = 0.017) indicating a shift in cardiac autonomic control towards sympathetic predominance whereas there was no significant change during cathodal (n = 8) and sham (n = 17) tDCS. There was a significant difference between anodal and cathodal groups (interaction, p = 0.005). (B) Illustration of electrode placements for anodal and cathodal tDCS (* = significantly different from pre-stimulation).

Table 5.2 Absolute values of heart rate variability. Results are presented as mean ± S.E.M.

	LF/HF Pre-Stimulation	LF/HF Stimulation	LF/HF Post-Stimulation
Anodal tDCS	0.81 ± 0.13	1.56 ± 0.51	1.28 ± 0.28
Cathodal tDCS	0.93 ± 0.13	0.84 ± 0.11	0.75 ± 0.13
Sham tDCS	0.85 ± 0.14	0.87 ± 0.18	0.81 ± 0.15

.

Table 5.3 HRV values for anodal, cathodal and sham tDCS groups. There was a significant increase in LF power in the anodal tDCS group during stimulation (Friedman's test, p = 0.011) and an increase in LF/HF ratio that reached significance in the post-stimulation period (Friedman's test, p = 0.017; * = significantly different from pre-stimulation).

	Anodal			**Cathodal**			**Sham**		
	Pre-stimulation	**Stimulation**	**Post-stimulation**	**Pre-stimulation**	**Stimulation**	**Post-stimulation**	**Pre-stimulation**	**Stimulation**	**Post-stimulation**
Total power (ms^2)	3047.61 ± 975.71	3426.90 ± 685.25	3252.25 ± 778.14	2459.42 ± 485.35	2324.99 ± 603.41	2638.58 ± 578.64	2903.44 ± 539.23	2554.96 ± 451.05	3052.56 ± 624.31
LF power (ms^2)	992.15 ± 324.43	**1316.01 ± 307.35***	1216.51 ± 360.53	700.92 ± 136.68	539.36 ± 115.29	715.57 ± 214.48	827.24 ± 199.48	711.23 ± 189.51	802.82 ± 187.68
HF power (ms^2)	1426.25 ± 2069.54	1386.01 ± 1443.43	1254.48 ± 1443.02	948.99 ± 224.53	764.08 ± 202.51	924.93 ± 164.37	1137.28 ± 1226.28	887.23 ± 754.38	1263.31 ± 1744.50
LF (nu)	40.49 ± 3.59	**48.12* ± 5.88**	**49.08* ± 4.65**	45.92 ± 3.77	43.52 ± 3.26	40.99 ± 4.65	40.54 ± 3.37	38.23 ± 3.78	38.86 ± 3.67
HF (nu)	59.50 ± 3.59	51.81 ± 5.88	**50.92* ± 4.65**	54.08 ± 3. 77	56.48 ± 3.26	59.01 ± 4.65	59.05 ± 3.41	61.36 ± 3.72	61.14 ± 3.67
LF/HF	1.00	2.07 ± 0.47	**2.50 ± 0.14***	1.00	0.94 ± 0.09	0.95 ± 0.18	1.00	1.00 ± 0.14	0.97 ± 0.14

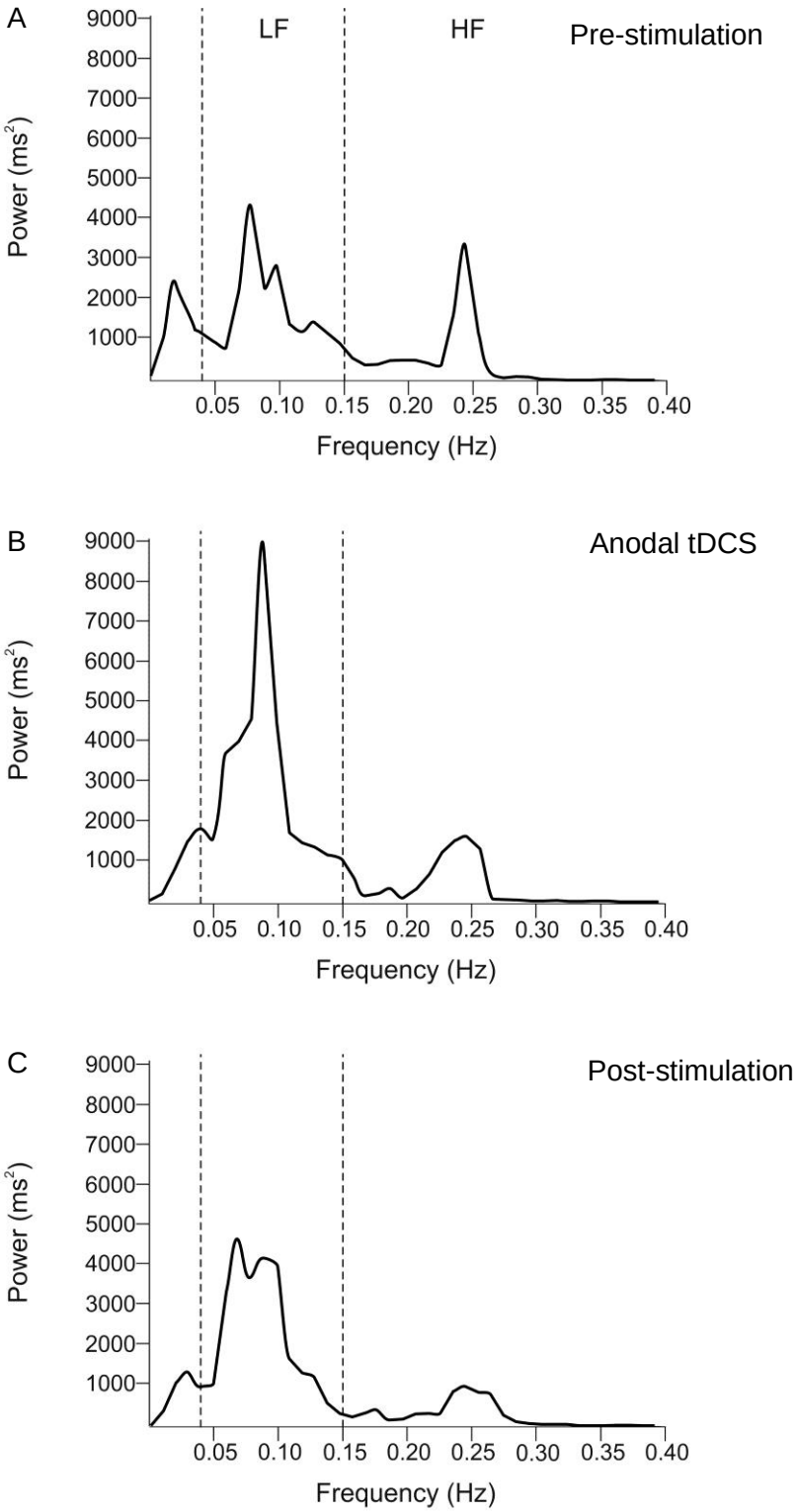


Figure 5.3 Example of the effects of anodal tDCS on HRV power spectra in one individual. There is an increase in LF power during anodal tDCS whereas there is no significant change in HF power. (A) pre-stimulation, (B) anodal tDCS, (C) post-stimulation.

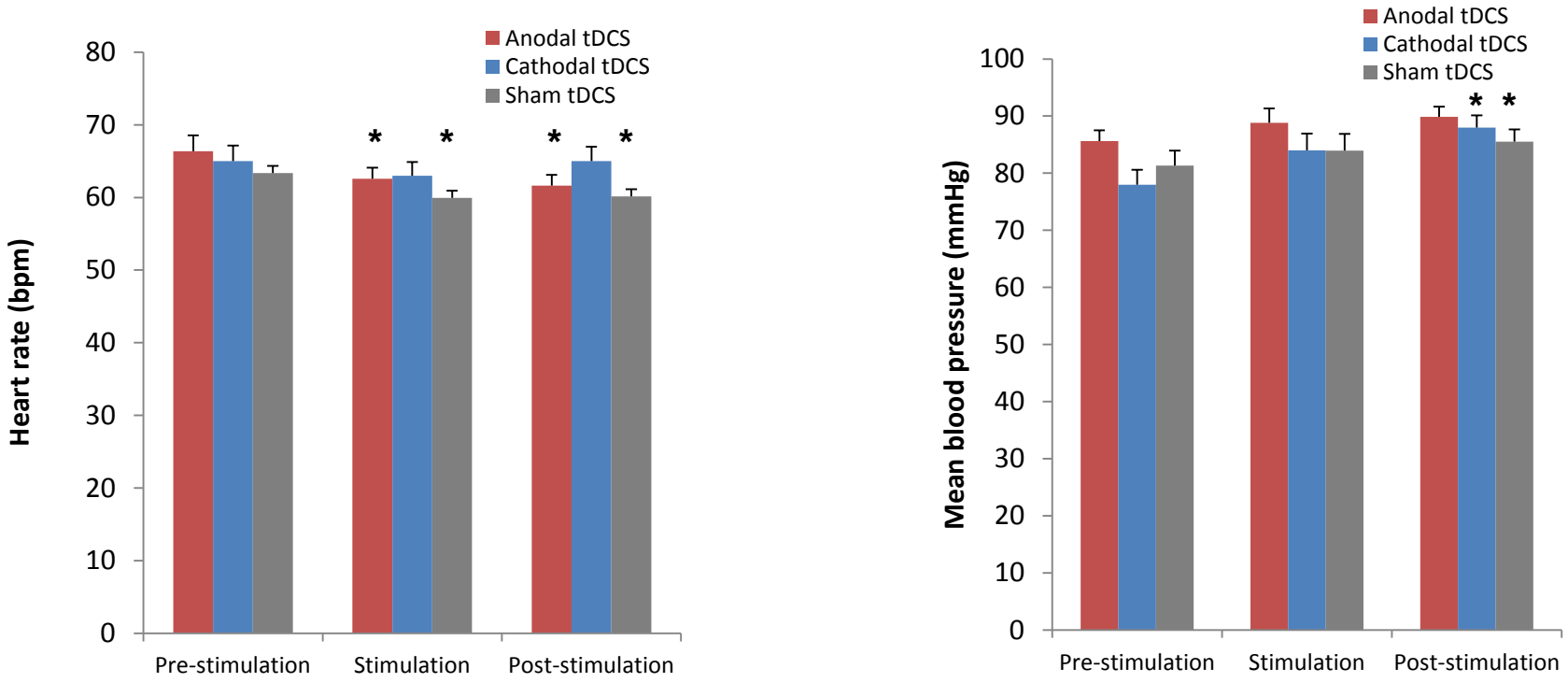


Figure 5.4 Heart rate and mean blood pressure for anodal, cathodal and sham tDCS groups. There was a significant decrease in heart rate (time effect, $p = 0.010$) and a significant increase in BP (time effect, $p < 0.0005$; measured using a Finometer). Mode of stimulation (anodal or cathodal) had no impact on heart rate or blood pressure (main effect for mode of stimulation, $p > 0.05$) and there was no interaction between mode of stimulation and time (* = significantly different from pre-stimulation).

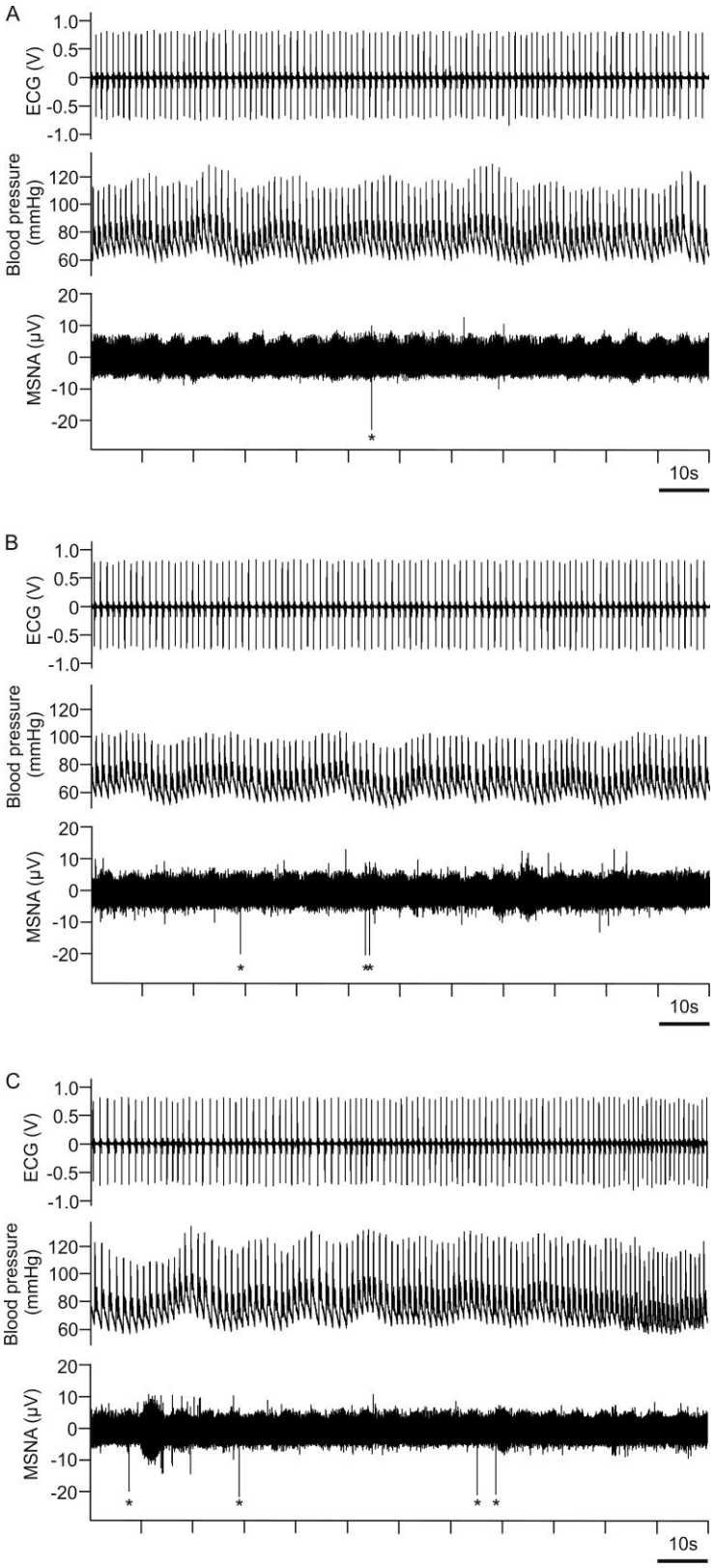


Figure 5.5 The effects of anodal tDCS on muscle sympathetic nerve activity. Example recordings of ECG, blood pressure and MSNA at baseline (A), during anodal tDCS (B) and recovery (C) from one individual (* = single unit).

5.5.2 Transcranial direct current stimulation increases sympathetic nerve activity

Vasoconstrictor muscle sympathetic nerve activity was recorded directly using microneurography in participants receiving anodal tDCS (Figure 5.5 and Figure 5.6; n = 5). There was a significant increase in single unit frequency and incidence during the stimulation phase which increased and persisted into the post-stimulation phase (time effect, p = 0.046 and p = 0.029 respectively; Figure 5.7 ; Table 5.4). There was no significant change in heart rate or blood pressure during tDCS (Table 5.5).

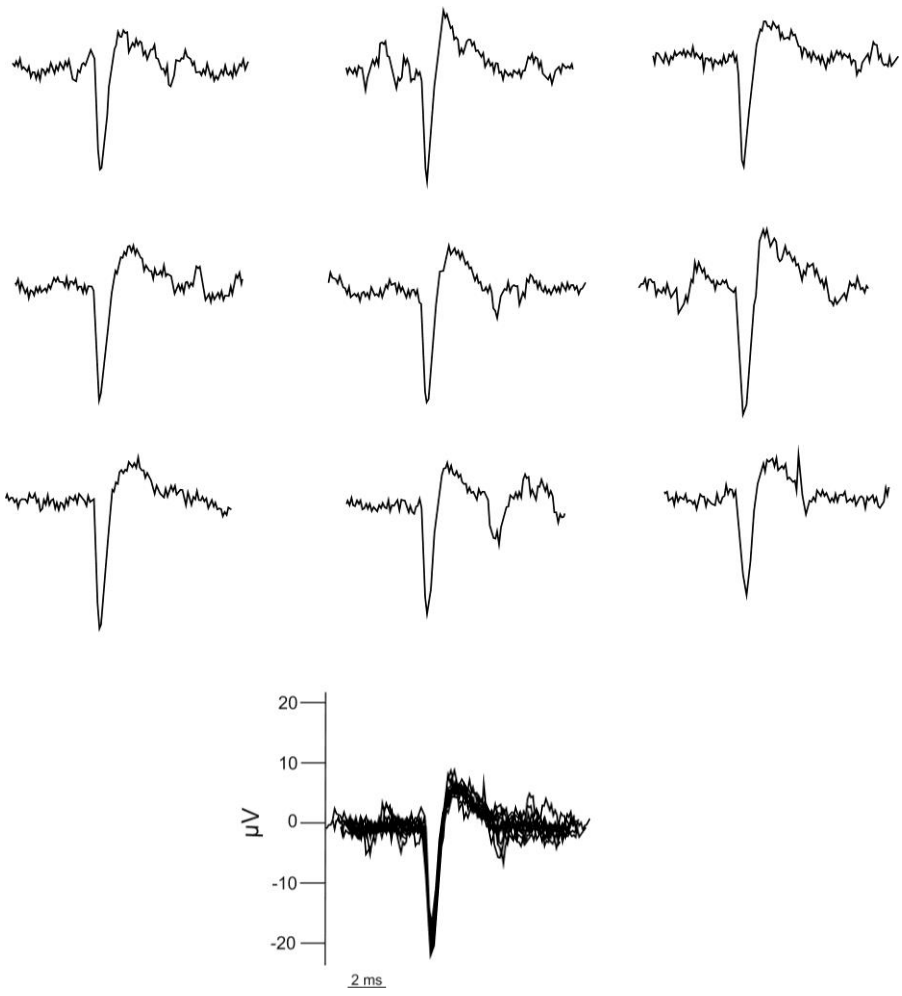


Figure 5.6 Examples of individual single units from MSNA recordings shown in Figure 5.5. MSNA units have a similar morphology as demonstrated by superimposition.

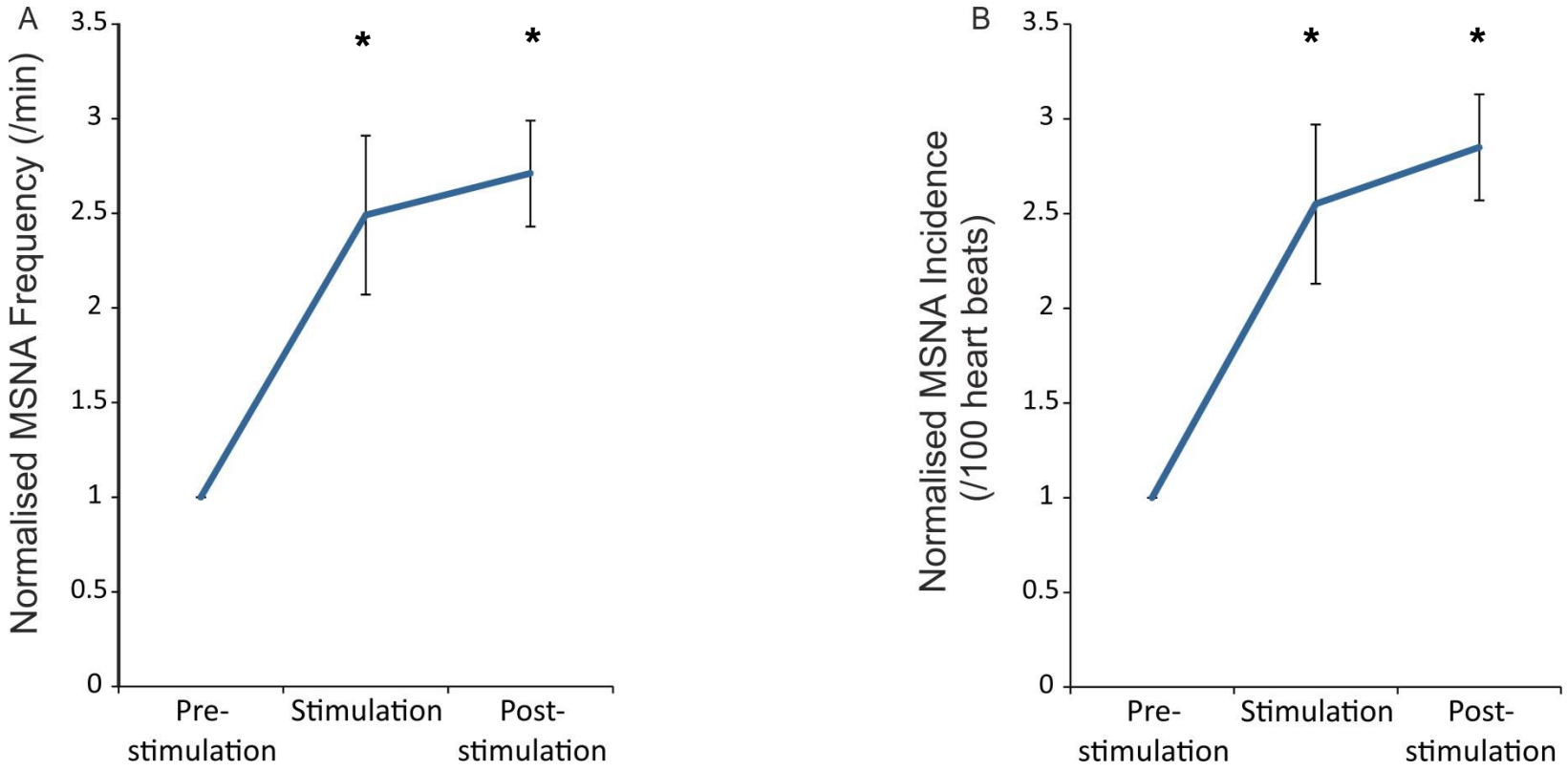


Figure 5.7 There is a significant increase in MSNA frequency and incidence during anodal tDCS and the post-stimulation phase (time effect, p = 0.046 and p = 0.029; normalised group data n = 5; * = significantly different from pre-stimulation).

Table 5.4 Raw microneurography values. Results are presented as mean ± S.E.M.

	Baseline	Stimulation	Recovery
MSNA frequency (units/min)	0.32 ± 0.05	0.68 ± 0.21	0.82 ± 0.12
MSNA incidence (units/100 heart beats)	0.52 ± 0.07	1.12 ± 0.3	1.40 ± 0.20

Table 5.5 Effects of anodal tDCS on cardiovascular and HRV variables of participants who underwent microneurography (n = 5). There is no significant change in heart rate, BP, BRS or HRV.

	Baseline	Stimulation	Recovery
Total Power (ms^2)	4902 ± 2480	4692 ± 1607	1310 ± 1936
LF Power	1676 ± 758	2243 ± 603	1957 ± 808
HF Power	2642 ± 1457	2041 ± 1005	1938 ± 1037
LF/HF	0.82 ± 0.26	2.58 ± 1.35	1.62 ± 0.64
Heart rate (bpm)	62 ± 3.4	61 ± 3.0	59 ± 2.9
Mean BP (mmHg)	86 ± 3.1	91 ± 5.8	93 ± 3.8
BRS (ms/mmHg)	15.6 ± 4.8	19.1 ± 4.25	14.37 ± 4.0

5.6 Discussion

This double-blind sham controlled study provides evidence that anodal tDCS of the motor cortex can shift HRV towards sympathetic predominance in healthy humans in the post-stimulation period. This study shows, for the first time, that tDCS also increases vasoconstrictor sympathetic nerve activity measured directly using microneurography. This is the first direct evidence that tDCS can affect sympathetic nervous activity and thus reveals potential implications for future use of tDCS in a therapeutic setting.

5.6.1 tDCS and autonomic control

Since the reports in the 1960s that tDCS may affect respiration, surprisingly few studies have investigated the potential effects of tDCS on the brainstem further. The original study found that anodal tDCS over the frontal cortex caused respiratory depression in a healthy volunteer, however this was using a current of 3 mA and small electrodes (1/2 inch diameter or 1.3 cm) (Lippold and Redfearn, 1964; Redfearn et al., 1964) with a current density of 0.564 mA/cm^2, much higher than the recommended 0.029 mA/cm^2 (Nitsche et al., 2003a). In addition, the electrode montage consisted of an extra-cephalic cathodal electrode unlike the majority of studies that use a bi-cephalic montage (Lippold and Redfearn, 1964; Redfearn et al., 1964). It was thought that this particular montage may pass more electrical current through the brainstem, however, modelling of electric fields during tDCS suggests that this is not the case (Im et al., 2012). Another extra-cephalic electrode montage, with an electrode placed on the neck and the other over the occipital cortex, has subsequently been found to have no effect on heart rate, blood pressure, body temperature, or respiratory frequency during both anodal and cathodal stimulation (Accornero et al., 2007), however, these are crude measures of autonomic function. Vandermeeren et al., (2010) also found that anodal tDCS over the frontal cortex with an extra-cephalic electrode had no significant effect on these indices but also included the analysis of HRV. They reported no significant effect, however, they did note a small increase in the LF/HF ratio during anodal, cathodal and sham tDCS suggesting an increase in sympathetic activity. As this occurred in all three groups, including sham, it may be that this was a result of anxiousness experienced by the volunteers during the study. Only one study has looked at the autonomic effects of the more commonly used bi-cephalic montage for tDCS and reported that anodal tDCS over the motor cortex had no significant effect on blood pressure, body temperature, respiratory rate or cortisol levels (Raimundo et al., 2012). The present study also observed no effect on respiratory rate although this was not formally analysed. Importantly, this study provides the only direct recording of sympathetic

nerve activity and shows that anodal tDCS over the motor cortex may indeed influence autonomic control in healthy humans.

Since bi-cephalic tDCS over the motor cortex can increase sympathetic nervous activity it may prove a useful tool to modify autonomic activity. Interestingly, the increase in LF/HF ratio and MSNA continued after tDCS ceased. tDCS has been reported to have residual effects on motor cortical excitability (increased amplitude of MEPs) outlasting stimulation by up to 90 minutes in humans (Nitsche and Paulus, 2000, 2001) and this may account for the continued sympathoexcitation observed in this study. Whether the increase in sympathetic nerve activity is maintained for a similar duration of 90 minutes post stimulation merits further attention. In addition, whether the effects of tDCS on autonomic function are influenced by repeated application may warrant investigation.

tDCS over other areas of the cortex may have different effects on autonomic function. Bi-cephalic anodal tDCS over the temporal lobe has been reported to increase the HF component of HRV and decrease LF/HF ratio indicating an increase in parasympathetic influence on heart rate (Montenegro et al., 2011). The potential for tDCS to alter autonomic function towards either parasympathetic (temporal lobe placement) or sympathetic predominance (motor cortex placement) is especially pertinent as tDCS has recently been applied in the context of stroke rehabilitation (Schulz et al., 2013). Stroke patients often have compromised autonomic function and the degree of autonomic dysfunction is predictive of mortality (Robinson et al., 2003; Mäkikallio et al., 2004; Sörös and Hachinski, 2012). Decreased HRV indicating increased sympathetic predominance is reported after stroke and has been reported to last up to 6 months post stroke (Korpelainen et al., 1996). Reduced BRS and elevated plasma noradrenaline levels are also observed following stroke (Myers et al., 1981; Robinson et al., 1997). tDCS could have beneficial or detrimental effects in stroke patients depending on the sympathovagal balance of each individual. It may be possible to tailor tDCS therapy to improve autonomic function by stimulating different areas of the cortex e.g. anodal tDCS over the temporal lobe for patients with reduced parasympathetic activity. Individual autonomic function could be assessed on a case by case basis and would be easily implemented in clinics by using

non-invasive measures of autonomic function such as HRV. Further research into the use of tDCS in stroke patients may therefore be justified, including examining the duration of effects.

5.6.2 Potential pathways involved in cortical modulation of autonomic function by tDCS

Since tDCS is known to influence cortical structures, it may indirectly affect autonomic outflow through these structures. Krogh and Lindhard (1913) first proposed higher control of autonomic function, later termed ‘central command’ to account for the rapid increase in heart rate at the start of exercise. Since then numerous studies have detailed areas of the cortex that influence autonomic function including the medial prefrontal cortex (mPFC) (Bacon and Smith, 1993; Owens et al., 1999; Gabbott et al., 2005), insular cortex (Verberne and Owens, 1998) and motor (Schlindwein et al., 2008) cortex.

5.6.3 Influence of the medial prefrontal cortex on autonomic function

The medial prefrontal cortex is of particular interest in this study as it may have been inhibited by the cathodal electrode placed over the supraorbital area. Several lines of evidence indicate that such inhibition of the mPFC can explain the sympathoexcitation detected in this study. Stimulation of the mPFC in anaesthetised rats resulted in a decrease in blood pressure and concomitantly reduced sympathetic nerve activity, suggesting that the mPFC has a sympathoinhibitory effect (Owens et al., 1999). Furthermore, inhibiting NTS neurones by injecting muscimol ($GABA_A$ receptor agonist) bilaterally into the NTS attenuated the depressor and sympathoinhibitory effects of mPFC stimulation (Owens et al., 1999). The depressor effect of mPFC stimulation was also reversed by injecting bicuculline ($GABA_A$ receptor blocker) into the RVLM (which contains sympathetic premotor neurones) or by injecting kynurenic acid (ionotropic glutamate receptor antagonist) into the CVLM (which inhibits the RVLM), suggesting that these areas have an

important role in mediating the autonomic effects of mPFC stimulation (Owens and Verberne, 2000). The mPFC has been shown to project to areas of autonomic control including the NTS and RVLM using injection of the retrograde neuronal tracer wheat germ agglutinin conjugated to horseradish peroxidase (WGA-HRP) in the rat (Gabbott et al., 2005). Injecting anterograde neuronal tracer into the mPFC also revealed projections to autonomic regions of the spinal cord where sympathetic pre-ganglionic neurones are located (Bacon and Smith, 1993) therefore the mPFC could also influence sympathetic activity through this pathway.

Imaging studies have provided evidence that the mPFC is involved in autonomic control in humans. Combining fMRI and isometric handgrip exercise, causing sympathetic activation, revealed a decrease in blood oxygen level dependent (BOLD) signal in the ventral mPFC that correlated with an increase in heart rate (Wong et al., 2007). The mPFC was also deactivated during baroreceptor unloading induced by the application of lower body negative pressure (Kimmerly et al., 2005). It can therefore be envisaged that one possible route by which tDCS induced sympathoexcitation is through inhibition of the mPFC.

5.6.4 Influence of the motor cortex on autonomic function

There is also evidence that the motor cortex may play a role in autonomic regulation. Stimulating the motor cortex in rats induced expression of the activity marker c-fos protein in the NTS, DVN and RVLM (Sequeira et al., 2000). Furthermore, injecting fluorogold (FG) into the NTS/DVN and dextran-tetramethylrhodamine (DR) into the RVLM revealed direct projections to the motor cortex (Sequeira et al., 2000). Anterograde tracing with WGA-HRP or DR from the motor cortex confirmed a reciprocal projection to the NTS, DVN, CVLM and RVLM. Moreover, by injecting DR into the ventrolateral spinal cord (T2-T4) and FG into the NTS/DVN or RVLM, double labelled neurones were found in the motor cortex indicating that corticospinal fibres provided collaterals to brainstem autonomic centres. This indicates that there is integration between the somatic and autonomic nervous systems in relation to movement (Viltart et al., 2003). Interestingly, the densest labelled cortical

projection was to the RVLM indicating that the motor cortex plays an important role in regulating sympathetic activity. Viltart et al. (2003) confirmed that stimulation of the motor cortex in the rat induced c-fos protein activation in the CVLM and RVLM. Motor cortex stimulation also decreased blood pressure and plasma noradrenaline levels, supporting a role for the motor cortex in autonomic regulation (Viltart et al., 2003). The presence of c-fos protein in the CVLM indicates activation which would, in turn, cause GABAergic inhibition of the RVLM and would account for the decrease in BP and plasma noradrenaline observed during motor cortex stimulation. Conversely, as there was also c-fos protein immunoreactivity in the RVLM this would imply activation of the RVLM which would lead to increased sympathetic activity, contrary to the autonomic changes observed. However, there is a small sub-population of RVLM neurones which, when stimulated, caused inhibition of sympathetic preganglionic neurones in the spinal cord (Deuchars et al., 1997). This response could be abolished by bicuculline ($GABA_A$ receptor antagonist) indicating a direct GABAergic projection from the RVLM to sympathetic preganglionic neurones (Deuchars et al., 1997). This may account for the sympathetic inhibition observed by Vitart et al. (2003) upon motor cortex stimulation. The electrical stimulation was performed using a bipolar electrode implanted directly onto the motor cortex which delivered pulsed stimulation, the polarity of which was reversed every 2 s. The difference in stimulation modalities used may explain why sympathoinhibition was observed by Viltart et al. (2003) whereas we report sympathoexcitation. In addition, the rats were under anaesthesia which may also have affected the results.

The motor cortex also influences autonomic function in humans. fMRI during lower body negative pressure revealed an increase in BOLD signal in the motor cortex that was correlated with increased heart rate (Kimmerly et al., 2005). Perhaps the most elegant study of cortical contribution to autonomic activity used positron emission tomography with labelled glucose to assess cerebral metabolism at rest and relate this to spontaneous changes in heart rate and plasma noradrenaline levels. This revealed a positive correlation between plasma noradrenaline levels and increased regional cerebral glucose metabolism in the motor cortex (Schlindwein et al.,

2008) supporting a role for the motor cortex in sympathoexcitation. Direct activation of the motor cortex by the anodal electrode may also, therefore, contribute to the increased sympathetic nervous activity observed in this study.

Currently, few studies have investigated the potential of tDCS to modulate autonomic function. These studies are preliminary in nature and report mixed results. Further investigation of the effects of tDCS on autonomic function is warranted, including electrode positioning and any residual effects. The results of this study require consideration when using tDCS in patients groups with altered autonomic function e.g. stroke patients but also represent a potential therapeutic avenue to target autonomic control that may have been overlooked in the past.

Chapter 6
General Discussion

6.1 Summary of findings

6.1.1 H- tVNS altered cardiovascular autonomic function towards parasympathetic predominance in healthy humans and heart failure patients

The physiological evidence presented in this thesis indicates that H-tVNS can alter cardiovascular autonomic function in both healthy humans and heart failure patients. In healthy subjects there was a shift in cardiac autonomic control towards parasympathetic predominance, however, this was dependent on the stimulation parameters used. Only high pulse width (200 μs) and frequency (30 Hz) tVNS caused a significant decrease in LF/HF ratio. The effect of these stimulation parameters on sympathetic vasoconstrictor nerve activity was also investigated. This revealed that H-tVNS caused a significant reduction in MSNA. Based on these results, a pilot study of the effects of H-tVNS on cardiac autonomic control in heart failure patients was conducted. There was a significant decrease in LF/HF ratio indicating a shift in cardiac autonomic control towards parasympathetic predominance. This is remarkable as heart failure patients were on optimal medical therapy including beta-blockers and ACE inhibitors. H-tVNS was also well tolerated by heart failure patients and was not associated with any side effects.

6.1.2 Anodal tDCS over the motor cortex increased sympathetic nerve activity in healthy humans

Anodal tDCS over the motor cortex had the opposite effect on cardiovascular autonomic function compared to H-tVNS. There was an increase in LF/HF ratio during anodal tDCS indicating a shift towards sympathetic predominance that continued and reached significance in the post-stimulation period. This was supported by a significant increase in MSNA during anodal tDCS that continued after stimulation had ceased.

6.2 Potential mechanisms of H-tVNS cardiovascular autonomic effects

The mechanisms underlying the effects of H-tVNS on autonomic function require further elucidation, however, there is evidence through retrograde neuronal tracing in cats and dogs that the ABVN projects to the NTS (Nomura and Mizuno, 1984; Chien et al., 1996). The NTS is integral to autonomic regulation and stimulating the ABVN may alter neuronal processing here. Interestingly, the ABVN projects to the dorsomedial part of the caudal NTS which also receives baroreceptor afferent projections from the carotid sinus and aortic arch (Ciriello et al., 1981). Therefore, stimulating the ABVN may activate the baroreceptor pathway through the NTS to the CVLM which inhibits the RVLM and decreases sympathetic output (Figure 6.1). This could account for the microneurography findings of this thesis that revealed a decrease in MSNA during H-tVNS in healthy humans. This study attempted to define the central projections of the ABVN in humans by applying the neuronal tracer DiI to the ABVN in cadaveric tissue, however, this was unsuccessful. By adjusting the protocol e.g. applying a direct current to the tissue to increase the diffusion rate, it should be possible to define the neuroanatomy of the ABVN in humans. This would clarify, at least in part, the mechanisms involved in H-tVNS cardiovascular autonomic effects.

The NTS also projects to the DVN and NA (containing parasympathetic preganglionic neurones), therefore, H-tVNS could also modulate parasympathetic output through this pathway. Unlike sympathetic nerve activity, parasympathetic nerve activity cannot be recorded directly in humans. The HF component of HRV represents cardiac vagal modulation and may provide an indication of H-tVNS effects on HRV. There was no significant change in HF power during H-tVNS in healthy humans suggesting that H-tVNS does not alter parasympathetic output, however, these participants were healthy and stimulation was applied during rest. These conditions would result in high parasympathetic tone at baseline and this may mask potential effects of tVNS on the parasympathetic nervous system. In heart failure patients, there was an increase in HF power, however, this

did not reach significance. The heart failure group was small (n = 8), therefore, further investigation is required to explore the possible effects of H-tVNS on parasympathetic activity in populations with diminished parasympathetic activity.

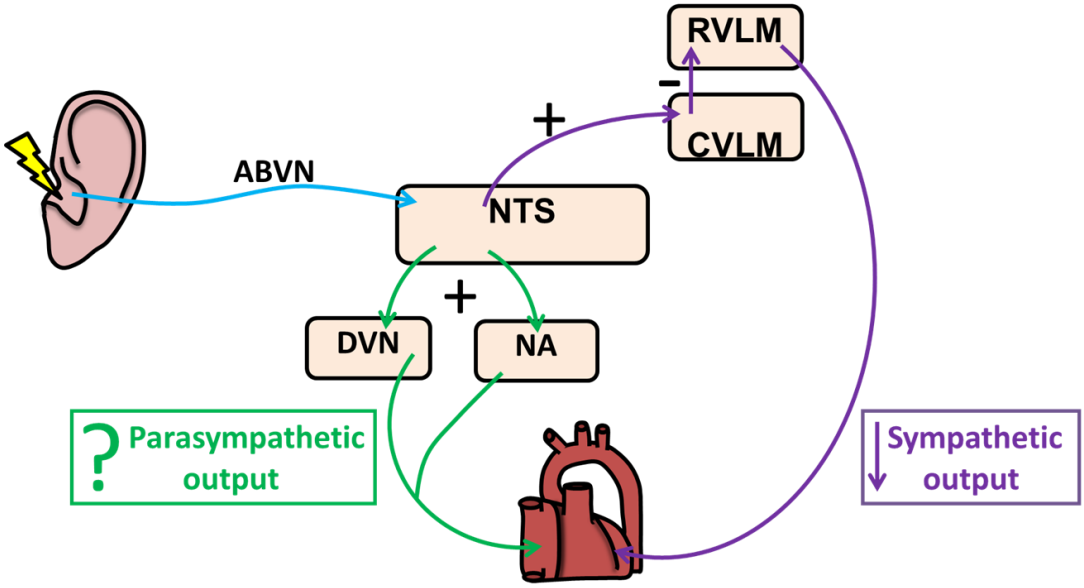


Figure 6.1 Potential mechanisms of H-tVNS effects on cardiovascular autonomic function. Stimulation of the ear could excite the NTS through projections of the ABVN. This could lead to activation of the CVLM which, in turn, would inhibit the RVLM and reduce sympathetic output. The NTS also projects to the DVN and NA therefore tVNS could alter parasympathetic output through this pathway.

6.4.1 Potential mechanisms of tDCS cardiovascular autonomic effects

The diffuse nature of tDCS stimulation, as revealed by computational models (Im et al., 2012), hinders interpretation of the mechanisms underlying the changes in cardiovascular autonomic function during anodal tDCS over the motor cortex. However, the greatest current density amplitude is under the

surface electrodes (Im et al., 2012), therefore, these areas are likely to be most affected by the stimulation. This study found that placing the anodal electrode over the motor cortex and the cathodal electrode over the contralateral supraorbital region increased LF/HF ratio and MSNA. Neuronal tracing studies have revealed projections from the motor cortex to areas of autonomic control including the NTS, DVN, CVLM and RVLM (Sequeira et al., 2000). PET studies in humans have also revealed a correlation between increased plasma noradrenaline levels and increased glucose metabolism in the motor cortex indicating that the motor cortex plays a role in sympathoexcitation (Schlindwein et al., 2008). Stimulation of the motor cortex by the anodal electrode may therefore have contributed to the increase in sympathetic activity observed in this study. In addition, the cathodal electrode may have influenced activity in the medial prefrontal cortex. The mPFC has also been found to project to areas of autonomic control such as the NTS and RVLM using neuronal tracers (Bacon and Smith, 1993; Gabbott et al., 2005). Furthermore, stimulation of the mPFC in anaesthetised rats decreased blood pressure and sympathetic nerve activity (Owens et al., 1999) suggesting that the mPFC has a sympathoinhibitory role. Cathodal stimulation over the mPFC may therefore have caused disihbition of sympathetic activity that may have contributed to the sympathoexcitation reported in Chapter 5. In the future, fMRI during tDCS may aid investigation of the mechanisms of tDCS effects on cardiovascular function.

6.3 Neuromodulation of the autonomic nervous system

Neuromodulation, the alteration of nerve activity through targeted electrical or pharmaceutical interventions, is a rapidly expanding field with applications in a plethora of conditions. Many of these interventions alter activity of the autonomic system e.g. renal sympathetic denervation for the treatment of hypertension (Esler et al., 2010) (see Section 6.5.1 below), carotid sinus stimulation and deep brain stimulation, however many of these techniques are invasive and expensive, limiting application to patient populations.

6.3.1 Carotid sinus stimulation

Electrical stimulation of the carotid sinus to activate baroreceptors and/or baroreceptor afferents has been proposed as a possible therapy for hypertension (Jordan et al., 2012). This technique aims to mimic activation of the arterial baroreflex by increased blood pressure to cause a reflex decrease in sympathetic nerve activity and blood pressure. This has proven effective in clinical trials in treatment resistant hypertension patients with a significant decrease in blood pressure at 3 and 12 months follow-up (Bisognano et al., 2011). The acute effects of carotid sinus stimulation on MSNA have been evaluated in treatment resistant hypertension patients. There was a significant decrease in MSNA burst frequency, incidence and area during stimulation with a concomitant decrease in blood pressure and plasma renin concentration (Heusser et al., 2010). Interestingly, carotid sinus stimulation has also been reported to reduce left ventricular mass and wall thickness indicating improved left ventricular structure (Bisognano et al., 2011). This, coupled with reduced MSNA, suggests that carotid sinus stimulation may also be beneficial in heart failure. Indeed, carotid sinus stimulation for 3 months in a canine heart failure model attenuated the decrease in left ventricular ejection fraction compared to untreated dogs (Sabbah et al., 2011b). In addition, carotid sinus stimulation attenuated the increase in plasma noradrenaline levels and cardiac remodelling with a reduction in interstitial fibrosis and cardiomyocyte hypertrophy. Studies of carotid sinus stimulation and the mechanisms involved are few, however, these remarkable findings warrant further investigation.

6.3.2 Deep brain stimulation

Deep brain stimulation of the periaqueductal grey (PAG) matter is performed in cases of chronic pain, however, this also affects central autonomic control (Carter et al., 2011). The PAG projects to areas of autonomic control as revealed by neuronal tracing studies. Injecting neuronal tracer into the PAG revealed projections to the NTS, DVN and NA (Farkas et al., 1997) indicating that these areas of the PAG influence parasympathetic activity.

The PAG was also found to project to sympathetic premotor neurones in the RVLM (Farkas et al., 1998). Stimulation of the PAG produces both pressor and depressor responses depending on the location of the stimulating electrode within the PAG. Stimulation of the dorsal PAG in anaesthetised rats increased blood pressure and heart rate (Lovick, 1985) whereas microinjection of an excitatory amino acid (D,L-homocysteic acid) into the ventral PAG caused bradycardia and reduced blood pressure (Lovick, 1992). Similar results have since been reported in humans. Stimulation of the ventral PAG in conscious humans caused a significant decrease in blood pressure whereas stimulation of the dorsal PAG increased blood pressure, however, stimulation at either site had no effect on heart rate (Green et al., 2005). These effects on blood pressure merit consideration when selecting patients for PAG stimulation and during long term follow-up, however, this also offers another potential method to therapeutically alter blood pressure e.g. stimulation of the ventral PAG may beneficial in reducing blood pressure in hypertension whereas stimulation of the dorsal PAG may alleviate symptoms of orthostatic hypotension.

6.4 Residual effects of neuromodulation

Both neuromodulatory techniques investigated in this thesis exhibited a residual effect on cardiovascular autonomic function to some degree. LF/HF ratio did not return to baseline levels after H-tVNS but remained slightly reduced. There was a trend towards a return to baseline levels, however, this was not reached in the 15 minute period following stimulation. After tDCS, there was a continued increase in LF/HF ratio and MSNA. These residual effects imply that continuous stimulation may not be necessary to maintain changes in autonomic function which would facilitate the use of these techniques by patients and reduce disruption to daily life. This is supported by a previous finding that auricular acupuncture (25 minutes) produced changes in HRV that persisted for at least 60 minutes post-stimulation (Haker et al., 2000). Anodal tDCS over the motor cortex (13

minutes) is also reported to cause changes in cortical excitability that lasted for 90 minutes (Nitsche and Paulus, 2000, 2001).

Interestingly, chronic, daily administration of both tVNS and tDCS produce longer residual effects (Zamotrinsky et al., 1997; Zamotrinsky et al., 2001; Boggio et al., 2007). 10 consecutive days of auricular electroacupuncture (15 minutes duration) in patients with coronary artery disease decreased the use of vasodilator medication for up to three weeks after stimulation ceased (Zamotrinsky et al., 1997; Zamotrinsky et al., 2001). Similarly, 5 consecutive days of tDCS (20 minutes duration) improved motor function in stroke patients that lasted 2 weeks after stimulation ended whereas 4 tDCS sessions administered weekly had no residual effect (Boggio et al., 2007). These are the only studies investigating chronic tVNS and tDCS stimulation and the residual effects. The effects of chronic H-tVNS, as described in this thesis, on cardiovascular autonomic function remain to be determined, however, based on these studies it seems likely that repeated H-tVNS interventions could lengthen the residual effect on cardiovascular autonomic function. This would enable short H-tVNS therapy times that would be amenable to patients rather than continuous stimulation.

6.5 Clinical Implications of H-tVNS

H-tVNS reduced MSNA in healthy participants and therefore may be therapeutic in other conditions characterised by sympathoexcitation in addition to heart failure. Many conditions, that at first may seem unrelated , exhibit underlying sympathoexcitation e.g. hypertension, obstructive sleep apnoea and obesity (Charkoudian and Rabbitts, 2009).

6.5.1 H-tVNS and treatment resistant hypertension

Hypertension is the leading attributable cause of mortality worldwide causing 7.5 million deaths every year and is characterised by increased levels of sympathetic activity (Simplicity HTN-1 Investigators, 2011). Approximately half of hypertensive patients are treatment resistant (elevated blood

pressure despite lifestyle modifications and ≥3 antihypertensive drugs) (Esler et al., 2010). Recently, renal nerve denervation has been trialled in treatment resistant hypertensive patients. This neuromodulatory technique attempts to attenuate sympathetic activation in hypertension by denervating the renal artery using radiofrequency energy delivered through a catheter placed in the renal artery (Esler et al., 2010). At 6 months follow-up after renal nerve ablation, blood pressure was significantly reduced compared to the control group (Esler et al., 2010) and this effect was sustained at 24 months follow-up (Investigators, 2011). This is encouraging as these effects were obtained in a patient population that was resistant to conventional anti-hypertension treatments. However, the technique is invasive and the investigators report several cases of femoral pseudoaneurysm and one case of renal artery dissection (Investigators, 2011) therefore a non-surgical approach is desirable. Furthermore, a recent study of renal nerve ablation in treatment resistant hypertension patients reported no significant change in blood pressure at 1 and 6 months follow-up (Hart et al., 2013). These results are based on a small sample group (n = 8), however, this indicates that further investigation of the efficacy of this technique is required.

The mechanisms of the potential antihypertensive effects of this technique require further investigation. Renal nerve ablation reduced renal noradrenaline spill-over and MSNA indicating a decrease in regional and systemic sympathetic nerve activity (Schlaich et al., 2009). This could be mediated by attenuation of efferent sympathetic nerve activity, reducing vasoconstriction of the renal artery and thereby increasing renal perfusion. This would reduce renin production which would lead to a decrease in angiotensin II levels. Angiotensin II acts at peripheral and central sites to increase sympathetic nerve activity and blood pressure, including receptors in the vasculature to promote vasoconstriction and in the brainstem to increase sympathetic outflow (Arnold et al., 2013). This is similar to medical therapies utilising angiotensin converting enzyme (ACE) to reduce the production of angiotensin II. However, these results are based on one case study (Schlaich et al., 2009). Contrary to this, a recent study reported that MSNA was not altered at 1 and 6 months follow-up of 8 patients treated with renal nerve ablation (Hart et al., 2013). This same study also investigated

the effects of renal nerve ablation in spontaneously hypertensive rats and found a consistent decrease in MSNA and blood pressure. The difference in responses of rats and humans to renal nerve ablation may be attributed to the greater destruction of renal nerves (90%) in rats compared to humans suggesting that the procedure of renal nerve ablation requires further refinement.

Alternatively/additionally, renal nerve ablation may affect afferent renal signalling and alter central autonomic control. Stimulation of renal afferent nerves in rats revealed c-fos immunoreactivity in areas of autonomic control including the insular cortex, paraventricular nucleus of the hypothalamus and the NTS (Solano-Flores et al., 1997). Activation of renal afferent nerves can reflexly increase or decrease efferent renal nerve activity (Johns and Abdullah, 2013). Stimulation of renal mechanoreceptors by dilating the renal pelvis in anaesthetised rats decreased efferent renal sympathetic nerve activity (Kopp et al., 1985) whereas activation of renal afferents throughout the kidney by infusing bradykinin increased efferent renal sympathetic nerve activity (Smits and Brody, 1984). Given the lack of understanding of afferent renal signalling and the effects of renal nerve ablation on renal afferent activity, further research is required to elucidate the mechanisms of renal denervation on blood pressure and autonomic function.

This thesis has demonstrated that H-tVNS can reduce MSNA, therefore, H-tVNS merits consideration as an antihypertensive therapy. tVNS or cervical VNS have not yet been investigated in humans or animals as potential therapies in systemic hypertension. Investigating the effects of VNS on hypertension in humans would be limited by the invasive nature and expense of the procedure, however, H-tVNS offers a non-invasive method that could easily be investigated in hypertension patients and may provide an alternative to both cervical VNS and renal nerve ablation.

6.5.2 Obesity

The worldwide prevalence of obesity doubled between 1980 to 2008 to approximately 500 million obese adults and is expected to continue rising over the next two decades (Malik et al., 2013). Obesity is characterised by

elevated MSNA and the degree of sympathetic activation is related to increased visceral abdominal fat, independent of total fat mass (Alvarez et al., 2002). Moreover, weight loss through a low calorie diet and exercise decreased sympathetic nerve activity and plasma noradrenaline levels (Grassi et al., 1998; Trombetta et al., 2003). Obesity is an important risk factor for cardiovascular disease and this may be related to the underlying sympathoexcitation. Although dieting and exercise can decrease sympathetic activity in obese individuals, patient compliance is problematic (Makris and Foster, 2011). An incidental finding of VNS in epilepsy is weight loss and this has been investigated in animal models of obesity (Introduction 1.3.1.5), however, these studies have not assessed any changes in sympathetic activity. Further investigation is required, however, VNS could potentially reduce weight and sympathetic activity in obesity, reducing the risk of developing cardiovascular disease. Conversely, reducing sympathetic activity could decrease metabolic rate and energy expenditure leading to further weight gain (Spraul et al., 1993), therefore, further animal studies are required to clarify the effects of VNS in obesity before trialling in obese humans.

6.5.3 Obstructive sleep apnoea and H-tVNS

Obstructive sleep apnoea (OSA) is estimated to affect 1-3% of the general population (Carlson et al., 1993), however, this increases to 40% of the obese population (Narkiewicz and Somers, 2003). OSA is associated with increased sympathetic activity demonstrated by increased MSNA and plasma noradrenaline levels in OSA patients (Carlson et al., 1993). Episodes of apnoea cause repeated periods of hypoxia and hypercapnia that activate the chemoreflex to increase sympathetic activity leading to increased blood pressure (Narkiewicz et al., 1999). Patients with OSA have an increased risk of cardiovascular disease and elevated levels of sympathetic nerve activity may contribute to this (Narkiewicz et al., 1999). One therapy for OSA is continuous positive airway pressure. This reduced episodes of apnoea and decreased MSNA in OSA patients (Narkiewicz et al., 1999), however, it requires patients to wear a mask while sleeping and is not well tolerated.

One study reported that 10/25 OSA patients recruited for a microneurography study refused continuous positive airway pressure treatment (Narkiewicz et al., 1999). H-tVNS may provide a more tolerable method to reduce sympathetic activity in OSA and decrease the risk of developing cardiovascular disease.

6.5.4 Polycystic ovary syndrome

Polycystic ovary syndrome (PCOS) is an endocrine condition that affects approximately 6-10% of women of reproductive age and is associated with an increased risk of cardiovascular disease (Yildirir et al., 2006; Lansdown and Rees, 2012). PCOS is also associated with obesity and OSA which are associated with sympathetic activation and this led to speculation that chronic sympathoexcitation may contribute to the pathophysiology of PCOS. An investigation of HRV in women with PCOS revealed a significantly elevated LF/HF ratio compared to age-matched controls, indicative of increased cardiac sympathetic predominance in PCOS patients (Yildirir et al., 2006). Heart rate recovery was also diminished in PCOS patients, compared to healthy controls, supporting evidence of autonomic imbalance. Indeed, impaired HRR suggests that there is parasympathetic withdrawal in PCOS (Giallauria et al., 2008). Microneurography has provided direct evidence of autonomic dysfunction in PCOS. MSNA burst frequency and incidence were significantly higher in PCOS patients compared to matched controls (Sverrisdóttir et al., 2008). These studies indicate underlying sympathoexcitation in PCOS, however, it is not known if this contributes to the pathophysiology of the condition. It is therefore difficult to predict if reducing sympathetic nerve activity would alleviate the symptoms of PCOS. Nevertheless, H-tVNS is a simple, non-invasive and safe technique to investigate this further. Importantly, H-tVNS may reduce sympathetic nerve activity in PCOS and thereby attenuate the risk of cardiovascular disease in this patient population.

6.5.5 The potential beneficial effects of H-tVNS post-stroke

VNS may also benefit stroke patients by reducing infarct size. Cervical VNS applied 30 minutes after the initiation of cerebral ischaemia and lasting 1 hour reduced infarct size by 50% in rats (Ay et al., 2011). There was no significant difference between VNS performed ipsilateral or contralateral to the area of ischaemia. The mechanisms behind VNS in reducing infarct size are unknown. It was hypothesised that VNS may increase cerebral blood flow through vasodilation of the cerebral arteries, however, measures of cerebral blood flow using Doppler ultrasound revealed no significant changes (Ay et al., 2011). Systemic inflammation and neuroinflammation occur as a result of stroke and the degree of inflammation is associated with poor prognosis post-stroke. VNS may, therefore, exert a beneficial effect through the anti-inflammatory reflex, however, this requires further investigation (General Introduction 1.3.1.3) (Cheyuo et al., 2011). Despite positive results in animal models of stroke, VNS in its current form is not a feasible acute intervention in human stroke patients due to the need for surgery. However, H-tVNS could be applied by paramedics or accident and emergency staff if a stroke is suspected. The effects of tVNS in stroke have not yet been investigated in animal models, however, it could potentially decrease the size of the infarct and thus could preserve a greater degree of neurological function. Furthermore, H-tVNS could reduce the risk of sudden death post stroke by reducing sympathetic activity (Chapter 5 Section 5.6.1)

6.5.6 Vagus nerve stimulation and neurogenesis

It has been postulated that VNS may facilitate adult neurogenesis in the subgranular zone of the hippocampus. Serotonin and noradrenaline have both been implicated in adult neurogenesis (Rajendran et al., 2009). The firing rates of locus coeruleus (LC) noradrenergic neurones and dorsal raphe serotonergic neurones recorded extracellularly *in vivo* in rats were increased by vagus nerve stimulation (Dorr and Debonnel, 2006), therefore, VNS may promote neurogenesis in the adult brain. Indeed, stimulation of the left cervical vagus nerve in rats for 24 hours increased hippocampal progenitor proliferation by 50% compared to sham stimulation (Revesz et al., 2008).

Vagus nerve stimulation also activates the central cholinergic pathway through the LC. The central cholinergic pathway projects to the hippocampus (Cheyuo et al., 2011) and acts on nicotinic acetylcholine receptors. Alpha 7 containing nicotinic ACh receptors play an important role in the survival, maturation and integration of new-born neurones in the hippocampus (Campbell et al., 2010). Rats in which alpha-7 containing nicotinic receptors were knocked down by injection of a retrovirus into the hippocampus exhibited reduced survival of new-born neurones and those that survived had truncated dendrites with reduced arborisation (Campbell et al., 2010). The hippocampus is involved in learning and memory processing therefore increasing neurogenesis and facilitating the integration of new-born neurones in this area may attenuate some of the symptoms of Alzheimer's disease and other dementias. Indeed, a pilot study of VNS in 17 Alzheimer's patients found that 70% showed no decline in cognitive function at 1 year follow-up and 40% showed an improvement in cognitive function (Merrill et al., 2006). These results are remarkable considering the progressive nature of Alzheimer's disease, however, no other trials have since been conducted. This may be due to the invasiveness and expense of VNS, therefore, H-tVNS may provide a non-invasive route to investigate the effects of VNS on cognition as a possible therapy to attenuate the progression of Alzheimer's disease and dementia.

6.5.7 Ageing and autonomic function

Currently, 10 million people in the UK are over 65 years old and this is estimated to increase to around 19 million by 2050 (Cracknell, 2010). Good health is essential if older people are to remain independent and play an active role in family and community life. However, ageing is associated with an increase in the prevalence of many chronic conditions, which can contribute to loss of independence. Common chronic conditions affecting the elderly are those concerning the cardiovascular system (North and Sinclair, 2012) and mental health (Luppa et al., 2012). One element linking these areas is altered function of the autonomic nervous system.

Age is the most important factor in determining cardiovascular health (North and Sinclair, 2012). Ageing is associated with alterations in cardiovascular autonomic control. Respiratory sinus arrhythmia is reduced, suggesting a decrease in parasympathetic influence on sinus node function (Kuo et al., 1999) and sympathetic activity is increased, evidenced by increased plasma noradrenaline levels (Esler et al., 1995) and muscle sympathetic nerve activity (Ebert et al., 1992; Ng et al., 1993). These parasympathetic and sympathetic changes contribute to a continuous decline in baroreflex sensitivity (BRS) and heart rate variability (HRV) in ageing males and females (Barantke et al., 2008), which is related to predisposition to cardiovascular disease (Umetani et al., 1998; Felber Dietrich et al., 2006; Abhishekh et al., 2013). Indeed reduced HRV is an indicator of poor prognosis in both healthy and patient populations (Kleiger et al., 1987; Nunan et al., 2010). Improving cardiovascular autonomic function in the elderly may, therefore, prevent or slow the development of cardiovascular disease.

Depression is one of the most common mental health disorders in the elderly and is associated with functional decline, decreased quality of life, increased health care costs and increased mortality (Luppa et al., 2012). Indeed, depression increases the risk of developing cardiovascular disease by 1.5 in the general population (Luppa et al., 2012). Furthermore, patients with both cardiovascular disease and depression are 2-3 times more likely to suffer a cardiac event. Depression is also an independent indicator of poor prognosis after myocardial infarction. Similarly to cardiovascular disease, patients with depression exhibit reduced parasympathetic and enhanced sympathetic activity, evidenced by reduced heart rate variability (Taylor, 2010), increased noradrenaline spill-over (Barton et al., 2007) and increased muscle sympathetic nerve activity (Scalco et al., 2009). Cervical VNS is currently approved for treatment resistant depression in the USA (General Introduction 1.3.1.2) and is therefore restricted to a small subset of patients. tVNS may provide a non-invasive adjunctive therapy for depression that could also attenuate associated cardiovascular risks and improve quality of life in the elderly. The invasive nature and expense of cervical VNS does not

justify its use as a prophylactic treatment, however, H-tVNS may provide an alternative solution.

6.6 Clinical Implications of tDCS

Sympathetic activation is associated with a wide range of conditions as discussed above. Furthermore, the degree of sympathoexcitation has been correlated with poor prognosis, including post stroke (Cohn et al., 1984; Kleiger et al., 1987; Kaye et al., 1995; Robinson et al., 2003; Barretto et al., 2009). tDCS is being investigated as a potential therapy to aid stroke rehabilitation by promoting cortical plasticity, however, the possible effects on autonomic function may be underestimated. The results of this study indicate that tDCS, using a similar electrode montage and parameters to those used in stroke motor rehabilitation studies, increased MSNA. The effects of a single tDCS treatment are transient, however, chronic stimulation caused long-lasting effects on cortical excitation such that 5 daily treatments produced effects that lasted 2 weeks (Boggio et al., 2007). The effects of chronic tDCS on autonomic function are unknown, however, based on these results, caution is warranted before applying chronic tDCS over the motor cortex in stroke patient populations. It would be advisable to monitor autonomic function in patients undergoing such a trial e.g. regular analyses of HRV, to ensure there was no worsening of autonomic function that could potentially impact on patient prognosis.

Additionally, tDCS is being investigated as a potential therapy for depression which is also associated with sympathoexcitation. The electrode montage utilised in studies of tDCS in depression differs from that used in the present study with the anode and cathode placed over the left and right dorsolateral prefrontal cortex. The effects of this set-up on autonomic function are unknown, however, this merits investigation. Recently, combining fMRI and microneurography revealed a positive correlation between spontaneous MSNA burst activity and activation of the left and right dorsolateral prefrontal cortex (Macefield et al., 2013). This suggests that the dorsolateral prefrontal cortex may be involved in sympathoexcitation. This

finding warrants caution in using tDCS to treat depression until the potential effects on autonomic function are better understood.

6.7 Limitations of the study

A major limitation of this study is the small number of heart failure patients recruited. The high incidence of atrial fibrillation and other arrhythmias in patients attending the heart failure clinic hampered recruitment. Furthermore, many patients were recruited who had no diagnosis of arrhythmia but presented with regular ectopic beats that prevented acquisition of 5 minute long ECG recordings with < 2 ectopics beats as required for HRV analyses. In addition, recruitment and testing of patients during clinic restricted the time available for each experiment - hence recovery recordings to explore any residual effect of H-tVNS in heart failure were not made. In the future, patients could be recruited and undergo a screening ECG at the clinic prior to returning on a convenient day to take part in the study. This would allow adequate time for the study and the incorporation of microneurography into the protocol which would provide vital information on the effects of H-tVNS on MSNA in heart failure patients.

The initial investigation of H-tVNS in healthy participants and heart failure patients relied on non-invasive measures that provide an estimate of autonomic balance rather than direct measurements. Two non-invasive measures of autonomic function, HRV and BRS analyses, were utilised in order to verify any changes during H-tVNS and tDCS. Whilst there were significant changes in HRV during H-tVNS and tDCS there was no significant change in BRS during either condition. This could be due to recruiting healthy volunteers with optimal reflex blood pressure control that was not affected by either intervention. Unfortunately, it was not possible to analyse BRS in the majority of the heart failure patients due to poor coherence between spontaneous changes in heart rate and blood pressure. Many of the heart failure patients were also excluded due to the frequent occurrence of ectopic heart beats. Heart rate turbulence could be used in future studies to both overcome the difficulty of obtaining ECG records free

of ectopic beats and also as an alternative to spontaneous BRS analysis in heart failure patients. The lack of effect of L-tVNS, cathodal tDCS and sham stimulation for both interventions implies that changes in HRV during H-tVNS and anodal tDCS were valid. Using microneurography to record MSNA in healthy participants partially overcame the restriction of non-invasive autonomic measures, however, this was not performed in heart failure patients and did not provide information on any regional changes in autonomic function. Regional changes in sympathetic activity could be explored in the future using the noradrenaline spill-over technique to examine specific changes in cardiac sympathetic nerve activity. Furthermore, the reproducibility of HRV and MSNA responses during both H-tVNS and tDCS has not been evaluated.

Another limitation of this study is that tDCS was only performed in healthy participants and has not yet been investigated stroke patients. Given the results of this study and the clinical implication of potentially exacerbating sympathoexcitation in this group, determining the effects of tDCS on autonomic function in stroke patients is crucial. It may be that tDCS has no effect on autonomic function in stroke patients due to pre-existing sympathoexcitation and only increases MSNA in populations in which baseline levels are relatively low. Despite this, it is not ethical to apply tDCS to stroke patients at this stage without confirmation or refutation of the results presented in this study. A larger study of tDCS in healthy participants is required before contemplating a study of autonomic function in stroke patients.

The findings of the neuronal tracing study of the ABVN were limited by restrictions on collecting the superior vagal ganglion for analysis as the cell bodies of the neurones that comprise the ABVN reside in the superior ganglion. It would have been useful to section this to determine if DiI had reached this point. Unfortunately, this procedure was not included in the original ethics application and tissue transfer agreement and there was not sufficient time to amend these and gain approval from the ethics committee and human tissue license holder. This could have provided information that would have informed the design of future experiments and will be included in subsequent studies.

6.8 Future studies

6.8.1 H-tVNS in heart failure patients

The preliminary data presented in Chapter 3 indicate that H-tVNS can improve cardiac autonomic function in heart failure patients, however, a larger sample group of heart failure patients is required to confirm this. It is also necessary to perform microneurography on heart failure patients during H-tVNS to establish if H-tVNS reduces MSNA in this population.

The residual effect of H-tVNS on HRV in healthy participants represents an interesting phenomenon that may preclude the need for continuous stimulation. The residual effect was not explored in heart failure patients due to time constraints in the clinic therefore this requires investigation in the future. Heart failure patients would undergo a similar protocol as described in Chapter 3 (with the addition of microneurography) and would then undergo further recordings to establish the duration of the effect of H-tVNS on HRV and MSNA. Recordings would be made every 15 minutes for two hours post-stimulation. If HRV and MSNA had still not returned to baseline levels recordings would continue every 15 minutes until they reach baseline levels.

Chronic H-tVNS may have a cumulative effect on cardiovascular autonomic control. To investigate this, heart failure patients would be recruited to undergo H-tVNS twice daily for 1 month. To facilitate this, patients would be provided with a device and instructions to take home and perform H-tVNS. To monitor patient compliance, TENS machines that record a log of when the device was used would be utilised. A similar protocol to that used in Chapter 3 would be used on day 1 of the study. This would establish the baseline values of HRV and the acute effects of H-tVNS in each patient. In addition, microneurography would also be conducted. Patients would then undergo physiological recordings every fortnight for 2 months to monitor the effects of chronic H-tVNS and any residual effects. This would provide data on cardiovascular autonomic function halfway through the chronic stimulation phase and on the last day of stimulation plus 2 weeks and 4 weeks after H-tVNS had ceased. Clinical tests, such as the 6 minute walking test and quality of life scores, would also be incorporated to

establish the effectiveness of H-tVNS in heart failure. If a residual effect was still present after one month, it may be necessary to extend the duration of the study to continue monitoring.

The experiments described above would establish the efficacy of H-tVNS in heart failure patients and would inform the design of future studies. In particular, determining the duration of the residual effect of H-tVNS and the impact of chronic stimulation may alter the length and number of H-tVNS periods per day. Positive results - a significant reduction in MSNA and LF/HF ratio - may lead to a clinical trial. A randomised controlled clinical trial would allow comparison of the effects of H-tVNS therapy in heart failure to a control group of heart failure patients receiving standard clinical care. To achieve this, the patients would be asked to utilise H-tVNS twice daily for 6 months with follow-up sessions at 1 month, 3 months and 6 months. At baseline and follow-up sessions, physiological measures (e.g. microneurography, HRV) and clinical tests (6 minute walking test, quality of life scores) would be used to establish the effectiveness of H-tVNS in heart failure. A multi-centre trial of cervical VNS in heart failure patients reported a significant increase in left ventricular ejection fraction at 3 months that was maintained at 1 year follow-up (De Ferrari et al., 2011) therefore echocardiography should be included in future clinical studies of tVNS to investigate any effects on cardiac function.

6.8.2 Elucidating the mechanisms of H-tVNS

The central mechanisms of the cardiovascular autonomic effects of H-tVNS require further investigation. The working heart brainstem preparation (WHBP) provides an ideal method to explore this further in rats or mice. The WHBP is an intra-arterially perfused decerebrate *in situ* preparation. This eliminates the need for anaesthetic while preserving brainstem autonomic neuronal circuits and allowing intracellular neuronal recordings (Paton, 1996). The preservation of autonomic circuits in this preparation can be demonstrated by increasing perfusion pressure. This stimulates baroreceptors and leads to a decrease in heart rate (Lall et al., 2012). Similarly, injecting sodium cyanide into the perfusate results in a decrease in heart rate and an increase in phrenic nerve discharge characteristic of

chemoreceptor activation (Lall et al., 2012). By adapting the electrodes used for tVNS, it would be possible to stimulate the ABVN in rats and record efferent activity of the vagus nerve directly. This would clarify the effects, if any, of tVNS on parasympathetic nerve activity. Moreover, to determine the effects of H-tVNS on neuronal activity in the NTS, intracellular recordings of neuronal activity would be made. By applying a neuronal tracer to the ABVN approximately 5 days prior to the experiment and including a fluorescent label (e.g.rhodamine) in the electrode utilised for intracellular recordings, it would then be possible to subsequently identify the NTS neurone that was recorded from and observe any close appositions from the ABVN.

The consequences of H-tVNS on cardiac remodelling could also be investigated *in vivo* in an animal model of heart failure. There are many animal models of heart failure and one of the most commonly used involves ligation of the anterior interventricular coronary artery to cause myocardial infarction. However, this causes rapid onset heart failure which differs from the gradual development of heart failure in humans (Patten and Hall-Porter, 2009). Aortic banding is an alternative technique in which a stricture is placed around the ascending aorta in young rats (3-4 weeks). As the rats grow, flow through the aorta is increasingly impeded leading to pressure overload in the left ventricle. At 8 weeks after banding there is left ventricular hypertrophy and symptoms of heart failure, such as dyspnoea, manifest at 18 weeks post-banding (Patten and Hall-Porter, 2009). Aortic banding is a less invasive technique than coronary artery ligation and the gradual development of heart failure is more consistent with human disease. This model could be used to compare the effects of H-tVNS therapy to untreated rats with aortic banding. The development of heart failure can be monitored *in vivo* using echocardiology to assess left ventricular hypertrophy. After 18 weeks the animals would be sacrificed and histological changes in cardiac tissue such as cardiomyocyte diameter and the degree of fibrosis could be assessed. This would elucidate the effects of H-tVNS on the pathophysiological remodelling of the heart.

6.8.3 Distribution of the ABVN

This study attempted to determine the central projections of the ABVN in humans in order to clarify the potential mechanisms of H-tVNS effects on cardiovascular autonomic function. The results of this study were inconclusive and future studies to improve the protocol are outlined in Section 4.6. In addition to the central projections of the ABVN, the peripheral distribution to the external ear also merits investigation. This has only been investigated in one study in which the ABVN was dissected in 7 cadavers (Peuker and Filler, 2002). Dissection of the terminal branches of the ABVN would be technically challenging due to their small size. DiI travels anterogradely as well as retrogradely, therefore application to the distal end of the ABVN would label the distribution of the ABVN to the ear which could then be sectioned and viewed under a microscope as described in Chapter 4. It would be beneficial to determine if there is any anatomical variation in the distribution of the ABVN between individuals as this may account for any non-responders to H-tVNS and may also inform future tailoring of H-tVNS electrode placement to maximise success rates.

6.8.4 Autonomic effects of tDCS in stroke patients

This thesis reports that anodal tDCS over the motor cortex, as proposed for stroke motor rehabilitation therapy, increased MSNA in healthy participants. This result requires confirmation, however, it may warrant consideration before tDCS is utilised in stroke patients. The effects of tDCS on autonomic function in stroke patients may differ from healthy participants, however, investigating this poses ethical dilemmas based on results presented in this thesis. It may be that a future collaboration with researchers investigating tDCS in stroke patients would minimise the number of patients exposed to tDCS and as the effects of tDCS are temporary, it may have no long-lasting effect. Despite this, an investigation of tDCS in an animal model of stroke would be a valuable initial investigation. The most common type of stoke in humans is caused by occlusion of the middle cerebral artery, therefore, performing a similar occlusion of the middle cerebral artery in rodents is frequently used to model stroke (Howells et al., 2010). Middle cerebral artery

occlusion in rats and mice can be performed by introducing a filament to the internal carotid artery and advancing this superiorly into the middle cerebral artery (Braeuninger et al., 2012). There are a number of established behavioural correlates that could be used to assess the effectiveness of tDCS on motor rehabilitation e.g. the ladder rung test (Balkaya et al., 2013). Telemetry could also be used to continuously record heart rate and blood pressure to allow HRV and BRS analyses. The consistency of infarct size could be assessed at the end of the experiment by sectioning the brain and using triphenyltetra-zolium chloride (TTC) to stain non-infarcted tissue red, leaving infarcted tissue pale (Braeuninger et al., 2012). By investigating the cardiovascular autonomic effects of tDCS over different areas of the cortex it may be possible to tailor this technique to fine tune central autonomic control in patients.

6.9 Conclusion

This thesis has demonstrated that it is possible to modulate autonomic function using tVNS and tDCS. Non-invasive neuromodulation of the autonomic nervous system could provide an adjunctive therapy to redress autonomic imbalance in many conditions and improve prognosis. These techniques do not require surgery and are therefore applicable to a wider cohort of patients than surgical techniques such as cervical VNS. Furthermore, tVNS and tDCS are simple and inexpensive techniques. Based on the results presented in this thesis, these techniques merit further investigation to determine the exact mechanisms involved and to explore their application to other conditions that exhibit autonomic imbalance such as hypertension and polycystic ovary syndrome. This may aid refinement of these techniques and enable translation to clinical practice.

List of References

Abbott SBG, Stornetta RL, Socolovsky CS, West GH, Guyenet PG (2009) Photostimulation of channelrhodopsin-2 expressing ventrolateral medullary neurons increases sympathetic nerve activity and blood pressure in rats. The Journal of Physiology 587:5613-5631.

Abhishekh H, Nisarga P, Kisan R, Meghana A, Chandran S, Trichur R, Sathyaprabha T (2013) Influence of age and gender on autonomic regulation of heart. J Clin Monit Comput:1-6.

Abraham PJ, Capobianco DJ, Cheshire WP (2003) Facial Pain as the Presenting Symptom of Lung Carcinoma With Normal Chest Radiograph. Headache: The Journal of Head and Face Pain 43:499-504.

Accornero N, Li Voti P, La Riccia M, Gregori B (2007) Visual evoked potentials modulation during direct current cortical polarization. Exp Brain Res 178:261-266.

Adrian ED, Bronk DW, Phillips G (1932) Discharges in mammalian sympathetic nerves. The Journal of Physiology 74:115-133.

Agarwal SK, Gelsema AJ, Calaresu FR (1990) Inhibition of rostral VLM by baroreceptor activation is relayed through caudal VLM. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 258:R1271-R1278.

Akselrod S, Gordon D, Ubel FA, Shannon DC, Berger AC, Cohen RJ (1981) Power spectrum analysis of heart rate fluctuation: a quantitative probe of beat-to-beat cardiovascular control. Science 213:220-222.

Alam M, Smirk FH (1937) Observations in man upon a blood pressure raising reflex arising from the voluntary muscles. Journal of Physiology 89:372-383.

Allen GV, Cechetto DF (1994) Serotoninergic and nonserotoninergic neurons in the medullary raphe system have axon collateral projections to autonomic and somatic cell groups in the medulla and spinal cord. The Journal of Comparative Neurology 350:357-366.

Alvarez GE, Beske SD, Ballard TP, Davy KP (2002) Sympathetic Neural Activation in Visceral Obesity. Circulation 106:2533-2536.

Amirhaeri S, Spencer, D. (2010) Myocardial infarction with unusual presentation of otalgia: a case report. International Journal of Emergency Medicine 3:459-460.

Arnold AC, Gallagher PE, Diz DI (2013) Brain renin-angiotensin system in the nexus of hypertension and aging. Hypertens Res 36:5-13.

Aubert AE, Ramaekers D, Beckers F, Breem R, Denef C, Van de Werf F, Ector H (1999) The analysis of heart rate variability in unrestrained rats. Validation of method and results. Computer Methods and Programs in Biomedicine 60:197-213.

Ay I, Sorensen AG, Ay H (2011) Vagus nerve stimulation reduces infarct size in rat focal cerebral ischemia: An unlikely role for cerebral blood flow. Brain Research 1392:110-115.

Bacon SJ, Smith AD (1993) A monosynaptic pathway from an identified vasomotor centre in the medial prefrontal cortex to an autonomic area in the thoracic spinal cord. Neuroscience 54:719-728.

Badawy RAB, Harvey AS, Macdonell RAL (2009) Cortical hyperexcitability and epileptogenesis: Understanding the mechanisms of epilepsy – Part 1. Journal of Clinical Neuroscience 16:355-365.

Baigi A, Oden, Anders., Almlid-Larsen, V., Barrens, M., Holgers, K., (2011) Tinnitus in the general population with a focus on noise and stress: a public health study. Ear and Hearing 32:787-789.

Bajwa A, Hollerbach S, Kamath MV, Upton AR, Fitzpatrick D, Fallen EL, Tougas G (1997) Neurocardiac response to esophageal electric stimulation in humans: effects of varying stimulation frequencies. Am J Physiol Regul Integr Comp Physiol 272:R896-901.

Balkaya M, Krober JM, Rex A, Endres M (2013) Assessing post-stroke behavior in mouse models of focal ischemia. J Cereb Blood Flow Metab 33:330-338.

Barantke M, Krauss T, Ortak J, Lieb W, Reppel M, Burgdorf C, Pramstaller PP, Schunkert H, Bonnemeier H (2008) Effects of Gender and Aging on Differential Autonomic Responses to Orthostatic Maneuvers. Journal of Cardiovascular Electrophysiology 19:1296-1303.

Barretto ACP, Santos AC, Munhoz R, Rondon MUPB, Franco FG, Trombetta IC, Roveda F, de Matos LNJ, Braga AMW, Middlekauff HR, Negrão CE (2009) Increased muscle sympathetic nerve activity predicts mortality in heart failure patients. International Journal of Cardiology 135:302.

Barton DA, Dawood T, Lambert EA, Esler MD, Haikerwal D, Brenchley C, Socratous F, Kaye DM, Schlaich MP, Hickie I, G.W L (2007) Sympathetic activity in major depressive disorder: identifying those at increased cardiac risk? Journal of Hypertension 25:2117-2124.

Bauer A, Zurn CS, Schmidt G (2010) Heart rate turbulance to guide treatment for prevention of sudden death. Journal of cardiovascular pharmacology 55:531-538.

Bekelman DB, Havranek EP, Becker DM, Kutner JS, Peterson PN, Wittstein IS, Gottlieb SH, Yamashita TE, Fairclough DL, Dy SM (2007)

Symptoms, Depression, and Quality of Life in Patients With Heart Failure. Journal of Cardiac Failure 13:643-648.

Beleza P (2009) Refractory Epilepsy: A Clinically Oriented Review. European Neurology 62:65-71.

Berlim MT, Van den Eynde F, Daskalakis ZJ (2013) Clinical utility of transcranial direct current stimulation (tDCS) for treating major depression: A systematic review and meta-analysis of randomized, double-blind and sham-controlled trials. Journal of Psychiatric Research 47:1-7.

Berthoud H-R (2008) The vagus nerve, food intake and obesity. Regulatory Peptides 149:15-25.

Berthoud H-R, Neuhuber WL (2000) Functional and chemical anatomy of the afferent vagal system. Autonomic Neuroscience 85:1-17.

Bieger D, Hopkins DA (1987) Viscerotopic representation of the upper alimentary tract in the medulla oblongata in the rat: The nucleus ambiguus. The Journal of Comparative Neurology 262:546-562.

Billinton N, Knight AW (2001) Seeing the Wood through the Trees: A Review of Techniques for Distinguishing Green Fluorescent Protein from Endogenous Autofluorescence. Analytical Biochemistry 291:175-197.

Bindman LJ, Lippold OCJ, Redfearn JWT (1964) The action of brief polarizing currents on the cerebral cortex of the rat (1) during current flow and (2) in the production of long-lasting after-effects. The Journal of Physiology 172:369-382.

Bindoff L, Heseltine D (1988) Unilateral facial pain in patients with lung cancer: a referred pain via the vagus? The Lancet 331:812-815.

Bishop VS, Peterson DF (1978) The circulatory influences of vagal afferents at rest and during coronary occlusion in conscious dogs. Circulation Research 43:840-847.

Bisognano JD, Kaufman CL, Bach DS, Lovett EG, de Leeuw P (2011) Improved Cardiac Structure and Function With Chronic Treatment Using an Implantable Device in Resistant Hypertension: Results From European and United States Trials of the Rheos System. Journal of the American College of Cardiology 57:1787-1788.

Blau JN (1989) Ear pain referred by the vagus. BMJ 299:1569-1570.

Blessing WW (1988) Depressor neurons in rabbit caudal medulla act via GABA receptors in rostral medulla. American Journal of Physiology - Heart and Circulatory Physiology 254:H686-H692.

Boggio PS, Nunes A, Rigonatti SP, Nitsche MA, Pascual-Leone A, Fregni F (2007) Repeated sessions of noninvasive brain DC stimulation is

associated with motor function improvement in stroke patients. Restorative Neurology and Neuroscience 25:123-129.

Boghossian S, Alkarmi DA, Boueini K (2010) Asystole after ear suctioning. Journal of primary health care 2:70-71.

Bolognini N, Fregni F, Casati C, Olgiati E, Vallar G (2010) Brain polarization of parietal cortex augments training-induced improvement of visual exploratory and attentional skills. Brain Research 1349:76-89.

Borckardt JJ, Romagnuolo J, Reeves ST, Madan A, Frohman H, Beam W, George MS (2011) Feasibility, safety, and effectiveness of transcranial direct current stimulation for decreasing post-ERCP pain: a randomized, sham-controlled, pilot study. Gastrointestinal Endoscopy 73:1158-1164.

Borovikova LV, Ivanova S, Zhang M, Yang H, Botchkina GI, Watkins LR, Wang H, Abumrad N, Eaton JW, Tracey KJ (2000) Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature 405:458-462.

Braeuninger S, Kleinschnitz C, Nieswandt B, Stoll G (2012) Focal Cerebral Ischemia. In: Platelets and Megakaryocytes (Gibbins JM, Mahaut-Smith MP, eds), pp 29-42: Springer New York.

Bratton BO, Martelli D, McKinley MJ, Trevaks D, Anderson CR, McAllen RM (2012) Neural regulation of inflammation: no neural connection from the vagus to splenic sympathetic neurons. Experimental Physiology 97:1180-1185.

Brunoni AR, Valiengo L, Baccaro A, Zanao TA, de Oliveira JF, Goulart A, Boggio PS, Lotufo PA, Bensenor IM, Fregni F (2013a) The sertraline vs electrical current therapy for treating depression clinical study: Results from a factorial, randomized, controlled trial. JAMA Psychiatry:1-9.

Brunoni AR, L. V, A. B, Zanão TA, De Oliveira JF, Goulart A, Boggio PS, Lotufo PA, Bensenor IM, Fregni F (2013b) The sertraline vs electrical current therapy for treating depression clinical study: Results from a factorial, randomized, controlled trial. JAMA Psychiatry 70:383-391.

Bui AL, Horwich TB, Fonarow GC (2011) Epidemiology and risk profile of heart failure. Nat Rev Cardiol 8:30-41.

Burneo JG, Faught E, Knowlton R, Morawetz R, Kuzniecky R (2002) Weight loss associated with vagus nerve stimulation. Neurology 59:463-464.

Busch V, Zeman F, Heckel A, Menne F, Ellrich J, Eichhammer P (2013) The effect of transcutaneous vagus nerve stimulation on pain perception – An experimental study. Brain Stimulation 6:202-209.

Campbell NR, Fernandes CC, Halff AW, Berg DK (2010) Endogenous Signaling through α7-Containing Nicotinic Receptors Promotes

Maturation and Integration of Adult-Born Neurons in the Hippocampus. The Journal of Neuroscience 30:8734-8744.

Carlson JT, Hedner J, Elam M, Ejnell H, Sellgren J, Wallin BG (1993) AUgmented resting sympathetic activity in awake patients with obstructive sleep apnea. CHEST Journal 103:1763-1768.

Carter HH, Dawson EA, Cable NT, Basnayake S, Aziz TZ, Green AL, Paterson DJ, Lind CRP, Thijssen DHJ, Green DJ (2011) Deep Brain Stimulation of the Periaqueductal Grey Induces Vasodilation in Humans. Hypertension 57:e24-e25.

Chapleau M, Sabharwal R (2011) Methods of assessing vagus nerve activity and reflexes. Heart Failure Reviews 16:109-127.

Charkoudian N, Rabbitts JA (2009) Sympathetic Neural Mechanisms in Human Cardiovascular Health and Disease. Mayo Clinic Proceedings 84:822-830.

Chen BK, Miller SM, Mantilla CB, Gross L, Yaszemski MJ, Windebank AJ (2006) Optimizing conditions and avoiding pitfalls for prolonged axonal tracing with carbocyanine dyes in fixed rat spinal cords. Journal of Neuroscience Methods 154:256-263.

Cheyuo C, Jacob A, Wu R, Zhou M, Coppa GF, Wang P (2011) The parasympathetic nervous system in the quest for stroke therapeutics. J Cereb Blood Flow Metab 31:1187-1195.

Chidsey CA, Braunwald E, Morrow AG (1965) Catecholamine excretion and cardiac stores of norepinephrine in congestive heart failure. The American Journal of Medicine 39:442-451.

Chien C, Shieh J, Ling E, Tan C, Wen C (1996) The composition and central projections of the internal auriculae nerves of the dog. Journal of Anatomy 189:349-362.

Ciriello J, Hrycyshyn AW, Calaresu FR (1981) Horseradish peroxidase study of brain stem projections of carotid sinus and aortic depressor nerves in the cat. Journal of the Autonomic Nervous System 4:43-61.

Clancy B, Cauller LJ (1998) Reduction of background autofluorescence in brain sections following immersion in sodium borohydride. Journal of Neuroscience Methods 83:97-102.

Clancy JA, Deuchars SA, Deuchars J (2013) 2012 GL Brown Lecture 'The Wonders of the Wanderer'. Experimental Physiology 98:38-45.

Coffey RJ (2001) Deep Brain Stimulation for Chronic Pain: Results of Two Multicenter Trials and a Structured Review. Pain Medicine 2:183-192.

Cohen Kadosh R, Soskic S, Iuculano T, Kanai R, Walsh V (2010) Modulating Neuronal Activity Produces Specific and Long-Lasting Changes in Numerical Competence. Current Biology 20:2016-2020.

Cohn JN, Levine TB, Olivari MT, Garberg V, Lura D, Francis GS, Simon AB, Rector T (1984) Plasma norepinephrine as a guide to prognosis in patients with chronic congestive heart failure. New England Journal of Medicine 311:819-823.

Cole CR, Blackstone EH, Pashkow FJ, Snader CE, Lauer MS (1999) Heart-Rate Recovery Immediately after Exercise as a Predictor of Mortality. N Engl J Med 341:1351-1357.

Communal C, Singh K, Pimentel DR, Colucci WS (1998) Norepinephrine Stimulates Apoptosis in Adult Rat Ventricular Myocytes by Activation of the β-Adrenergic Pathway. Circulation 98:1329-1334.

Connor DE, Nixon M, Nanda A, Guthikonda B (2012) Vagal nerve stimulation for the treatment of medically refractory epilepsy: a review of the current literature. Neurosurgical Focus 32:E12.

Cook S, Togni M, Schaub MC, Wenaweser P, Hess OM (2006) High heart rate: a cardiovascular risk factor? Eur Heart J 27:2387-2393.

Corning JL (1884) Electrization of the sympathetic and pneumogastric nerves, with simultaneous bilateral compression of the carotids. New York Medical Journal 39:212-215.

Cracknell R (2010) The ageing population. In: Key issues of the new parliament 2010, pp 44-45. UK: House of Commons Library Research.

Creutzfeldt OD, Fromm GH, Kapp H (1962) Influence of transcortical d-c currents on cortical neuronal activity. Experimental Neurology 5:436-452.

d'Elia G OJSL (1983) PResent practice of electroconvulsive therapy in scandinavia. Archives of General Psychiatry 40:577-581.

Dale HH, Feldberg W (1934) The chemical transmission of secretory impulses to the sweat glands of the cat. The Journal of Physiology 82:121-128.

Dampney RA (1994) Functional organization of central pathways regulating the cardiovascular system. Physiological Reviews 74:323-364.

Dampney RA, Moon EA (1980) Role of ventrolateral medulla in vasomotor response to cerebral ischemia. American Journal of Physiology - Heart and Circulatory Physiology 239:H349-H358.

De Ferrari GM, Crijns HJGM, Borggrefe M, Milasinovic G, Smid J, Zabel M, Gavazzi A, Sanzo A, Dennert R, Kuschyk J, Raspopovic S, Klein H, Swedberg K, Schwartz PJ (2011) Chronic vagus nerve stimulation: a new and promising therapeutic approach for chronic heart failure. European Heart Journal 32:847-855.

De Herdt V, Bogaert S, Bracke KR, Raedt R, De Vos M, Vonck K, Boon P (2009) Effects of vagus nerve stimulation on pro- and anti-inflammatory cytokine induction in patients with refractory epilepsy. Journal of Neuroimmunology 214:104-108.

de Luna AB, Coumel P, Leclercq JF (1989) Ambulatory sudden cardiac death: Mechanisms of production of fatal arrhythmia on the basis of data from 157 cases. American Heart Journal 117:151-159.

de Vries MH, Barth ACR, Maiworm S, Knecht S, Zwitserlood P, Flöel A (2009) Electrical Stimulation of Broca's Area Enhances Implicit Learning of an Artificial Grammar. Journal of Cognitive Neuroscience 22:2427-2436.

Deuchars SA, Spyer KM, Gilbey MP (1997) Stimulation Within the Rostral Ventrolateral Medulla Can Evoke Monosynaptic GABAergic IPSPs in Sympathetic Preganglionic Neurons In Vitro. Journal of Neurophysiology 77:229-235.

Dorr AE, Debonnel G (2006) Effect of Vagus Nerve Stimulation on Serotonergic and Noradrenergic Transmission. Journal of Pharmacology and Experimental Therapeutics 318:890-898.

DuBois FS, Foley JO (1936) Experimental studies on the vagus and spinal accessory nerves in the cat. The Anatomical Record 64:285-307.

Dunlap ME, Bibevski S, Rosenberry TL, Ernsberger P (2003) Mechanisms of altered vagal control in heart failure: influence of muscarinic receptors and acetylcholinesterase activity. Am J Physiol Heart Circ Physiol 285:H1632-1640.

Ebert TJ, Morgan BJ, Barney JA, Denahan T, Smith JJ (1992) Effects of aging on baroreflex regulation of sympathetic activity in humans. American Journal of Physiology - Heart and Circulatory Physiology 263:H798-H803.

Eckberg DL, Drabinsky M, Braunwald E (1971) Defective Cardiac Parasympathetic Control in Patients with Heart Disease. New England Journal of Medicine 285:877-883.

Eckberg DL, Nerhed C, Wallin BG (1985) Respiratory modulation of muscle sympathetic and vagal cardiac outflow in man. The Journal of Physiology 365:181-196.

Eisma R, Lamb C, Soames RW (2013) From formalin to thiel embalming: what changes? One anatomy department's experiences. Clinical Anatomy 26:564-571.

Elliott RE, Morsi A, Kalhorn SP, Marcus J, Sellin J, Kang M, Silverberg A, Rivera E, Geller E, Carlson C, Devinsky O, Doyle WK (2011) Vagus nerve stimulation in 436 consecutive patients with treatment-resistant epilepsy: Long-term outcomes and predictors of response. Epilepsy & Behavior 20:57-63.

Endler NS (1988) The origins of electroconvulsive therapy. Convulsive Therapy 4:5-23.

Engineer ND, Riley JR, Seale JD, Vrana WA, Shetake JA, Sudanagunta SP, Borland MS, Kilgard MP (2011) Reversing pathological neural activity using targeted plasticity. Nature 470:101-104.

Engleman K, Portnoy B, Lovenberg W (1968) A sensitive ans specific double-isotope derivative method for the determination of catecholamines in biological specimens. American journal of medical sciences 255:259-268.

Erlanger J, Gasser HS (1930) THE ACTION POTENTIAL IN FIBERS OF SLOW CONDUCTION IN SPINAL ROOTS AND SOMATIC NERVES. American Journal of Physiology -- Legacy Content 92:43-82.

Esler M (2010) The 2009 Carl Ludwig Lecture: pathophysiology of the human sympathetic nervous system in cardiovascular diseases: the transition from mechanisms to medical management. Journal of Applied Physiology 108:227-237.

Esler M, Kaye D, Thompson J, Jennings G, Cox H, Turner A, Lambert G, Seals D (1995) Effects of aging on epinephrine secretion and regional release of epinephrine from the human heart. Journal of Clinical Endocrinology & Metabolism 80:435-442.

Esler MD, Krum H, Sobotka PA, Symplicity HTN-2 Investigators (2010) Renal sympathetic denervation in patients with treatment-resistant hypertension (The Symplicity HTN-2 Trial): a randomised controlled trial. The Lancet 376:1903-1909.

Esler MD, Hasking GJ, Willet IR, Leonard PW, Jennings GL (1985) Noradrenaline release and sympathetic nervous system activity. Journal of Hypertension 3:117-129.

Fagius J, Karhuvaara S, Sundlof G (1989) The cold pressor test: effects on sympathetic nerve activity in human muscle and skin nerve fascicles. Acta Physiologica Scandinavica 137:325-334.

Fagius J, Wallin BG, Sundloff G, Nerhed C, Englesson S (1985) Sympathetic outflow in man after anaesthesia of the glossopharyngeal nerves and vagus nerves. Brain 108:423-438.

Fagraeus L, Linnarsson D (1976) Autonomic origin of heart rate fluctuations at the onset of muscular exercise. Journal of Applied Physiology 40:679-682.

Faller H, Störk S, Schowalter M, Steinbüchel T, Wollner V, Ertl G, Angermann CE (2007) Depression and survival in chronic heart failure: Does gender play a role? European Journal of Heart Failure 9:1018-1023.

Fallgatter AJ, Neuhauser B, Herrmann MJ, Ehlis AC, Wagener A, Scheuerpflug P, Reiners K, Riederer P (2003) Far field potentials from the brain stem after transcutaneous vagus nerve stimulation. Journal Of Neural Transmission 110:1437-1443.

Farkas E, Jansen ASP, Loewy AD (1997) Periaqueductal gray matter projection to vagal preganglionic neurons and the nucleus tractus solitarius. Brain Research 764:257-261.

Farkas E, Jansen ASP, Loewy AD (1998) Periaqueductal gray matter input to cardiac-related sympathetic premotor neurons. Brain Research 792:179-192.

Felber Dietrich D, Schindler C, Schwartz J, Barthélémy J-C, Tschopp J-M, Roche F, von Eckardstein A, Brändli O, Leuenberger P, Gold DR, Gaspoz J-M, Ackermann-Liebrich U (2006) Heart rate variability in an ageing population and its association with lifestyle and cardiovascular risk factors: results of the SAPALDIA study. Europace 8:521-529.

Fenton BW, Palmieri PA, Boggio P, Fanning J, Fregni F (2009) A preliminary study of transcranial direct current stimulation for the treatment of refractory chronic pelvic pain. BRAIN STIMULATION: Basic, Translational, and Clinical Research in Neuromodulation 2:103-107.

Fisher JP, Seifert T, Hartwich D, Young CN, Secher NH, Fadel PJ (2010) Autonomic control of heart rate by metabolically sensitive skeletal muscle afferents in humans. The Journal of Physiology 588:1117-1127.

Fisher JP, Adlan AM, Shantsila A, Secher A, Sørensen H, Secher NH (2013) Muscle metaboreflex and autonomic regulation of heart rate in humans. Journal of Physiology Epub ahead of print.

Flanigan RM, DiGiovanni BF (2011) Peripheral Nerve Entrapments of the Lower Leg, Ankle, and Foot. Foot and Ankle Clinics 16:255-274.

Folan-Curran J, Cooke FJ (2001) Contribution of cranial nerve ganglia to innervation of the walls of the rat external acoustic meatus. Journal of the Peripheral Nervous System 6:28-32.

Folan-Curran J, Hickey K, Monkhouse WS (1994) Innervation of the Rat External Auditory Meatus: A Retrograde Tracing Study. Somatosensory & Motor Research 11:65-68.

Fornai F, Ruffoli R, Giorgi FS, Paparelli A (2011) The role of locus coeruleus in the antiepileptic activity induced by vagus nerve stimulation. European Journal of Neuroscience 33:2169-2178.

Franco F, Thomas GD, Giroir B, Bryant D, Bullock MC, Chwialkowski MC, Victor RG, Peshock RM (1999) Magnetic Resonance Imaging and Invasive Evaluation of Development of Heart Failure in Transgenic Mice With Myocardial Expression of Tumor Necrosis Factor-α. Circulation 99:448-454.

Freeman R (2006) Assessment of cardiovascular autonomic function. Clinical Neurophysiology 117:716-730.

Fregni F, Pascual-Leone A (2007) Technology Insight: noninvasive brain stimulation in neurology[mdash]perspectives on the therapeutic potential of rTMS and tDCS. Nat Clin Pract Neuro 3:383-393.

Fregni F, Boggio PS, Nitsche M, Pascual-Leone A (2005) Transcranial direct current stimulation. The British Journal of Psychiatry 186:446-447.

Fregni F, Boggio PS, Nitsche MA, Marcolin MA, Rigonatti SP, Pascual-Leone A (2006a) Treatment of major depression with transcranial direct current stimulation. Bipolar Disorders 8:203-204.

Fregni F, Boggio PS, Lima MC, Ferreira MJL, Wagner T, Rigonatti SP, Castro AW, Souza DR, Riberto M, Freedman SD, Nitsche MA, Pascual-Leone A (2006b) A sham-controlled, phase II trial of transcranial direct current stimulation for the treatment of central pain in traumatic spinal cord injury. Pain 122:197-209.

Fregni F, Gimenes R, Valle AC, Ferreira MJL, Rocha RR, Natalle L, Bravo R, Rigonatti SP, Freedman SD, Nitsche MA, Pascual-Leone A, Boggio PS (2006c) A randomized, sham-controlled, proof of principle study of transcranial direct current stimulation for the treatment of pain in fibromyalgia. Arthritis & Rheumatism 54:3988-3998.

Freidland DR, Eden AR, Laitman JT (1996) Use of the novel carbocyanine tracer fast-DiI for investigating upper respiratory tract cranial nerves in prenatal rats. Laboratory animal science 46:220-225.

Fu Q, Sugiyama Y, Kamiya A, Shamsuzzaman ASM, Mano T (1998) Responses of muscle sympathetic nerve activity to lower body positive pressure. American Journal of Physiology - Heart and Circulatory Physiology 275:H1254-H1259.

Gabbott PLA, Warner TA, Jays PRL, Salway P, Busby SJ (2005) Prefrontal cortex in the rat: Projections to subcortical autonomic, motor, and limbic centers. The Journal of Comparative Neurology 492:145-177.

Gao X-Y, Zhang S-P, Zhu B, Zhang H-Q (2008) Investigation of specificity of auricular acupuncture points in regulation of autonomic function in anesthetized rats. Autonomic Neuroscience 138:50-56.

Gao XY, Li YH, Liu K, Rong PJ, Ben H, Li L, Zhu B, Zhang SP (2011) Acupuncture-like stimulation at auricular point Heart evokes cardiovascular inhibition via activating the cardiac-related neurons in the nucleus tractus solitarius. Brain Research 1397:19-27.

Gebber GL (1980) Central oscillators responsible for sympathetic nerve discharge. American Journal of Physiology - Heart and Circulatory Physiology 239:H143-H155.

Giallauria F, Palomba S, Manguso F, Vitelli A, Maresca L, Tafuri D, Lombardi G, Colao A, Vigorito C, Orio F (2008) Abnormal heart rate recovery after maximal cardiopulmonary exercise stress testing in young overweight women with polycystic ovary syndrome. Clinical Endocrinology 68:88-93.

Gieroba ZJ, Li YW, Wesselingh SL, Blessing WW (1991) Transneuronal labeling of neurons in rabbit brain after injection of herpes simplex virus type 1 into the aortic depressor nerve. Brain Research 558:264-272.

Gil K, Bugajski A, Thor P (2011) Electrical vagus nerve stimulation decreases food concumption and weight gain in rats fed a high-fat diet. Journal of physiology and pharmacology 62:637-646.

Gilbey MP, Spyer MK (1993) Essential organization of the sympathetic nervous system. Baillière's Clinical Endocrinology and Metabolism 7:259-278.

Godement P, Vanselow J, Thanos S, Bonhoeffer F (1987) A study in developing visual systems with a new method of staining neurones and their processes in fixed tissue. Development 101:697-713.

Grassi G, Seravalle G, Colombo M, Bolla G, Cattaneo BM, Cavagnini F, Mancia G (1998) Body Weight Reduction, Sympathetic Nerve Traffic, and Arterial Baroreflex in Obese Normotensive Humans. Circulation 97:2037-2042.

Green AL, Wang S, Owen SLF, Xie K, Liu X, Paterson DJ, Stein JF, Bain PG, Aziz TZ (2005) Deep brain stimulation can regulate arterial blood pressure in awake humans. NeuroReport 16:1741-1745.

Greenwood JP, Stoker JB, Mary DASG (1999) Single-Unit Sympathetic Discharge. Circulation 100:1305-1310.

Gupta D, Verma S, Vishwakarma S (1986) Anatomic basis of Arnold's ear-cough reflex. Surgical and Radiologic Anatomy 8:217-220.

Guyenet PG (2000) Neural structures that mediate sympathoexcitation during hypoxia. Respiration Physiology 121:147-162.

Guyenet PG (2006) The sympathetic control of blood pressure. Nat Rev Neurosci 7:335-346.

Guyenet PG, Stornetta RL, Abbott SB, Depuy SD, Fortuna MG, Kanbar R (2010) Central CO2 chemoreception and integrated neural mechanisms of cardiovascular and respiratory control. Journal of Applied Physiology 108:995-1002.

Hagbarth KE, Vallbo ÅB (1968) Pulse and Respiratory Grouping of Sympathetic Impulses in Human Muscle Nerves. Acta Physiologica Scandinavica 74:96-108.

Haker E, Egekvist H, Bjerring P (2000) Effect of sensory stimulation (acupuncture) on sympathetic and parasympathetic activities in healthy subjects. Journal of the Autonomic Nervous System 79:52.

Harden CL, Pulver MC, Ravdin LD, Nikolov B, Halper JP, Labar DR (2000) A Pilot Study of Mood in Epilepsy Patients Treated with Vagus Nerve Stimulation. Epilepsy & Behavior 1:93-99.

Hart EC, McBryde FD, Burchell AE, Ratcliffe LEK, Stewart LQ, Baumbach A, Nightingale A, Paton JFR (2013) Translational Examination of Changes in Baroreflex Function After Renal Denervation in Hypertensive Rats and Humans. Hypertension.

Hasking GJ, Esler MD, Jennings GL, Burton D, Johns JA, Korner PI (1986) Norepinephrine spillover to plasma in patients with congestive heart failure: evidence of increased overall and cardiorenal sympathetic nervous activity. Circulation 73:615-621.

Hein E, Nowak M, Kiess O, Biermann T, Bayerlein K, Kornhuber J, Kraus T (2013) Auricular transcutaneous electrical nerve stimulation in depressed patients: a randomized controlled pilot study. Journal Of Neural Transmission 120:821-827.

Heusser K, Tank J, Engeli S, Diedrich A, Menne J, Eckert S, Peters T, Sweep FCGJ, Haller H, Pichlmaier AM, Luft FC, Jordan J (2010) Carotid Baroreceptor Stimulation, Sympathetic Activity, Baroreflex Function, and Blood Pressure in Hypertensive Patients. Hypertension 55:619-626.

Hilz MJ, Dutsch M (2006) Quantitative studies of autonomic function. Muscle & Nerve 33:6-20.

Howells DW, Porritt MJ, Rewell SSJ, O'Collins V, Sena ES, van der Worp HB, Traystman RJ, Macleod MR (2010) Different strokes for different folks: the rich diversity of animal models of focal cerebral ischemia. J Cereb Blood Flow Metab 30:1412-1431.

Im CH, Park JH, Shim M, Chang WH, Kim YH (2012) Evaluation of local electric fields generated by transcranial direct current stimulation with an extracepahilic electrode based on realistic 3D body modeling. Physics in Medicine and Biology 57:2137-2150.

Imai K, Sato, H., Hori, M., Kusuoka, H., Ozaki, H., Yokoyama, H., Inoue, M., Kamada, T. (1994) Vagally mediated heart rate recovery after exercise is accelerated in athletes but blunted in patients with chronic heart failure. Journal of the American College of Cardiology 24:1529-1535.

Investigators SH- (2011) Catheter-Based Renal Sympathetic Denervation for Resistant Hypertension: Durability of Blood Pressure Reduction Out to 24 Months. Hypertension 57:911-917.

Izzo PN, Deuchars J, Spyer KM (1993) Localization of cardiac vagal preganglionic motoneurones in the rat: Immunocytochemical evidence of synaptic inputs containing 5-hydroxytryptamine. The Journal of Comparative Neurology 327:572-583.

Jacobsen TN, Morgan BJ, Scherrer U, Vissing SF, Lange RA, Johnson N, Ring WS, Rahko PS, Hanson P, Victor RG (1993) Relative contributions of cardiopulmonary and sinoaortic baroreflexes in causing sympathetic activation in the human skeletal muscle circulation during orthostatic stress. Circulation Research 73:367-378.

Johns EJ, Abdullah MH (2013) Renal nerves in blood pressure regulation. Current Opinion in Nephrology and Hypertension Epub ahead of print.

Jordan J, Heusser K, Brinkmann J, Tank J (2012) Electrical carotid sinus stimulation in treatment resistant arterial hypertension. Autonomic Neuroscience 172:31-36.

Jose AD, Collison D (1970) The normal range and determinants of the intrinsic heart rate in man. Cardiovascular Research 4:160-167.

Jouven X, Empana J-P, Schwartz PJ, Desnos M, Courbon D, Ducimetiere P (2005) Heart-Rate Profile during Exercise as a Predictor of Sudden Death. N Engl J Med 352:1951-1958.

Kalia M, Mei SS, Kao FF (1981) Central projections from ergoreceptors (C fibres) in muscle involved in cardiopulmonary responses to static exercise. Circulation Research 48:I48-62.

Kamath MV, Upton AR, Talalla A, Fallen EL (1992) Neurcardiac responses to vagoafferent electrostimulation in humans. Pacing and Clinical Electrophysiology 15:1581-1587.

Katsouras G, Sakabe M, Comtois P, Maguy A, Burstein B, Guerra PG, Talajic M, Nattel S (2009) Differences in atrial fibrillation properties under vagal nerve stimulation versus atrial tachycardia remodeling. Heart Rhythm 6:1465-1472.

Katz ML, Robison Jr WG (2002) What is lipofuscin? Defining characteristics and differentiation from other autofluorescent lysosomal storage bodies. Archives of Gerontology and Geriatrics 34:169-184.

Kaye DM, Lefkovits J, Jennings GL, Bergin P, Broughton A, Esler MD (1995) Adverse consequences of high sympathetic nervous activity in the failing human heart. Journal of the American College of Cardiology 26:1257-1263.

Kellaway P (1946) The Part Played by Electric Fish in the Early History of Bioelectricity and Electrotherapy. Bulletin of the History of Medicine 20:112.

Kellner CH, Greenberg RM, Murrough JW, Bryson EO, Briggs MC, Pasculli RM (2012) ECT in treatment-resistant depression. Treatment in Psychiatry 169:1238-1244.

Kimmerly DS, O'Leary DD, Menon RS, Gati JS, Shoemaker JK (2005) Cortical regions associated with autonomic cardiovascular regulation during lower body negative pressure in humans. The Journal of Physiology 569:331-345.

Kirchner A., Birklein F., Stephan H., Handwerker HO (2000) Left vagus nerve stimulation suppresses experimentally induced pain. Neurology 55:1167-1171.

Kitamura S, Ogata K, Nishiguchi T, Nagase Y, Shigenaga Y (1991) Location of the motoneurons supplying the rabbit pharyngeal constrictor muscles and the peripheral course of their axons: A study using the retrograde HRP or fluorescent labeling technique. The Anatomical Record 229:399-406.

Kleiger RE, Miller JP, Bigger Jr JT, Moss AJ (1987) Decreased heart rate variability and its association with increased mortality after acute myocardial infarction. The American Journal of Cardiology 59:256-262.

Kopp UC, Smith LA, DiBona GF (1985) Renorenal reflexes: neural components of ipsilateral and contralateral renal responses. American Journal of Physiology - Renal Physiology 249:F507-F517.

Koren MS, Holmes MD (2006) Vagus nerve stimulation does not lead to significant changes in body weight in patients with epilepsy. Epilepsy & Behavior 8:246-249.

Korpelainen JT, Sotaniemi KA, Huikuri HV, Myllylä VV (1996) Abnormal Heart Rate Variability as a Manifestation of Autonomic Dysfunction in Hemispheric Brain Infarction. Stroke 27:2059-2063.

Koshiya N, Huangfu D, Guyenet PG (1993) Ventrolateral medulla and sympathetic chemoreflex in the rat. Brain Research 609:174-184.

Krahl SE, Senanayake SS, Handforth A (2001) Destruction of Peripheral C-Fibers Does Not Alter Subsequent Vagus Nerve Stimulation-Induced Seizure Suppression in Rats. Epilepsia 42:586-589.

Krahl SE, Clark KB, Smith DC, Browning RA (1998) Locus Coeruleus Lesions Suppress the Seizure-Attenuating Effects of Vagus Nerve Stimulation. Epilepsia 39:709-714.

Kraus T, Hosl K, Kiess O, Schanze A, Kornhuber J, Forster C (2007) BOLD fMRI deactivation of limbic and temporal brain structures and mood enhancing effect by transcutaneous vagus nerve stimulation. Journal Of Neural Transmission 114:1485-1493.

Kraus T, Kiess O, Hösl K, Terekhin P, Kornhuber J, Forster C (2013) CNS BOLD fMRI effects of sham-controlled transcutaneous electrical nerve stimulation in the left outer auditory canal – A pilot study. Brain Stimulation Epub ahead of print.

Kregel KC, Seals DR, Callister R (1992) Sympathetic nervous system activity during skin cooling in humans: relationship to stimulus intensity and pain sensation. The Journal of Physiology 454:359-371.

Kreuzer PM, Landgrebe M, Husser O, Resch M, Schecklmann M, Geisreiter F, Poeppl TB, Prasser SJ, Hajak G, Langguth B (2012) Transcutaneous vagus nerve stimulation: retrospective assessment of cardiac safety in a pilot study. Frontiers in Psychiatry 3.

Krogh A, Lindhard J (1913) The regulation of respiration and circulation during the initial stages of muscular work. The Journal of Physiology 47:112-136.

Kubo T, Parker JD, Azevedo ER, Atchison DJ, Newton GE, Picton P, Floras JS (2005) Vagal heart rate responses to chronic beta-blockade in human heart failure relate to cardiac norepinephrine spillover. Eur J Heart Fail 7:878-881.

Kuo M-F, Nitsche MA (2012) Effects of Transcranial Electrical Stimulation on Cognition. Clinical EEG and Neuroscience 43:192-199.

Kuo TBJ, Lin T, Yang CCH, Li C-L, Chen C-F, Chou P (1999) Effect of aging on gender differences in neural control of heart rate. American Journal of Physiology - Heart and Circulatory Physiology 277:H2233-H2239.

La Rovere MT, Pinna GD, Raczak G (2008) Baroreflex Sensitivity: Measurement and Clinical Implications. Annals of Noninvasive Electrocardiology 13:191-207.

La Rovere MT, Mortara A, Pinna GD, Bloomfield E, Aliot E, Schwartz PJ (1997) Quatification of baroreflex sensitivity by FINAPRES in patients after myocardial infarction: the ATRAMI experience. (Abstract). Journal of the American College of Cardiology 29:470A-470A.

La Rovere MTL, Bigger JJT, Marcus FI, Mortara A, Schwartz PJ (1998) Baroreflex sensitivity and heart-rate variability in prediction of total cardiac mortality after myocardial infarction. The Lancet 351:478-484.

La Marca R, Nedeljkovic M, Yuan L, Maercker A, Ehlert U (2010) Effects of auricular electrical stimulation on vagal activity in healthy men: evidence from a three-armed randomized trial. Clinical Science 118:537-546.

Lahiri MK, Kannankeril PJ, Goldberger JJ (2008) Assessment of Autonomic Function in Cardiovascular Disease: Physiological Basis and Prognostic Implications. Journal of the American College of Cardiology 51:1725.

Lall VK, Dutschmann M, Deuchars J, Deuchars SA (2012) The anti-malarial drug Mefloquine disrupts central autonomic and respiratory control in the working heart brainstem preparation of the rat. Journal of Biomedical Science 19:103.

Lampert R, Ickovics JR, Viscoli CJ, Horwitz RI, Lee FA (2003) Effects of propranolol on recovery of heart rate variability following acute myocardial infarction and relation to outcome in the Beta-Blocker Heart Attack Trial. The American Journal of Cardiology 91:137-142.

Lanciego JL, Wouterlood FG (2011) A half century of experimental neuroanatomical tracing. Journal of Chemical Neuroanatomy 42:157-183.

Lang N, Siebner HR, Ward NS, Lee L, Nitsche MA, Paulus W, Rothwell JC, Lemon RN, Frackowiak RS (2005) How does transcranial DC stimulation of the primary motor cortex alter regional neuronal activity in the human brain? European Journal of Neuroscience 22:495-504.

Lansdown A, Rees DA (2012) The sympathetic nervous system in polycystic ovary syndrome: a novel therapeutic target? Clinical Endocrinology 77:791-801.

Lehtimäki J, Hyvärinen P, Ylikoski M, Bergholm M, Mäkelä JP, Aarnisalo A, Pirvola U, Mäkitie A, Ylikoski J (2012) Transcutaneous vagus nerve stimulation in tinnitus: a pilot study. Acta Oto-laryngologica 0:1-5.

Lesman-Leegte I, van Veldhuisen DJ, Hillege HL, Moser D, Sanderman R, Jaarsma T (2009) Depressive symptoms and outcomes in patients with heart failure: data from the COACH study. European Journal of Heart Failure 11:1202-1207.

Li M, Zheng C, Sato T, Kawada T, Sugimachi M, Sunagawa K (2004) Vagal Nerve Stimulation Markedly Improves Long-Term Survival After Chronic Heart Failure in Rats. Circulation 109:120-124.

Li Y-W, Wesselingh SL, Blessing WW (1992) Projections from rabbit caudal medulla to C1 and A5 sympathetic premotor neurons, demonstrated with phaseolus leucoagglutinin and herpes simplex virus. The Journal of Comparative Neurology 317:379-395.

Liebetanz D, Nitsche MA, Tergau F, Paulus W (2002) Pharmacological approach to the mechanisms of transcranial DC stimulation induced after effects of human motor cortex excitability. Brain 125:2238-2247.

Lima MC, Fregni F (2008) Motor cortex stimulation for chronic pain: systematic review and meta-analysis of the literature. Neurology 70:2329-2337.

Lippold OCJ, Redfearn JWT (1964) Mental Changes Resulting from the Passage of Small Direct Currents Through the Human Brain. The British Journal of Psychiatry 110:768-772.

Loewy AD, Burton H (1978) Nuclei of the solitary tract: Efferent projections to the lower brain stem and spinal cord of the cat. The Journal of Comparative Neurology 181:421-449.

Loo CK, Alonzo A, Martin D, Mitchell PB, Galvez V, Sachdev P (2012) Transcranial direct current stimulation for depression: 3-week, randomised, sham-controlled trial. The British Journal of Psychiatry 200:52-59.

Lovick TA (1985) Ventrolateral medullary lesions block the antinociceptive and cardiovascular responses elicited by stimulating the dorsal periaqueductal grey matter in rats. Pain 21:241-252.

Lovick TA (1992) Inhibitory modulation of the cardiovascular defence response by the ventrolateral periaqueductal grey matter in rats. Exp Brain Res 89:133-139.

Luedtke K, Rushton A, Wright C, Geiss B, Juergens TP, May A (2012) Transcranial direct current stimulation for the reduction of clinical and experimentally induced pain: a systemic review and meta-analysis. The Clinical Journal of Pain 28:452-461.

Luppa M, Sikorski C, Luck T, Ehreke L, Konnopka A, Wiese B, Weyerer S, König HH, Riedel-Heller SG (2012) Age- and gender-specific prevalence of depression in latest-life – Systematic review and meta-analysis. Journal of Affective Disorders 136:212-221.

Macefield VG, Wallin BG, Vallbo AB (1994) The discharge behaviour of single vasoconstrictor motoneurones in human muscle nerves. The Journal of Physiology 481:799-809.

Macefield VG, Elam M, Wallin BG (2002) Firing properties of single postganglionic sympathetic neurones recorded in awake human subjects. Autonomic Neuroscience 95:146-159.

Macefield VG, James C, Henderson LA (2013) Identification of sites of sympathetic outflow at rest and during emotional arousal: Concurrent recordings of sympathetic nerve activity and fMRI of the brain. International Journal of Psychophysiology Epub ahead of print.

Macefield VG, Rundqvist B, Sverrisdottir YB, Wallin BG, Elam M (1999) Firing Properties of Single Muscle Vasoconstrictor Neurons in the Sympathoexcitation Associated With Congestive Heart Failure. Circulation 100:1708-1713.

Mäkikallio AM, Mäkikallio TH, Korpelainen JT, Sotaniemi KA, Huikuri HV, Myllylä VV (2004) Heart rate dynamics predice post stroke mortality. Neurology 62:1822-1826.

Makris A, Foster GD (2011) Dietary Approaches to the Treatment of Obesity. Psychiatric Clinics of North America 34:813-827.

Malik VS, Willett WC, Hu FB (2013) Global obesity: trends, risk factors and policy implications. Nat Rev Endocrinol 9:13-27.

Malliani A (2005) Heart rate variability: from bench to bedside. European Journal of Internal Medicine 16:12.

Mandell DA, Schreihofer AM (2006) Central respiratory modulation of barosensitive neurones in rat caudal ventrolateral medualla. Journal of Physiology 572:881-896.

Mano T, Iwase S, Toma S (2006) Microneurography as a tool in clinical neurophysiology to investigate peripheral neural traffic in humans. Clinical Neurophysiology 117:2357-2384.

Mark AL, Victor RG, Nerhed C, Wallin BG (1985) Microneurographic stuides of the mechanisms of sympathetic nerve responses to static exercise in humans. Circulation Research 57:461-469.

Marrosu F, Serra A, Maleci A, Puligheddu M, Biggio G, Piga M (2003) Correlation between GABAA receptor density and vagus nerve stimulation in individuals with drug-resistant partial epilepsy. Epilepsy Research 55:59-70.

Martin JLR, Martín-Sánchez E (2012) Systematic review and meta-analysis of vagus nerve stimulation in the treatment of depression: Variable results based on study designs. European Psychiatry 27:147-155.

McAllen RM, Spyer KM (1978) The baroreceptor input to cardiac vagal motoneurones. The Journal of Physiology 282:365-374.

McCall RB (1984) Evidence for a serotonergically mediated sympathoexcitatory response to stimulation of medullary raphe nuclei. Brain Research 311:131-139.

McManus DD, Saczynski JS, Lessard D, Kinno M, Pidikiti R, Esa N, Harrington J, Goldberg RJ (2013) Recent Trends in the Incidence, Treatment, and Prognosis of Patients With Heart Failure and Atrial Fibrillation (the Worcester Heart Failure Study). The American Journal of Cardiology 111:1460-1465.

Members ATF et al. (2012) ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2012: The Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association (HFA) of the ESC. European Heart Journal 33:1787-1847.

Merrill CA, Jonsson MAG, Minthon L, Ejnell H, Silander HC, Blennow K, Karlsson M, Nordlund A, Rolstad S, Warkentin S, Ben-Marachem E, Sjorgen MJC (2006) Vagus nerve stimulation in patients with Alzheimer's disease: additional follow-up results of a pilot study through 1 year. Journal of clinical Psychiatry 67:1171-1178.

Milby AH, Halpern CH, Baltuch GH (2008) Vagus Nerve Stimulation for Epilepsy and Depression. Neurotherapeutics 5:75-85.

Minisi AJ (1998) Vagal Cardiopulmonary Reflexes After Total Cardiac Deafferentation. Circulation 98:2615-2620.

Minson JB, Llewellyn-Smith IJ, Arnolda LF, Pilowsky PM, Oliver JR, Chalmers JP (1994) Disinhibition of the rostral ventral medulla increases blood pressure and Fos expression in bulbospinal neurons. Brain Research 646:44-52.

Mischel NA, Mueller PJ (2011) (In)activity-dependent alterations in resting and reflex control of splanchnic sympathetic nerve activity. Journal of Applied Physiology 111:1854-1862.

Mitchell JH, Victor RG (1996) Neural control of the cardiovascular system: insights from muscle sympathetic nerve recordings in humans. Medicine and Science in Sports and Exercise 28:60-69.

Molnár Z, Blakey DL, Bystron I, Carney RSE (2006) Tract-tracing in developing systems and in postmortem human material using carbocyanine dyes. In: Neuroanatomical Tract-Tracing 3: Molecules, Neurons, and Systems (Zaborszky L, Wouterlood FG, Lanciego JL, eds), pp 366-393. New York: Springer.

Montenegro RA, Farinatti PdTV, Fontes EB, Soares PPdS, Cunha FAd, Gurgel JL, Porto F, Cyrino ES, Okano AH (2011) Transcranial direct current stimulation influences the cardiac autonomic nervous control. Neuroscience Letters 497:32-36.

Mortara A, La Rovere MT, Pinna GD, Prpa A, Maestri R, Febo O, Pozzoli M, Opasich C, Tavazzi L (1997) Arterial Baroreflex Modulation of Heart Rate in Chronic Heart Failure: Clinical and Hemodynamic Correlates and Prognostic Implications. Circulation 96:3450-3458.

Myers DE (2008) Vagus nerve pain referred to the craniofacial region. A case report and literature review with implications for referred cardiac pain. Br Dent J 204:187-189.

Myers MG, Norris JW, Hachniski VC, Sole MJ (1981) Plasma norepinephrine in stroke. Stroke 12:200-204.

Napadow V, Edwards RR, Cahalan CM, Mensing G, Greenbaum S, Valovska A, Li A, Kim J, Maeda Y, Park K, Wasan AD (2012) Evoked Pain Analgesia in Chronic Pelvic Pain Patients Using Respiratory-Gated Auricular Vagal Afferent Nerve Stimulation. Pain Medicine 13:777-789.

Narang R, Cleland JGF, Erhardt L, Ball SG, Coats AJS, Cowley AJ, Dargie HJ, Hall AS, Hampton JR, Poole-Wilson A (1996) Mode of death in chronic heart failure: A request and proposition for more accurate classification. European Heart Journal 17:1390-1403.

Narkiewicz K, Somers VK (2003) Sympathetic nerve activity in obstructive sleep apnoea. Acta Physiologica Scandinavica 177:385-390.

Narkiewicz K, Kato M, Phillips BG, Pesek CA, Davison DE, Somers VK (1999) Nocturnal Continuous Positive Airway Pressure Decreases Daytime Sympathetic Traffic in Obstructive Sleep Apnea. Circulation 100:2332-2335.

Neff RA, Mihalevich M, Mendelowitz D (1998) Stimulation of NTS activates NMDA and non-NMDA receptors in rat cardiac vagal neurons in the nucleus ambiguus. Brain Research 792:277-282.

Ng AV, Callister R, Johnson DG, Seals DR (1993) Age and gender influence muscle sympathetic nerve activity at rest in healthy humans. Hypertension 21:498-503.

Ng G, Brack K, Coote J (2001) Effects of direct sympathetic and vagus nerve stimulation on the physiology of the whole heart--a novel model of isolated Langendorff perfused rabbit heart with intact dual autonomic innervation. Experimental Physiology 86:319-329.

Ng GA, Brack KE, Patel VH, Coote JH (2007) Autonomic modulation of electrical restitution, alternans and ventricular fibrillation initiation in the isolated heart. Cardiovascular Research 73:750-760.

Nitsche MA, Paulus W (2000) Excitability changes induced in the human motor cortex by weak transcranial direct current stimulation. The Journal of Physiology 527:633-639.

Nitsche MA, Paulus W (2001) Sustained excitability elevations induced by transcranial DC motor cortex stimulation in humans. Neurology 57:1899-1901.

Nitsche MA, Liebetanz D, Lang N, Antal A, Tergau F, Paulus W (2003a) Safety criteria for transcranial direct current stimulation (tDCS) in humans. Clinical Neurophysiology 114:2220-2222.

Nitsche MA, Fricke K, Henschke U, Schlitterlau A, Liebetanz D, Lang N, Henning S, Tergau F, Paulus W (2003b) Pharmacological modulation of cortical excitability shifts induced by transcranial direct current stimulation in humans. The Journal of Physiology 553:293-301.

Nolan J, Batin PD, Andrews R, Lindsay SJ, Brooksby P, Mullen M, Baig W, Flapan AD, Cowley A, Prescott RJ, Neilson JMM, Fox KAA (1998) Prospective Study of Heart Rate Variability and Mortality in Chronic Heart Failure : Results of the United Kingdom Heart Failure Evaluation and Assessment of Risk Trial (UK-Heart). Circulation 98:1510-1516.

Nomura S, Mizuno N (1984) Central distribution of primary afferent fibers in the Arnold's nerve (the auricular branch of the vagus nerve): A transganglionic HRP study in the cat. Brain Research 292:199-205.

North BJ, Sinclair DA (2012) The Intersection Between Aging and Cardiovascular Disease. Circulation Research 110:1097-1108.

Nunan D, Sandercock GRH, Brodie DA (2010) A Quantitative Systematic Review of Normal Values for Short-Term Heart Rate Variability in Healthy Adults. Pacing and Clinical Electrophysiology 33:1407-1417.

Ogoh S, Fisher JP, Fadel PJ, Raven PB (2007) Increases in central blood volume modulate carotid baroreflex resetting during dynamic exercise in humans. Journal of Physiology 581:405-418.

Ohn SU, Park C, Yoo W, Ko M, Choi KP, Kim G, Lee YT, Kim YH (2008) Time-dependent effect of transcranial direct current stimulation on the enhancement of working memory. NeuroReport 19:43-47.

Ohtaki H, Ohara K, Song D, Miyamoto K, Tsumuraya T, Yofu S, Dohi K, Tanabe S, Sasaki S, Uchida S, Matsunaga M, Shioda S (2012) Accumulation of autofluorescent storage material in brain is accelerated by ischemia in chloride channel 3 gene-deficient mice. Journal of Neuroscience Research 90:2163-2172.

Owens NC, Verberne AJM (2000) Medial prefrontal depressor response: involvement of the rostral and caudal ventrolateral medulla in the rat. Journal of the Autonomic Nervous System 78:86-93.

Owens NC, Sartor DM, Verberne AJM (1999) Medial prefrontal cortex depressor response: role of the solitary tract nucleus in the rat. Neuroscience 89:1331-1346.

Pagani M, Montano N, Porta A, Malliani A, Abboud FM, Birkett C, Somers VK (1997) Relationship Between Spectral Components of Cardiovascular Variabilities and Direct Measures of Muscle Sympathetic Nerve Activity in Humans. Circulation 95:1441-1448.

Palkovits M (1999) Interconnections between the Neuroendocrine Hypothalamus and the Central Autonomic System: Geoffrey Harris Memorial Lecture, Kitakyushu, Japan, October 1998. Frontiers in Neuroendocrinology 20:270-295.

Palm U, Schiller C, Fintescu Z, Obermeier M, Keeser D, Reisinger E, Pogarell O, Nitsche MA, Möller HJ, Padberg F (2012) Transcranial direct current stimulation in treatment resistant depression: A randomized double-blind, placebo-controlled study. Brain Stimulation 5:242-251.

Palmieri A (2006) Lung Cancer Presenting With Unilateral Facial Pain: Remission After Laryngeal Nerve Palsy. Headache: The Journal of Head and Face Pain 46:813-815.

Parati G, Di Rienzo M, Mancia G (2000) How to measure baroreflex sensitivity: from the cardiovascular laboratory to daily life.7-19.

Parati G, Ongaro G, Bilo G, Glavino F, Castiglioni P, Di Rienzo M, Mancia G (2003) Non-invasive beat-to-beat blood pressure monitoring: new developments. Blood Pressure Monitoring 8:31-36.

Pardo JV, Sheikh SA, Kuskowski MA, Surerus-Johnson C, Hagen MC, Lee JT, Rittberg BR, Adson DE (2007) Weight loss during chronic, cervical vagus nerve stimulation in depressed patients with obesity: an observation. Int J Obes 31:1756-1759.

Paton JFR (1996) A working heart-brainstem preparation of the mouse. Journal of Neuroscience Methods 65:63-68.

Patten RD, Hall-Porter MR (2009) Small Animal Models of Heart Failure: Development of Novel Therapies, Past and Present. Circulation: Heart Failure 2:138-144.

Patton HD (1948) SECRETORY INNERVATION OF THE CAT'S FOOTPAD. Journal of Neurophysiology 11:217-227.

Penry JK, Dean JC (1990) Prevention of Intractable Partial Seizures by Intermittent Vagal Stimulation in Humans: Preliminary Results. Epilepsia 31:S40-S43.

Pereira JB, Junqué C, Bartrés-Faz D, Martí MJ, Sala-Llonch R, Compta Y, Falcón C, Vendrell P, Pascual-Leone Á, Valls-Solé J, Tolosa E (2013) Modulation of verbal fluency networks by transcranial direct current stimulation (tDCS) in Parkinson's disease. Brain Stimulation 6:16-24.

Petersson M, Friberg P, Eisenhofer G, Lambert G, Rundqvist B (2005) Long-term outcome in relation to renal sympathetic activity in patients with chronic heart failure. European Heart Journal 26:906-913.

Peuker ET, Filler TJ (2002) The nerve supply of the human auricle. Clinical Anatomy 15:35-37.

Pinna GD, La Rovere MT, Maestri R, Mortara A, Bigger JT, Schwartz PJ (2000) Comparison between invasive and non-invasive measurements of baroreflex sensitivity. Implications for studies on risk stratification after a myocardial infarction. European Heart Journal 21:1522-1529.

Pinna GD, Maestri R, Capomolla S, Febo O, Robbi E, Cobelli F, La Rovere MT (2005) Applicability and Clinical Relevance of the Transfer Function Method in the Assessment of Baroreflex Sensitivity in Heart Failure Patients. Journal of the American College of Cardiology 46:1314-1321.

Polak T, Markulin F, Ehlis A-C, Langer J, Ringel T, Fallgatter A (2009) Far field potentials from brain stem after transcutaneous Vagus nerve stimulation: optimization of stimulation and recording parameters. Journal Of Neural Transmission 116:1237-1242.

Porter BA, Khodaparast N, Fayyaz T, Cheung RJ, Ahmed SS, Vrana WA, Rennaker RL, Kilgard MP (2011) Repeatedly Pairing Vagus Nerve Stimulation with a Movement Reorganizes Primary Motor Cortex. Cerebral Cortex.

Portillo F, Carrasco M, Juan Vallo J (1998) Separate populations of neurons within the paraventricular hypothalamic nucleus of the rat project to vagal and thoracic autonomic preganglionic levels and express c-Fos protein induced by lithium chloride. Journal of Chemical Neuroanatomy 14:95-102.

Prasad KS (1984) Cardiac depression on syringing the ear. The Journal of Laryngology & Otology 98:1013-1013.

Priori A (2003) Brain polarization in humans: a reappraisal of an old tool for prolonged non-invasive modulation of brain excitability. Clinical Neurophysiology 114:589-595.

Purpura DP, McMurtry JG (1965) Intracellular activities and evoked potential changes during polarization of motor cortex. Journal of Neurophysiology 28:166-185.

Pyner S (2009) Neurochemistry of the paraventricular nucleus of the hypothalamus: Implications for cardiovascular regulation. Journal of Chemical Neuroanatomy 38:197-208.

Pyner S, Coote JH (2000) Identification of branching paraventricular neurons of the hypothalamus that project to the rostroventrolateral medulla and spinal cord. Neuroscience 100:549-556.

Raimundo RJS, Uribe CE, Brasil-Neto JP (2012) Lack of clinically detectable acute changes on autonomic or thermoregulatory functions in healthy subjects after transcranial direct current stimulation (tDCS). Brain Stimulation 5:196-200.

Rajendran R, Jha S, Fernandes KA, Banerjee SB, Mohammad F, Dias BG, Vaidya VA (2009) Monoaminergic regulation of Sonic hedgehog signaling cascade expression in the adult rat hippocampus. Neuroscience Letters 453:190-194.

Redfearn JWT, Lippold OCJ, Costain R (1964) Preliminary Account of the Clinical Effects of Polarizing the Brain in Certain Psychiatric Disorders. The British Journal of Psychiatry 110:773-785.

Revesz D, Tjernstrom M, Ben-Menachem E, Thorlin T (2008) Effects of vagus nerve stimulation on rat hippocampal progenitor proliferation. Experimental Neurology 214:259-265.

Ricardo JA, Koh ET (1978) Anatomical evidence of direct projections from the nucleus of the solitary tract to the hypothalamus, amygdala, and other forebrain structures in the rat. Brain Research 153:1-26.

Riccio ML, Koller ML, Gilmour RF (1999) Electrical Restitution and Spatiotemporal Organization During Ventricular Fibrillation. Circulation Research 84:955-963.

Ristuccia HL, Grossman P, Watkins LL, Lown B (1997) Incremental Bias in Finapres Estimation of Baseline Blood Pressure Levels Over Time. Hypertension 29:1039-1043.

Robbe HW, Mulder LJ, Rüddel H, Langewitz WA, Veldman JB, Mulder G (1987) Assessment of baroreceptor reflex sensitivity by means of spectral analysis. Hypertension 10:538-543.

Robinson TG, James M, Youde J, Panerai R, Potter J (1997) Cardiac Baroreceptor Sensitivity Is Impaired After Acute Stroke. Stroke 28:1671-1676.

Robinson TG, Dawson SL, Eames PJ, Panerai RB, Potter JF (2003) Cardiac Baroreceptor Sensitivity Predicts Long-Term Outcome After Acute Ischemic Stroke. Stroke 34:705-712.

Rogers J, Silver MA, Shoemaker WJ, Bloom FE (1980) Senescent changes in a neurobiological model system: Cerebellar Purkinje cell electrophysiology and correlative anatomy. Neurobiology of Aging 1:3-11.

Rosas-Ballina M, Olofsson PS, Ochani M, Valdés-Ferrer SI, Levine YA, Reardon C, Tusche MW, Pavlov VA, Andersson U, Chavan S, Mak TW, Tracey KJ (2011) Acetylcholine-Synthesizing T Cells Relay Neural Signals in a Vagus Nerve Circuit. Science 334:98-101.

Rosin DL, Chang DA, Guyenet PG (2006) Afferent and efferent connections of the rat retrotrapezoid nucleus. Journal of Comparative Neurology 499:64-89.

Ross C, Ruggiero D, Park D, Joh T, Sved A, Fernandez-Pardal J, Saavedra J, Reis D (1984a) Tonic vasomotor control by the rostral ventrolateral medulla: effect of electrical or chemical stimulation of the area containing C1 adrenaline neurons on arterial pressure, heart rate, and plasma catecholamines and vasopressin. The Journal of Neuroscience 4:474-494.

Ross CA, Ruggiero DA, Reis DJ (1981) Afferent projections to cardiovascular portions of the nucleus of the tractus solitarius in the rat. Brain Research 223:402-408.

Ross CA, Ruggiero DA, Reis DJ (1985) Projections from the nucleus tractus solitarii to the rostral ventrolateral medulla. The Journal of Comparative Neurology 242:511-534.

Ross CA, Ruggiero DA, Joh TH, Park DH, Reis DJ (1984b) Rostral ventrolateral medulla: Selective projections to the thoracic autonomic cell column from the region containing C1 adrenaline neurons. The Journal of Comparative Neurology 228:168-185.

Rothwell PM (1993) Angina and mycardial infarction presenting with pain confined to the ear. Postgraduate Medical Journal 69:300-301.

Ruffoli R, Giorgi FS, Pizzanelli C, Murri L, Paparelli A, Fornai F (2011) The chemical neuroanatomy of vagus nerve stimulation. Journal of Chemical Neuroanatomy 42:288-296.

Rush AJ, Marangell LB, Sackeim HA, George MS, Brannan SK, Davis SM, Howland R, Kling MA, Rittberg BR, Burke WJ, Rapaport MH, Zajecka J, Nierenberg AA, Husain MM, Ginsberg D, Cooke RG (2005a) Vagus Nerve Stimulation for Treatment-Resistant Depression: A Randomized, Controlled Acute Phase Trial. Biological Psychiatry 58:347-354.

Rush AJ, Sackeim HA, Marangell LB, George MS, Brannan SK, Davis SM, Lavori P, Howland R, Kling MA, Rittberg B, Carpenter L, Ninan P, Moreno F, Schwartz T, Conway C, Burke M, Barry JJ (2005b) Effects of 12 Months of Vagus Nerve Stimulation in Treatment-Resistant Depression: A Naturalistic Study. Biological Psychiatry 58:355-363.

Sabbah H, Ilsar I, Zaretsky A, Rastogi S, Wang M, Gupta R (2011a) Vagus nerve stimulation in experimental heart failure. Heart Failure Reviews 16:171-178.

Sabbah HN, Gupta RC, Imai M, Irwin ED, Rastogi S, Rossing MA, Kieval RS (2011b) Chronic Electrical Stimulation of the Carotid Sinus Baroreflex Improves Left Ventricular Function and Promotes Reversal of Ventricular Remodeling in Dogs With Advanced Heart Failure. Circulation: Heart Failure 4:65-70.

Saito M, Naito M, Mano T (1990) Different responses in skin and muscle sympathetic nerve activity to static muscle contraction. Journal of Applied Physiology 69:2085-2090.

Scalco AZ, Rondon MUPB, Trombetta IC, Laterza MC, Azul JBCCS, Pullenayegum EM, M.Z. S, Kuniyoshi FHS, Wajngarten M, Negrao E, Lotufo-Neto F (2009) Muscle sympathectic nerve activity in depressed patients before and after treatment with sertraline. Journal of Hypertension 27:2429-2436.

Schambra HM, Abe M, Luckenbaugh DA, Reis J, Krakauer JW, Cohen LG (2011) Probing for hemispheric specialization for motor skill learning: a transcranial direct current stimulation study. Journal of Neurophysiology 106:652-661.

Schlaich MP, Sobotka PA, Krum H, Lambert E, Esler MD (2009) Renal Sympathetic-Nerve Ablation for Uncontrolled Hypertension. New England Journal of Medicine 361:932-934.

Schlindwein P, Buchholz HG, Schreckenberger M, Bartenstein P, Dieterich M, Birklein F (2008) Sympathetic activity at rest and motor brain areas: FDG–PET study. Autonomic Neuroscience 143:27-32.

Schmidt G, Malik M, Barthel P, Schneider R, Ulm K, Rolnitzky L, Camm AJ, Bigger Jr JT, Schömig A (1999) Heart-rate turbulence after ventricular premature beats as a predictor of mortality after acute myocardial infarction. The Lancet 353:1390-1396.

Schnupp J (2011) Auditory Neuroscience: How to Stop Tinnitus by Buzzing the Vagus. Current Biology 21:R263-R265.

Schosser A, Serretti A, Souery D, Mendlewicz J, Zohar J, Montgomery S, Kasper S (2012) European Group for the Study of Resistant Depression (GSRD) — Where have we gone so far: Review of clinical and genetic findings. European Neuropsychopharmacology.

Schulz R, Gerloff C, Hummel FC (2013) Non-invasive brain stimulation in neurological diseases. Neuropharmacology 64:579-587.

Schwaber J, Kapp B, Higgins G, Rapp P (1982) Amygdaloid and basal forebrain direct connections with the nucleus of the solitary tract and the dorsal motor nucleus. The Journal of Neuroscience 2:1424-1438.

Schwartz PJ, De Ferrari GM, Sanzo A, Landolina M, Rordorf R, Raineri C, Campana C, Revera M, Ajmone-Marsan N, Tavazzi L, Odero A (2008) Long term vagal stimulation in patients with advanced heart failure First experience in man. Eur J Heart Fail 10:884-891.

Schweitzer A, Wright S (1937) The anti-strychnine action of acetylcholine, prostigmine and related substances, and of central vagus stimulation. The Journal of Physiology 90:310-329.

Seals DR, Chase PB, Taylor JA (1988) Autonomic mediation of the pressor responses to isometric exercise in humans. Journal of Applied Physiology 64:2190-2196.

Sequeira H, Viltart O, Ba-M'Hamed S, Poulain P (2000) Cortical control of somato-cardiovascular integration: neuroanatomical studies. Brain Research Bulletin 53:87-93.

Shahwan A, Bailey C, Maxiner W, Harvey AS (2009) Vagus nerve stimulation for refractory epilepsy in children: More to VNS than seizure frequency reduction. Epilepsia 50:1220-1228.

Shaper AG, Wannamethee G, Macfarlane PW, Walker M (1993) Heart rate, ischaemic heart disease, and sudden cardiac death in middle-aged British men. British Heart Journal 70:49-55.

Sharifov OF, Fedorov VV, Beloshapko GG, Glukhov AV, Yushmanova AV, Rosenshtraukh LV (2004) Roles of adrenergic and cholinergic stimulation in spontaneous atrial fibrillation in dogs. Journal of the American College of Cardiology 43:483-490.

Shen MJ, Choi E-K, Tan AY, Lin S-F, Fishbein MC, Chen LS, Chen P-S (2012) Neural mechanisms of atrial arrhythmias. Nat Rev Cardiol 9:30-39.

Shen MJ, Shinohara T, Park H-W, Frick K, Ice DS, Choi E-K, Han S, Maruyama M, Sharma R, Shen C, Fishbein MC, Chen LS, Lopshire JC, Zipes DP, Lin S-F, Chen P-S (2011) Continuous Low-Level Vagus Nerve Stimulation Reduces Stellate Ganglion Nerve Activity and Paroxysmal Atrial Tachyarrhythmias in Ambulatory Canines. Circulation 123:2204-2212.

Sherwood A, Blumenthal JA, Trivedi R, Johnson MPH, O'Connor CM, Adams KF, Dupree CS, Waugh RA, Bensimhon DR, Gaulden L, Christenson RH, Koch GG, Hinderliter AL (2007) Relationship of depression to death or hospitalization in patients with heart failure. Archives of Internal Medicine 167:367-373.

Shetler K, Marcus R, Froelicher VF, Vora S, Kalisetti D, Prakash M, Do D, Myers J (2001) Heart rate recovery: validation and methodologic issues. Journal of the American College of Cardiology 38:1980.

Smits JF, Brody MJ (1984) Activation of afferent renal nerves by intrarenal bradykinin in conscious rats. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 247:R1003-R1008.

Solano-Flores LP, Rosas-Arellano MP, Ciriello J (1997) Fos induction in central structures after afferent renal nerve stimulation. Brain Research 753:102-119.

Soler MD, Kumru H, Pelayo R, Vidal J, Tormos JM, Fregni F, Navarro X, Pascual-Leone A (2010) Effectiveness of transcranial direct current stimulation and visual illusion on neuropathic pain in spinal cord injury. Brain 133:2565-2577.

Somers VK, L. MA, Zavala DC, Abboud FM (1989) Contrasting effects of hypercapnia on ventilation and sympathetic activity in humans. Journal of Applied Physiology 67:2101-2106.

Sörös P, Hachinski V (2012) Cardiovascular and neurological causes of sudden death after ischaemic stroke. The Lancet Neurology 11:179-188.

Spraul M, Ravussin E, Fontvieille AM, Rising R, Larson DE, Anderson EA (1993) Reduced sympathetic nervous activity. A potential mechanism predisposing to body weight gain. The Journal of Clinical Investigation 92:1730-1735.

Spuck S, Tronnier, V., Orosz, I., Schonweiler, R., Sepehrnia, A., Nowak, G., Sperner, J. (2010) Operative and techincal complications of vagus nerve stimulator implantation. Neurosurgery 67 489-494.

Stagg CJ, Nitsche MA (2011) Physiological Basis of Transcranial Direct Current Stimulation. The Neuroscientist 17:37-53.

Stagg CJ, Best JG, Stephenson MC, O'Shea J, Wylezinska M, Kincses ZT, Morris PG, Matthews PM, Johansen-Berg H (2009) Polarity-Sensitive

Modulation of Cortical Neurotransmitters by Transcranial Stimulation. The Journal of Neuroscience 29:5202-5206.

Standish A, Enquist L, Escardo J, Schwaber J (1995) Central neuronal circuit innervating the rat heart defined by transneuronal transport of pseudorabies virus. The Journal of Neuroscience 15:1998-2012.

Stefan H, Kreiselmeyer G, Kerling F, Kurzbuch K, Rauch C, Heers M, Kasper BS, Hammen T, Rzonsa M, Pauli E, Ellrich J, Graf W, Hopfengärtner R (2012) Transcutaneous vagus nerve stimulation (t-VNS) in pharmacoresistant epilepsies: A proof of concept trial. Epilepsia 53:e115-e118.

Strack AM, Sawyer WB, Platt KB, Loewy AD (1989) CNS cell groups regulating the sympathetic outflow to adrenal gland as revealed by transneuronal cell body labelling with pseudorabies virus. Brain Research 491:274-296.

Sverrisdóttir YB, Mogren T, Kataoka J, Janson PO, Stener-Victorin E (2008) Is polycystic ovary syndrome associated with high sympathetic nerve activity and size at birth? American Journal of Physiology - Endocrinology And Metabolism 294:E576-E581.

Swift MJ, Crago PE, Grill WM (2005) Applied electric fields accelerate the diffusion rate and increase the diffusion distance of DiI in fixed tissue. Journal of Neuroscience Methods 141:155-163.

Task Force of the European Society of Cardiology and the North American Society of Pacing Electrophysiology (1996) Heart Rate Variability : Standards of Measurement, Physiological Interpretation, and Clinical Use. Circulation 93:1043-1065.

Taylor CB (2010) Depression, heart rate related variables and cardiovascular disease. International Journal of Psychophysiology 78:80-88.

Tekdemir I, Aslan A, Elhan A (1998) A clinico-anatomic study of the auricular branch of the vagus nerve and Arnold's ear-cough reflex. Surgical and Radiologic Anatomy 20:253-257.

Terry R, Tarver WB, Zabara J (1990) An Implantable Neurocybernetic Prosthesis System. Epilepsia 31:S33-S37.

Thomas JA, Marks BH (1978) Plasma norepinephrine in congestive heart failure. The American Journal of Cardiology 41:233-243.

Torre-Amione G, Kapadia S, Benedict C, Oral H, Young JB, Mann DL (1996) Proinflammatory cytokine levels in patients with depressed left ventricular ejection fraction: A report from the studies of left ventricular dysfunction (SOLVD). Journal of the American College of Cardiology 27:1201-1206.

Tracey KJ (2009) Reflex control of immunity. Nat Rev Immunol 9:418-428.

Triposkiadis F, Karayannis G, Giamouzis G, Skoularigis J, Louridas G, Butler J (2009) The Sympathetic Nervous System in Heart Failure: Physiology, Pathophysiology, and Clinical Implications. Journal of the American College of Cardiology 54:1747.

Trombetta IC, Batalha LT, Rondon MUPB, Laterza MC, Kuniyoshi FHS, Gowdak MMG, Barretto ACP, Halpern A, Villares SMF, Negrão CE (2003) Weight loss improves neurovascular and muscle metaboreflex control in obesity. American Journal of Physiology - Heart and Circulatory Physiology 285:H974-H982.

Tsuji H, Larson MG, Venditti FJ, Manders ES, Evans JC, Feldman CL, Levy D (1996) Impact of Reduced Heart Rate Variability on Risk for Cardiac Events: The Framingham Heart Study. Circulation 94:2850-2855.

Tsutsumi T, Ide T, Yamato M, Kudou W, Andou M, Hirooka Y, Utsumi H, Tsutsui H, Sunagawa K (2008) Modulation of the myocardial redox state by vagal nerve stimulation after experimental myocardial infarction. Cardiovasc Res 77:713-721.

Umetani K, Singer DH, McCraty R, Atkinson M (1998) Twenty-Four Hour Time Domain Heart Rate Variability and Heart Rate: Relations to Age and Gender Over Nine Decades. Journal of the American College of Cardiology 31:593-601.

Val-Laillet D, Biraben A, Randuineau G, Malbert CH (2010) Chronic vagus nerve stimulation decreased weight gain, food consumption and sweet craving in adult obese minipigs. Appetite 55:245-252.

van der Kooy D, Koda LY, McGinty JF, Gerfen CR, Bloom FE (1984) The organization of projections from the cortes, amygdala, and hypothalamus to the nucleus of the solitary tract in rat. The Journal of Comparative Neurology 224:1-24.

Vandermeeren Y, Jamart J, Ossemann M (2010) Effect of tDCS with an extracephalic reference electrode on cardio-respiratory and autonomic functions. BMC Neuroscience 11:38.

Vanneste S, Kilgard M, Engineer N, Tarver B, DeRidder D (2012) Paired vagus nerve stimulation for tinnitus – pilot study results. 6th International Conference on Tinnitus Abstract:Brugge, Belgium.

Vanoli E, De Ferrari GM, Stramba-Badiale M, Hull SS, Jr., Foreman RD, Schwartz PJ (1991) Vagal stimulation and prevention of sudden death in conscious dogs with a healed myocardial infarction. Circ Res 68:1471-1481.

Veith RC, Lewis N, Linares OA, Barnes RF, Raskind MA, Villacres EC, Murburg MM, Ashleigh A, Castillo S, Peskind ER, Pascualy M, Halter JB (1994) Sympathetic nervous system activity in major depression:

Basal and desipramine-induced alterations in plasma norepinephrine kinetics. Archives of General Psychiatry 51:411-422.

Verberne AJM, Owens NC (1998) Cortical Modulation of theCardiovascular System. Progress in Neurobiology 54:149-168.

Victor RG, Leimbach WN, Seals DR, Wallin BG, Mark AL (1987) Effects of the cold pressor test on muscle sympathetic nerve activity in humans. Hypertension 9:429-436.

Viltart O, Mullier O, Bernet F, Poulain P, Ba-M'Hamed S, Sequeira H (2003) Motor cortical control of cardiovascular bulbar neurones projecting to spinal autonomic areas. Journal of Neuroscience Research 73:122-135.

Vissing SF, Scherrer U, Victor RG (1989) Relation between sympathetic outflow and vascular resistance in the calf during perturbations in central venous pressure. Evidence for cardiopulmonary afferent regulation of calf vascular resistance in humans. Circulation Research 65:1710-1717.

Wallin BG, Sundlöf G, Delius W (1975) The effect of carotid sinus nerve stimulation on muscle and skin nerve sympathetic activity in man. Pflugers Arch 358:101-110.

Weiss ML, Chowdhury SI (1998) The renal afferent pathways in the rat: a pseudorabies virus study. Brain Research 812:227-241.

Wong SW, Massé N, Kimmerly DS, Menon RS, Shoemaker JK (2007) Ventral medial prefrontal cortex and cardiovagal control in conscious humans. NeuroImage 35:698-708.

Yang Z, Coote JH (1998) Influence of the hypothalamic paraventricular nucleus on cardiovascular neurones in the rostral ventrolateral medulla of the rat. The Journal of Physiology 513:521-530.

Yildirir A, Aybar F, Kabakci G, Yarali H, Oto A (2006) Heart Rate Variability in Young Women with Polycystic Ovary Syndrome. Annals of Noninvasive Electrocardiology 11:306-312.

Yu L, Scherlag BJ, Li S, Fan Y, Dyer J, Male S, Varma V, Sha Y, Stavrakis S, Po SS (2013) Low-level transcutaneous electrical stimulation of the auricular branch of the vagus nerve: A noninvasive approach to treat the initial phase of atrial fibrillation. Heart Rhythm 10:428-435.

Zagon A, Smith AD (1993) Monosynaptic projections from the rostral ventrolateral medulla oblongata to identified sympathetic preganglionic neurons. Neuroscience 54:729-743.

Zamotrinsky A, Afanasiev S, Karpov RS, Cherniavsky A (1997) Effects of electrostimulation of the vagus afferent endings in patients with coronary artery disease. Coronary Artery Disease 8:551-558.

Zamotrinsky AV, Kondratiev B, de Jong JW (2001) Vagal neurostimulation in patients with coronary artery disease. Autonomic Neuroscience 88:109.

Zanchetti A, Wang SC, Moruzzi G (1952) The effect of vagal afferent stimulation on the EEG pattern of the cat. Electroencephalography and Clinical Neurophysiology 4:357-361.

Zhang Y, Mazgalev T (2011) Arrhythmias and vagus nerve stimulation. Heart Failure Reviews 16:147-161.

Zhang Y, Ilsar I, Sabbah HN, Ben David T, Mazgalev TN (2009a) Relationship between right cervical vagus nerve stimulation and atrial fibrillation inducibility: therapeutic intensities do not increase arrhythmogenesis. Heart Rhythm 6:244-250.

Zhang Y, Popovic Z, Bibevski S, Fakhry I, Sica D, Van Wagoner D, Mazgalev TN (2009b) Chronic Vagus Nerve Stimulation Improves Autonomic Control and Attenuates Systemic Inflammation and Heart Failure Progression in a Canine High-Rate Pacing Model. Circulation Heart Failure 2:692-699.

Zhao YX, He W, Jing XH, Liu JL, Rong PJ, Ben H, Liu K, Zhu B (2012) Transcutaneous Auricular Vagus Nerve Stimulation Protects Endotoxemic Rat from Lipopolysaccharide-Induced Inflammation. Evidence-Based Complementary and Alternative Medicine 2012:10.

Appendix
Ethics Approvals

Research Support

3 Cavendish Road
University of Leeds
Leeds LS2 9JT

Tel: 0113 3434873
e-mail: j.m.blaikie@adm.leeds.ac.uk

Biological Sciences Faculty Research Ethics Committee
University of Leeds

21 April 2010

Miss Jennifer Clancy
Institute of Membrane and Systems Biology
Garstang Building 5.53
University Of Leeds

Dear Jennifer

Title of Study	**An investigation of the effects of ear stimulation on the nervous control of the heart**
Ethics Reference	**BIOSCI 09-021**

The above project has been reviewed by the Biological Sciences Faculty Research Ethics Committee at its meeting on 20th April 2010.

The following documentation was considered:

Document	*Version*	*Date*
BIOSCI 09-021 Ethics App.doc	1	12/04/10
BIOSCI 09-021 HEALTH QUESTIONNAIRE.doc	1	12/04/10
BIOSCI 09-021 Subject Info.doc	1	12/04/10

On the basis of the information provided, the Committee approves this project.

Yours sincerely

Jennifer Blaikie

Jennifer Blaikie
Research Ethics Administrator
Research Support
On Behalf of
Professor Eric Blair
Chair, Biological Sciences FREC.

NHS
Health Research Authority

NRES Committee Yorkshire & The Humber - Leeds Central
North East REC Centre
Room 002
TEDCO Business Centre
Viking Industrial Park
Rolling Mill Road
Jarrow
NE32 3DT

Tel: 0191 4283545

10 April 2013

John Greenwood
Academic Unit of Cardiovascular Medicine
G Floor
Jubilee Wing
Leeds General Infirmary
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 H Tyrrell

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

Research Support
3 Cavendish Road
University of Leeds
Leeds LS2 9JT

Tel: 0113 343 4873
e-mail: j.m.blaikie@adm.leeds.ac.uk

UNIVERSITY OF LEEDS

Jennifer Clancy
IMSB
G5.53 Garstang Building,
University of Leeds
Leeds LS2 9JT

Biological Sciences Faculty Research Ethics Committee
University of Leeds

18 October 2011

Dear Jenny

Title of study: **An investigation of the distribution and central connections of the auricular branch of the vagus nerve**

Ethics reference: **BIOSCI 11-001**

I am pleased to inform you that the above application for ethical review has been reviewed by the Biological Sciences Faculty Research Ethics Committee and I can confirm a favourable ethical opinion on the basis of the information provided in the following documents:

Document	*Version*	*Date*
BIOSCI 11-001 Ethics application human tissue.doc	2	30/09/11
BIOSCI 11-001 J_Clancy_letter.doc	1	30/09/11

Please notify the Committee if you intend to make any amendments to the original research as submitted at date of this approval. This includes recruitment methodology and all changes must be ethically approved prior to implementation.

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.

Yours sincerely

J Blaikie

Jennifer Blaikie
Research Ethics Administrator
Research Support
On Behalf of Professor Eric Blair
Chair, BIOSCI Faculty Research Ethics Committee

CC: Student supervisor(s)

Performance, Governance and Operations
Research & Innovation Service
Charles Thackrah Building
101 Clarendon Road
Leeds LS2 9LJ Tel: 0113 343 4873
Email: j.m.blaikie@leeds.ac.uk

UNIVERSITY OF LEEDS

Biological Sciences Faculty Research Ethics Committee
University of Leeds

Jennifer Clancy
IMSB
University of Leeds
Leeds LS2 9JT

16 April 2012

Dear Jennifer

Title of study: **The influence of transcranial direct current stimulation on cardiovascular autonomic function in healthy human subjects**
Ethics reference: **BIOSCI 11-019**

I am pleased to inform you that the above application for ethical review has been reviewed by the Biological Sciences Faculty Research Ethics Committee and following receipt of your response to the Committee's initial comments, I can confirm a favourable ethical opinion on the basis of the information provided in the following documents:

Document	*Version*	*Date*
BIOSCI 11-019 Response to ethics tDCS questions.docx	1	11/04/12
BIOSCI 11-019 Ethics App tDCS.doc	1	16/03/12
BIOSCI 11-019 Information Sheet tDCS.docx	1	16/03/12
BIOSCI 11-019 Consent for tDCS.docx	1	16/03/12
BIOSCI 11-019 Health questionnaire micro.docx	1	16/03/12

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 www.leeds.ac.uk.

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.

Yours sincerely

Jennifer Blaikie
Senior Research Ethics Administrator
Research & Innovation Service
On Behalf of Karen Birch
Chair, BIOSCI Faculty Research Ethics Committee
CC: Student supervisor(s)