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Vagus Nerve Stimulation

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Preface to the Series

Experimental life sciences have two basic foundations: concepts and tools. The *Neuromethods* series focuses on the tools and techniques unique to the investigation of the nervous system and excitable cells. It will not, however, shortchange the concept side of things as care has been taken to integrate these tools within the context of the concepts and questions under investigation. In this way, the series is unique in that it not only collects protocols but also includes theoretical background information and critiques which led to the methods and their development. Thus it gives the reader a better understanding of the origin of the techniques and their potential future development. The *Neuromethods* publishing program strikes a balance between recent and exciting developments like those concerning new animal models of disease, imaging, in vivo methods, and more established techniques, for example, immunocytochemistry and electrophysiological technologies. New trainees in neurosciences still need a sound footing in these older methods in order to apply a critical approach to their results.

Under the guidance of its founders, Alan Boulton and Glen Baker, the *Neuromethods* series has been a success since its first volume published through Humana Press in 1985. The series continues to flourish through many changes over the years. It is now published under the umbrella of Springer Protocols. While methods involving brain research have changed a lot since the series started, the publishing environment and technology have changed even more radically. *Neuromethods* has the distinct layout and style of the Springer Protocols program, designed specifically for readability and ease of reference in a laboratory setting.

The careful application of methods is potentially the most important step in the process of scientific inquiry. In the past, new methodologies led the way in developing new disciplines in the biological and medical sciences. For example, physiology emerged out of anatomy in the nineteenth century by harnessing new methods based on the newly discovered phenomenon of electricity. Nowadays, the relationships between disciplines and methods are more complex. Methods are now widely shared between disciplines and research areas. New developments in electronic publishing make it possible for scientists that encounter new methods to quickly find sources of information electronically. The design of individual volumes and chapters in this series takes this new access technology into account. Springer Protocols makes it possible to download single protocols separately. In addition, Springer makes its print-on-demand technology available globally. A print copy can therefore be acquired quickly and for a competitive price anywhere in the world.

Preface

Vagus nerve stimulation (VNS) was once limited in application to the treatment of refractory epilepsy. With its remarkable safety record, over 100,000 patients carry implanted VNS devices. Moreover, 30,000 of these patients are children, demonstrating the applicability of VNS across the lifespan [1]. With the advent of non-invasive methods for stimulation, even more individuals may benefit from stimulation of the vagus nerve. Research and clinical applications for invasive and non-invasive VNS now span across many disciplines, including neuroscience, neurology, psychiatry, and psychology as well as infectious, inflammatory, and metabolic disease research.

The expansion of invasive and non-invasive VNS research has resulted in the creation of multiple promising preclinical and clinical models. Preclinical and clinical research applications of VNS include an extensive range of psychiatric, cognitive, inflammatory, and neurological disorders. Similarly, research in both invasive and non-invasive VNS attempts to understand the mechanisms by which VNS may elicit its effects within a particular model. These include modulation of inflammation, neurotransmitter systems, and the autonomic nervous system, among other mechanisms. In this book, the editors and authors attempt to cover the wide range of current research and development in the area of VNS in the exciting new field of bioelectronic medicine. The book approaches VNS by asking two major questions: How does it work and what can we do with it? Both questions cannot be answered without asking the intimately coupled question of where does VNS have a measurable and meaningful effect?

The ensuing chapters review research in invasive and non-invasive VNS, including methodological considerations (e.g., study design, stimulation parameters, use of heart rate variability as a biomarker of vagal activity), the evolution of the vagus nerve via polyvagal theory, mechanisms of action (e.g., autonomic regulation, immune plasticity), and disorders in which VNS approaches may be therapeutic (e.g., migraine/cluster headaches, mood disorders, trauma-related disorders, cognitive enhancement, and language learning). While mechanisms of action are still largely unknown, each chapter delves into research on potential mechanisms by which VNS may elicit its therapeutic benefits. While the chapters in this book do not provide an exhaustive list of applications or mechanisms, the editors and authors intend for this book to serve as a strong foundation of knowledge about VNS. Given the many uses of VNS, this book is written for a broad, multidisciplinary audience to enjoy. The audience includes preclinical and clinical scientists, clinicians, physicians, and scholars, both new and familiar with VNS. As a versatile and up-and-coming intervention technique, VNS offers many opportunities for further research, methodological refinement, and therapeutic benefits across a diverse range of disorders. We hope that the pages of this book will inspire new or additional interest in VNS so that together we may continue to unlock the exciting potential for VNS to improve the lives of many.

Scoping Table of Contents for All Chapters

Chapter number, title, and first author	Key messages
Chapter 1 Introduction *Shortell et al.*	This chapter provides an overview of the book's focus, including a summary of content and the intended audience. The chapter reviews the anatomy of the vagus nerve, including its parasympathetic, noradrenergic, metabolic, and anti-inflammatory functions. It also reviews the types of vagus nerve stimulation and associated research.
Chapter 2 Peripheral Targets *Özden*	Autonomic dysregulation underlies many disorders, such as fibromyalgia, irritable bowel syndrome (IBS), migraine, excess inflammation, obesity, chronic fatigue syndrome, depression, and anxiety. Parasympathetic and sympathetic branches of the autonomic nervous system work synergistically, and their activity can vary across parts of the body. The vagus nerve comprises the largest portion of the parasympathetic branch. Stimulation of the vagus nerve can balance autonomic dysregulation by modulating central and peripheral nervous systems. This chapter also reviews VNS for the gastrointestinal system, the respiratory system, the endocrine system, the peripheral circulatory system, obesity, chronic pain, and peripheral disorders.
Chapter 3 Polyvagal Theory *Porges*	Polyvagal theory describes evolutionary changes in the autonomic nervous system that enabled mammals to suppress defensive strategies, express sociality, and optimize homeostatic functions through a ventral vagal circuit. This circuit facilitates capacities to self-calm, socially engage others, and mitigate internal and external threat reactions in ourselves and others with social cues. The chapter further describes how the ventral vagal complex regulates aspects of social engagement. Mental and physical health problems arise when the uniquely mammalian ventral vagal pathway is chronically disrupted. The neuroanatomy and neurophysiology of

(continued)

Chapter number, title, and first author	Key messages
	important vagal pathways can be objectively monitored via non-invasive techniques (i.e., measuring components of heart rate variability) and potentially targeted for neuromodulation. This chapter describes the various vagal pathways and their evolution. It highlights that metrics evaluating vagal activity and devices designed to stimulate activity need to indicate which pathways are being monitored or stimulated since the vagal pathways evolved at different stages of vertebrate evolution and support distinct adaptive functions.
Chapter 4 HRV as a Biomarker *Borges & Laborde*	This chapter focuses on heart rate variability as a biomarker for VNS. Results for the acute effects of transcutaneous cervical and auricular VNS modulation on cardiac vagal activity (CVA) are mixed. Some studies show no effect, and others show increased activity. CVA results for medium to long-term administration of VNS are also mixed, while acute effects show increased activity. Ideal stimulation parameters to increase CVA are unclear. RMSSD, pNN50, and HF are common indices of vagally mediated heart rate variability and are considered reliable indices of CVA. The chapter concludes with considerations for proper cardiac vagal activity measurement and study design, including a checklist for these considerations.
Chapter 5 Microglial plasticity *Courchesne et al.*	This chapter details a preclinical study using pregnant sheep as a model of human fetal development, with a focus on brain development, especially microglia. Vagotomy and selective efferent or afferent VNS were performed to manipulate fetal inflammatory response to lipopolysaccharide (LPS). This chapter reports the methodology and findings in hippocampal brain regions, highlighting the impact of VNS on microglial function as well as discussing the neurodevelopmental implications. Microglial morphometry and traditional immunohistochemistry are discussed as

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Chapter number, title, and first author	Key messages
	important measures of the effects of VNS on microglial plasticity.
Chapter 6 Perinatal Physiology *Castel et al.*	This chapter briefly reviews VNS and inflammation and presents an approach to VNS in late-onset neonatal sepsis modeled in piglets. Connection is drawn between direct VENG and HRV as representations of brain-body communication via the vagus nerve and how VNS alters this. A generic signature of inflammatory response via vagus nerve reflected in HRV is proposed, which is exciting as HRV can be obtained non-invasively.
Chapter 7 Cognitive Enhancement *Bumanglag et al.*	VNS has been shown to enhance neuroplasticity and cognition in human and animal models. Optimal cognition is supported by a balance of excitation and inhibition in specific brain regions. Therapeutic strategies that can modulate excitability may restore or improve cognitive function. This chapter reviews a manuscript on enhanced reversal learning in rats with VNS. The chapter presents experimental guidelines and considerations for testing the effects of VNS on cognition in preclinical models, particularly aged rats.
Chapter 8 Emotion and Depression *Bottari et al.*	Early studies on VNS and depression occurred in individuals with VNS for the treatment of epilepsy. In 2005, VNS became FDA-approved for the treatment of treatment-resistant major depressive disorder. This chapter reviews studies of VNS and self-reported depressive symptoms as well as studies on the impact of VNS on brain areas relevant to depression. The chapter explores other potential mechanisms by which VNS may impact depression (e.g., inflammation, neurogenesis, and effects on neurotransmitters). The chapter also discusses VNS for anxiety and sleep disorders, which are common comorbidities with depression.
Chapter 9 Trauma Spectrum	This chapter reviews the effects of VNS on neurobiology and applications to patients with posttraumatic stress disorder (PTSD)

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Chapter number, title, and first author	Key messages
Psychiatric Disorders *Bremner et al.*	and other stress-related or trauma spectrum psychiatric disorders, including major depression, borderline personality disorder, and dissociative disorders. VNS may modulate common underlying alterations in stress-responsive systems, including the sympathetic nervous system, inflammatory biomarkers, and brain areas, such as the medial prefrontal cortex, insula, and hippocampus, that mediate symptoms of these disorders. Further research is needed in non-invasive VNS, which may allow for the widespread use of vagus nerve-based interventions.
Chapter 10 Migraine/cluster headache *Boch & Mauskop*	VNS is a promising treatment for aborting and preventing migraines and episodic cluster headaches. Early interest in VNS for headache treatment arose after patients with VNS for epilepsy reported decreased headache symptoms. This chapter reviews the pathophysiology of migraines and cluster headaches, the mechanism by which VNS may confer treatment benefits, and studies of VNS for migraine and cluster headaches.
Chapter 11 Language *Kaan & Lin*	VNS has been shown to affect cognitive and perceptual mechanisms important for language processing and learning. Language learning is a new area of research in non-invasive transcutaneous vagus nerve stimulation (tVNS). The chapter reviews the few studies that have examined the effects of tVNS on language learning. The chapter discusses experimental design considerations for examining language learning.

Introduction to the Vagus Nerve

Understanding the clinical implications and mechanisms of VNS first requires an appreciation of the complex anatomy of the vagus nerve. As the tenth and longest cranial nerve, the vagus nerve derives its name from its extensive efferent and afferent projections between the brainstem and the viscera. These projections include sensory, motor, and parasympathetic fibers. The ventrolateral medulla oblongata in the brainstem houses several cell bodies for

the vagus nerve, including the nucleus ambiguus, spinal trigeminal nucleus, nucleus tractus solitarius (NTS), and the dorsal motor nucleus of the vagus nerve. Vagal projections exit or enter the brainstem through the jugular foramen, passing through the subarachnoid space before (if efferent) or after (if afferent) the medulla oblongata. Approximately 80% of vagus fibers are afferent, transmitting sensory information about the physiological states of various organs to the brainstem. The remaining 20% are efferent and transmit motor information from the brainstem to the viscera [2]. Efferent vagal fibers in the nucleus ambiguus project to the palate, pharynx, upper esophagus, and larynx and are important for branchial motor control, the gag reflex, and swallowing. General somatic sensory afferent fibers transmit sensory information from the pharynx, larynx, trachea, esophagus, meninges, and external ear to the spinal trigeminal nucleus. Special visceral sensory afferent ganglions of the vagus nerve transmit taste from the epiglottis and pharynx to the rostral NTS, while the general visceral sensory afferent ganglions transmit sensation from chemoreceptors and baroreceptors of the aortic arch, cardiorespiratory system, and digestive tract to the caudal NTS [3–5]. The NTS also contains afferent projections to various areas of the brain, including the locus coeruleus, which is the principal site of norepinephrine synthesis in the brain [6, 7]. Through this afferent pathway, the vagus nerve has the potential to modulate the release of cortical norepinephrine, which has diffuse effects throughout the brain, including on subcortical structures, such as the basal forebrain, thalamus [8–10], and cerebral cortex as well as the hippocampus, the hypothalamus, and the amygdala [2, 11]. In addition, the NTS projects to the nucleus ambiguus and the dorsal motor nucleus, both of which contain parasympathetic fibers [4].

Most parasympathetic nerve fibers in the autonomic nervous system (ANS) belong to the vagus nerve. As a result, the vagus nerve is heavily involved in the parasympathetic control of physiological states associated with rest, digestion, and recuperation [2]. Efferent preganglionic motor and parasympathetic fibers modulate parasympathetic control of the heart, lungs, esophagus, and parts of the gastrointestinal tract down to the splenic flexure, beyond which innervation occurs from parasympathetic nuclei in the sacral spinal cord [3, 4].

The vagus nerve is also an important regulator of metabolic homeostasis and immune functioning via feedback loops between efferent and afferent fibers. General visceral sensory afferent ganglions transmit levels of lipids, leptin, insulin, glucose, and other molecules from the gastrointestinal tract and liver to the NTS. These fibers form a feedback loop with efferent vagus fibers from the dorsal motor nucleus, which sense metabolic alterations in the brainstem and hypothalamus. Efferent fibers signal for changes in gastrointestinal motility and secretion, glycogen synthesis, hepatic glucose production, and pancreatic insulin secretion in order to maintain homeostasis [12].

The vagus nerve affects immune function and exerts anti-inflammatory effects through several pathways, including via the cholinergic anti-inflammatory pathway in combination with the spleen and the hypothalamic-pituitary-adrenal (HPA) axis. In the early 2000s, Kevin Tracey first proposed the potential for electrical or pharmacological stimulation of the vagus nerve to prevent inflammation via inhibiting release of cytokines through the cholinergic anti-inflammatory pathway (CAIP; Tracey [13, 14]). CAIP activation occurs when sensory afferent vagus projections detect increasing levels of pro-inflammatory molecules and transmit the information to brainstem nuclei [15, 16]. From brainstem nuclei, the information is transmitted to efferent motor vagus projections. Stimulation of the efferent vagus nerve releases acetylcholine on the following efferent targets: the liver, spleen, kidneys, and gastrointestinal tract. Acetylcholine binds to receptors on the target organ, which

deactivates macrophages and thus inhibits cytokine release of tumor-necrosis factor-alpha (TNF-α), interleukin-1, and high mobility group B1 [13–16]. Subsequent research demonstrates that macrophages in the spleen are an important part of the CAIP, as they are required for the vagus to attenuate production of TNF [17–20].

The HPA axis is involved in the body's response to stressors and releases glucocorticoid hormones, such as the stress hormone cortisol, into the bloodstream. Afferent vagus fibers project to the locus coeruleus, which has numerous projections to areas of the brain involved in emotion and stress, including the amygdala and the paraventricular nucleus of the hypothalamus (PVH). Neurons in the locus coeruleus then project to corticotropin-releasing factor neurons in the PVH, which release adrenocorticotropic hormone (ACTH). ACTH ultimately stimulates the release of glucocorticoids from the adrenal gland to inhibit the release of inflammatory glucocorticoids from the HPA axis and decrease peripheral inflammation [6, 21].

Invasive and Non-invasive Vagus Nerve Stimulation

Considering the vagus nerve's widespread projections and its ability to modulate physiological state, stimulation of the vagus nerve has been explored as an intervention for numerous disorders. Currently, invasive vagus nerve stimulation devices are Food and Drug Administration (FDA) approved for the treatment of treatment-resistant epilepsy and drug-resistant depression [22, 23]. Research also suggests VNS may be effective in the treatment of obesity, headaches, stroke, rheumatoid arthritis, sepsis, heart disease, and other conditions [23, 24]. Invasive VNS generally involves the surgical implantation of a pulse generator in the chest and stimulating electrodes on the cervical branch of the vagus nerve in the neck, which contains afferent and efferent projections [22]. VNS implantation and stimulation traditionally occur on the left vagus nerve in order to avoid right vagal projections to the sinoatrial node of the heart [4, 25]. Through its stimulation of efferent projections, VNS has direct consequences on structures innervated by the vagus; as a result, common side effects of VNS include hoarseness, cough, and voice alterations [23, 26–28]. While relatively safe, VNS implantation surgery also poses risks. A sample of 247 patients with VNS followed over a mean time of 12 years demonstrated an overall complication rate of 12.4% due to both hardware and surgical complications. The most common surgical complications included hematoma, infection, and vocal cord palsy, while the most common hardware complication was lead fracture/replacement. Considering VNS may be used as a lifelong treatment, patients may also require additional surgeries beyond initial implantation, including for battery and lead replacements [29]. While VNS clinical research is limited to individuals with disorders necessitating VNS implantation, VNS research can also be conducted preclinically via implantation in preclinical models.

An alternative method by which to engage the vagus nerve is transcutaneous, or non-invasive, vagus nerve stimulation (tVNS). tVNS is safe and well-tolerated with a low side effect profile, as suggested by a meta-analysis of tVNS safety that found the most common complaint was skin irritation from electrodes [30]. Generally, tVNS involves stimulation of either the afferent auricular branch of the vagus nerve at the left external ear or the cervical branch of the vagus at the neck. Minimally invasive, percutaneous VNS has also been applied at the external ear or cervical branch of the vagus nerve. Common external ear stimulation locations include the concha and tragus [25, 30].

While direct evidence of cortical norepinephrine release has not been reported from tVNS conducted in humans, several functional magnetic resonance imaging studies have measured blood oxygen level-dependent (BOLD) signal changes in the brain, suggestive of activation or deactivation patterns, to determine the neuromodulatory effects of tVNS. One found decreased BOLD signal in limbic areas, such as the amygdala, hippocampus, and parahippocampus, during tVNS similar to VNS [31]. Two studies in healthy individuals reported increased BOLD signal in the locus coeruleus during in-scanner tVNS [32, 33]. Similarly, another study found BOLD-signal activation in the brainstem, the region housing the epicenter of the vagus nerve, following anterior tVNS stimulation when compared to sham stimulation [34]. Further results from another study detected vagus somatosensory evoked potentials, a measure of vagus brainstem activity, on the scalps of participants following tVNS [35]. While promising, many of the studies have low sample sizes, prompting the need for additional investigations into the cortical consequences of tVNS.

Salivary alpha amylase (sAA), pupillary responses, and P300 event-related potentials have all been measured as proxies of noradrenergic functioning in the context of tVNS, with variable results. Some studies have found acutely increased pupillary responses [36–38], larger P300 values with easy stimuli [39], and increased sAA in healthy individuals [39, 40]. Another study in individuals with migraine found exhalatory-gated tVNS increased activation in noradrenergic nuclei (i.e., locus coeruleus) in response to trigeminal sensory afference [41]. Conversely, other studies have found no effect of tVNS on P300 [40, 42], pupil response [40, 43–45], and sAA [36, 45]. The challenges of assessing the effects of tVNS on the noradrenergic system have recently been reviewed [46]. Some of these challenges include between-study heterogeneity in stimulation devices, parameters, and sites; heterogeneity in locus coeruleus/noradrenergic measurement methods (e.g., pupillometry, P300, sAA); potential differences in the anatomy of the outer ear and the auricular branch of the vagus nerve between individuals; and potential age and disease-related changes in the locus coeruleus/noradrenergic system's structure and function [46]. Additional research on invasive VNS and the norepinephrine system has been captured elsewhere in a review [47].

While auricular tVNS only directly stimulates afferent vagus nerve fibers, it may also affect efferent vagal pathways. Research on patients undergoing open abdominal surgeries demonstrated increased muscle activity of the stomach during tVNS and increased gastrin levels, a vagus-dependent process, 3 h after tVNS compared to baseline [48]. Another study has since demonstrated that high-frequency tVNS increases gastric motility in healthy participants more than low-frequency stimulation [49]. Additionally, cardiac vagus efferent pathways are well known to modulate heart rate, and some research demonstrates tVNS modulation of cardiac function [25, 50–52]. One of the most common methods to measure the activity of the vagus nerve (i.e., vagal tone) is through projections to the sinoatrial node of the heart [53], through which the vagus nerve modulates heart rate and heart-rate variability (HRV; i.e., variation in the beat-to-beat intervals of heart rate). Importantly, the heart rhythm is controlled by numerous influences, including the sympathetic and parasympathetic divisions of the autonomic nervous system as well as other internal and external factors, such as catecholamine release, hormones, posture, and activity level, among others [54]. The effect of vagus activity on the heart is rapid and is reflected in the high frequency power component of HRV [54–56]. Despite clear evidence of the role of the vagus nerve in HRV, results vary as to whether tVNS impacts HRV [57, 58]. Differences in HRV measurement, tVNS application [57], and the health of participant samples may account for some of the variability between studies.

Taken together, these studies suggest the afferent auricular tVNS may be able to modulate organs in the viscera through both afferent and efferent projections. As tVNS research continues to grow, standardization efforts are underway. Common stimulation parameter terms and ranges across animal models and human neurological and psychiatric disorders have been reviewed previously [59]. More information on tVNS applications and targets is outlined in a 2020 consensus conference review paper [25].

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Chapter 1

Vagus Nerve Stimulation in Peripheral Targets

Ali Veysel Özden

Abstract

Although vagus nerve stimulation (VNS) is nowadays frequently used in the treatment of neuropsychiatric disorders including epilepsy, depression, and chronic pain such as migraine, an increasing number of studies mention its peripheral effects. Central effects of VNS as a cranial neuromodulation method can be prioritized, but it can cause physiological changes in many peripheral organs as well by the autonomic nervous system (ANS) activity modification. It can be argued that the peripheral effects of VNS can occur through many mechanisms such as reducing hyperinflammation, regulating circulation, determining the level of muscle tone, and controlling endocrine and exocrine secretions. In addition, the role of the vagus nerve on the gut-brain axis should always be kept in mind. However, the wide distribution of the vagus nerve and its extensive connections including those in the central nervous system complicate our understanding of its peripheral effects. VNS appears to have effects on the body (metabolism, neuronal activity, immune status, etc.) but we need more randomized placebo-controlled studies to fully understand and demonstrate the effects of VNS on peripheral targets. Biofeedback systems can be useful both for testing different stimulation parameters and for better understanding of peripheral effects.

Key words Vagus nerve stimulation, Peripheral effects, Peripheral organs, Autonomic nervous system, Sympathetic hyperactivity

1 Introduction

Autonomic nervous system (ANS) dysfunction (nowadays dysregulation) is related with many disorders such as fibromyalgia, irritable bowel syndrome (IBS), migraine, excess inflammation, obesity, chronic fatigue syndrome, depression, anxiety, etc. in the body. Sometimes sympathetic-parasympathetic imbalance is the etiologic factor, sometimes it develops after the primary problem. Chronic stress mostly is the underlying cause and increases the activity of the sympathetic nervous system (SNS) and creates the imbalance between the branches [1]. Simply, we can think of ANS dysregulation as the root of a tree. When there is a disorder in the root of the tree, as time goes on, we can see diseases or disorders in its branches according to genetic and environmental effects (*see* Fig. 1). ANS dysregulation also contributes to the chronicization

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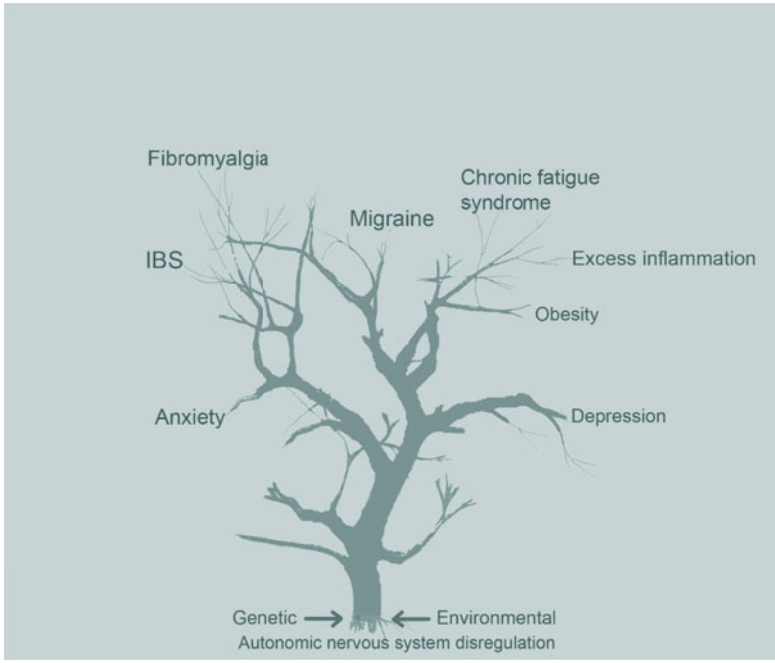


Autonomic nervous system dysfunction (dysregulation) gives rise to different related disorders under the effects of aging, genetic and environmental conditions. IBS: Irritable Bowel Syndrome

Fig. 1 Autonomic nervous system dysregulation and related disorders

of diseases that are seen commonly in society, increases morbidity and treatment costs. Although vagus nerve stimulation (VNS) is very new in terms of medical history, it has great potential to correct this imbalance and restore homeostasis.

It is generally thought that the branches of the ANS work oppositely like a seesaw but actually they work synergistically. Their activity can vary in different parts of the body, for example someone who has excessive sweating in his/her hands (sympathetic hyperactivity) may have bradycardia (sympathetic hypoactivity / parasympathetic hyperactivity). It is hard to evaluate the localized and overall activity of the ANS because of its activity difference in distinct body parts and its adaptive conformance to environmental changes. This makes it difficult to find the best stimulation parameters for the vagus nerve, even if preliminary assessment is made before or a closed-loop feedback system is used during administration. In their article, Bernston et al. explained the activities of the ANS in its complexity as shown in Table 1. Functional activities and functional responses of the organs depend on both the autonomic starting point together with direction and magnitude of the progress [2]. Heart rate variability (HRV) is a clinical measure presumed to assess ANS activity and it is mostly accepted as a gold standard method; however, vagal activity may not correlate with HRV

Table 1
Autonomic nervous system activities

Sympathetic Response	Parasympathetic Response		
	Increase ↑	No change ↔	Decrease ↓
Increase ↑	Coactivation	Uncoupled sympathetic activation	Reciprocal sympathetic activation
No change ↔	Uncoupled parasympathetic activation	Baseline	Uncoupled parasympathetic withdrawal
Decrease ↓	Reciprocal parasympathetic activation	Uncoupled sympathetic withdrawal	Coinhibition

Complex interrelation of the branches of the Autonomic Nervous System

[3]. Sympathetic or parasympathetic pathways to specific target tissues generally can be activated tonically or phasically, depending on current physiological requirements [4].

The ANS has a large distribution in the body and has many connections. Vagus nerve constitutes a big portion of it, provides brain-gut connection and modulation of the vagal activity can lead to many consequences. In addition to neurostimulation by electricity, there are many ways to affect the ANS such as meditation, biofeedback, yoga, etc. Electrical (transcutaneous auricular) or physiological (deep slow breathing) stimulation of the vagus can increase gastroduodenal motility and decrease somatic pain sensitivity [5]. So, if you rebalance the system to its optimum level and cure the problem in the root of the tree, you may solve many disorders in the body related to ANS dysfunction. Perhaps the absolute treatment will not be achieved but improvements in health can take place. In this chapter, electrical VNS in peripheral targets will be discussed in detail.

2 Peripheral Nervous and Musculoskeletal System Disorders

VNS, like the other cranial nerve stimulations, carries the potential of cerebral neuromodulation due to direct connection to the brain. On the other hand, peripheral stimulations are processed via the spinal cord before the stimulus enters the brain. In addition to that property, because the vagus nerve constitutes the huge portion of the parasympathetic nervous system (PNS) and it has wide distribution in the body, its stimulation can balance the disrupted ANS.

In chronic painful disorders (central and/or peripheral originated) VNS can decrease stress perception and sympathetic hyperactivity which increase pain sensation, also can diminish excess inflammation which contributes to pain production. VNS can modulate cerebral activity related with pain, and may modulate spinal cord secondly concluding with two results: (1) sympathetic discharge control; (2) decrement in neuronal hyperexcitability [6]. The antinociceptive effects of VNS (both cervical and auricular) seem to be primarily dependent on the nucleus tractus solitarius (NTS) and its projections to the locus coeruleus and raphe nuclei, followed by the subsequent activation of the descending noradrenergic and serotonergic systems in the spinal cord, including spinal opioid receptors, all of which inhibit second order nociceptive neurons in the spinal cord. VNS-induced activation of an ascending pain inhibitory pathway from the periaqueductal gray matter and raphe nuclei to the ventral posteromedial nucleus of the thalamus can contribute to the process. VNS can improve pain perception and mood concomitantly. This shows us the potential of VNS for various indications for one person and the different effects in apart parts of the body [7]. VNS could potentially mediate modulation of nociplastic pain including the inhibition of inflammation, the SNS and the pain neuromatrix.

Fibromyalgia is a syndrome in which chronic, widespread muscle tenderness exists as a result of central sensitization. Hypothalamic-hypophyseal-adrenal (HPA) axis distortion together with ANS dysfunction may contribute to the process or besides central sensitization, ANS dysfunction may play a pivot role in etiopathogenesis of musculoskeletal pain or in pain survival. HPA axis and ANS functioning showed hyporeactivity to applied stress (because already hyperactivated). This altered neuroendocrine responsiveness seems to be due to changes in hypothalamic function, not to a primary adrenal defect. It is unknown whether these neuroendocrine alterations are involved in the pathophysiology of fibromyalgia and contribute to its ongoing symptomatology or are a consequence of pain and its associated symptoms (e.g., fatigue, low physical fitness, sleep, and mood disorder) or both [8]. Exercise intolerance is a common problem in patients with fibromyalgia. Hyporeactive sympatho-adrenal system and the HPA-axis during exercise is seen [9]. This condition may be because of chronic muscle hypoperfusion in fibromyalgia and/or due to SNS which is already hyperactive, so cannot respond suitably to exercise in a normal physiologic manner. Fibromyalgia is characterized by heightened somatic pain sensitivity and there are deficits in descending pain inhibition. Fibromyalgia patients have hyperalgesic responses to painful stimuli. Thus, sympathetic hyperactivity may be associated with pain inhibitory pathways [10].

There are few studies on the use of VNS in pain, especially in peripherally located ones. VNS may have potential for the treatment of different painful conditions, peripheral or central, acute or chronic. Lange et al. investigated the effects of invasive VNS in fibromyalgia patients. Participants had the disease for at least 2 years and all were refractory to conventional pharmacological treatment. After 3 months of implantation, 2 of the 14 patients no longer met widespread pain or tenderness criteria for the diagnosis of fibromyalgia. The therapeutic effect seemed to increase over time in that additional participants attained both criteria at 11 months. Two patients did not tolerate stimulation [11]. Kutlu et al. used transcutaneous auricular VNS for 20 days in addition to exercise program in fibromyalgia patients. The group experiencing the VNS had better scores than the exercise only group but not statistically significant. The two groups had statistically significant improvements in pain, depression, anxiety, functionality, and life quality scores at the end of the treatment. They did the stimulation from both ears and declared no side effects [12].

When we look at the literature, it is seen that the effect of VNS on pain varies according to the selected participants (painless or painful; animal or human), the method applied (invasive or noninvasive VNS, type of painful stimulus) and stimulation parameters. Transcutaneous VNS can produce both anti- and pro-nociceptive effects within an experimental pain model in healthy volunteers and can modulate the cerebral response to experimental heat pain, without having a direct effect on pain thresholds [13, 14]. In rats, invasive VNS has an antinociceptive effect but the alteration in stimulation parameters change the response time [15]. In humans VNS (applied at the cymba conchae) was indistinguishable from sham (applied at the earlobes) and placebo (inactive device) in the reduction of experimental heat pain. Pain intensity decreased during all interventions as compared to no intervention [16]. In 48 healthy volunteers, 1-h left-sided auricular VNS increased evoked pain threshold according to sham VNS (no stimulation) whereas the non-noxious somatosensory perception was not affected. The results suggest an impact of VNS on central pain processing rather than on peripheral nociceptor activity [17]. Peripheral antinociception might play a role in pain reducing effect of VNS but it seems limited [18]. Icco et al. claimed that cervical noninvasive VNS has an effect on pain control by modulation of central descending pathways [19]. However, Alt et al. pointed out in their randomized sham-controlled cross-over study that noninvasive cervical VNS does not have an acute effect on pain intensity, perception, and nociception in healthy adults. But it reduces pain unpleasantness [20]. Central and peripheral autonomic neural circuits should be considered in the mechanism of VNS for reducing the pain perception. Noxious thermal stimulus increases sympathetic activity but noninvasive VNS modulates the

central nervous system response by the medulla that relay autonomic responses [21]. Studies of VNS effects on patients without pain have again conflicting results. Kirchner et al. tested the invasive VNS effect on experimental pain in epilepsy patients before and after the implantation. The stimulation decreased the pain and it was independent of the acute on-off cycles of the technique [22]. Borckardt et al. made their investigation on chronic depressed patients. Average duration of the VNS therapy among the participants was 35.33 months. On the day of the testing, they found that different VNS parameters may cause different effects on experimental pain perception in patients with chronic depression [23]. However, in their case report, Borckardt et al. mentioned that VNS decreases pain and depression in chronic usage but after 7 years of implantation, acute increases in experimental pain perception during VNS device activation were detected in the same patient [24].

So, VNS can be used for painful conditions such as chronic pelvic pain, fibromyalgia, trigeminal allodynia, and chronic headaches and migraines. Increasing evidence of VNS points to anti-inflammatory effects working in conjunction with both central and peripheral pain pathways [25]. Compared to non-vagal auricular stimulation, transcutaneous stimulation of concha reduces evoked deep pain intensity and temporal summation of mechanical pain in patients with chronic pelvic pain due to endometriosis [26]. VNS is most likely effective in inflammatory musculoskeletal disorders (psoriatic arthritis, ankylosing spondylitis, rheumatoid arthritis) by reducing inflammation [27, 28]. VNS is recommended for the treatment of fatigue associated with Sjögren's syndrome [29]. Short-term transcutaneous auricular VNS reduces pain and fatigue in systemic lupus erythematosus patients with no significant changes in cytokine levels; however, VNS still has a great potential in the treatment of this disease [30, 31].

There is limited evidence for the usage of VNS in disorders of peripheral nervous system, muscle tonus and bone. Percutaneous auricular VNS was applied to a therapy resistant cervical dystonia patient for 20 months and significant improvements were obtained in muscle pain and dystonic symptoms. Remarkable reduction of muscle tone was also achieved [32]. It is stated that VNS can increase bone mineral density in epileptic patients so VNS can have an effect on bone remodeling [33]. In rats, invasive VNS reduces hyperalgesia in chemotherapy induced neuropathy and improves recovery of somatosensory and motor function after peripheral nerve injury [34, 35]. VNS can improve limb rehabilitation after ischemic stroke [36, 37].

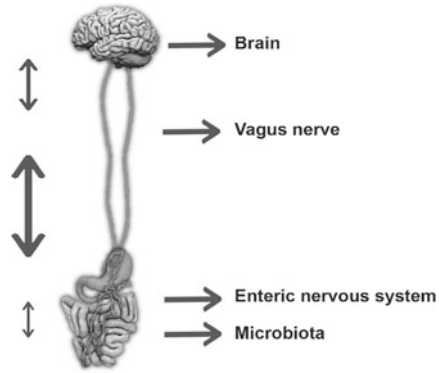
As a result, beyond its autonomic functions, VNS displays anti-inflammatory and analgesic properties. Therefore, it has a potential for the treatment of musculoskeletal diseases with inflammation and/or pain [38]. It may have important contributions in the treatment of peripheral musculoskeletal disorders with sympathetic

hyperactivity such as complex regional pain syndrome and bruxism [39–41]. Furthermore, it can be used to reduce inflammation and increase healing in disorders of the peripheral nervous system. It can be argued that VNS specifically affects the nociceptive mechanisms of clinical pain rather than experimental pain. Also, peripheral effects might be limited if there is not enough inflammation to be reduced. As a cranial neuromodulation method, it can be suggested that VNS will probably be more effective in cases where the sympathetic activity is continually high and neuroplastic changes are needed in the central nervous system.

3 Digestive System

In this part we will consider the usage of VNS in digestive system problems. There are studies and opinions about the use of the VNS in inflammatory bowel diseases (IBD) such as ulcerative colitis, but since inflammation is the subject of another section, disorders other than inflammation will be more emphasized here.

Visceral information is carried by the ANS to the brainstem and then transferred to upper centers in the brain (*see* Fig. 2). ANS related brain regions constitute the central autonomic network and establish homeostasis. Gut microbiota lie beneath this interaction and can be thought as an external milieu inside the body but affect the vagus nerve through its metabolites (microbiota-gut-brain axis). In return, the vagus nerve could modulate the gut microbiota through modifications of intestinal permeability and local immunity. Brain-gut bidirectional communication is maintained mostly by the vagus nerve which is involved in the transmission of visceral pain and has anti-inflammatory properties. This feature could be



The brain communicates with the microbiota through the vagus nerve and enteric nervous system. A problem in any of the parts will disrupt the pathway up and down.

Fig. 2 Components of the brain-gut axis

used to reduce inflammation in the body as observed in Crohn's disease, and ulcerative colitis. VNS has the potential to affect all this structure. VNS can alter stress related metabolic, hormonal changes and ANS imbalance. Stress modifies gastrointestinal motility and secretion together with immunity and gut blood flow, increases visceral sensitivity and intestinal permeability, causes dysbiosis; all these modifications may play a role in the pathophysiology of IBS and IBD. VNS could be used in IBS patients due to its antinociceptive, antidepressive, and anti-inflammatory properties. It is currently difficult to predict which patients will respond to VNS therapy for the gastrointestinal tract and to what extent. It is unclear which one is the cause or result of the faulty communication; the microbiota, the gut (enteric nervous system), the vagus, or the brain [42–46]? Autonomic vagal dysfunction has indeed been observed in functional and inflammatory digestive disorders. Given its extensive innervation of the gastrointestinal tract and its predominant role in parasympathetic regulation of inflammation and motility, the vagus nerve may be utilized as a powerful target for the treatment of gastrointestinal dysfunction and associated pain [47].

Bioelectric neuromodulation might be a valuable treatment for several gastrointestinal disorders but further investigations into the underlying mechanisms, placement of stimulating electrodes, stimulus parameters and patient populations to optimize effectiveness are still required [48]. VNS has the potential to affect the brain, ANS, enteric nervous system and microbiota but again there are very few and conflicting results in the literature. Haney et al. found that in mice, 1-h right-cervical invasive VNS did not affect the gastrointestinal microbiota when controlled 7 days after stimulation [49]. On the other hand, in the study of Campbell et al., left- or right-sided invasive cervical VNS for 50 days in pigs prevented abnormal gut flora following aortic constriction, which could contribute to its beneficial effects in heart failure patients. Heart function together with gut flora was preserved in animals receiving VNS therapy following pressure overload induction. The result in flora may be a direct effect of VNS or may be the result of improved heart function by VNS [50]. Based on this, it can be thought that the VNS effect is more visible in pathological conditions. It is noteworthy that the focus of attention of researchers on VNS and the digestive system is mostly the gastrointestinal tract motility. Stimulation of the afferent or efferent fibers of the vagus nerve can vary the result. Primarily afferent left-cervical VNS increased antral motility whereas primarily efferent VNS decreased it in anesthetized rats. Afferent pathway seems to be potentially more effective for facilitating occlusive contractions than the efferent pathway. Stimulation of the efferent pathway could induce multiple effects on gastric physiology. As mentioned, these effects were mostly dependent on the dose (pulse amplitude × pulse width) of the stimulation [51]. Due to the co-stimulation of afferent and efferent

fibers, the final result in cervical stimulation will be the sum of the effects of descending and ascending stimuli. Four-hour invasive left-sided cervical VNS promoted gastric emptying, caused dilation of the pyloric sphincter, increased antral contraction amplitude, and peristaltic velocity in rats [52]. In another study in rats, left-cervical VNS caused significant contractions in the middle colon and distal colon; however, in the proximal colon the contractions were less prominent [53]. Stakenborg et al. applied 5 min abdominal VNS in postoperative ileus model of mice and showed that VNS significantly improved intestinal transit and reduced intestinal inflammation [54].

In the study of Li et al., bilateral percutaneous auricular VNS for 45 min in anesthetized rats improved gastric emptying which was delayed due to cutaneous burn injury. VNS also lowered increased plasma noradrenaline [55]. Auricular VNS also improves constipation by enhancing colon motility in opioid-induced constipated rats. In colon tissues, VNS increased the protein expression of choline acetyltransferase and glial cell line-derived neurotrophic factor in myenteric interstitial cells of Cajal but decreased the protein expression of neural nitric oxide synthase [56]. Cajal's intramuscular interstitial cells play an important role in the formation of inhibitory responses that occur in the stomach after electrical stimulation of the vagal trunks in mice [57]. It can be stated that VNS not only increases neuronal activity, but also causes molecular and cellular changes, and can even affect genetic expression. In pigs, stimulation of the celiac branch of the abdominal vagus nerve increases contractions in the entire colon and transcutaneous auricular VNS may affect gastric motility differently depending on the protocol and/or baseline activity [58, 59]. Regardless of whether it is done from the ear, neck or abdomen, the VNS seems to affect the enteric nervous system and gastrointestinal tract motility. However, the characteristics of stimulation (pulse amplitude, pulse width, frequency, and polarity) may alter this effect [51].

Human studies about motility are less but promising. Thirty-minute left cymba concha transcutaneous auricular VNS reduced gastric myoelectric frequency, but did not affect resting energy expenditure in healthy adults [60]. Hong et al. enrolled patients requiring elective, open laparotomy in their study and performed a transcutaneous right-sided auricular VNS for 10 min after laparotomy before surgery. Muscle activity of the stomach was measured by electromyography before and during VNS. VNS decreased action potential frequency and increased action potential amplitude in the stomach compared to control [61]. Four hours of transcutaneous auricular VNS increases gastric motility in healthy participants. It was determined that the increase in motility was higher at 25 Hz than at 1 Hz [62]. Paulon et al. suggest that cervical noninvasive VNS may be beneficial for treating drug-refractory gastroparesis [63]. Whereas in their study with major colorectal

surgery patients, Chapman et al., did not find noninvasive cervical VNS as clinically effective [64]. In a small sized study, researchers found that transcutaneous VNS can improve gastroenteric symptoms in Parkinson's disease. Treatment was applied from the neck for 4 weeks with four stimulations per day [65].

VNS has been found to be effective in reducing pain and inflammation in research so far. In patients with Crohn's disease, at 6 and 12-month follow-ups, invasive left-cervical VNS induced remission, reduced disease activity and inflammation, rebalanced autonomic activity, and restored homeostatic vagal tone [66, 67]. Thirty-minute auricular VNS prevents the development of, and reverses established, acid-induced esophageal hypersensitivity by increasing parasympathetic tone in healthy participants [68]. There are also trials showing that VNS reduces the severity of injuries and improves wound healing in the gastrointestinal tract of animals. Ten-minute right-cervical VNS before burn injury improved intestinal barrier integrity with improved expression of the intestinal tight junction protein occludin in mice [69]. Ten-minute left-cervical VNS before hemorrhagic shock decreased gut and lung permeability in rats. This effect was seen also in splenectomized animals [70, 71]. In mice postinjury right-cervical VNS decreased intestinal permeability after burn when performed within 90 min [72]. Ten-minute right-cervical VNS prior to burn injury increased enteric glia activation and prevented burn induced intestinal barrier injury and permeability in mice. VNS also elevated intestinal glial fibrillary acidic protein expression to a greater degree than burn alone [73]. Invasive VNS reduces disease activity in acute pancreatitis models in mice but 2 weeks noninvasive cervical VNS has no effect on pain in patients with chronic pancreatitis. Nevertheless, both active and sham treatments showed improvement according to baseline [74, 75]. Regardless of the purpose or indication used, the central effects of VNS should always be kept in mind [76]. Beyond its central effects, VNS can lead to significant consequences even at the molecular level in the digestive system. These effects of VNS, starting from the brain and extending to the microbiota, seem to be dependent on many factors. Having too many variables (ANS baseline activity, stimulation site and parameters, etc.) makes it difficult to predict the expected result. Closed-loop systems controlled by artificial intelligence can offer a solution to this problem. Identifying suitable candidates for VNS treatment should perhaps be the first step in solving this.

4 Respiratory System

The studies concerning VNS effect on respiratory system and its disorders constitute a small number. Mostly related with allergic-inflammatory conditions and injury-healing episodes. A report with

very few cases shows us that the noninvasive VNS may be a feasible nonpharmacological treatment option for the management of acute asthma [77]. Miner et al. investigated VNS treatment for moderate to severe acute asthma exacerbations in patients not responding to at least 1 h of initial standard care therapy. Percutaneous electrode near the right carotid sheath was used for 60 min of VNS. Improvements in forced expiratory volume in 1 s (FEV1) and perceived dyspnea were detected with no serious adverse events. It is assumed that the bronchodilation is through afferent nerves [78]. However, in rats, electrical stimulation of both vagus nerves induces bronchoconstriction and this is potentiated by hyperinsulinemia accompanying obesity [79]. In anesthetized pigs low-voltage VNS ($\leq$2 V) attenuated histamine-induced bronchoconstriction. Afferent nerves are required (but not efferent) for this effect and systemic elevation in catecholamines is seen together. Stimulation of the vagus nerve with relatively high voltages (10–25 V) results in direct bronchoconstriction due to the release of acetylcholine from efferent parasympathetic nerves traveling in the cervical vagus nerve to the lung [80]. Cervical VNS causes tachypnea in anesthetized rats. The results are more pronounced on the left side than the right [81]. As can be seen, VNS has different effects on the respiratory system depending on the subject and method applied. VNS can increase bronchoconstriction with efferent fibers in asthma patients but can also reduce inflammation. So, the benefit or harm will vary depending on which is more dominant in the asthma patient; bronchoconstriction or decreasing inflammation?

In injuries of the lung, VNS seems beneficial possibly because of decreasing inflammation. Right-cervical VNS improves pulmonary function and regulates ANS activity in chemically induced acute lung injury in rabbits [82]. In rats, invasive left-cervical VNS is protective in lipopolysaccharide induced acute lung injury [83]. Because of the wide distribution of the vagus nerve and its extensive connections from the brain to the gut, it is difficult to precisely understand how VNS-related changes occur and where they begin. Krzyzaniak et al. showed that right-cervical VNS can diminish acute lung injury following skin burn and abdominal vagotomy can reverse this protective effect. Gut-lung axis is important for lung injury prevention [84]. Perhaps VNS first affected the gut and microbiota, and then visible changes took place in the lung. Lastly, it is suggested that VNS may be a useful treatment for idiopathic persistent hiccups, but it is fair to assume that it will not be effective in all patients [85]. There is a case report of invasive VNS being effective in this disorder [86]. Currently, we do not know exactly which patients will benefit or will benefit more from VNS therapy not only for hiccups but also for other diseases and disorders.

5 Obesity and Endocrine System

Obesity, which is a candidate to be the biggest problem of our age, continues to grow inevitably with the advancement of urbanization and technology. The sedentary lifestyle, whose duration is increasing thanks to television, computers, mobile phones and the internet, is leading us towards obesity. The development of synthetic or refined foods and the food industry also increases the incidence of obesity. Obese people with or without type II diabetes have higher sympathetic activity and they have an increased risk of developing type II diabetes in proportion to autonomic dysfunction [87–89]. Satiety signals are transmitted through the vagus nerve to the nucleus tractus solitarius in the brain stem [90]. Decreased vagus nerve activity or function can lead to decreased ability to respond to the transition between "hungry" and "fullness" state, continued sensitivity to orexigenic signals in the presence of nutrition, or decreased ability to respond to satiety hormones. Eventually, the infrastructure for obesity is established or preserved. So, the vagus nerve is a crucial component of appetite regulation via the gut-brain axis [91]. In the studies conducted in mice, it is stated that intra-abdominal fat is reduced by cutting the vagus nerve. With the cutting of the vagus, gastric emptying slows down, gastric capacity and enlargement decrease, and the feeling of hunger decreases [92, 93]. It seems contradictory but the vagus nerve is responsible for hunger, digestion, and satiety. Organ responses may not always progress in a consistent manner (such as a flat increase or decrease) with changes in vagal activity. As evidence, the study by Alkan et al., in which they stated that cervical invasive vagal inhibition leads to more weight loss than vagal stimulation, can be shown. In addition, the mechanism of the VNS is mediated through the fasting center; but, the mechanism of the vagal inhibition is thought to occur through the center of satiety [94]. On the other hand, in a small group of patients which include anorexia nervosa and bulimia nervosa, Melis et al. found that transcutaneous auricular VNS can provide weight gain with insignificant changes in HRV [95]. Since VNS can increase weight in anorexia and decrease it in obesity, it can be considered as a method to approach ideal body mass.

While a few decades ago, cutting the vagus nerve represented a well-accepted treatment for peptic ulcer disease; it is now clear that this nerve is extremely precious not only for the homeostasis of a variety of organ systems, but also for the regulation of appetite, mood, and inflammation [96]. The VNS effect on obesity is a new and intriguing area for researchers, medical device companies and policy makers for public health, although the last one is not so obvious. Kansagra et al. mentioned in their retrospective analysis that, VNS did not have a major clinically significant effect on body mass index (BMI) percentile in pediatric epilepsy patients during an

average follow-up of more than 4 years [97]. However, Burneo et al. showed that invasive VNS causes weight loss in epilepsy patients in their retrospective analysis [98]. In an uncontrolled observation, more than 2 years of invasive cervical VNS for severe, treatment-resistant depression was associated with significant, gradual weight loss despite no dieting or exercise. The weight loss was proportional to the initial BMI, that is, the more severe the obesity, the greater the weight loss. Weight loss did not correlate with changes in mood symptoms [99].

In high fat diet rats, bilateral auricular VNS reduced body weight, visceral fat and increased serum norepinephrine, β3-adrenoceptor expression in the brown adipose tissue but had no effect on food intake. Electrical VNS was applied 30 min daily for 6 weeks [100]. Additionally, in a case report, significant weight loss was seen in an epilepsy patient after VNS device implantation. So, VNS stopped for a while and then started again. In the second VNS therapy, the patient's weight and appetite improved. Desensitization to the initial effect on appetite occurred but seizure control was the same as in the first time [101]. In rats, the 27-day bilateral abdominal VNS was more effective in body weight and food intake reduction than unilateral abdominal VNS with the same duration. The result was associated with elevated vagal afferent signals [102]. In the study of Gil et al. rats were fed with a high-fat diet for 42 days and left abdominal VNS was applied too. Pulsating magnetic field was used as an external source of the current in the microstimulator wires connected to the vagus nerve. VNS significantly decreased food intake, body weight gain and adipose tissue weight according to control groups. Serum concentrations of ghrelin were increased, while serum levels of leptin were decreased [103]. In obese rats, Ziomber et al. applied pulsating electromagnetic field to the left abdominal vagus nerve with solenoid's electrodes placed on it. After 15 days of magnetic VNS, reduction of food intake and body weight was observed. The researchers explained that the magnetic field also affects feeding behavior by unknown mechanisms [104]. Auricular electrical stimulation, not specifically vagus, with the electrodes placed as anode at the ear lobe and cathode at the ear apex increased weight loss with a low-fat diet in obese rats. Stimulation was done 20 min a day, and 15 sessions. Researchers explained the effect by the increased subcutaneous white adipose tissue browning to augment energy expenditure [105]. Four weeks of left-cervical VNS reduced the body weight and the amount of mesenteric adipose tissue in rats. Both lower dietary intake and elevated energy expenditure independently contributed to this effect. VNS also increased the level of non-esterified fatty acids in plasma and mesenteric adipose tissue together with the increased expression of the gene for brain-derived neurotrophic factor in the hypothalamus [106]. Tseng et al. reported that left invasive cervical VNS reduced calorie intake in rats without weight

loss by central mechanisms [107]. In rats, battery free abdominal VNS with the electricity produced by the stomach movement decreased the body weight 38% less than the control groups within 100 days [108]. Beside VNS, gastric electrical stimulation has also been targeted to increase satiety and decrease obesity [109]. Although the vagus is not specifically stimulated, it does not seem possible to accept that the nerve is not affected. In pigs, 2 weeks of very high-frequency stimulation applied bilaterally at the abdominal vagus reduced daily food intake and chronic (14 weeks) bilateral thoracic VNS decreased weight gain, food consumption and sweet craving in adult obese minipigs [110, 111]. However, ventral abdominal VNS had no effect on food intake or body weight in swine after 4 weeks of implantation [112]. It can be seen that different applications of VNS may lead to different results in obesity.

VNS can also affect plasma concentrations of the glucose, insulin, and other hormones. In their study, Li et al. showed that daily 30 min bilateral transcutaneous auricular VNS for 27 days effectively inhibited the development of nociceptive hypersensitivity in Zucker diabetic fatty rats. This beneficial effect in nociceptive behavior is related to an elevated serotonin (5-HT) plasma concentration and an upregulated expression of 5-HT receptor type 1A (5-HT1AR) in hypothalamus [113]. Wang et al. used a similar stimulation protocol and animal group for 5 consecutive weeks. They showed that VNS triggers melatonin release in a tidal manner, reduces the glucose concentration and inhibits the progression of type II diabetes [114]. In high fat diet rats, 12-week left-cervical VNS decreased peripheral and brain insulin resistance [115]. Similarly, in obese rats, left-cervical VNS for 12 weeks significantly decreased plasma insulin, total cholesterol, triglyceride, low-density lipoprotein (LDL), visceral fat and significantly increased serum adiponectin [116]. In diabetic fatty rats, once daily 30 min right-sided transcutaneous auricular VNS was applied under anesthesia for 34 consecutive days. VNS improved insulin receptor reduction, and hyperglycemia progression [117].

In normal rats, left-cervical invasive VNS reduced corticotropin releasing factor induced adrenocorticotropic hormone (ACTH) release, but corticosterone levels were not significantly altered [118]. However, again in rats, the same VNS, acutely or chronically, did not activate the stress axis or affect the HPA axis or sympatho-adrenal system homeostasis. Chronic VNS (10 days) improved stress-induced HPA axis responses (plasma ACTH) in returning to baseline values [119]. In anesthetized rats, 2-h right-cervical afferent VNS caused a significant increase in blood glucose concentration that was not accompanied by an increase in serum insulin level. In contrast, right-cervical efferent VNS increased insulin levels [120]. In conscious rats, right- or left-sided cervical vagal nerve stimulation (1 h on 1 h off) was applied. Overnight,

VNS did not alter mean levels of blood pressure or heart rate, but increased fasted blood glucose levels. VNS impairs glucose tolerance possibly by inhibition of glucose induced insulin release [121]. Abdominal VNS in rats modulates glucose, insulin, and glucagon levels; however, the outcome depends on the direction (afferent or efferent) and frequency of the current. 15 Hz stimulation significantly increases glucose and glucagon while 40 kHz stimulation significantly decreases glucagon with no changes in glucose level [122]. Yin et al. showed that in rats, various abdominal VNS parameters (pulse width, frequency, intermittent or continuous) can affect blood glucose levels differently. There was no difference between unilateral and bilateral VNS [123]. In adult mini-pigs, 12-week bilateral abdominal VNS improves whole-body insulin sensitivity, increases ghrelin concentrations, reduces fasting glucose and insulin levels in diet-induced obesity by both peripheral and central mechanisms. VNS decreased weight gain 25% less than the non-stimulated group. Total fat mass reduced by 24%, but not visceral fat, which was not altered by vagal stimulation [124]. Apovian et al. claimed that in obesity intermittent abdominal vagal nerve blocking by electrical stimulation through 2 years causes weight loss and significant improvements in LDL and high-density lipoprotein (HDL) cholesterol, triglyceride, Hemoglobin A1c (HbA1c), systolic, and diastolic blood pressure levels [125]. VNS may affect the levels of glucose, insulin, and other molecules in different ways depending on the subject's condition at the time of administration. Under normal conditions and in a healthy organism, the VNS may not need to make any changes. However, as a neuromodulation method, the central effects of VNS should always be kept in mind [76].

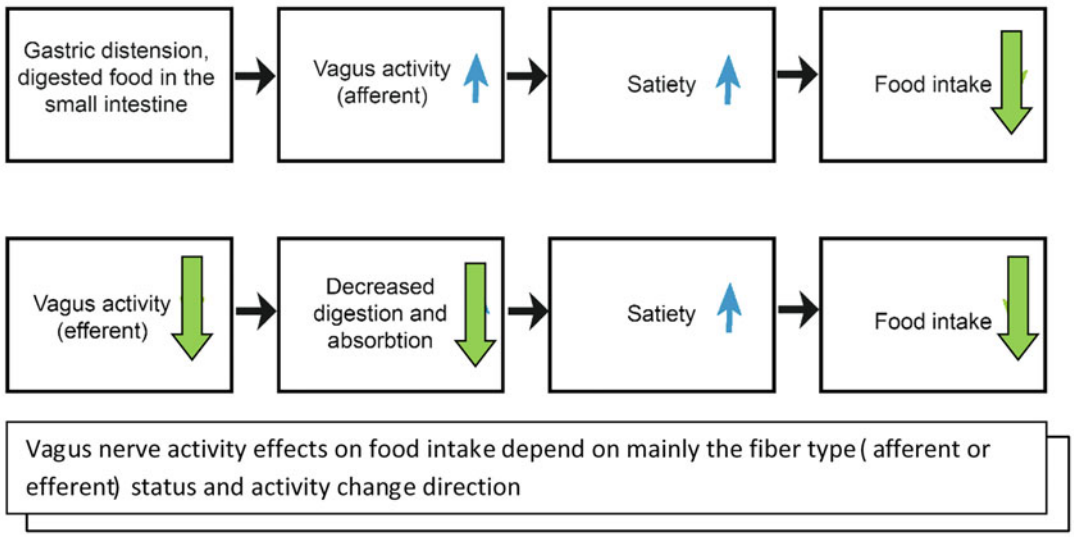
In healthy lean men undergoing an oral glucose tolerance test, short-term transcutaneous auricular VNS has no effect on HRV, plasma catecholamine and glucose levels, insulin sensitivity and insulin secretion [126]. Fourteen-minute left-sided transcutaneous auricular VNS in fasted healthy humans did not affect circulating glucose, free fatty acids, insulin, glucagon, or pancreatic polypeptide. It also had no effect on hepatic insulin sensitivity, lipid, and energy metabolism. No changes in HRV were detected [127]. In impaired glucose tolerance patients, 20 min, twice daily (total 40 min) 12-week noninvasive auricular VNS treatment decreased systolic blood pressure, glycosylated hemoglobin (HbA1c) and fasting plasma glucose [128]. A retrospective analysis was done in epilepsy patients taking chronic cervical VNS therapy. Blood glucose levels were found to be elevated in patients with long on and short off periods of stimulation, whereas blood glucose did not change or even decreased in patients with short on and long off periods. It is mentioned that chronic cervical VNS in patients with epilepsy is unlikely to induce glucose intolerance or hyperglycemia with commonly used stimulation parameters. However, stimulation

on times of longer than 25 s may bear a risk for hyperglycemia, especially if the stimulation off time is shorter than 200 s [129]. Liu et al. defined that chronic cervical VNS may elevate fasting blood glucose levels within normal limits with commonly used stimulation parameters in epilepsy patients. The changes in fasting blood glucose concentrations are negatively correlated with baseline levels [130]. In rheumatoid arthritis patients that have been treated with implanted VNS device for at least 3 months, acute VNS reduced early postprandial insulin secretion, but not other hormone levels (growth hormone, thyroid stimulating hormone, ACTH, prolactin, follicle-stimulating hormone, luteinizing hormone, cortisol, and catecholamine) and did not affect postprandial metabolism [131]. As with the aforementioned uses, when the features of VNS change, its effects may differ.

VNS can also affect centers related with food intake and calorie burn. Alicart et al. showed that 1-h left-sided cymba concha VNS has the ability to affect food desire in healthy participants [132]. In their study, Bodenlos et al. investigated whether left-cervical VNS might temporarily affect food cravings in patients with chronic, treatment-resistant depression. Devices of the patients were turned on and off to see the acute effect of VNS. VNS device activation was associated with a significant change in cravings-ratings for sweet foods according to control groups. VNS device settings, depression scores and BMI affect this desire [133]. They also analyzed the association among BMI, VNS device settings, and caloric intake during VNS on versus VNS off sessions in 16 adult patients using left-cervical VNS therapy for either epilepsy or depression. Activated VNS may suppress caloric intake in lean people. Higher output current was associated with consumption of more calories when the device was on versus off [134]. In chronic depression patients, olfactory and gustatory functions were investigated while the VNS device was on or off after the implantation of it. The patients were medication-free during the study. There were no statistically relevant differences concerning olfactory perception; however, statistically significant increases in the perception intensity of the flavors “sweet” and “bitter” during stimulation was noticed [135]. Vijgen et al. included refractory epilepsy patients and measured their basal metabolic rate (BMR) when VNS was turned on and off. VNS significantly increases BMR and energy expenditure. Brown adipose tissue activity possibly contributes to this change. VNS induced thermogenesis suggests that VNS may affect sympathetic mechanisms about that [136].

Despite the above studies, the efficacy and mechanisms of VNS in obesity remain unclear [137]. Obesity, chronic inflammation, insulin resistance, and type II diabetes mellitus are all interlinked and have a connection with ANS dysregulation. Vagus nerve signaling has an important role in the regulation of feeding behavior and metabolic homeostasis [138]. Sympathetic activation is

Table 2
Vagus nerve effects on food intake



associated with metabolic syndrome. VNS could benefit in the management of metabolic syndrome and insulin resistance [139]. The vagus nerve is closely linked to the control of body weight and provides communication between the gut and the brain. Interestingly, increasing or decreasing vagus activity can cause weight loss. Because it controls food intake, satiety, digestion, and absorption (Table 2).

But it must be kept in mind that vagus afferent and efferent signaling can affect each other. Cervical or abdominal (except auricular) VNS can induce both afferent and efferent pathways. The acute and chronic effects of VNS may differ [140]. Although VNS is considered to reduce sympathetic activity, rarely it can result in stimulation of the adrenal medulla and increased release of catecholamines in the systemic circulation, either via afferent vagus nerve fibers and activation of central pathways, or more directly via efferent vagus nerve fibers [141]. Chronic low-grade inflammation and metabolic derangements are closely related in obesity [142]. ANS dysregulation can be added to this relationship. Mainly, sympathetic hyperactivity dominates in this imbalance. As a part of ANS, vagus nerve cholinergic signaling plays a major role in controlling metabolic and immune homeostasis. Activation of cholinergic signaling by VNS carries the potential to rebalance ANS and control the obesity and obesity-associated disorders. Brain-imaging methods or other biofeedback systems together with computational models of VNS can help control obesity [143]. These methods may also facilitate our understanding of the mechanism of VNS.

6 Peripheral Circulatory System

In this part we will consider peripheral circulation, tissue injury and healing in other systems. ANS is closely related with the regulation of circulation including peripheral tissues and ANS dysfunction may cause disorders like Raynaud's phenomenon. Sympathetic hyperreactivity to stressful situations such as exposure to cold causes vasoconstriction and initiates the process in this disease. This condition is accompanied by the impairment of parasympathetic modulation and central impairment of autonomic function [144–146]. VNS could potentially help stabilize this ANS dysfunction and restore circulation. VNS may also have circulatory-enhancing effects in the absence of ANS dysfunction. Noninvasive auricular VNS can cause body fluid shifts between extracellular and intracellular spaces which can be detected by bioimpedance spectroscopy [147]. VNS also appears to have effects on bleeding and thrombosis. Czura et al. resected the ears of the anesthetized pigs partially and respectively before and after the invasive cervical VNS. VNS significantly decreased bleeding time and blood loss according to sham procedure (no stimulation). Reduced bleeding time after VNS was independent of changes in heart rate or blood pressure and correlated with increased local thrombin/antithrombin III complex generation. It is important that VNS directly or indirectly modulates thrombin activity at the site of injury, not in the systemic circulation as compared to the femoral arterial blood [148].

More research has been done on ischemia/reperfusion (I/R) models to see the effects of VNS on peripheral circulation and recovery. Xia et al. investigated whether left-cervical VNS has a protective effect on I/R injury in rat liver. One-hour vessel occlusion is followed with 24 h reperfusion. VNS was initiated 15 min after ischemia and continued 30 min. In the VNS group, reduction of the histological damage and reduction of the apoptosis rate of the hepatocytes were seen in the ischemic area. VNS increased the production of glutathione synthetase (GSS) and glutathione S-transferase (GST) proteins, plasma levels of glutathione and glutathione peroxidases [149]. In another study, 1 h ischemia 6 h reperfusion was applied to the rat liver with left-cervical VNS throughout the whole I/R process in the experiment group. VNS significantly decreased the necrosis in the I/R area. Serum levels of alanine aminotransferase, aspartate aminotransferase, and lactate dehydrogenase were also reduced by VNS. VNS significantly enhanced the nuclear factor erythroid 2-related factor 2/heme oxygenase-1 signaling in the liver [150]. Except for the liver, VNS seems to be beneficial for I/R injury of other organs. Wang et al. applied 45 min ischemia 6 h reperfusion to the rat left kidney, the right one is nephrectomized, with left-cervical VNS throughout the whole I/R process in the experiment group. The VNS

treatment reduces serum creatinine and blood urea nitrogen levels. VNS also significantly decreases nuclear factor kappa B, inducible nitric oxide synthase, nitrite/nitrate, myeloperoxidase levels and increases the superoxide dismutase level. The VNS treatment ameliorates renal injury [151]. Inoue et al. showed that left-cervical VNS 24–48 h before ischemia protects the kidney from I/R injury. Stimulation of vagal afferents or efferents is equally effective and attenuates the injury through α7nAChR-positive splenocytes [152]. Occlusion of the left femoral artery for 2.5 h followed by reperfusion for 2 h was performed in the rats. Left-cervical VNS was performed during the whole I/R process. VNS reduced cellular apoptosis, necrosis, and inflammatory cell infiltration in skeletal muscle and decreased serum creatine kinase and lactate dehydrogenase levels compared to sham VNS [153]. In a rat model of intestinal I/R injury, cervical invasive VNS can reduce arachidonic acid concentration and nuclear factor kappa B activation level in mesenteric lymph with reduced histological damage to the intestine [154]. Cervical VNS evoked action potentials in the abdominal vagus nerve and protected against trauma/hemorrhagic shock induced gut injury. VNS decreased the circulating noradrenaline levels and increased the intestinal blood flow [155]. Ortiz-Pomales et al. performed cervical VNS prior to 30% total body surface area burn in mice. VNS led to a reduction in burn-induced vascular permeability both in the skin and the lung compared to sham. The protective effects of VNS were independent of the spleen [156]. In rats, serum lactic acid level increased after burn injury. Twelve-minute electrical stimulation of the left vagal trunk in addition with bilateral cervical vagotomy decreased the level of lactic acid in burn injury [157].

In the light of studies, VNS appears to have an effect that improves circulation and accelerates wound healing. Although there are studies of VNS in central nervous system circulatory problems such as stroke, additional studies involving peripheral vessels and tissue perfusion are needed. Improved microcirculation may be one of the underlying causes of the effects of VNS.

7 Other Peripheral Disorders

Since the ANS is associated with many vital functions, VNS can be considered as a treatment option, especially in peripheral disorders accompanied by sympathetic hyperactivity. Normal ageing is associated with increases in sympathetic prevalence and/or decreases in vagal tone and overall variability. Two weeks of daily transcutaneous VNS improved measures of autonomic function, and some aspects of quality of life, mood, and sleep. Importantly, findings showed that improvements in measures of autonomic balance were more pronounced in participants with greater baseline sympathetic

prevalence [158]. As a neuromodulation method, VNS (either invasive or noninvasive) can affect the interoception, in other words, the body's perception by the brain [159]. So it can improve the proprioception and may reduce falls in the elderly. There is no clear data yet on the role of the vagus nerve in thermoregulation [160]. However, in freely moving rats, 2-h left-cervical VNS decreased brain and body temperature together with peripheral (tail) vasodilatation which is associated with heat dissipation [161]. In rats, electrical stimulation of cervical vagal afferent fibers inhibited the increases in brown adipose tissue sympathetic nerve activity and brown adipose tissue thermogenesis evoked by cold exposure [162].

VNS may promote anticancer immunity, slow tumorigenesis and reduce metastasis because tumor growth and metastasis is strongly associated with ANS activity levels [163, 164]. As stated earlier, VNS may be beneficial in allergic conditions, and in epilepsy patients, invasive VNS decreases histamine induced itching [165]. In rats with incomplete cervical spinal cord injury, left-cervical VNS improves forelimb strength recovery. VNS-dependent benefits can also be seen 1 week after the cessation of stimulation [166]. Cervical invasive VNS has an impact on voice as a side effect [167]. There is no data yet on the effect of noninvasive VNS on voice. If it is found to have an effect on the voice, it can be used in the future for voice rehabilitation, speech therapy, or stuttering. VNS could be used also for genitourinary system disorders. Auricular VNS seems beneficial in reducing endometriosis in mice [168]. Although the direct connection of the testicles with the vagus nerve has not been clearly demonstrated, there are also opinions suggesting that VNS may be beneficial through anti-inflammatory and neurohormonal pathways in male infertility. It should also be kept in mind that the desire for sexual intercourse is related to the activity of the SNS [169].

8 Conclusion

We need more randomized placebo-controlled studies involving a large number of participants to understand and put forth the effects of VNS on peripheral targets. When the studies done so far are evaluated, it can be argued that VNS can cause different effects on humans, ranging from positive and negative to no effect. It should be kept in mind that the sum of the different responses that occur in individuals after VNS may overshadow the individual effect of the application.

It seems that biofeedback systems are needed more than other neuromodulation methods to provide effective and targeted treatment in VNS. One of the reasons for that is the wide distribution of the vagus nerve and its extensive connections including those in the

central nervous system. Also, stimulating different locations (auricular VNS vs. cervical VNS) by different stimulation parameters may cause activation of different nerve fibers, and therefore have disparate potential effects. Co-stimulation of the afferent and efferent fibers complicates the process, and it becomes more difficult to assess whether the result is due to central or peripheral mechanisms. VNS appears to have effects on the body (metabolism, neuronal activity, immune status, etc.) and these effects are likely to depend on the subject's baseline ANS activity and body functions. It can be said that the vagus nerve and hence the VNS have a feature that tries to normalize the activities of live and complex organisms (especially mammals), which are necessary to maintain their lives other than fight or flight, and to bring the body to homeostasis. It may be clearer by explaining that the recovery after exercise, which is a type of stress with sympathetic hyperactivity, is accompanied by parasympathetic system activity. Diseases / disorders / health problems often lead to a situation in favor of sympathetic hyperactivity with the stress they create on the body. VNS may contribute to recovery by reducing increased sympathetic activity.

We can expect to see more research about VNS and its relations with microbiota, dysbiosis in the future. Although it is stated in the article that the most important nerve forming the gut-brain axis is the vagus, there are almost no studies examining the effect of VNS on microbiota. However, as with ANS dysfunction, it is possible to find many studies in which dysbiosis is associated with many diseases.

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Chapter 2

Vagal Nerve Stimulation Through the Lens of the Polyvagal Theory: Recruiting Neurophysiological Mechanisms to Dampen Threat Reactions and Promote Homeostatic Functions

Stephen Porges

Abstract

Vagal nerve stimulation, when viewed through the lens of the Polyvagal Theory, emphasizes three points. First, it emphasizes the link between the functions of the ventral vagal complex and symptoms reduced by vagal nerve stimulation, which functionally enhance mental and physical health. Second, it shifts the emphasis of vagal nerve stimulation from the entire nerve to select afferent pathways that communicate with brainstem areas that regulate both somatomotor and visceromotor efferent pathways originating in the ventral vagal complex. Third, by documenting the positive impact of vagal nerve stimulation, it acknowledges that trauma and chronic stress can "retune" autonomic function and disrupt the adaptive function of the ventral vagal complex in mitigating threat reactions and optimizing homeostatic functions of health, growth, and restoration. It is anticipated that methods and clinical targets of vagal nerve stimulation will evolve as we become more informed about the specific anatomical pathways traveling through the vagus. As this knowledge becomes integrated into technologies and procedures, there may be a category of vagal neuromodulators that function as neural exercises that would result in a more resilient autonomic nervous system and would not require chronic use.

Key words Vagal nerve stimulation, Ventral vagal complex, Social engagement system, Polyvagal Theory, Special efferent nerves

1 Introduction: Insights into Why Vagal Nerve Stimulation Optimizes Homeostatic Functions and Reduces Mental Health Symptoms

Polyvagal Theory highlights the evolutionary journey from asocial reptiles to social mammals during which the autonomic nervous system was repurposed and reorganized with unique structural and functional changes in the vagus. These changes in the autonomic nervous system enabled mammals to suppress defensive strategies, allowing both expressions of sociality and optimizing homeostatic functions. The product of this transition is an autonomic nervous system with a ventral vagal circuit that facilitates capacities to

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self-calm, to socially engage others, and to mitigate internal and external threat reactions in ourselves and others through social cues. When this uniquely mammalian ventral vagal pathway is chronically disrupted, mental and physical health problems arise.

The ventral vagus is part of a circuit that evolved, as a core mechanism, to mitigate threat, enhance sociality, and optimize visceral organ regulation (i.e., homeostasis). The neuroanatomy and neurophysiology of important vagal pathways can be objectively monitored via noninvasive techniques (i.e., measuring components of heart rate variability) and potentially targeted for neuromodulation with vagal nerve stimulation techniques. By exploring new frontiers within neuroanatomy and neurophysiology, we gain insights into other portals that may functionally enhance vagal communication, through both direct and indirect neural pathways to support more optimal health and performance.

For mammals, whose survival is dependent on their sociality to cooperate, to connect, and to co-regulate, the primordial defense programs dependent on sympathetic activation to support fight/flight behaviors, and vagal activation to support immobilization (e.g., death feigning), had to be harnessed and repurposed. Ultimately, this evolutionary process resulted in a reorganized brainstem network known as the ventral vagal complex, from which a discrete branch of the vagus nerve enabled the expression of several uniquely mammalian features—such as the ability to calm and to signal safety to conspecifics. As a consequence, sociality and feelings of safety became intrinsically linked with specific neurobiological processes capable of mitigating threats and supporting mental and physical health.

When this "calming" system is disrupted, markers of chronic stress and core features shared by several psychiatric conditions are expressed (e.g., flat facial affect, poor vocal prosody, hypervigilance, hyper-reactivity, and hypersensitivities to auditory, visual, and tactile stimuli). These features are not unique to a specific diagnosis but rather reflect an adaptive adjustment of the autonomic nervous system to support strategies of defense. Moreover, not only are the psychological and behavioral features of calmness and sociality disrupted, but there are also frequent disruptions in the function of visceral organs reflected in cardiopulmonary and digestive disorders. The commonly observed "parallel" symptoms manifest in mental health challenges and end organ disease are often misunderstood as unrelated comorbidities. From a Polyvagal perspective, somatic and mental health conditions share a dependency on vagal regulation of the autonomic nervous system. Dampened vagal regulation, often detected as low heart rate variability, appears to be a common neurobiological marker of both mental health conditions (e.g., depression, PTSD) and visceral disorders (e.g., IBS, heart disease, diabetes). It is this common feature of an

apparent diminished vagal influence that has directed interest in remediating and optimizing vagal function via methods of vagal nerve stimulation.

Anatomically, the vagus is a mixed nerve containing both efferent and afferent fibers originating in three primary brainstem nuclei: the dorsal nucleus of the vagus, the nucleus of the solitary tract, and the nucleus ambiguus, often described as the ventral nucleus of the vagus. A fourth nucleus, the spinal trigeminal nucleus that receives primary input from the trigeminal nerve, has a minor input from the vagus nerve. The complexity of the neuronal activity traveling through the nerve can render concepts like vagal tone and vagus nerve stimulation confusing and ambiguous. Since the various vagal pathways evolved at different stages of vertebrate evolution and support distinct adaptive functions, metrics evaluating vagal activity and devices designed to stimulate activity need to indicate which pathways are being monitored or stimulated.

The dorsal vagal nucleus is a structure shared with virtually all vertebrate species. In mammals, the dorsal vagus is predominantly an unmyelinated motor pathway that exits the dorsal side of the brainstem and primarily regulates organs below the diaphragm. This does not preclude the observation that some dorsal vagal fibers influence organs above the diaphragm such as the heart. For example, dorsal vagal pathways are presumed to be responsible for the clinical bradycardia observed in high-risk newborns [1]. Developmentally, the vagal nuclei parallel evolution, with the dorsal vagus becoming functional prior to the ventral vagus. The nucleus of the solitary tract functions as the sensory portal for the organs regulated by the dorsal vagus, although there is reported afferent activity traveling through the solitary tract originating in organs regulated by the ventral vagus. The third vagal nucleus, nucleus ambiguus, serves as the source nucleus for the efferent pathways of the ventral vagus. The ventral vagal pathways are myelinated and originate in the brainstem ventral to both the dorsal vagal nucleus and the nucleus of the solitary tract. All vagal nuclei have a viscerotropic representation in which different visceral organs are topographically linked to different portions of the nucleus. Unlike other ancestral vertebrates, the cardioinhibitory ventral vagus originating in the nucleus ambiguus is uniquely mammalian and is a marker of a repurposed autonomic nervous system that integrates sociality as a portal for enhancing cardioinhibitory vagal function.

2 The Emergence of the Ventral Vagal Complex and the Social Engagement System

The ventral vagus emerges from a brainstem area known as the ventral vagal complex. The ventral vagal complex contains the source nuclei of a subset of special visceral efferent pathways

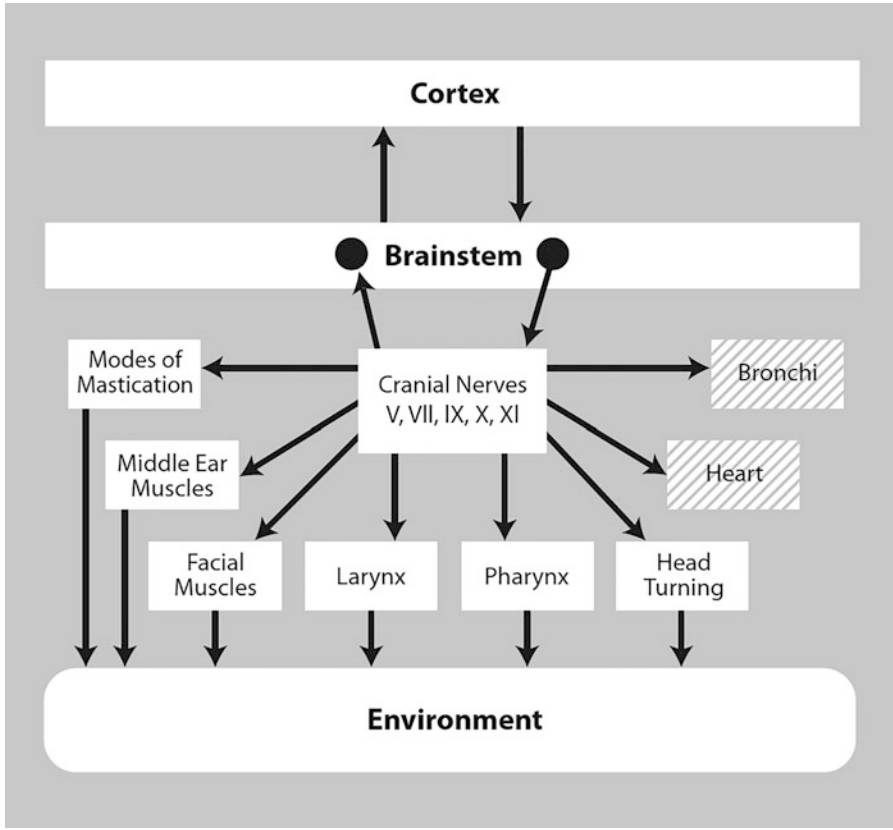

Fig. 1 The social engagement system consists of a somatomotor component (solid blocks) and a visceromotor component (dashed blocks). The somatomotor component involves special visceral efferent pathways that regulate the striated muscles of the face and head, while visceromotor component involves the myelinated vagus that regulates the heart and bronchi

traveling through five cranial nerves (i.e., trigeminal, facial, glossopharyngeal, vagus, and accessory) that innervate the striated muscles of the face and head. These pathways regulate aspects of social engagement (e.g., intonation of voice, facial expression) and permit the coordination of sucking, swallowing, vocalizing, and breathing. As illustrated in Fig. 1, the outputs of the social engagement system consist of motor pathways regulating: striated muscles of the face and head (i.e., somatomotor) and smooth and cardiac muscles of the heart and bronchi (i.e., visceromotor). The somatomotor component involves a subset of special visceral efferent pathways that regulate the striated muscles of the face and head. The visceromotor component involves the myelinated supradiaphragmatic ventral vagal pathway that regulates the heart and bronchi. Functionally, the social engagement system emerges from a face-heart connection that coordinates the heart with the muscles of the face and head. The initial function of the system is to coordinate sucking, swallowing, breathing, and vocalizing. Atypical coordination of this

system early in life may be life threatening and is an indicator of subsequent health related difficulties as well as problems in social behavior and emotional regulation.

Since the nerves included in the social engagement system are exclusively special visceral efferent, the exclusion of the hypoglossal nerve might be questioned]. A deeper explanation of PVT notes that although the Social Engagement System is composed of special visceral efferent pathways, being classified as special visceral efferent is not the sole criterion for inclusion. Given that PVT has its roots in evolution and embryology, cranial nerves are viewed from an embryological and not solely from an anatomical perspective. In structuring the functional social engagement system and its anatomical substrate, the ventral vagal complex, the inclusion of specific special visceral efferent nerves was based on two criteria: (1) the nerve arises from pharyngeal arches during embryonic development, and (2) there is evidence of neural communication between the nerve and the vagus. Applying these criteria resulted in clustering cranial nerves V, VII, IX, X, and XI, while excluding XII, the hypoglossal nerve. Consistent with these features, the sensory feedback into the motor centers regulating these specific special visceral pathways nerves may, via direct connections, provide additional portals to regulate the ventral vagus and functionally may act as a vagal nerve stimulator.

Ingestion and social engagement share common underlying neural structures. Links between ingestion and sociality are observable through human development and within other social mammals. Both ingestion and sociality are essential for survival as evidenced by the profound consequences of malnutrition and social isolation (e.g., failure to thrive) on physical and mental well-being. Signs indicating dysfunction of the processes dependent on the ventral vagal complex appear in several clinical disorders in which patients commonly present with flat facial affect, low prosodic vocalizations, and an autonomic nervous system in a state of high sympathetic activation (e.g., autism spectrum disorders, PTSD, depression, anxiety).

When fully developed, this system expresses two important biobehavioral features. First, bodily state is efficiently regulated to promote growth and restoration (e.g., visceral homeostasis). Functionally, this is accomplished by increasing the influence of myelinated vagal motor pathways on the cardiac pacemaker to calm the bodily state by slowing heart rate, inhibiting the sympathetic nervous system support of fight-or-flight mechanisms, dampening the stress response system of the hypothalamic-pituitary-adrenal axis (responsible for cortisol release), and reducing inflammation by modulating immune reactions (e.g., [2]). Second, the phylogenetically mammalian face-heart connection functions to convey a calm physiological state via facial expression and prosody

(intonation of voice), as well as regulate the middle-ear muscles to optimize species-specific listening within the frequency band used for social communication [2–6].

The brainstem source nuclei of the social engagement system are influenced by higher brain structures (i.e., top-down influences) and by sensory pathways from visceral organs (i.e., bottom-up influences). Direct pathways from the cortex to the brainstem (i.e., corticobulbar) reflect the influence of frontal areas of the cortex (i.e., upper motor neurons) on the medullary source nuclei of this system. Bottom-up influences occur via feedback through the sensory pathways of the vagus (e.g., tractus solitarius), conveying information from visceral organs to medullary areas (e.g., the nucleus of the solitary tract). In addition, these bottom-up pathways influence both the source nuclei of this system and the forebrain areas, via the insula, which are assumed to be involved in several psychiatric disorders, including depression and anxiety [7, 8]. In addition, the anatomical structures involved in the social engagement system have neurophysiological interactions with the hypothalamic-pituitary-adrenal axis, the social neuropeptides (e.g., oxytocin and vasopressin), and the immune system [9, 10].

3 Sensory Pathways from the Target Organs of the Social Engagement System Provide Potent Input to the Source Nuclei Regulating Both the Visceral and Somatic Components of the Social Engagement System

The source nucleus of the facial nerve forms the border of the nucleus ambiguus, and sensory pathways from both the facial and trigeminal nerves provide a primary sensory input to the nucleus ambiguus [2, 11]. Thus, the ventral vagal complex, consisting of the nucleus ambiguus and the nuclei of the trigeminal and facial nerves, is functionally related to the expression and experience of affective states and emotions. Activation of the somatomotor component (e.g., listening, ingestion, vocalizations, facial expressions) could trigger visceral changes that would support social engagement while modulating visceral state. Depending on whether there is an increase or decrease in the influence of the myelinated ventral vagal motor fibers on the sinoatrial node (i.e., increasing or decreasing the influence of the vagal brake), social engagement behaviors would be either promoted or impeded [2, 11]. For example, stimulation of visceral states that promote mobilization (i.e., fight-or-flight behaviors) would impede the ability to express social engagement behaviors.

The face-heart connection enables mammals to detect whether a conspecific is in a calm physiological state and safe to approach, or in a highly mobilized and reactive physiological state during which engagement would be dangerous. The face-heart connection

concurrently enables an individual to signal "safety" through patterns of facial expression and vocal intonation, which could potentially calm an agitated conspecific to form a social relationship. When the newer mammalian vagus is optimally functioning in social interactions, emotions are well regulated, vocal prosody is rich, and the autonomic nervous system supports calm, spontaneous social engagement behaviors. The face-heart system is bidirectional, with the newer myelinated vagal circuit influencing social interactions and positive social interactions influencing vagal function to optimize health, dampen stress-related physiological states, and support growth and restoration. Social communication and the ability to co-regulate interactions, via reciprocal social engagement systems, lead to a sense of connectedness and are important defining features of the human experience.

4 Vagal Pathways

The ventral vagus provides the primary vagal regulation to the organs above the diaphragm. In addition, this efferent vagal pathway may inform all aspects of the autonomic nervous system, including the enteric system [12, 13]. to optimize homeostatic function. This is distinct from the vagal pathways originating in the dorsal vagal nucleus, which are unmyelinated and provide the primary vagal regulation of organs below the diaphragm. The ventral vagal complex also regulates the striated muscles of the face and head and is greatly influenced by *afferent pathways traveling through the vagus, trigeminal, and facial nerves.* This point informs us that stimulation of specific pathways of the trigeminal and facial nerves may function similarly to vago-vagal reflexes. In neurophysiological terms, there are functional trigeminal-vagal and facial-vagal pathways that can efficiently and directly influence the cardioinhibitory actions of the ventral vagus on the heart, potentially without involving afferent pathways involving the nucleus of the solitary tract.

A functional ventral vagus, representing a restructuring of the autonomic nervous system in mammals, provides adaptive advantages that enable autonomic state to dynamically interface with the environment to downregulate threat reactivity to optimize survival by supporting sociality and nurturance. The dependence of survival on the ventral vagus is evident in the study of high-risk preterm infants, who may be born without a functional ventral vagus to support the coordination of suck-swallow-breath-vocalize processes.

In mammals, the brainstem areas regulating the heart and bronchi are interconnected with the areas regulating ingestion, facial expression, listening, breathing, and vocalizations, to form an integrated social engagement system. In fact, intonations of

vocalizations are mediated by the vagus, enabling prosodic features of voice to convey a relatively accurate index of vagal regulation of the heart [11, 14]. Understanding the anatomy and neurophysiology of the ventral vagal complex informs strategies to "retune" autonomic function through vagal nerve stimulation and highlights the different neuroanatomical portals that may provide access to target specific functions through neuromodulation.

It is helpful to get an estimate of the distribution of different types of vagal fibers. Although the vagus is a mixed nerve, it is primarily a sensory nerve with approximately 80% of its fibers being afferent [15, 16]. An estimated 80%–90% of vagal afferents are unmyelinated, multimodal C-fibers, conveying mainly chemical information. The remaining 10–20% are B- and A-fibers that convey mainly mechanical information [17]. Most efferent fibers involved in parasympathetic regulation are thinly myelinated or unmyelinated B- or C-fibers that arise from the dorsal vagal motor nucleus and the nucleus ambiguus [18]. In mammals, the current literature suggests that all myelinated vagal efferent fibers appear to originate in the nucleus ambiguus. Of the 20% of vagal fibers that are efferent, based on autopsy data with infants [19], approximately one-fifth are myelinated. In mammals, virtually all myelinated vagal efferent fibers appear to originate in the nucleus ambiguus. Thus, if we assume stability in the distribution ratio of myelinated to unmyelinated efferent fibers over the lifespan, then about 4% of all vagal fibers are myelinated efferent fibers with their primary targets being the heart and bronchi.

5 Vagal Nerve Stimulation: Assumed Mode of Action

Vagal nerve stimulation assumes that stimulation of vagal afferents has a direct effect on the regulation of higher brain structures. The source nucleus of the vagal afferents is the nucleus of the solitary tract. This medullary nucleus plays an important role in the regulation of homeostatic functions (e.g., behavioral state, digestion, respiration, and blood pressure) and in conveying information to higher brain structures. The nucleus of the solitary tract relays the incoming sensory information via three primary pathways: (1) feedback to regulate the periphery, (2) direct projections to the nucleus ambiguus and dorsal nucleus of the vagus, and (3) ascending projections to the forebrain, primarily through the parabrachial nucleus and the locus ceruleus. The parabrachial nucleus and the locus ceruleus send direct connections to all levels of the forebrain (e.g., the hypothalamus, amygdala, thalamic regions that control the insula, orbitofrontal, and prefrontal cortices), areas that have been implicated in neuropsychiatric disorders. Thus, vagal afferent stimulation has direct input to both the lower motor neurons in the brainstem and the upper motor neurons in the cortex that regulate

the social engagement system. Early reviews provide a detailed description of the neurophysiologic basis for the intervention [20] and explain the neural mechanisms involved in treating depression with vagal nerve stimulation [21]. Missing from these explanations is an acknowledgment of the communication between ventral vagal efferents and the source nuclei of the nerves that regulate striated muscles of the face and head (i.e., special visceral efferent pathways), which collectively form the motor part of the social engagement system. It is this interaction that is emphasized in the Polyvagal Theory may provide insights into identifying portals for vagal nerve stimulation in addition to the well-documented vago-vagal pathways [2, 10].

Extrapolating from the vagal nerve stimulation model, one might speculate that other forms of vagal stimulation might have beneficial effects. In an earlier review [22], the link between vagal nerve stimulation and the reduction of autistic behaviors was described. Murphy et al. [23] reported that vagal nerve stimulation reduced autistic-like behaviors. In their study, vagal stimulation was administered to six patients with hypothalamic hamartoma, a congenital brain malformation that is associated with medically refractory epilepsy and injurious autistic behavior. Four of the six patients had autistic behaviors that included poor communication, ritualisms, compulsions, poor social skills, and injury to self and others. The authors report that during vagal nerve stimulation. All four participants showed impressive improvements in behavior. In one subject, the behavioral improvements were immediately reversed when the vagal nerve stimulation was temporarily discontinued without worsening of seizure frequency.

Clues about the clinical effects of vagal nerve stimulation become more apparent when the neuroanatomy of the vagal pathways is understood. For example, vagal pathways from the dorsal nucleus of the vagus have powerful influences on the function of subdiaphragmatic organs. Spontaneous reactions to life threats, which trigger a massive dorsal vagal surge associated with syncope or diarrhea, have often been experienced by individuals who have been diagnosed with PTSD or clinical disorders related to the gut or sexual function. Similar reactions have been reported in response to implantable vagal nerve stimulators.

Unlike the direct effect of dorsal vagal pathways on visceral organs located below the diaphragm via dorsal vagal pathways, the effects of the ventral vagus impact on a neurophysiological substrate that is involved in social communication, ingestion, and feelings of safety derived from a calmer autonomic nervous system (e.g., slower heart rate and optimized oxygen saturation). For example, auricular vagal nerve stimulation has been used to effectively treat acute asthma [24, 25] and to increase peripheral blood oxygenation in diabetics [26].

6 Ventral Vagal Complex: A Portal for Vagal Nerve Stimulation

Neuroanatomical studies have demonstrated that visceromotor functions regulated by the ventral part of the nucleus ambiguus provide parasympathetic support for the somatomotor projections from the nucleus ambiguus as well as the trigeminal and facial nuclei. Neuroanatomical studies suggest that, unlike the dorsal vagal nucleus, which receives primary sensory input through the nucleus of the solitary tract, the nucleus ambiguus receives important sensory input from the trigeminal nerve. Moreover, the rostral region of the nucleus ambiguus communicates directly with the facial nucleus. This coupling of nucleus ambiguus with facial and trigeminal nuclei provides a plausible anatomical substrate supporting the observed coordination between visceromotor (cardioinhibitory ventral vagal) and somatomotor (striated muscles of the face and head) pathways regulating observable behavioral functions such as swallowing [27], sucking [28, 29], vocal intonation [3, 6] and, perhaps, facial expressions.

The neuroanatomical mapping of the ventral vagal complex provides insight into potential portals of neuromodulation that would, by having a positive impact on autonomic regulation, produce an autonomic state that would promote calmness, sociality, and optimal mental and physical health. Specifically, Polyvagal Theory explains why engagement of the special visceral efferent pathways, regulating the striated muscles of the face and head, could function as a "neural exercise" dynamically influencing cardioinhibitory pathways originating in the nucleus ambiguus. Moreover, the theory would suggest that there might be two synergistic pathways involving afferent input to enhance systemic autonomic regulation via ventral vagal efferent outflow.

7 Role of Afferents in Vagal Nerve Stimulation

These two pathways, one via input from the nucleus of the solitary tract and the other via afferents providing feedback to the function of special visceral efferent pathways, travel through the vagus, accessory, trigeminal, and facial nerves that directly impact the nucleus ambiguus. Recall that the source nuclei of the facial and trigeminal nerves provide primary sensory input to the nucleus ambiguus. The synergism of the functions of special visceral efferent pathways and the autonomic nervous system is observable in the complimentary expression and experience of emotion and forms the basis of the Social Engagement System [14]. Thus, the neuroanatomy provides two plausible routes for effect. First, the well-documented neuroanatomical communication between the nucleus of the solitary tract and both vagal motor nuclei.

Second, the neuroanatomical documentation of neural communication between nucleus ambiguus and both the trigeminal and facial nuclei. In addition, the cranial portion of the accessory nerve arises directly from the nucleus ambiguus.

Noninvasive technologies that target the trigeminal afferents on the forehead have had positive effects. For example, the Monarch eTNS System has been approved for pediatric ADHD [30]. Interestingly, the mode of action described on the website (https://www.monarch-etns.com/) does not acknowledge direct trigeminal-vagal connections. In the description, the company states that "*The trigeminal nerve projects directly or indirectly to specific areas of the brain, such as the locus coeruleus, nucleus tractus solitarius, thalamus, and the cerebral cortex, which are involved in attention-deficit hyperactivity disorder (ADHD) and other disorders.*" Although this statement is consistent with the neuroanatomy literature, it is incomplete. Another form of auricular neurostimulation via percutaneous electrical nerve field stimulation reduced functional abdominal pain in adolescents [31]. Interestingly, only participants who had poor vagal efficiency (the dynamic relationship between cardiac vagal tone, measured by respiration sinus arrhythmia, and heart rate) benefited from the stimulation. Vagal efficiency was quantified as the dynamic relationship between cardiac vagal tone (measured by respiratory sinus arrhythmia) and heart rate. Moreover, when the stimulation was terminated, the pain returned. These observations suggest in vagal efficiency may identify a feature that would optimize the effectiveness of auricular neurostimulation [32].

It appears that noninvasive technologies for vagal nerve stimulation have fewer side effects. A plausible explanation of the fewer side effects relative to invasive technologies is that stimulation through a select system of afferents avoids the profound possibility of directly stimulating the efferents—primarily the dorsal vagal nucleus. Functionally, a noninvasive approach of stimulating the auricular afferents of the vagus, facial, and trigeminal nerves provide an efficient means of targeting the ventral vagal pathways that neurophysiologically support homeostasis and not adaptive defense systems dependent on either access or conservation of metabolic resources. This would be observed as an increase in sympathetic-adrenal activation to support fight/flight behavior or a conservation reaction of greatly reducing metabolic needs through a dorsal vagal reaction. Also, by stimulating the afferent limb, the endogenous neural regulation circuit may have endogenous constraints that hypothetically could evaluate the characteristics of the input stimulation and titrate the output to be more consistent with naturally occurring signaling. Thus, indirect vagal nerve stimulation through the afferent limb is likely not a simple pass-through circuit in which more input (i.e., stimulation) produces more outflow.

This focus on afferents that have direct and potentially indirect, via the nucleus of the solitary tract, input to the ventral vagal complex is consistent with Polyvagal Theory. It provides a theoretical conceptualization of exploring other portals to the ventral vagal complex that could potentially enhance and optimize the outflow of the 4% of vagal fibers that appear to be cardioinhibitory while providing an organizing signal to many attributes of the autonomic nervous system that support the homeostatic functions of health, growth, and restoration. These points support the conclusion that by refining our understanding of the component neural pathways within the vagus, we can refine the protocol to more be more efficient and more directly targeted.

8 Emphasis on Vagal Afferents: Aortic Depressor Nerve

Additional support for targeting afferent pathways to stimulate vagal efferent outflow is documented in publications that contrast direct electrical stimulation of the dorsal nucleus of the vagus with stimulation of the aortic depressor nerve, an afferent pathway going to the nucleus of the solitary tract. The aortic depressor nerve is often used in studies of baroreceptor function since the aortic depressor nerve contains almost exclusively afferent vagal fibers [33] that travel directly to the nucleus of the solitary tract. In rabbits, the aortic depressor nerve is morphologically separated from the vagus in the cervical region and is easily accessible for stimulation.

In the anesthetized rabbit, stimulation of the dorsal nucleus of the vagus produced significant bradycardia without an increase in respiratory sinus arrhythmia. In contrast stimulation of the aortic depressor nerve, which communicates with both the ventral and dorsal vagal nuclei via the nucleus of the solitary tract, produced even greater bradycardia with a substantial increase in respiratory sinus arrhythmia (5). These findings suggest that vagal fibers discharging following stimulation of the dorsal nucleus of the vagus did not have a respiratory rhythm, while the bradycardia was immediate and similar in latency to that observed following aortic depressor nerve stimulation. Interestingly, the magnitude of bradycardia in response to stimulation of the dorsal nucleus of the vagus was about 50% of the magnitude elicited by aortic nerve stimulation, a technique assumed to recruit vagal fibers from both the ventral and dorsal vagal nuclei. The attribution of 50% of the magnitude of the heart rate component of the baroreceptor reflex to each vagal system is consistent with Machado and Brody [34].

The powerful selective effect of vagal afferent stimulation via the aortic depressor nerve is detailed in a systematic study [35]. Following the onset of aortic depressor nerve stimulation, heart rate, and arterial blood pressure decreased, and respiratory rate and

amplitude remained unchanged. In addition, the amplitude of respiratory sinus arrhythmia increased dramatically. All variables returned to pre-stimulation levels during the post-stimulation period. These studies provide insight into the unique influence of the ventral vagal pathway in producing respiratory sinus arrhythmia and the potential cumulative influences of both vagal nuclei in producing bradycardia.

9 Recruiting the Acoustic Efferent Limb of the Social Engagement System: A Portal to Stimulate Vagal Efferent Activity

The mechanisms mediating sound sensitivities, autonomic and behavioral state regulation, social engagement, and auditory processing are generally assumed to represent disparate response systems. From an empirical perspective, behavioral state regulation and social engagement are manifested in observable behaviors; autonomic regulation is observed in peripheral physiology; sound sensitivities are manifested through subjective experiences; and auditory processing is manifested in expressive and/or receptive language skills. Polyvagal Theory leads us to think about a common brainstem mechanism regulating both autonomic state and the structures involved in listening. The theory proposes a strategy applying evolution as an organizing principle to understand a link between sound sensitivities, behavioral and autonomic state regulation, social engagement, and auditory processing.

According to the Polyvagal Theory, the well-documented phylogenetic shift in neural regulation of the autonomic nervous system provided mammals with a neural circuit that promotes social interactions in safe contexts. Functionally, sociality evolved by the mechanisms that supported calm physiological states and an ability to "broadcast" physiological state through facial expressions and especially vocalizations within a frequency band distinct from the lower frequencies associated with reptilian predators. This "mammalian" circuit functions as the neural substrate for an integrated, social engagement system that dampens the functional impact of sounds outside the frequency band of vocalizations employed for social communication and regulates the neural circuits that optimize autonomic regulation, behavioral state, social engagement, and auditory processing.

As illustrated in Fig. 1, the social engagement system includes a somatomotor component with special visceral efferent pathways traveling through five cranial nerves (trigeminal nerve (V), facial nerve (VII), glossopharyngeal nerve (IX), vagus nerve (X) and accessory nerve (XI)) that regulate the striated muscles of the face and head (e.g., middle-ear muscles, laryngeal muscles, muscles of mastication, facial muscles, pharyngeal muscles, and head turning

muscles). The somatomotor component regulates the pitch of vocalizations via pharyngeal and laryngeal vagal pathways, the tension on the middle-ear muscles to enhance detection and processing of vocalizations, and facial expressions that supplement communicated messages and allow a listener to provide feedback to a vocalizer. But can this system be recruited to function as an acoustic simulator enhancing the function of the neural circuits dependent on the ventral vagal complex?

Polyvagal Theory proposes that the social engagement system is a portal to the ventral vagus, an important detail relevant to the field of vagal nerve stimulation. Specifically, the structures of the social engagement system include a visceromotor component that adjusts an individual's physiological state to complement facial and vocal signals of social communication. In effect, an individual functionally broadcasts their physiological state of calmness or defense through voice and face. Coincident with this projection of physiological state, the middle-ear muscles change muscle tone to either facilitate the processing of vocalizations (e.g., by dampening the transfer of acoustic energy from low frequencies in the background) or enhance the processing of low-frequency acoustic energy at the expense of the ability to extract the acoustic information of vocalizations. Because, via evolution, low-frequency acoustic information signals the proximity of predators or environmental danger.

Based on the Polyvagal Theory, sound sensitivities and deficits in autonomic state regulation, social engagement, and auditory processing may be paralleled by reduced vagal influences on the heart and bronchi via myelinated vagal pathways. Such a reduction is an adaptive response strategy to support mobilization in dangerous environments (i.e., fight-flight behaviors). Since the Polyvagal Theory articulates a hierarchy of neural circuits, the metabolic resources necessary for fight-flight behaviors are not efficiently available unless there is a retraction of the cardioinhibitory ventral vagal influences.

The cardioinhibitory ventral vagal pathway functions as a powerful vagal bake on the heart's pacemaker. As a vagal brake, this pathway provides an efficient calming mechanism that functions to slow heart rate, optimize oxygenation of the blood, and downregulate the sympathetic nervous system. This neurophysiological calming mechanism downregulates defensive states and enables social engagement behaviors to spontaneously occur. Thus, the neural mechanisms defining the social engagement system provide a plausible model to explain why sound sensitivities and difficulties in both auditory processing and autonomic state regulation are prevalent in individuals with clinical symptoms that are, in part, due to an autonomic nervous system locked in a state of defense. Consistent with this model, features of the social engagement system may serve as efficient pathways to the ventral vagus. *Due to*

the integrated nature of the social engagement system, its structural components of the system may provide portals to stimulate vagal activity.

In our research, we hypothesized that an accessible portal to the ventral vagus and the entire integrated social engagement system could be accessed by presenting acoustic stimulation that dynamically challenged the neural regulation of the middle-ear muscles. The middle ear muscles facilitate the extraction of human speech by dampening the transmission of low-frequency noise from the external environment to the inner ear. Sound enters the outer ear and travels, through the external auditory canal, to the eardrum where it is transduced by the structures of the middle ear (i.e., small bones comprising the ossicular chain) that connect the eardrum with the cochlea. Complementing the ascending pathways are descending pathways that regulate the middle-ear muscles. The descending pathways functionally adjust the transfer function of the middle ear structures to determine the energy (i.e., attenuate, pass, or amplify) of specific frequencies that reach the inner ear [3, 6, 36].

Hypothetically, modulation of acoustic frequencies should dynamically adjust middle ear muscle tone by actively recruiting special visceral efferent pathways that travel through the trigeminal and facial nerves. Changes in middle ear muscle tone, should via feedback, through the sensory branches of the trigeminal and facial nerves, impact directly on the ventral vagal complex including influencing the cardioinhibitory ventral vagal pathways. The product of this feedback circuit could function as a neural exercise enhancing the regulation of both middle ear muscles and cardioinhibitory ventral vagal pathways (i.e., vagal brake).

Polyvagal Theory has led to the plausible hypothesis that the recruitment and "exercise" of the middle ear muscles can rehabilitate both visceromotor and somatomotor components of the integrated social engagement system. Thus, by engaging the descending auditory processing limb (via facial and trigeminal special visceral efferent pathways) to enhance neural regulation of the middle ear muscles, one could also increase cardioinhibitory ventral vagal tone. The clinical products from this type of neuromodulation would function to reduce sound sensitivities, behavioral and autonomic state regulation deficiencies, auditory processing and social engagement deficits, chronic pain and associated conditions, anxiety disorders, and other conditions for which vagal nerve stimulation treatment has demonstrated efficacy. Functionally, this technology would function as a noninvasive acoustic vagal nerve stimulator to increase cardioinhibitory vagal activity to the heart via an auditory pathway.

Our research has documented [37, 38] that modulation of acoustic stimulation within the frequency band defined by the Speech Intelligibility Index (American National Standards Institute [39], a metric used to define the acoustic frequencies necessary to

understand human speech, results in enhanced function of the social engagement system. These studies reported improved auditory processing, reduced auditory hypersensitivities, changes in autonomic state characterized by increased vagal regulation of the heart (e.g., increases in respiratory sinus arrhythmia), and increased spontaneous social behaviors (e.g., sharing). The research supports the theoretical expectations that variations in acoustic stimulation may trigger neural mechanisms that regulate the entire social engagement system, including enhanced vagal regulation of bodily organs. The use of modulated acoustic stimulation to optimize functions dependent on the Social Engagement System has been patented [40] and includes an approved claim for application for acoustic vagal nerve stimulation.

10 Summary

Vagal nerve stimulation, when viewed through the lens of the Polyvagal Theory, emphasizes three points. First, it emphasizes the link between the functions of the ventral vagal complex and symptoms reduced by vagal nerve stimulation, which functionally enhance mental and physical health. Second, it shifts the emphasis of vagal nerve stimulation from the entire nerve to select afferent pathways that communicate with brainstem areas that regulate both somatomotor and visceromotor efferent pathways originating in the ventral vagal complex. Third, by documenting the positive impact of vagal nerve stimulation, it acknowledges that trauma and chronic stress can "retune" autonomic function and disrupt the adaptive function of the ventral vagal complex in mitigating threat reactions and optimizing homeostatic functions of health, growth, and restoration.

Understanding that the core neurophysiological mechanisms involved in brainstem structures regulated by the ventral vagal complex are expressed in a variety of clinical disorders provides an important neurophysiological justification for the application of vagal stimulation and identifies functions (e.g., voice, heart rate patterns, facial expression, ingestion, digestion) that can be quantified as objective outcome variables. Polyvagal Theory, by defining an integrated social engagement system dependent on the ventral vagal complex, provides an organizing principle to understand how and why vagal nerve stimulation is effective in reducing apparently disparate symptoms. Through the lens of the theory, the observed disparate effects reflect processes dependent on the ventral vagal complex that are expressed through special visceral and ventral vagal pathways (*see* Fig. 1). The theory leads to a shift in focus from higher brain structures to the foundational lower brainstem processes related to mammalian survival. This does not preclude upstream influences from the ventral vagal complex and

communication with the nucleus of the solitary tract [41, 42]. When these survival systems are disrupted by chronic illness and adversity history (trauma), the autonomic nervous system is "retuned" and frequently locked in a state that supports defense and threat reactions, while disrupting homeostatic functions. The theory proposes that effective neuromodulation via vagal nerve stimulation enables a return to an autonomic state requiring the ventral vagus that supports more optimal mental and physical health.

Through the polyvagal lens, new questions about vagal nerve stimulation emerge. For example, if the positive effects are due to the processing of afferent information by brainstem structures regulating processes mediated by the ventral vagal complex, then it is important to explore other potential afferent pathways that may form the basis for neuromodulation of vagal efferent pathways. In the future, there may be a category of vagal neuromodulators that function as neural exercises that would result in a more resilient autonomic nervous system and would not require chronic use. By emphasizing afferent limbs in vagal stimulation, numerous opportunities emerge. These may include strategies that systematically change vagal outflow through posture, movement, vocalization, behavior, and even mental effort. Functionally, any manipulation that reliably changes cardioinhibitory vagal regulation of the heart could be conceptualized as a vagal exercise.

It is anticipated that methods and clinical targets of vagal nerve stimulation will evolve as we become more informed about the specific anatomical pathways traveling through the vagus. As this knowledge becomes integrated into technologies and procedures, the science will lead to new forms and applications of vagal stimulation. The short history of clinical vagal nerve stimulation has already moved from placing an electrode around the vagus to a more functional portal leveraging an easily accessible afferent on the ear and face. There are several benefits in emphasizing the afferent limb as a target for vagal nerve stimulation: (1) there is no possibility of directly stimulating efferent pathways, especially those going to organs below the diaphragm resulting in adverse effects on digestive and sexual functions, (2) stimulation of the afferent limb of a feedback system does not bypass the central regulatory functions of brainstem nuclei in the interpretation of the afferent input and may provide a neural signal that is more functional to the target organ.

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Chapter 3

Heart Rate Variability as a Biomarker for Electrical Vagus Nerve Stimulation

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Abstract

Electrical vagus nerve stimulation (eVNS) comprises an array of non-pharmacologic techniques used to stimulate afferent fibers of the vagus nerve. Despite the increasing use of eVNS both in clinical settings and in research, there is still a need for a valid biological marker that can be used to indicate the action of eVNS in the central as well as in the peripheral nervous system. The present chapter focuses on one of the most prominent candidates for such a biomarker, namely, heart rate variability (HRV). We provide arguments for the s of specific HRV parameters to this aim, namely, vagally-mediated HRV, which reflects cardiac vagal activity. We describe the mechanism of action underlying the expected effects of eVNS on cardiac vagal activity and perform a literature review, which shows mixed evidence on the increase of cardiac vagal activity due to eVNS. Furthermore, we point out relevant methodological caveats of current literature on eVNS and discuss how to avoid them in a study designed to investigate the relationship between eVNS and cardiac vagal activity. Based on this, we describe what is necessary to properly measure cardiac vagal activity during the use of eVNS, and discuss considerations that need to be observed when it comes to designing studies that aim to address this relationship.

Key words Vagus nerve stimulation, Transcutaneous vagus nerve stimulation, Heart rate variability, Cardiac vagal activity, Neurovisceral integration model, Biomarkers

1 Introduction

Electrical vagus nerve stimulation (eVNS) comprises an array of non-pharmacologic techniques used to stimulate afferent fibers of the vagus nerve. In particular, implanted vagus nerve stimulation (iVNS), transcutaneous cervical vagus nerve stimulation (tcVNS), and transcutaneous auricular vagus nerve stimulation (taVNS) have increasingly received attention in the last years [1]. Currently, eVNS has predominantly been applied as adjunctive therapy of refractory epilepsy and resistant depression, and has shown potential for the treatment of refractory migraine, cluster headache, Alzheimer's disease, treatment-resistant anxiety disorders, bipolar disorder, and obesity [2, 3]. Moreover, eVNS, especially taVNS, has recently found use in psychophysiological research because of

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its potential to infer a causal relationship between stimulated brain areas and their related cognitive as well as affective functions [4]. Despite these promising applications, research with eVNS, especially with taVNS and tcVNS, is still at an early stage. Consequently, the precise mechanisms of action underlying its effects on cognitive, affective, and on related physiological processes are still unknown, and the evidence provided to date is predominantly heterogeneous. With the aim to further develop the state of evidence on the effectiveness of eVNS on certain pathological and psychophysiological processes, there is a need for a valid biological marker that can be reliably used to indicate whether the vagus nerve is being stimulated both afferently and efferently. The present chapter focuses on one of the most prominent candidates for a biomarker that is thought to be affected by eVNS, namely, heart rate variability (HRV) [5]. In the following paragraphs, we describe the possible working mechanisms of eVNS on heart rate variability, review the state of evidence on this putative relationship, and summarize important considerations that need to be observed for further investigating and using this potential candidate as a biomarker in the context of eVNS.

1.1 Working Mechanisms of Electrical Vagus Nerve Stimulation Underlying Effects on Heart Rate Variability

The vagus nerve efferently provides the primary parasympathetic regulation of the heart. This regulation occurs through the vagal projections to the sinoatrial node, atrioventricular node, and atrial cardiac muscle [6, 7]. Efferent vagal activity releases acetylcholine, which binds to the muscarinic receptors in the heart, thus reducing heart rate [7]. The heart is innervated differently by both sides of the vagus nerve: Whereas fibers originating from the left vagus nerve supply the atrioventricular node, causing decremental conduction, those from the right vagus nerve innervate the sinoatrial node, which is mainly responsible for depolarization rates and for triggering bradycardia [8].

In a healthy human organism, the heart rate is under the tonic influence of the neural output of the vagus nerve, being responsible for decreasing heart rate, and the sympathetic nerves, which accelerate it. At rest, both the vagus and sympathetic nerves are tonically active, with vagal activity being dominant [9]. This interaction between vagus and sympathetic nerves results in an oscillation in the time interval between adjacent heartbeats, which is called heart rate variability [10]. The term coined to refer to the activity of the vagus nerve regulating cardiac functioning is cardiac vagal activity (CVA), and specific parameters derived from HRV measurements are considered reliable indices of CVA [10, 11]. For this reason, they are often called vagally-mediated HRV (vmHRV) parameters [11]. Because vmHRV reflects CVA (*see*Subheading 2), in the present chapter, we focus on the relationship between these specific HRV parameters and eVNS, and refer to them hereafter as CVA.

Integrative theories such as the neurovisceral integration model [9, 12] describe the brain areas that are an integral part of neuroanatomical pathways involving the vagus nerve, thus explaining the connection between the brain and the heart, which may be affected by eVNS. The neurovisceral integration model assumes a connection between the prefrontal cortex and the heart through the central autonomic network and the vagus nerve [9, 12]. The central autonomic network is an integrated component of an internal regulation system through which the brain controls visceromotor, neuroendocrine, and behavioral responses that are critical for self-regulation, and includes the anterior cingulate cortex, the insula, and the ventromedial prefrontal cortex [9]. The optimal activation of the neural pathways within this network is crucial for showing adaptive responses to a changing environment. This adaptation can affect executive performance, emotional responses, and physiological outcomes in which the vagus nerve is involved, with one prominent physiological outcome being CVA [9]. At rest, the medial prefrontal cortex exerts inhibitory control over the amygdala, indirectly enhancing cardiac control via the vagus nerve, which is reflected in an increase of CVA. Because of the common underlying neurovisceral self-regulation mechanisms, a higher resting CVA is associated with improved adaptability of the organism [9, 12].

Although the exact working mechanisms of eVNS in the brain are currently poorly understood, some functional resonance imaging (fMRI) studies provide evidence that eVNS can activate the vagal pathways as described by the neurovisceral integration model. In comparison to baseline measurements or sham stimulation—a stimulation that has the same characteristics as normal eVNS, but consists either of electrical stimulation of different nerves, a weaker stimulation, or an absence of stimulation—eVNS has shown to increase nucleus tractus solitarius activity. This finding provides evidence that an electrical signal applied to vagal fibers is projected to the medulla oblongata in the brainstem [13–18]. The activity of brain areas such as the amygdala has, in turn, shown heterogeneous results, i.e., in some studies activity of the amygdala increased while in others it decreased [13, 15, 16, 19–21]. Importantly, cortical areas such as cingulate and prefrontal cortices have been reported to show increased activity [13, 16, 21, 22]. These areas affected by eVNS are part of the central autonomic network, an internal regulation system through which the brain controls autonomic processes [23]. For this reason, besides the specified areas of the central nervous system, eVNS is also thought to modulate autonomic (peripheral) function, including CVA [5]. In the next section, we review the literature that investigated this relationship.

1.2 Effects of Electrical Vagus Nerve Stimulation on Cardiac Vagal Activity: State of Evidence

As described in Subheading 1.1, there is evidence supporting that eVNS can afferently upregulate brain areas known to be an integral part of vagal pathways within the central nervous system. However, little is known on the interrelation between the afferent and efferent vagal pathways resulting from this brain modulation. The mechanism influencing CVA—i.e., efferent vagal activation—may differ from the mechanistic target of eVNS, namely, the afferent vagal activation [1]. Similarly, the expected mechanism of action underlying iVNS, tcVNS, and taVNS may also strongly vary between one another: iVNS invasively targets the cervical branch of the vagus nerve, whereas both tcVNS and taVNS transcutaneously target branches of the vagus nerve, with the cervical branch being targeted by tcVNS and the auricular branch by taVNS [24]. Hence, the neuromodulating effect of these three technologies may differ considerably from one another [25]. For this reason, the state of evidence on the effects of eVNS on CVA is separately discussed in the following subsection. Furthermore, to avoid speculations due to possibly different mechanisms of action, the following literature review does not include findings from animal models, thus only focusing on studies with humans. Finally, we clearly state whether the findings have originated from a study with healthy participants or patients.

1.2.1 Transcutaneous Vagus Nerve Stimulation and Cardiac Vagal Activity

Evidence towards an increase of CVA during both tcVNS and taVNS is rather mixed. Some studies using taVNS have shown that taVNS both healthy participants and tinnitus patients, compared to sham stimulation, can increase CVA [26–28]. However, this enhancing effect of taVNS on CVA could not be shown in other studies with healthy participants [29–32]. Furthermore, three studies with healthy participants have shown that CVA can increase during both taVNS and sham stimulation, which raises questions about the current use of sham conditions in studies with taVNS [33–35]. A similar heterogeneity can be found in the sparse amount of studies using tcVNS to affect CVA: Whereas there is evidence in healthy participants towards an increase of CVA during tcVNS [36], this increase could not be found in another study [37]. These contradictory results might be due to varying study protocols, different tasks, or the use of different stimulation parameters in these studies, e.g., the use of varying positioning of the electrodes on the ear and different stimulation intensities. Standardized stimulation protocols are needed to help understand the relationship between transcutaneous VNS and CVA.

1.2.2 Implanted Vagus Nerve Stimulation and Cardiac Vagal Activity

Because of the clinical character of iVNS, studies on the relationship between iVNS and CVA usually take place in the context of clinical treatments, and literature comprises two main groups of studies: studies that test the acute effects of iVNS on CVA, and studies that investigate medium- to long-term effects of iVNS on CVA. Some of

these studies additionally provide information derived from supplementary analyses on the differences between responders and non-responders. This differentiation often aims at investigating whether CVA—as well as HRV in general—serves as a reliable predictor for treatment responsiveness [38–40].

To investigate the acute effects of iVNS on CVA, different vmHRV measurements have been considered within an iVNS' duty cycle, i.e., during the stimulation period and stimulation-free intervals [41, 42]. In patients with major depression, CVA was higher during the stimulation phase (60 s) compared to the stimulation-free phase (60 s out of 5 min), although the influence of medication, in particular antidepressants, could not be ruled out [41]. One study with epilepsy patients also showed an increase of CVA during the stimulation phase compared to a stimulation-free period [42].

In studies investigating medium- to long-term effects, usually the measurement time point before starting the clinical treatment with iVNS—termed baseline—is compared to the end of the use of this device in the treatment. This investigation led to mixed findings. Compared to baseline, permanent iVNS during 8 months on average evoked an increase in night CVA in epilepsy patients [43]. This improvement in night CVA has also been found in children with epilepsy, however, this improvement was transient, so that some rebound effects have been observed in the long-term follow-up [44]. In the opposite direction, iVNS over 3 years in patients with heart failure led to persistent improvements in CVA compared to baseline at 12, 24, and 36 months, although they did not reach normal levels [45]. In opposition to this evidence for the increase of CVA following iVNS, other studies failed to show increases in CVA in epilepsy patients as a consequence of iVNS [46, 47]. However, it is not clear whether these null results are due to very low sample sizes, with $N = 15$ [46] and $N = 8$ [47]. Another study even found a decrease of CVA after iVNS in children with epilepsy [48]. Similar to the mixed findings in the literature on tcVNS and taVNS, this heterogeneity may be due to major differences in the study protocols, for instance, the strong discrepancies in the length of iVNS application. Moreover, the literature reviewed presents relevant methodological caveats, such as low sample sizes, often a lack of control or sham conditions, and a lack of an appropriate statistical analysis (*see* Subheading 3).

Despite its limitations, the literature reviewed in the present subsection additionally provides insights into the potential use of CVA as a predictor of the patient's responsiveness to iVNS. Studies approaching this question showed that epilepsy patients predominantly have lower baseline CVA when compared to healthy participants, which may indicate an impaired adaptability of the autonomic nervous system in those patients [9, 38–40, 44, 48,

49]. Longer durations of epilepsy seem to be associated with a stronger decrease in CVA [44]. Moreover, studies that categorize participants as high-responders—e.g., participants who showed a reduction of at least 50% in seizure frequency during iVNS application [38]—and low-responders—less than 50% reduction in seizure frequency—point out the potential of CVA as a predictor for responsiveness to iVNS. High-responders have been found to have higher CVA than low-responders [38–40, 49], so that presurgical CVA measurements have shown to be positively associated with the patients' responsiveness to iVNS [39].

To summarize, two conclusions can be drawn based on the literature reviewed on the effects of eVNS on CVA: first, there is mixed evidence on the improving effects of tcVNS, taVNS, and iVNS on CVA, both acutely, which is the case for tcVNS and taVNS, and medium- to long-term, which is the case for iVNS. Second, low-responders may have a more pronounced impairment of cardiac autonomic control than high-responders, and epilepsy patients with higher baseline CVA are more likely to respond to iVNS treatment [39, 40]. The potential of preoperative CVA as a predictor of whether epilepsy patients will show a decreased number of seizures may help to optimize patient selection in an objective manner [39]. This potential role of CVA to index future iVNS responsiveness has been tested only in the context of refractory epilepsy, thus studies on other possible applications are needed.

In the following section, we summarize the materials that are necessary for studies that address eVNS and CVA.

2 Materials

2.1 Electrical Vagus Nerve Stimulation

Different devices have a dedicated use for either iVNS, tcVNS, or taVNS, and each of these types of stimulation tools presents advantages and disadvantages. Compared to the other tools, iVNS is well-established and its validity has been tested more widely. At the same time, iVNS is expensive and is associated with peri- and post-implantation risks. Furthermore, the stimulation is diffuse and can extend to efferent vagal fibers of the cervical vagus nerve, thus leading to adverse effects [1]. More affordable for end-users and free of chirurgical risks, tcVNS has been developed as a practical hand-held device, but provides strong currents, as the stimulation needs to pass through the skin barrier. Similarly to iVNS, this results in diffuse stimulation fields in the neck, leading to the stimulation of other cervical nerves besides the vagus nerve [1]. The target branch for taVNS, the auricular branch of the vagus nerve, primarily consists of afferent vagal fibers, thus taVNS is relatively free of direct efferent stimulation. However, taVNS is thought to recruit not only vagal but also non-vagal auricular nerves, which may lead to implications that remain unknown [50, 51].

Table 1
Overview of possible stimulation parameters for electrical vagus nerve stimulation

Parameter	Definition	Range found in the literature
Current intensity[a]	The magnitude of current relative to the isoelectric baseline, expressed in milliamperes (mA)	0.2 mA[b] (iVNS) [39] to 10 mA (taVNS) [55]
Pulse width[a]	The determined period of time elapsing from the beginning to the end of one pulse cycle, expressed in microseconds (μs)	250 μs (iVNS) [46] to 1 ms (taVNS) [56]
Pulse frequency[a]	The number of pulse cycles that are generated per unit of time (s), expressed in hertz (Hz)	0.5 Hz (taVNS) [57] to 100 Hz (taVNS) [58]
Duty cycle[a]	The ratio of ON time to OFF time, often expressed in seconds of ON and up to minutes of OFF times (s)	30 s ON, 5 min OFF (iVNS) [48] to continuous (taVNS) [35]
Session or intervention duration	Duration of a stimulation session, expressed in minutes for acute effects to months for chronic effects	10 min (taVNS) [28] to 40 months (iVNS) [38]
Side of stimulation	Choice between stimulating the right or the left vagus nerve	Left vagus nerve (iVNS) [48], right vagus nerve (taVNS) [28], bilateral stimulation (taVNS) [58]
Type of sham stimulation[c]	Application of sham stimulation as a control condition	Stimulation of another area (e.g., earlobe for taVNS) [59], no current (taVNS), lower frequency or intensity (tcVNS) [37]
Location of the stimulation[d]	The auricular area used for stimulation (taVNS)	Cymba conchae [60], tragus [35], ear canal [20]

[a]Definition from Bo et al. (2017)
[b]This was the chosen initial intensity which lasted 2 weeks until it was increased
[c]Mostly adopted in studies with both transcutaneous cervical and transcutaneous auricular vagus nerve stimulation
[d]Primarily relevant for transcutaneous auricular nerve stimulation

An array of stimulation parameters needs to be considered before the administration of eVNS. Parameters of eVNS can vary on stimulation intensity, pulse width, frequency, duty cycle, and session duration [52], stimulation side (right or left ear for taVNS, or left and right cervical branch of the vagus nerve for iVNS and tcVNS), the type of sham stimulation, and location of the stimulation (e.g., tragus or cymba conchae stimulation for taVNS) [25]. An overview of these stimulation parameters and their range presented in the literature can be found in Table 1. The impact of each of these stimulation parameters on psychophysiology and clinical outcomes is incompletely understood, as dose-response studies in humans are scarce [1]. Some of the reasons might be (1) the high number of combinations that are possible between

parameters, (2) the high number of cognitive and autonomic processes in which the vagus nerve is involved [9, 53], and (3) the lack of a gold-standard biomarker to assess the acute effects of eVNS on physiological, cognitive, and affective outcomes [54]. Despite first attempts to address the effects of parametrization on CVA [1], it is still unclear what an ideal combination of stimulation parameters for enhancing CVA could be, which could explain the high heterogeneity of findings in studies using eVNS. Thus, it is time to carry out further studies that aim at understanding the parametric-specific effects of eVNS on CVA in order to optimize this tool for its use along with CVA measurements as a biomarker.

Another important methodological question is which type of control should be adopted to measure the effects of eVNS on CVA. Studies using tcVNS often create a sham stimulation by producing a signal with a lower frequency. This change delivers a mild buzzing sensation similar to the tcVNS device but is not thought to result in stimulation of the vagus nerve, and consequently should not influence CVA [37]. Sham in studies with taVNS, in turn, mainly consists of placing the surface electrodes on an ear area that is thought to be free of vagal innervation, such as the earlobe [61]. Studies with iVNS often do not have any control group, and the majority of studies with a control group, instead of having a sham stimulation, tests CVA in healthy participants in order to compare them to the intervention group [41]. However, this test takes place without any relation to iVNS, as no iVNS device is attached to the healthy participants during the CVA measurements. For ethical reasons, a sham condition is possible when the aim is not to specifically compare patients with healthy individuals but with other patients. In this case, the sham condition can consist of a group of patients whose iVNS is off over the testing period, as done before [62]. Nonetheless, the use of a sham condition is crucial, as it may rule out a bias that derives from participants' expectations [1]. Furthermore, the use of a sham condition in conjunction with the measurement of at least baseline and post-phase allows for more expressive findings (*see* Subheading 3). For these reasons, the use of sham condition is highly recommendable.

2.2 Cardiac Vagal Activity

Studies that investigate the effect of eVNS on HRV often consider an array of parameters derived from an electrocardiogram (ECG) signal. However, as stated in Subheading 1.1, vmHRV parameters are especially promising candidates of biomarkers of iVNS because they are the output of efferent signals originating from the central autonomic network via the vagus nerve [9]. Thus, it is meaningful for both research and clinical purposes to differentiate HRV in general from CVA and to prefer HRV parameters that specifically reflect CVA.

As already stated, specific HRV parameters are considered reliable indices of CVA [10, 11]. Root mean square of successive differences (RMSSD) and the percentage of successive normal sinus RR intervals of more than 50 ms (pNN50) in the time domain, as well as high frequency (HF) in the frequency domain (when breathing frequency is comprised between 9 and 24 cpm), are commonly used vmHRV parameters. HF reflects respiratory sinus arrhythmia under certain conditions [63], whereas RMSSD is relatively free of respiratory influences [11]. The ratio between low and high frequencies of HRV (LF/HF) has often been used as an index of sympathovagal balance. However, the role of this HRV parameter in depicting sympathovagal balance has recently been discarded, because no real physiological conclusion can be drawn from this finding [64]. Both the low-frequency band of the frequency domain (LF) and heart rate have also often been used, but they do not allow for an accurate interpretation of the autonomic changes found during iVNS, as they reflect mixed inputs from the sympathetic and parasympathetic branches [10, 65]. Thus, LF and heart rate cannot be linked specifically to CVA.

To record values known to reflect CVA, several recording techniques can be used. These techniques measure HRV through an ECG signal, interbeat intervals (IBI), or photoplethysmography [11]. ECG tends to be more accurate than IBI and photoplethysmography because it allows for a more precise artifact correction, which is a very important step in signal pre-processing [66]. Photoplethysmography can have the disadvantage of not providing an accurate recording during stress because stress induces changes in pulse transit time, which result from changes in the elasticity of the arteries and cannot be detected through IBI [7]. Therefore, for research and clinical purposes we recommend using ECG to measure the effects of eVNS on CVA.

In the next section, we discuss what is necessary to properly measure CVA during eVNS, and discuss considerations that need to be taken into account to be able to evaluate the impact of eVNS on CVA.

3 Methods

3.1 Considerations for a Proper Cardiac Vagal Activity Measurement During Electrical Vagus Nerve Stimulation

As stated in Subheading 1.2.2, research with eVNS, especially iVNS, can present some major caveats that need to be avoided when it comes to testing whether CVA is a valid biomarker for eVNS. Low sample size—which likely leads to an underpowered study—the lack of a sham condition, the lack of the measurement of at least baseline and post-intervention phases, and a lack of an appropriate statistical analysis undermine the interpretation of many of the findings reported in the literature on this topic. The lack of a sham condition restricts the interpretation of the findings

because biases related to expectations, e.g., placebo effect, cannot be controlled for. On the measurement of different time points, the comparison at least between baseline and post-intervention CVA measurements is called phasic CVA. Phasic CVA differs from tonic (i.e., baseline) CVA and represents a change in CVA between two different time points, thus showing how the parasympathetic system reacts to a stimulus [11]. Both tonic and phasic CVA are important to consider to account for the adaptation abilities of the organism [67].

Going beyond the measurement of baseline and post-intervention phases, considering further measurement time points may be meaningful, depending on the research question the study is addressing. For instance, if a study aims at measuring how long the acute effects of taVNS on physiological processes last, then adding a post-intervention phase is strictly necessary [68]. If a cognitive study aims at investigating the effect of a build-up time—a time window in which only eVNS takes place and is supposed to be administered before a task phase combined with eVNS—on cognitive processes, like some studies already did [35, 69], then two different measurements must take place after the baseline measurement, namely, an "eVNS phase" and "eVNS & task phase". On statistical analysis, if repeated measures analysis of variance (rmANOVA) is used to analyze CVA measurements, then at least two factors need to be accounted for, namely, time (at least baseline and stimulation phase) and stimulation condition (at least eVNS and sham stimulation).

A study design that includes measurements of tonic and phasic CVA would have considerable advantages compared to a study design with only tonic measurements. Only comparing tonic measurements between eVNS and sham stimulation does not necessarily allow for concluding that eVNS led to a higher CVA. Similarly, a proper statistical analysis should be chosen, ideally a repeated-measures analysis, which considers the correlation between different time points [70]. Currently, many studies with eVNS either compare a post-intervention phase with baseline within each condition separately (in the few cases that are sham-controlled) and/or compare eVNS with sham condition using statistical analysis that does not account for differences between measurement time points. Such statistical analyses render it difficult to draw conclusions from the results. First, only comparing the stimulation phase with its baseline does not allow for quantifying the effects the stimulation eventually provoked in comparison to the sham condition. Second, testing the difference only between eVNS and sham stimulation does not necessarily mean that there was an increase in CVA; instead, there is a chance that the baseline CVA in the eVNS condition was already higher than the baseline CVA in the sham condition, and this measurement simply remained stable over time. If for instance rmANOVA is used to analyze CVA measurements,

then at least two factors need to be accounted for, namely, time and stimulation condition (at least eVNS and sham stimulation). In the case of the factor time, at least baseline and intervention phase need to be considered, but ideally also a measurement during stimulation should take place, because this way the three most important measurement time points for CVA according to the vagal tank theory [68] would be accounted for.

Besides the above-mentioned considerations that need to be made to conduct high-quality research with eVNS and CVA, it is also paramount to ponder which role CVA will play in the study. This decision depends highly on the research question, and two types of studies could be found that involve eVNS and CVA (*see* Subheading 1.2.2). Furthermore, the current state of evidence can be broken down into (a) findings on the acute effects of eVNS on CVA, (b) findings on the medium- to long-term effects of treatments with eVNS on CVA, and (c) findings from responder analyses. To summarize, studies with the aim to investigate the acute effects of iVNS often compare ON with OFF phases, although this approach may elicit carry-over effects [33]. Studies using tcVNS and taVNS, in turn, usually compare CVA during stimulation with CVA during sham stimulation [1]. Studies with the aim to investigate the medium- to long-term effects frequently compare CVA values before treatment with the ones after concluding treatment. On responder analysis, the use of a cut-off value to split the sample into high- and low-responders can be of relevance both for clinical use and for research. For clinical purposes, this clustering can be useful to predict the responsiveness of eVNS in treatments such as refractory epilepsy [39]. For research, the differentiation between high- and low-responders may help define possible parameter-depending effects of eVNS on CVA or other psychophysiological processes, thus leading to a further optimization of this tool. For instance, CVA can be split based on a cut-off value to investigate whether high- and low-responders show different performances in cognitive tasks. However, for this type of research question, there are other statistical approaches such as correlations and regression analyses [34].

Based on the presented methodological considerations, in the following subsection we point out considerations on how to properly design a study that allows for investigating the effects of eVNS on psychophysiological processes along with CVA as a biomarker.

3.2 Designing Studies on the Effects of eVNS with Cardiac Vagal Activity as a Biomarker

In this chapter, we described the state of evidence for three technologies that aim at electrically stimulating the afferent vagus nerve, namely, iVNS, tcVNS, and taVNS. Based on the literature review, an array of considerations can be pointed out that need to be contemplated when developing a study involving eVNS and CVA. The following list of considerations is summarized in the form of a checklist table to be used in the planning phase of the study design (Table 2):

Table 2
Checklist for considerations to be observed when designing studies with electrical vagus nerve stimulation and cardiac vagal activity

Consideration	Explanation
1 **Role of cardiac vagal activity in the study** *(predictor, dependent variable, or else (what?))*	
2 **Underlying theory to explain the expected mechanism of action** *(explanation on how the hypotheses fit into the underlying theory)*	
3 **Scope of the literature review** *(how narrow does the literature review need to be? Is it clearly defined in the literature review what kind of evidence (findings from iVNS, tcVNS, taVNS, or animal studies) is provided?)*	
4 **Power analysis** *(software, values, and justification with reference to the expected effect sizes[a])*	
5 **Stimulation parameters** *(current intensity, pulse width, pulse frequency, duty cycle, session/intervention duration, side of stimulation,[a] type of stimulation, and location of stimulation)*	
6 **Type of sham condition** *(stimulation of another area/nerve, no current, different parameters (please provide detailed information), or else (which one?))*	
7 **Measurement time points** *(additional measurement besides baseline & stimulation phase?)*	
8 **Repeated-measures statistical procedure** *(repeated-measure analysis of variance, linear/generalized mixed-models, or else (which one?))*	
9 **Control for confounders** *(how to avoid or control for carry-over effect, learning effect,* etc.*)*	

[a]When appropriate

1. The question of what kind of role CVA will have in the study needs to be reflected on prior to the beginning of the study. Whether CVA will be understood as a predictor or a dependent variable, or whether acute vs. medium- to long-term effects of eVNS on CVA will be addressed may influence the way to design the study.
2. Hypotheses should ideally be derived from an integrative theory that involves both the afferent and efferent functioning of the vagus nerve, as well as the dependent variable at stake. We focused here on the neurovisceral integration model [9, 12], but other theories such as the polyvagal theory [71] provide different perspectives on the connection between brain and heart via the vagus nerve. When the theory at hand does not refer to all dependent variables used in the study, the rationale for addressing these dependent variables should be coherent

with the theory. For instance, the studies using CVA as a biomarker to predict the responsiveness to eVNS are based on the hypothesis that the effects of eVNS depend on the degree to which the autonomic function of the patient is impaired [39]. This opens up a possibility to extend the theory at hand, or perhaps contribute to developing a theory that specifically describes the effects of eVNS on psychophysiological processes in the future.

3. To avoid justifying the hypotheses with possibly inconsistent speculations, the literature review on the state of evidence for the relationship between eVNS and CVA should remain within the scope of the study aim. That means, when citing previous research, the literature review needs to be clear whether this research has been done with humans (healthy or patients) or animals. Furthermore, the findings cited should make a clear reference to the type of stimulation that was used (iVNS, tcVNS, or taVNS).
4. Power analyses need to be performed to prevent underpowered studies due to very low sample sizes.
5. The stimulation parameters used in the study should be described in detail (Table 1). Additionally, if a well-known device is used but this device was modified, clearly stating the configuration in the methods section enables the reader to be aware of the use of divergent parameters and allows them to critically interpret the results in light of this divergence.
6. The use of sham stimulation to be compared to active stimulation is essential to enable a meaningful interpretation of the findings. The use of a sham condition is possible both in within-subject and in between-subjects designs. In the case of a within-subject design, all participants need to undergo both stimulation and sham condition in a counter-balanced order, to counteract order effects [72].
7. Phasic measurements of CVA are to be performed with the aim to measure the changes in CVA over time, at least from the baseline to the stimulation phase. Ideally, the study design should also include a post-stimulation phase as well as further measurement time points that are important according to the research question and hypothesis.
8. Statistical analysis needs to integrate both different stimulation conditions (e.g., eVNS vs. sham) and different time measurement points in order to provide an appropriate investigation of the effects of eVNS that have been hypothesized.
9. Potential confounders such as relaxation during the experimental session, task difficulty, and medication, especially neuromodulators, should be assessed if possible. This assessment can be

performed for instance using questionnaires at the end of each time measurement point, or at the end of the experiment.

This list of considerations is certainly not exhaustive on what is necessary to improve the quality of studies with eVNS, but it is an important step in this direction. There have recently been efforts to develop a consensus-based standardized study protocol for taVNS studies [1], as well as recommendations for experiment planning, data analysis, and data reporting for studies using HRV [11]. The minimum reporting standards proposed in both of these papers are also well suited for research that investigates the relationship between eVNS and CVA. Instead of replacing those or other recommendations and guidelines [73, 74], the list presented here should be seen as a complement for them, by additionally considering common caveats found in the literature on eVNS and CVA.

4 Conclusion

With the aim to investigate the effectiveness of eVNS on pathological and psychophysiological processes, research with eVNS has increasingly been using HRV as a biomarker. The present chapter focuses on HRV parameters known to reflect CVA and their relationship with eVNS. We point out common caveats present in this research area, and list considerations that are necessary when developing a study involving eVNS and CVA. Further optimizing stimulation protocols for study ideas with eVNS can promote the investigation of CVA as a promising biomarker for eVNS, and help future research to better understand the still unclear link between afferent and efferent vagal pathways.

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Chapter 4

Vagus Nerve Manipulation and Microglial Plasticity in the Prenatal Brain

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Abstract

The efferent and afferent effects of the vagus nerve on the developing brain have remained enigmatic. Here we review the evidence of such effects on microglial plasticity in the sheep model of human fetal development, one of the most recognized and deployed models of human fetal physiology. We show that vagotomy alters microglial phenotype and that this effect is hormetic under conditions of mild systemic inflammation, as may occur antepartum with chorioamnionitis. We present the methodology to assess not only biomarker-based microglial activation (Iba-1), but also the morphometric features of the microglia. Together, these assessments provide a more comprehensive toolbox of glial phenotypical characterizations, especially in the context of investigating the locoregional vagal control of glial function. The presented findings support the earlier discoveries in preclinical and clinical models of adult physiology whereby vagotomy appeared neuroprotective for Parkinson's, explained, at least in part, by the effects on microglia. In addition, we present the approach to measure and the findings on regional cerebral blood flow changes in relation to vagus nerve manipulation. Together, the body of evidence underscores the importance of both the efferent and the afferent vagal pathways, via the vagus nerve, in the programming of microglial phenotype in the developing brain. The significance of these relationships for the development and treatment of early susceptibility to neuroinflammatory and neurodegenerative disorders in later life requires further studies.

Key words Vagus nerve, Microglia, Brain development, Fetal sheep, Neuroinflammation, LPS, Cerebral blood flow

1 Introduction

The immune system was long believed to operate independently of the central nervous system (CNS). It was understood to operate autonomously of neural input, driven purely through cytokine response cascades. This dogma has been overturned in recent decades due to growing evidence of neuroimmune interactions, in part also known as the cholinergic anti-inflammatory pathway (CAP) [1].The CAP comprises both afferent and efferent vagal nerve signaling to modulate inflammatory responses largely through the control of cytokine release via cholinergic signaling, both in the

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periphery as well as in the brain [2, 3]. Pre-clinical and clinical evidence has established a role for the neuroimmune interactions in a wide variety of biological processes ranging from infection and autoimmunity to cardiovascular disease, cancer, neurodegeneration, and neuromuscular disease [1, 4–6].

1.1 Neuro-immunology: Quis Custodiet Ipsos Custodes?[1]

The traditional concept of CAP activity refers to the peripheral (i.e., outside the brain) effects of CAP on inflammatory homeostasis, with certain not yet well identified brain regions thought of as providing information processing, sometimes referred to as a neuroimmunological homunculus [7]. Simply put, as the inflammatory response is generated, cytokine receptors found on afferent vagus nerve fibers are activated, sending signals to the nucleus tractus solitarius (NTS) in the brainstem. CNS recognition triggers an efferent vagal response, delivering acetylcholine to the site of macrophage activation in the periphery [8]. Thus, afferent vagal stimulation by cytokine receptors permits CNS recognition and modulation of innate immune activity.

There is however also evidence for a version of the CAP playing a similar regulatory role in neuroinflammation, i.e., in the brain itself. An example of such central effects can be seen in chorioamnionitis, an inflammation typically originating in the mother and affecting the fetal brain [2, 9]. In a broad sense, peripheral inflammatory and central, neuroinflammatory, responses are largely precipitated by the activation of macrophages and microglial cells, the primary immune cell of the CNS, respectively. The activation occurs through pattern-recognition receptors (PRR) and toll-like receptors (TLR) found on the cell surface. Receptor activation then triggers an innate immune response, initiating signaling cascades for nuclear localization factors, namely, NF-kB, and subsequent production of pro-inflammatory cytokines such as TNF-α, IL-1ß, and IL-6 or HMGB1 [10].

In the brain, acetylcholine is capable of activating α7nAChR receptors found on the surface of microglia and astrocytes, subsequently inhibiting NF-kB (or HMGB1) nuclear translocation and limiting the generation of pro-inflammatory cytokines [2, 9–11]. The presence of a similarly centrally acting neuroinflammatory reflex is suggested by pre-clinical data in rats and fetal sheep [9, 12]. In animal models, CNS neuroinflammatory responses can be suppressed when immune cells in the CNS, such as microglia, are exposed to α7nAChR receptor agonists and acetylcholinesterase inhibitors triggering the same molecular anti-inflammatory cascade outlined above [1]. In this capacity, the CNS is capable of recognizing and limiting the activation of neuroinflammatory responses through CAP peripherally and likely centrally [1].

[1] Who watches the watchers?

Taken together, we propose that two complementary "brain CAP" systems exist that we subsume under the definition of CAP. The first system represents the peripheral afferent sensing of systemic or organ-specific inflammation which, in addition to the traditional central processing and vagal efferent regulatory outflow, provides the brain itself with information about peripheral inflammation in ways that modulate its local neuroinflammatory milieu via central cholinergic signaling on microglial and astrocytic α7nAChR receptors. The second system is central with regard to its afferent and efferent components and likely distributed with direct locoregional sensing and control of inflammation via glial cells and specific neuronal populations [13]. In this dual role, the watcher is watching itself together with the watched in a dynamic spatiotemporal relationship, if we are to circle back to the subtitle of this section.

For completeness' sake, it should be mentioned that the neural central control of CAP involves also cholinergic receptors other than α7nAChR, e.g., m1 and m2 muscarinic receptors [3]. The precise mechanisms of the brain's own sensing and control of neuroinflammation remain to be established, especially in the context of development. We review the significance of this neurodevelopmental perspective for lifetime health trajectories in more detail in Desplats et al. [14].

1.2 Cholinergic Anti-inflammatory Pathway and Microglia

In the following, we focus on the microglial behavior and leave astrocytes for future work. The impact of CAP on microglial cells is of interest due to their distinct role in the innate immune system. Microglial cells are a unique population of macrophages specific to the CNS. Aside from phagocytic activity, microglial cells are responsible for optimizing brain function and tissue maintenance [15]. Early in embryogenesis, microglial cells migrate to the embryonic brain to play a pivotal role in neurogenesis. Animal modeling has demonstrated that in the prenatal period microglial cells promote neuronal death, fasciculation formation, limit axonal outgrowth, regulate laminar positioning, and promote vascularization and myelination of the neuron. Perinatally, microglial cells largely drive neuronal death and survival. Postnatally, microglial cells promote synapse maturation, remodeling, and pruning as well as myelination maintenance [16]. Microglial cells reach a point of maturation 1 week postnatally. Throughout development and up until this point of maturation, microglial cells maintain an amoeboid morphology characterized by large, round cell bodies and short, thick branches. This developmentally activated phenotype is associated with the variety of functional roles microglial cells possess during the prenatal period. Of interest, upon immune challenge or postnatally in neurodegenerative disease processes, mature microglial cells morphologically take on an amoeboid structure reflective of that seen during prenatal

development [15]. This finding contradicts the traditional view that microglial cells adopt either a pro-inflammatory M1 phenotype or an anti-inflammatory M2 phenotype. As evidenced by morphological and genetic findings, it is now apparent that microglial cells have the capacity for a wide spectrum of activation states. This spectrum of activity, reflected by the amoeboid structure, is of interest as recent data have implicated microglial cells in a multitude of CNS pathologies. Moreso, with the presence of the CAP and the expansion of our capacity for vagal nerve modulation, there is enormous potential to take advantage of the functional diversity intrinsic to microglial cells by modulating CAP activity externally via VNS.

To better understand the interaction between CAP and microglial plasticity, our team employed a sheep model to model human fetal development. Sheep models serve as excellent proxies for human physiology due to similarities in blood gas profiles, hormone responses, organ development, and birthing profiles generally comprised of singletons or twins with weights mirroring those of human infants [11, 17, 18]. Using a biological model that so closely resembles human development allowed our team to explore the relationship between vagus nerve signaling and microglial activity. More specifically we investigated if and how vagotomy and VNS alter the morphometric phenotype of microglial cells, an aspect sometimes neglected in favor of the more readily accessible average area density metrics of a given immunohistological biomarker, such as Iba-1.

1.3 VNS and Cerebral Blood Flow: Is There a Link Between Neuroinflammation and rCBF?

VNS alters regional cerebral blood flow (rCBF) [19]. In adults, Conway et al. found VNS-induced increases in rCBF in the bilateral orbitofrontal cortex, bilateral anterior cingulate cortex, and right superior and medial frontal cortex. Decreases were found in the bilateral temporal cortex and right parietal area. Regions of change were consistent with brain structures associated with depression and the afferent pathways of the vagus nerve. As we mention above, Tracey et al. suggested the existence of a neuroimmunological homunculus which implies that neurovascular activity can be measured in certain brain regions reflecting neuroimmunological stimulation or information processing [7].

Enigmatically, recent studies also revealed that microglia sense and regulate rCBF [20]. The causal order of the connections between the afferent vagus nerve signaling, rCBF dynamics and microglial plasticity remains to be established. Here, we demonstrate the methodological approach and preliminary findings indicating that vagotomy and VNS modulate rCBF in the developing brain experiencing neuroinflammation.

In summary, this chapter provides an overview of the methodological approach to studying microglial plasticity in the developing brain as a function of vagus nerve activity. Next, we report key

results focusing on a vulnerable brain region, the hippocampus. We then conclude with a discussion of the implications of these insights for future research and clinical practice.

2 Methodology

Animal care followed the guidelines of the Canadian Council on Animal Care and the approval by the University of Montreal Council on Animal Care (protocol #10-Rech-1560).

2.1 Anesthesia and Surgical Procedure

Briefly, we instrumented 57 pregnant time-dated ewes at 126 days of gestation (dGA, ~0.86 gestation) with arterial, venous and amniotic catheters, fetal precordial ECG and cervical bilateral VNS electrodes; 19 animals received cervical bilateral vagotomy (Vx) during surgery of which eight animals received efferent VNS electrodes and VNS treatment and seven animals received afferent VNS electrodes and VNS treatment during the experiment [18, 21, 22].

Ovine singleton fetuses of mixed breed were surgically instrumented with sterile technique under general anesthesia (both ewe and fetus). In the case of twin pregnancy, the larger fetus was chosen based on palpating and estimating the intertemporal diameter. The total duration of the procedure was about 2 h. Antibiotics were administered to the mother intravenously (Trimethoprim sulfadoxine 5 mg/kg) as well as to the fetus intravenously and into the amniotic cavity (ampicillin 250 mg). Amniotic fluid lost during surgery was replaced with warm saline. The catheters exteriorized through the maternal flank were secured to the back of the ewe in a plastic pouch. For the duration of the experiment, the ewe was returned to the metabolic cage, where she could stand, lie and eat ad libitum while we monitored the non-anesthetized fetus without sedating the mother. During postoperative recovery antibiotic administration was continued for 3 days. Arterial blood was sampled for evaluation of the maternal and fetal condition and catheters were flushed with heparinized saline to maintain patency. We reported the detailed approach including Vx and VNS elsewhere [18, 21].

2.2 Experimental Protocol

Postoperatively, all animals were allowed 3 days to recover before starting the experiments. On these 3 days, at 9:00 am 3 mL arterial plasma sample was taken for blood gasses and cytokine analysis. Each experiment commenced at 9:00 am with a 1 h baseline measurement followed by the respective intervention as outlined below (*see* Fig. 1). FHR and arterial blood pressure was monitored continuously (CED, Cambridge, U.K., and NeuroLog, Digitimer, Hertfordshire, U.K). Blood and amniotic fluid samples (3 mL) were taken for arterial blood gasses, lactate, glucose and base excess

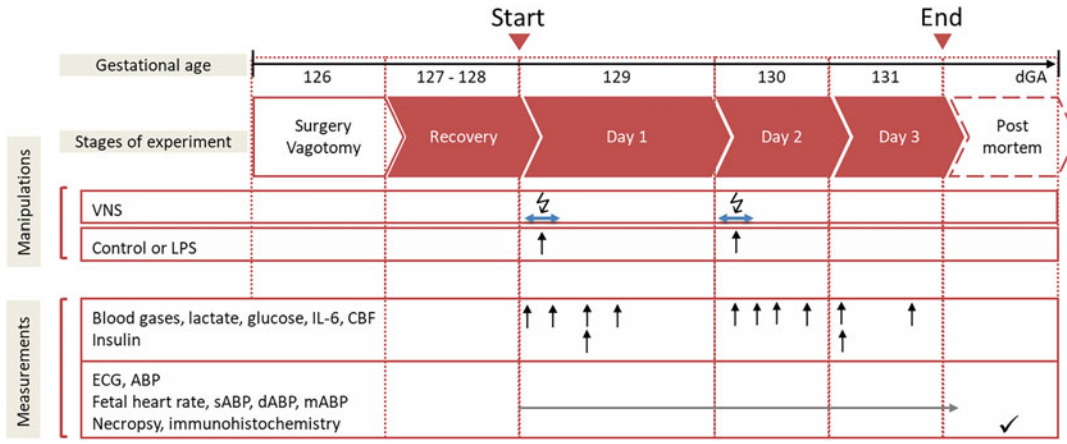


Fig. 1 Experimental design. Bilateral cervical vagotomy (Vx) was performed during surgery in Vx+LPS group animals. At Days 1 and 2, Vx+LPS animals received LPS dose 400 or 800 ng/fetus/day. Some Vx+LPS animals also received the efferent intermittent (to the periphery) VNS on Days 1 and 2 (Efferent or Afferent VNS groups)

(in plasma, ABL800Flex, Radiometer) and cytokines (in plasma and amniotic fluid) at the time points 0 (baseline), +1 (i.e., immediately after LPS administration), +3, +6, +12, +24, +48 and + 54 h (i.e., before sacrifice at day 3). For the cytokine analysis, plasma was spun at 4 °C (4 min, 4000 g force, Eppendorf 5804R, Mississauga, ON), decanted and stored at −80 °C for subsequent ELISAs. After the +54 h (day 3) sampling, the animals were sacrificed. Regional cerebral blood flow (rCBF) was measured at selected time points as outlined in the dedicated subsection. Fetal growth was assessed by body, brain, liver and maternal weights.

Lipopolysaccharide (LPS)-induced inflammation in fetal sheep is a well-established model of the human fetal inflammatory response to sepsis [23–26].

The experimental groups consisted of three following categories.

1. *Control and LPS groups*: Fifteen fetuses were used as controls receiving NaCl 0.9%. 23 fetuses received LPS (100 $n = 2$, 200 $n = 1$, 400 $n = 15$ or 800 $n = 5$ as ng/fetus/day) derived from E. coli (Sigma L5293, from E. coli O111:B4, readymade solution containing 1 mg/ml of LPS) were administered intravenously to fetuses on days 1 and 2 at 10:00 am to mimic high levels of endotoxin in fetal circulation over several days as it may occur in chorioamnionitis. As we identified that IL-6 response did not depend on the LPS dose in the applied range [26] these animals were all considered as one LPS group for statistical comparison purposes.
2. *Vx+LPS groups*: Eleven animals were vagotomized (Vx) and exposed, similar to the LPS group, to LPS400 ($n = 6$) or LPS800 ($n = 5$).

3. *VNS: Efferent (n = 8) or Afferent (n = 7) VNS groups*: Fifteen additional Vx animals were subjected to bilateral cervical VNS applied via NeuroLog's NL512/NL800A using pulse sequence pre-programmed in Spike 2 for 10 min prior to and 10 min after each injection of LPS. The VNS settings were as follows: DC rectangular 5 V, 100 uA, 2 ms, 1 Hz according to Borovikova et al. [27]. VENG was recorded at 10,000 Hz [21].

2.3 Immunofluorescence Staining and Microscopy

The microglial marker ionized calcium-binding adapter molecule 1 (Iba-1) expression was quantified. Fetal brains were perfusion-fixed during necropsy and then harvested and immediately fixed in 4% paraformaldehyde (PFA) for 72 h as reported elsewhere [28]. Samples were then rinsed daily for 3 days using phosphate-buffered saline (PBS), followed by storage in 70% ethanol at 4 °C. Brains were divided into thick coronal sections, processed with Leica TP 1020 Automatic Tissue Processor (Leica Instruments, Nussloch, Germany), and embedded in paraffin using the Leica EG 1160 Paraffin Embedding Center (Leica Microsystems, Nussloch, Germany). The Leica RM2145 Rotary Microtome (Leica Microsystems, Nussloch, Germany) was used to cut 5 μm sections collected onto Superfrost Plus microscope slides (Fisherbrand, Waltham, MA). Slides were deparaffinized with 35-min washes in xylene and rehydrated through a descending ethanol series (100%, 100%, 90%, 90% and 70%) for 2 min each followed by deionized water for 5 min. Slides were subjected to a heat-induced epitope retrieval process in 10 mM sodium citrate buffer (pH 6.0; Sigma-Aldrich, St. Louis, MO) at 90 °C in a 2100-Retriever pressure cooker (Electron Microscopy Sciences, Hatfield, PA) for 15 min, then rinsed with PBS three times for 5 min each. Non-specific protein binding was blocked with Background Sniper protein blocker (Biocare Medical, Pacheco, CA) for 10 min. Following a brief PBS rinse, the slides were incubated with the polyclonal rabbit anti-Iba-1 (1:1500 Dilution; Wako Pure Chemical Industries Ltd., Osaka, Japan; #019-19741) diluted in Dako diluent solution (Agilent, Santa Clara, CA) overnight at 4 °C in a humidity chamber. After another 3 × 5-min PBS rinse, slides were incubated for 40 min at room temperature with Invitrogen Alexa Fluor 647 goat anti-rabbit IgG (Thermo Fisher Scientific, Waltham, MA). 4′,6-diamidino-2-phenylindole (DAPI,1:300 Dilution in PBS; Thermo-Fisher Scientific, Mississauga, ON) was applied as a counterstain for 2 min to visualize cell nuclei. Finally, slides were mounted with Prolong Gold antifade mounting medium (Thermo-Fisher Scientific, Waltham, MA). Two negative controls were performed by replacing the primary antibody step with Dako diluent alone and also with Dako purified pre-immune mouse IgG to rule out non-specific binding.

Images were captured on a Zeiss AxioImager Z1 (Carl Zeiss Canada, North York, Ontario) with the user blinded to the treatment conditions. Using the Zeiss Zen stitch and tile and z-stack functions, whole-brain regions were delineated at low magnification and then imaged in their entirety with a 40× oil immersion lens, through a thickness of 3.96 um, which yielded seven layers through the section volume at optimal spacing. The regions of interest analyzed for this study were the CA1, CA2/3, and dentate gyrus (DG) of the hippocampus. Eight subset images of 300 × 300 μm were then isolated from each brain region and exported as seven-layer tiff images for analysis. This allowed us to include as full a range as possible of the processes associated with each cell body.

These analyses were performed in two batches, separated in time. We account for this in the quantification of the Iba-1 signal in Results section (cf. "Batch effects" subsection).

2.4 Quantification of Iba-1 Signal

All analysis was performed using Image Pro Premier version 9.3 software (Media Cybernetics, Rockland MD). First, the seven-layer 300 × 300 μm tiff images of (individually) both the multichannel RGB image and the greyscale Iba-1 channel alone were processed through the extended depth of focus (EDF) using the "large edges" algorithm, to yield single-plane images. This created images in which the processes associated with each cell body could be maximally measured in one single focal plane to help differentiate ramified from activated morphology. To allow the normalization of data to the actual tissue area in each field of view, any black, non-tissue area in the multichannel EDF image was first measured, and then subtracted from the total image area to yield the true total tissue area.

Subsequently, two copies of the EDF Iba-1 channel image were made. The first copy was background-corrected to eliminate lighting artifacts typical in widefield imaging that could affect cell recognition, while the second maintained its original intensity data. Using the OTSU minimum variance algorithm, the Iba-1 signal of the "whole-cell" (total of cell bodies cell plus processes) was counted on the corrected images, followed by a repeat count using size and roundness filtering to select only the cell bodies. A 2-pixel object growth at the perimeter was employed as well as a "morpho close" filter with a radius of 5 pixels to encourage the joining of adjacent processes to their cell body of origin, to improve morphological and fractal data. This made measurements more accurate, as the intensity difference tended to initially separate them. The outlines of these whole-cell and cell body counts were stored as geographical features, and re-applied in turn to the unaltered Iba-1 channel EDF images to yield the final data so that intensity data would not be affected by the background correction.

For each hippocampal region, the mean object intensity values from either "whole cells" or "cell bodies only" of all eight subset images were averaged. To derive the mean object intensity for only microglial processes, the difference in total sum intensity was divided by the difference in object counts of whole cells and cell bodies (*see* Fig. 2e). Similarly, cell counts derived from cell-body filtering from all eight subset images were summed and divided by the sum of "true total tissue area" to derive microglia density per region expressed as cells per mm^2. Average microglial soma size was determined by dividing the total "cell-body" area (in μm^2) by the cell body count of all eight subsets per region. Finally, the soma to whole cell ratios (%) were determined using respective sizes in μm^2 and used as a measurement of microglial activation as previously described by Hovens et al. [29].

2.5 Measurement of Cerebral Blood Flow

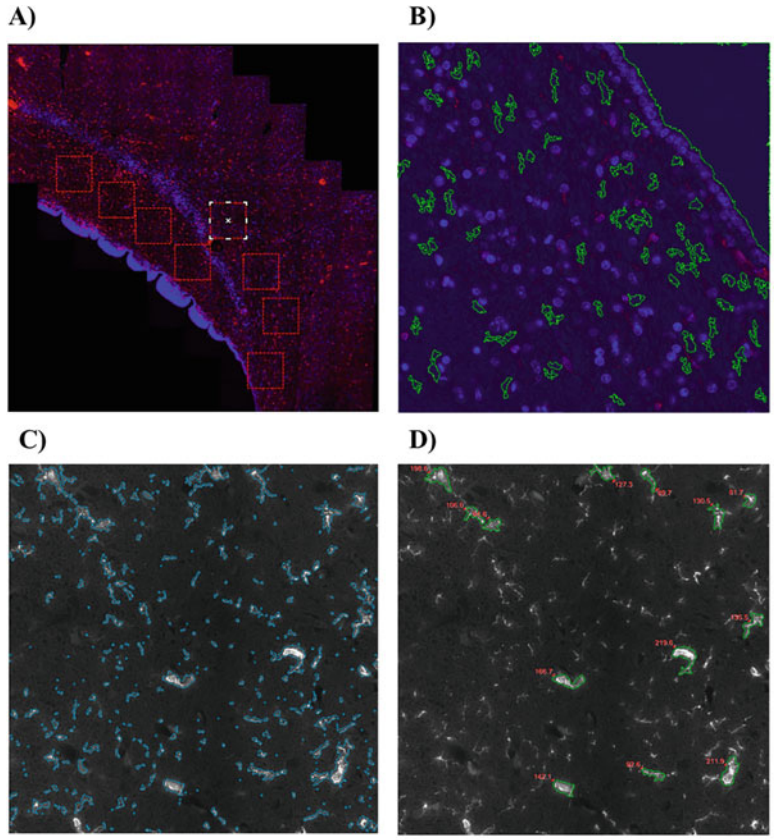
Briefly, rCBF was measured at four time points 0 (baseline), +6 (i.e., 6 h after first LPS), + 24 (i.e., 24 h after first LPS, just prior to second LPS) and + 48 h (i.e., 24 h after second LPS) with fluorescent microspheres of different colors (NuFLOW Hydro-Coat Microspheres, size 15.5 μm; the concentrations were 5 million/2 mL/vial, Interactive Medical Technologies (IMT), CA). To measure rCBF, we used the established fluorescent-colored microsphere (CMS) technique [30, 31]. For the purposes of this work, all cortical and subcortical regions were analyzed together.

In the following, we provide a detailed description of the CMS methodology.

To measure CBF, we adopted an established CMS technique using the reference sample method [32]. Microspheres were suspended by gently rolling or swirling the vial, followed by a brief sonication (30 s) with a low-power ultrasound probe, and drawn up into a sterile syringe immediately before injection. The number of injected colored microspheres was estimated to be sufficient to ensure an adequate number of microspheres per tissue sample (> 400) to meet the requirement of a systematic error of about 10% at the 95th confidence level [33]. The minimum number of microspheres per injection was calculated by the following formula:

$$\mathrm{CMS_{inj}} = \frac{\mathrm{CMSmin} * W\mathrm{organ}}{Q\mathrm{organ}/Q\mathrm{total}}$$

Where $\mathrm{CMS_{inj}}$ is the microsphere number per injection; $\mathrm{CMS_{min}}$ is the minimum number of microspheres required per sample; the value is 400 in the formula [33]. W_{organ} is the average weight of the target organ. In this study, the average fetal brain weight was 52 g (range 45–60 g); we chose the highest brain weight of 60 g for calculation. $Q_{\mathrm{organ}}/Q_{\mathrm{total}}$ is the percentage of blood flow distributed to the target organ, the cardiac output to the brain in fetal lamb is 15% [34]. So the $\mathrm{CMS_{inj}} = 400 \times 60/15\% = 160{,}000$, which is the minimum CMS number required



E)

$$\text{Object mean intensity} = \frac{Object\ Sum\ Intensity}{Object\ Count}$$

$$\text{Processes mean intensity} = \frac{(Whole\ Cell\ Sum\ Intensity - Soma\ Sum\ Intensity)}{(Total\ Object\ Count - Soma\ Count)}$$

$$\text{Microglial Density} = \frac{Cell\ Count\ (D)}{Tissue\ Area\ in\ mm^2 (B)}$$

$$\text{Microglia Whole Cell: Soma Size Ratio} = \frac{Sum\ Soma\ Size\ in\ um^2\ (D)}{Sum\ Whole\ Cell\ Size\ in\ um^2\ (C)}\ x\ 100$$

Fig. 2 Methodology for image analysis and quantification of the immunofluorescent signal. (**a**) Obtaining eight subsets of images from a single brain region. Red outlines indicate individual 300 × 300 μm images used for further analysis. (**b**) Removing empty spaces in tissue based on darkness threshold to obtain total tissue area. Green outlines indicate a non-tissue area excluded from further analysis. (**c**) Brightness threshold set to detect whole microglia (Soma and all processes shown with blue outlines. (**d**) Further thresholding (green outlines) to retain cell soma only. (**e**) Formulas used for quantification of Iba-1 object mean intensity, microglia density, and soma: whole cell size ratio

per injection; we optimized practically that an increase to 750,000 per injection would obtain the adequate microspheres count in the current study. Therefore, the volume of each injection (μl) was computed to be 750,000÷(5,000,000/2000 μl) = 300 μl.

Beginning 15–25 s before the injection of the CMS, a reference blood sample of 4 mL was withdrawn over 2 min from the ascending aorta into a heparinized glass syringe at a rate of 2 mL/min with a syringe pump (Harvard APP, 11 plus 70-2211, single syringe pump). The amount of blood withdrawn for each administration of the CMS for the reference flows (4 mL) was replaced by maternal venous blood. Reference blood samples were stored in the fridge for analysis.

Following the completion of the animal experiment, the right brain hemisphere tissues were processed by brain region, 0.5–2 g (average 1 g) fetal brain tissue samples were taken from frontal, parietal, occipital, and temporal cortices, white matter, brainstem, cerebellum, and thalamus. Brain tissues and five reference blood samples were sent to Interactive Medical Technologies (IMT, CA) for fluorescence analyses. For this work, all cortical and subcortical regions were analyzed together, and data was presented in one report for each animal. Then, group averages were calculated.

The rCBF was calculated as follows:

$$\mathrm{rCBF} = \frac{\text{Total Tissue Spheres}}{\text{Tissue Weight} * \text{Reference Spheres}}$$

Where rCBF is expressed in $\frac{\mathrm{mL}*\mathrm{gram}}{\mathrm{min}}$, tissue weight is measured in grams and reference spheres are expressed as $\frac{\mathrm{number}*\mathrm{min}}{\mathrm{mL}}$.

2.6 Statistical Approach

Statistical analysis of histomorphometric data was performed using Graphpad Prism 9.4.1 (GraphPad Software Inc., San Diego, CA). Differences in experimental groups were compared in pairs by performing unpaired nonparametric T-tests. The Mann-Whitney test was used to calculate two-tailed p values and differences were considered statistically significant if $p < 0.05$. No adjustments were made for multiple comparisons as recommended by Rothman [35].

Vertical box and whisker plots depict the median, 25th, 75th, and minimum/maximum values. Outliers were determined using Tukey's method and plotted as individual dots. These values are greater than 1.5 times the interquartile range (IQR) from the 75th and 25th percentile.

For cytokine analyses, general linear modeling (GLM) in Exploratory/R was used to assess the effects of treatment (LPS, Vx + LPS400, Vx + LPS800, Vx + efferent VNS + LPS, Vx + afferent VNS + LPS) while accounting for repeated measurements of fetal plasma IL-6 cytokine. Consequently, experimental groups and time points served as predictor variables; as base levels served the control group and the baseline time point, respectively. For cytokines, the results are presented as median ± IQR.

Generalized estimating equations (GEE) modeling was used to assess the effects of LPS while accounting for repeated measurements on fetal rCBF. We used a linear scale response model with time and LPS as predicting factors to evaluate their interactions using maximum likelihood estimate and Type III analysis with Wald Chi-square statistics. SPSS Version 21 was used for these analyses (IBM SPSS Statistics, IBM Corporation, Armonk, NY).

Not all measurements were obtained in each animal. In such a case, the sample size is reported explicitly.

3 Results

3.1 Systemic Inflammatory Response: IL-6

These findings were reported, in part, elsewhere, except for the effect of the afferent VNS intervention in the Vx + LPS400 treated animals [22]. We provide them here for the proper context in which the brain-specific inflammatory response needs to be interpreted.

LPS provoked a systemic inflammatory response with time effect, i.e., peaking at 3 and 6 h, as measured by fetal arterial IL-6 concentrations over 3 days (*see* Fig. 3). Surprisingly, Vx + LPS400

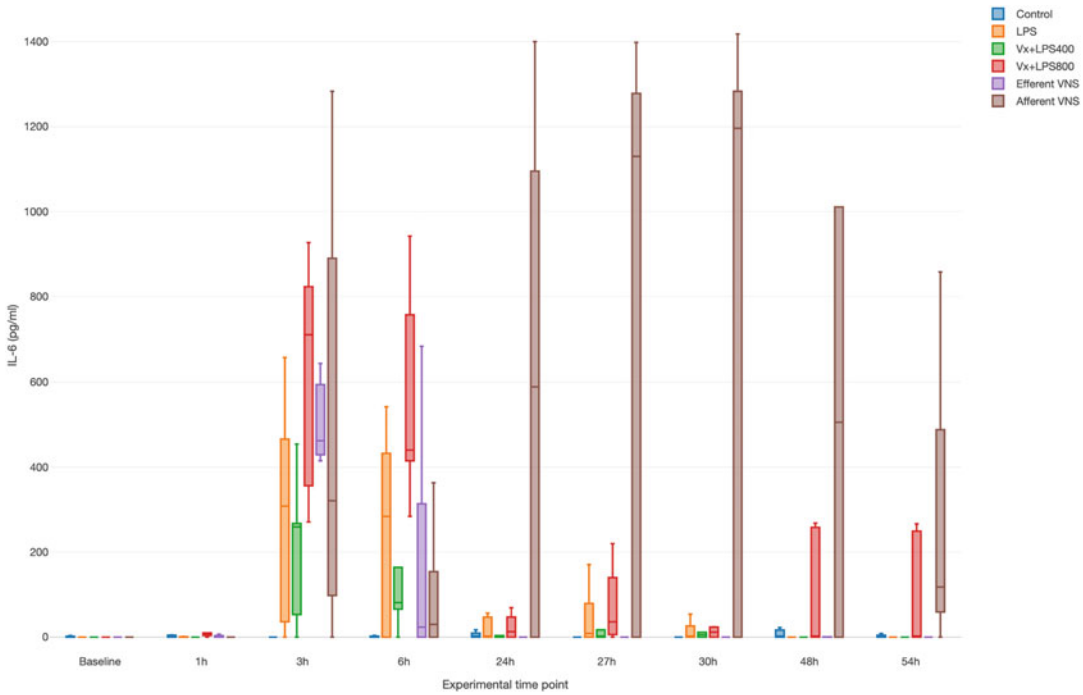


Fig. 3 Fetal systemic inflammatory response to intravenous LPS injection after baseline and at 24 h: the impact of vagus nerve manipulation. Vx, bilateral cervical vagotomy during surgical instrumentation; LPS400 and LPS800 indicate the respective intravenous dose of LPS in ng/fetus/day given after baseline and 24 h later; efferent and afferent VNS groups received Vx and LPS400 as the Vx+LPS400 group followed by respective targeted VNS treatment around the LPS administration at days 1 and 2. Based on the figure from [22]. (Reproduced with permission)

restored the levels of IL-6 to control levels, while treatment effects with elevated IL-6 were observed for afferent (but not efferent) VNS, Vx + LPS800, and LPS groups, in the decreasing order of magnitude.

It is important to note that in the efferent VNS group, the inflammatory response abated quicker than in other LPS-exposed groups, while in the Vx + LPS800 group and even more so in the afferent VNS group, the response persisted the longest, up until 54 h post LPS, the last experimental measurement time point.

Lastly, we observed a clearly diametrical temporal profile of the fetal inflammatory response to LPS in Vx + LPS400 versus Vx + LPS800 group, suggesting a sigmoid functional relationship between the vagal cervical denervation and the LPS dose-dependent magnitude of the fetal inflammatory response. We refer to this phenomenon as a hormetic response [22].

3.2 Vagus Nerve Signaling Modulates Microglial Phenotype

3.2.1 Effects of Vagus Nerve Manipulation on Iba-1 Expression in the Hippocampus

In this subsection, we focus on the object mean intensity of Iba-1 fluorescent signal in hippocampus subregions. The analyses of the experimental groups from two different batches of animals were separated to account for possible intensity differences. We present the analysis of the possible batch effects in the following subsection (*see* Fig. 4).

Quantification of fluorescent mean intensity of Iba-1-stained microglia was performed for the whole cell, soma, and processes in the CA1, CA2/3, and DG regions. Signal intensity was decreased for Vx + LPS400 treatment compared to controls ($p = 0.04$) for whole microglia only in the CA1. However, once microglial processes were isolated, Iba-1 signal was decreased for Vx + LPS400 relative to controls in the CA2/3 ($p = 0.05$) and DG ($p = 0.02$). Interestingly, Vx + LPS400 treatment also showed decreased Iba-1 signal intensity compared to LPS only for microglial processes in the CA2/3 ($p = 0.04$).

A hormetic effect was observed for vagotomized animals receiving LPS doses of 400 versus 800 ng/fetus/day. Iba-1 intensity was increased in Vx + LPS800 compared to Vx + LPS400 in whole microglia of the CA1 ($p = 0.04$), CA2/3 ($p = 0.03$), and DG ($p = 0.02$). For microglial soma only, there was a similar behavior whereby Iba-1 signal in Vx + LPS800 was greater than Vx + LPS400 in the CA1 ($p = 0.04$), CA2/3 ($p = 0.03$), and DG ($p = 0.02$). Finally, when microglial processes were isolated, signal for Vx + LPS800 was increased compared to Vx + LPS400 in the CA1 ($p = 0.04$), CA2/3 ($p = 0.03$), and DG ($p = 0.03$). Notably, no differences of Iba-1 signal mean intensity were observed between controls, efferent and afferent VNS within the same cohort.

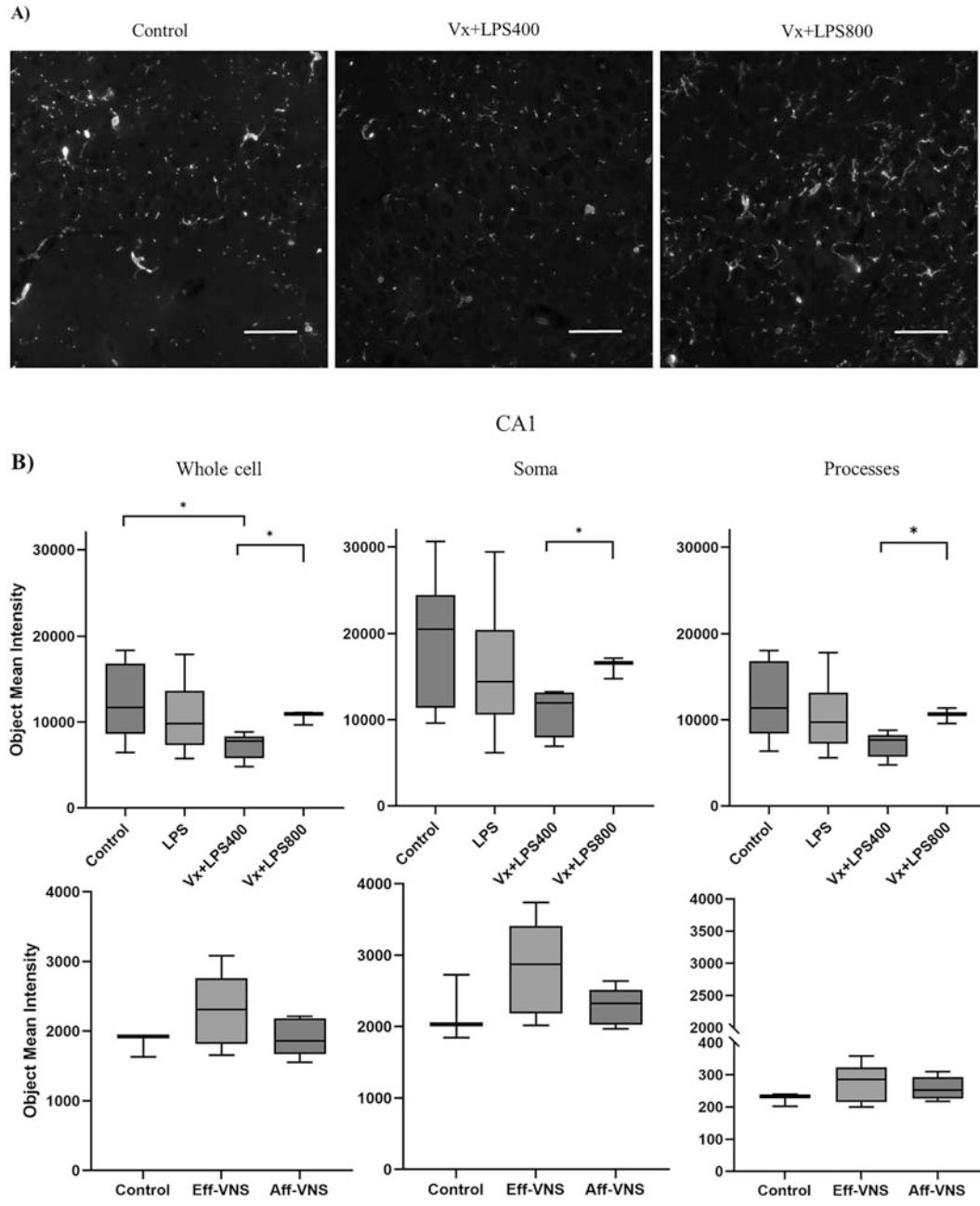


Fig. 4 Effects of systemic vagus nerve manipulation on Iba-1 expression in the hippocampus of near-term fetal sheep. (**a**) Immunofluorescent staining of Iba-1 on the hippocampal brain tissue of control and Vx+LPS400/Vx +LPS800-treated animals. Representative images of the CA1, CA2/3, and DG sub-regions are shown. Scale bar = 50 μm. Object mean intensity of Iba-1 fluorescent signal in the (**b**) CA1 (Control $n = 11$, LPS $n = 13$, Vx +LPS400 $n = 5$, Vx+LPS800 $n = 3$; Control $n = 3$, Eff-VNS $n = 7$, Aff-VNS $n = 7$), (**c**) CA2/3 (Control $n = 8$, LPS $n = 12$, Vx+LPS400 $n = 4$, Vx+LPS800 $n = 4$; Control $n = 3$, Eff-VNS $n = 7$, Aff-VNS $n = 6$), and (**d**) DG (Control $n = 11$, LPS $n = 14$, Vx+LPS400 $n = 4$, Vx+LPS800 $n = 5$; Control $n = 3$, Eff-VNS $n = 7$, Aff-VNS $n = 7$). *$p < 0.05$

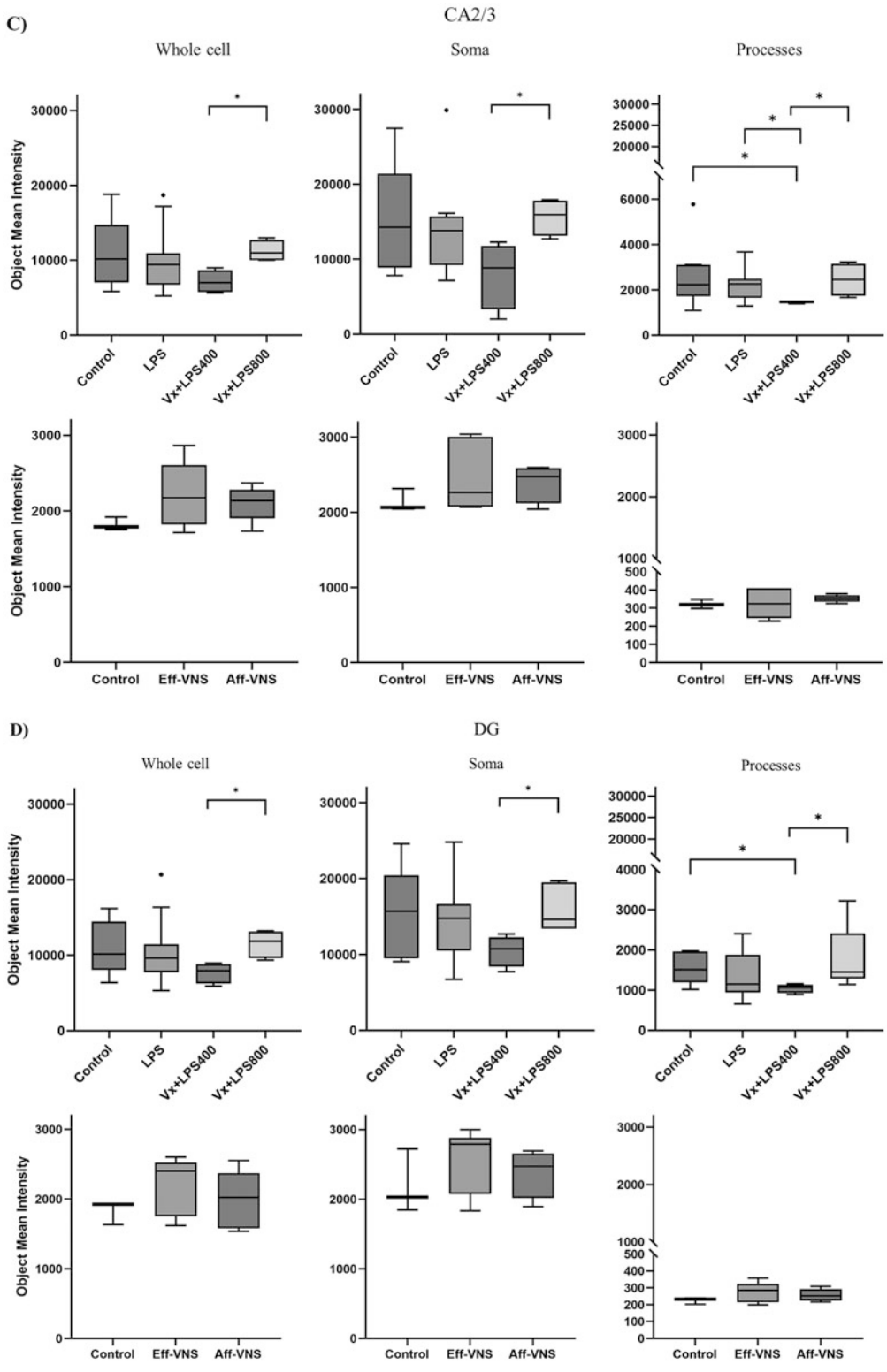


Fig. 4 (continued)

3.2.2 Effects of Vagus Nerve Manipulation on Microglial Morphometry in the Hippocampus

In this subsection, we focus on microglia morphometry data reflecting the cell activation state. Data presented includes cell count per tissue area, average soma size, and percentage of the whole cell occupied by soma only. Groups from two different batches of animals were combined. We present the analysis of the possible batch effects in the following subsection (*see* Fig. 5).

The microglial soma to whole cell ratio was used as a measure of microglial activation, expressed as a percentage of cell body area relative to the whole cell. A decreased ratio was observed with Vx + LPS400 treated animals compared to controls in the CA1 ($p = 0.01$) and DG ($p = 0.02$). In the CA2 subregion, Vx + LPS400 treatment was decreased compared to control ($p = 0.01$), LPS ($p = 0.01$), efferent ($p = 0.04$) and afferent ($p = 0.01$) VNS.

There was an increase in microglial density following efferent VNS compared to Vx + LPS400 ($p = 0.03$) only in the CA1, while no differences between groups were observed in the CA2/3 and DG.

In the CA1, both efferent and afferent VNS lead to a decrease in microglia soma size compared to Vx + LPS400 ($p = 0.02$, $p = 0.05$), Vx + LPS800 (both $p = 0.02$), and LPS only treatments ($p = 0.003$, $p = 0.02$). Additionally, soma sizes for efferent VNS animals were smaller compared to control ($p = 0.03$). In the CA2, Vx + LPS800 treated animals had larger microglia soma size compared to efferent ($p = 0.006$) and afferent ($p = 0.01$) VNS, while efferent VNS also decreased soma size relative to LPS ($p = 0.02$). Similarly, both efferent and afferent VNS lead to smaller soma size relative to LPS animals ($p = 0.03$, $p = 0.05$) in the DG. Efferent VNS treatment also decreased soma size relative to Vx + LPS800 ($p = 0.01$). We did not observe a difference in effects of efferent versus afferent VNS.

3.2.3 Batch Effects: Accounting for Measurements of Microglial Activity Made During Different Time Points

There was a concern about possible signal intensity differences between images captured in 2014 (batch 1) versus 2020 (batch 2), therefore potentially affecting soma size comparisons. However, there were no significant differences on soma size between groups of the same treatment (control & Vx + LPS400) (*see* Fig. 6). This consistency implies that a fair comparison of soma size can be made between these groups.

In summary, the key finding is that vagotomy influences microglial plasticity in an LPS dose-dependent hormetic manner.

Consistent with the earlier observations in systemic and organ-specific, regional effects in the terminal ileum [22], vagotomy exerted a hormetic effect on the fetal brain's microglial cell soma/whole body ratios. Interestingly, this effect was not apparent in whole cell density observations. Consistent with systemic reduction of IL-6 cytokine release, here we observed a reduction in soma/cell size ratio indicative of a less reactive microglial phenotype under conditions of Vx + LPS400, but not Vx + LPS800, below LPS or

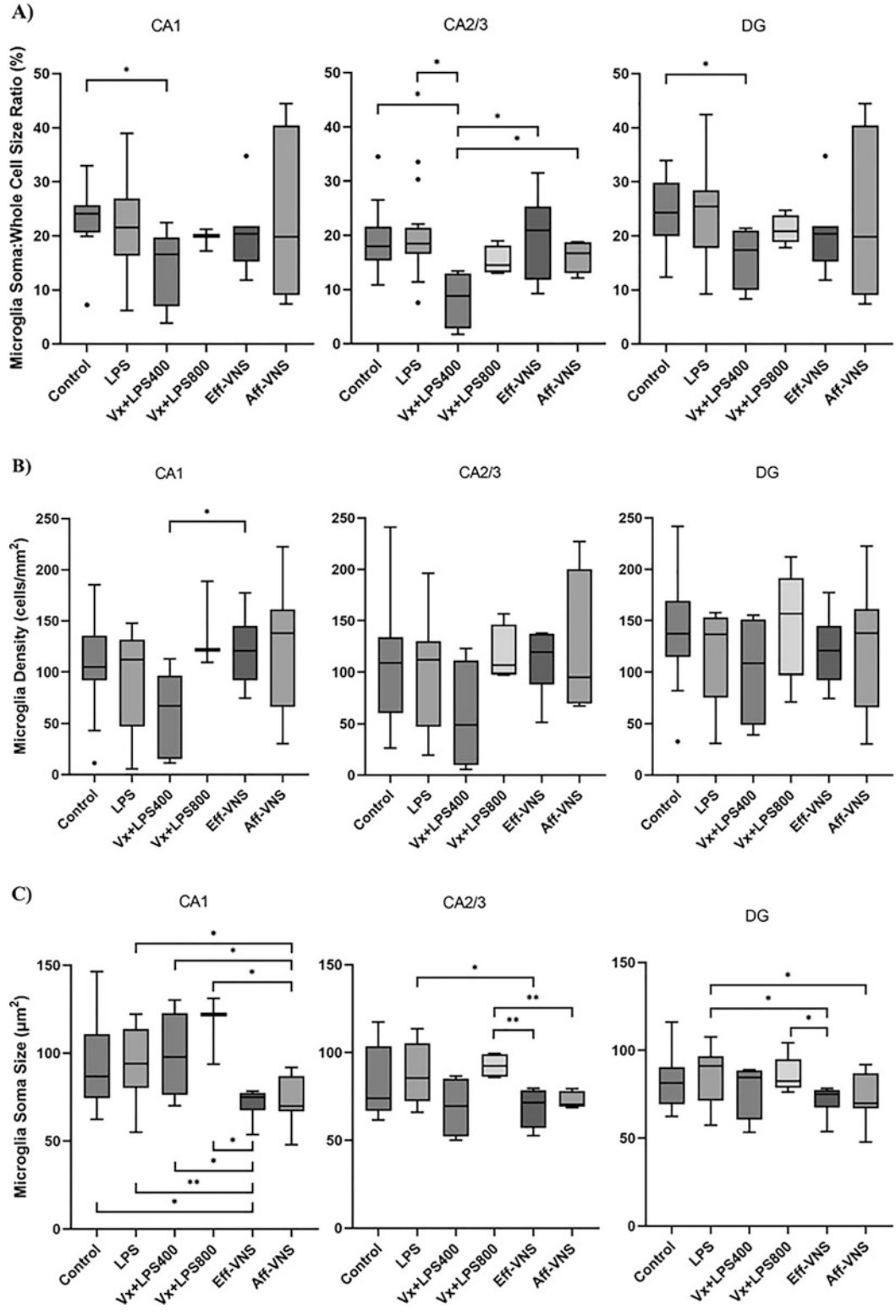


Fig. 5 Effects of systemic vagus nerve manipulation on microglial morphometry in the hippocampus of near-term fetal sheep. (**a**) Microglia soma to whole cell size ratio represented as a percentage of the whole

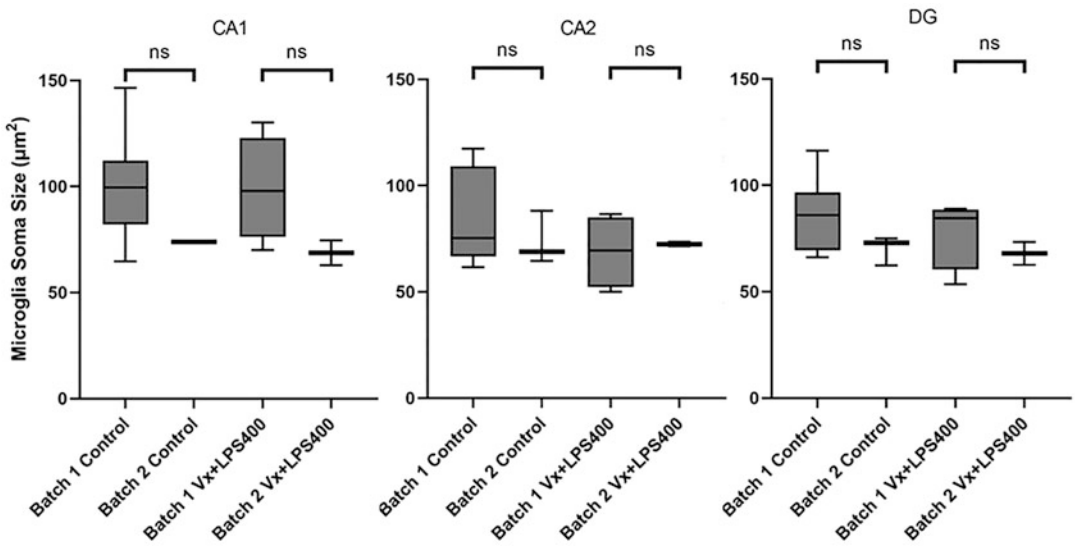


Fig. 6 No differences in microglial soma size were observed between batches. CA1 (Batch 1 control $n = 11$, Batch 2 control $n = 2$; Batch 1 Vx+LPS400 $n = 5$, Batch 2 Vx+LPS400 $n = 2$), CA2/3 (Batch 1 control $n = 8$, Batch 2 control $n = 3$; Batch 1 Vx+LPS400 $n = 4$, Batch 2 Vx+LPS400 $n = 2$), DG (Batch 1 control $n = 11$, Batch 2 control $n = 3$; Batch 1 Vx+LPS400 $n = 4$, Batch 2 Vx+LPS400 $n = 2$). n.s., $p > 0.05$

even control levels. This finding was consistent across all three hippocampus regions. In CA2/3, additional effects of efferent and afferent VNS were also observed as compared to Vx + LPS400.

We did not observe a difference in the effects of efferent versus afferent VNS. Both VNS regimes appeared to restore the microglial phenotype in these brain regions to control levels.

3.3 Vagus Nerve Activity Modulates rCBF

LPS resulted in a delayed (versus the peak of the inflammatory response) onset of hyperperfusion 2× compared to controls: this was seen at 24 h in the subcortical matter compared to cortical matter (*see* Fig. 7). Similar effects of intracerebral redistribution of blood flow have been observed in this species and gestational age due to umbilical cord occlusions that cause fetal neuroinflammation [2, 36].

Vx+LPS increased this effect threefold and appeared as early as 6 h. Efferent VNS reduced the rCBF to below control levels for the cortical matter, while afferent VNS diminished the rise of rCBF in the subcortical, but not cortical matter.

Fig. 5 (continued) microglia occupied by cell body only. (**b**) Microglia density expressed as cell body count per mm^2 of tissue within the CA1, CA2/3, and DG. (**c**) Average microglial soma size in µm^2. CA1 (Control $n = 14$, LPS $n = 13$, Vx+LPS400 $n = 5$, Vx+LPS800 $n = 3$, Eff-VNS $n = 7$, Aff-VNS $n = 7$), CA2/3 (Control $n = 11$, LPS $n = 12$, Vx+LPS400 $n = 4$, Vx+LPS800 $n = 4$; Eff-VNS $n = 7$, Aff-VNS $n = 6$), DG (Control $n = 14$, LPS $n = 14$, Vx+LPS400 $n = 4$, Vx+LPS800 $n = 5$, Eff-VNS $n = 7$, Aff-VNS $n = 7$).*$p < 0.05$

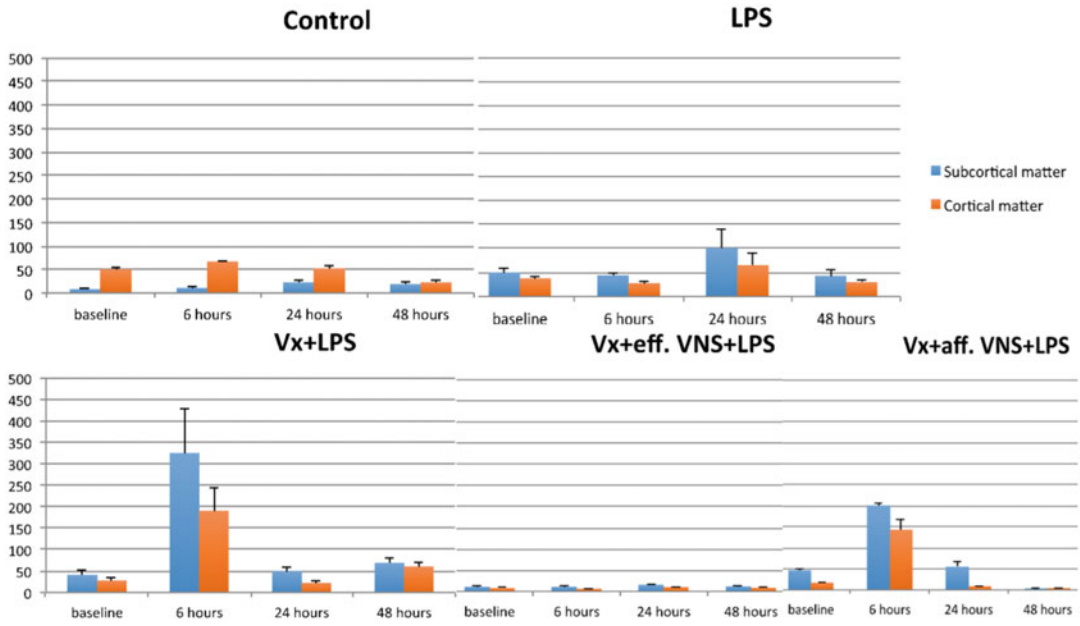


Fig. 7 Regional cerebral blood flow in cortical and subcortical brain regions of near-term fetal sheep is modulated by vagus nerve manipulation during 54 h following the induction of a fetal inflammatory response with lipopolysaccharide (LPS). Vx+LPS represents combined Vx+LPS400 and Vx+LPS800 groups, since no differences regarding rCBF patterns were observed between the two subgroups. Y axis, cerebral blood flow (mL/min/g). Sample sizes: Control, $n = 3$; LPS, $n = 4$; Vx+LPS, $n = 6$; Vx+efferent VNS, $n = 5$; Vx+afferent VNS, $n = 5$

The above-described changes in rCBF can be quantified as redistribution—a measure of redirected CBF from cortical to subcortical regions. We observed main effects for the treatment groups LPS, Vx + LPS400, Vx + LPS800 (i.e., Vx+LPS as shown in Fig. 6), and Vx + Efferent VNS+LPS ($p = 0.000$, $p = 0.03$, $p = 0.045$, and $p = 0.02$ respectively). In addition, we also found main effects for the time points "24 h" and "48 h" ($p = 0.000$ for each) (Fig. 7).

4 Discussion

Complete cervical vagal denervation alters microglial phenotype in an LPS-dose-dependent manner which we refer to as hormetic due to a pronounced reversal of the microglial properties with a doubling of the LPS dose. This behavior is in line with the other reported systemic and organ-specific responses to LPS under conditions of vagotomy [22]. VNS further modulates microglial morphology in a brain-region-specific, i.e., locoregional, manner, as we demonstrate for the subregions of the hippocampus, a key vulnerable region involved in neurodegenerative processes. Vagus nerve manipulations also alter rCBF further highlighting the complex effects VNS exerts on brain function.

4.1 Vagotomy Reduces the Activated State of Microglia Exposed to Systemic Inflammation Induced by LPS

Vagotomy combined with low-dose systemic LPS treatment appeared to reduce the "activated" phenotype in microglia of the fetal sheep hippocampus. Increased Iba-1 expression has been shown to correlate with microglia's membrane ruffling to morphologically change from "resting" to an activated amoeboid-shaped phenotype [37]. Here, we show that Vx + LPS400 treatment reduced Iba-1 mean fluorescent intensity for the whole microglia in the CA1 and for processes only in CA2/3 and DG, relative to control animals. Additionally, a decreased microglial soma to whole cell ratio was observed with Vx + LPS400 treated animals compared to controls in all three sub-regions. In the CA2 subregion, Vx + LPS400 treatment had this effect also compared to LPS only which underscores the impact of complete cervical vagal denervation on microglial morphology under conditions of neuroinflammation. To perform a variety of functional roles throughout development, prenatal microglia typically maintain an "activated" amoeboid morphology with rounder, larger soma and short branching. This result suggests that vagotomy and low-dose LPS treatment favors the "resting" microglia phenotype with smaller soma and more elaborate branching. While low-dose LPS exposure is sufficient to trigger functional microglial activation as presented in this model [28], fetal microglia acquire a "resting" phenotype in response to withdrawn cholinergic input from the vagus nerve. In a male adult rat model of subdiaphragmatic vagotomy, Gallaher et al. reported at 42 days following the vagotomy a mixed pattern of locoregional microglial activation (solitary tract (NTS), dorsal motor nucleus of the vagus nerve (DMV), and nodose ganglia (NG)) or silencing (spinal cord) measured by Iba-1 mean intensity [38]. Hofmann et al. identified the effects of unilateral right vagotomy after 7 days in adult male rats on astrocytes and microglia populations in NTS [39].

It is likely that there is a temporal profile to the locoregional activation pattern. This should be explored systematically in future studies: brain-regional, time-specific and developmental effects must be modeled in relation to vagus nerve activity and additional effects of systemic inflammation such as LPS exposure. Sex effects have been widely acknowledged for the inflammatory response patterns and will likely play a role in the physiology of vagal control of neuroinflammation as well [40, 41].

4.2 Under the Condition of Vagotomy, Iba-1 Expression in the Fetal Hippocampus Depends on LPS Dosage

For fetal sheep receiving vagotomy, Iba-1 expression in the hippocampus is dependent on LPS dosage. We demonstrated that Iba-1 signal intensity was increased in Vx + LPS800 compared to Vx + LPS400 in whole microglia, soma, and processes from all three sub-regions. It is possible that a higher dose of LPS triggers sufficient systemic inflammation and cytokine production that disrupts the blood-brain barrier, therefore prompting a more reactive

microglial phenotype. This hormetic effect under conditions of systemic inflammation with vagal withdrawal could impact neuroinflammatory response corresponding to the severity of chorioamnionitis [22].

4.3 Afferent or Efferent VNS Restores Microglial Phenotype

Compared to Vx + LPS400 treatment alone, intermittent efferent and afferent VNS on top of the condition of Vx + LPS400 restores the levels of microglial activation to an extent similar to control and LPS [28]. When efferent VNS was added compared to Vx + LPS400 only, an increased microglia cell count in the CA1 was observed, suggesting that more microglia have either migrated to this region or proliferated to regulate immunological activity. Similarly, Vx + LPS400 animals that received efferent or afferent VNS showed an increased microglial soma:cell ratio. Although increased microglial soma size has been suggested to be a typical sign of microglial reactivity, there were no significant differences in soma size between these three groups. Therefore, the increased ratio suggests that microglia are undergoing a stage of the activation process where processes have been retracted.

Afferent and efferent VNS treatments lead to smaller soma size compared to treatments expected to cause inflammation: LPS, Vx + LPS800. That is compatible with the general notion that VNS is anti-inflammatory, especially in the hippocampus, here shown for the VNS effect on neuroinflammation [42, 43]. It is important to note that the route of VNS had no measurable effect on the resulting microglial phenotype. This is important for the practical use of VNS where selective efferent or afferent stimulation can be more challenging to fully achieve, albeit progress has been made in this regard [44].

These findings are contrasted by the pronounced systemic inflammatory response seen with afferent VNS compared to efferent VNS. Future work will show if other brain regions may show differences from the hippocampal behavior in response to LPS with afferent VNS. The rCBF patterns were more aligned with the observed systemic inflammatory responses differences. Future studies should explore these effects further.

4.4 VNS Modulates rCBF

Vagus nerve manipulation results in changes of rCBF specific to cortical versus subcortical brain regions. This finding indicates that the vagus nerve exerts some control on the redistribution of rCBF, at least under conditions of unperturbed cerebral auto-regulation, as is likely the case in the presently described experiments: the blood pressure, especially the diastolic blood pressure did not drop to septic levels in these experiments as we reported elsewhere [22].

Specifically, vagotomy followed by induction of fetal inflammatory response results in an amplified redistribution of rCBF in favor of subcortical perfusion. In contrast, efferent VNS reduces the

rCBF to below control levels for the cortical matter, while afferent VNS diminishes the rise of rCBF in the subcortical, but not cortical matter.

Future research will attempt to causally relate the effects of the vagal activity on locoregional patterns of cerebral perfusion and neuroinflammation and how these patterns correlate to acute neurological and neurodevelopmental outcomes. The ability to modulate vagal activity via VNS provides here a novel therapeutic modality for early intervention to reduce sequelae from perinatal brain injury.

4.5 Therapeutic Implications for VNS in the Treatment of Neurodegenerative Disorders

Going beyond perinatal development, the interplay between the CAP and microglial cells offers enormous therapeutic potential. Early discoveries involving both preclinical and clinical data have implicated the CAP in neurodegenerative diseases such as Parkinson's, explained in part by interactions with microglial cells. The Braak hypothesis for Parkinson's pathogenesis has demonstrated that α-synuclein originating in the Meissner's and Auerbach's plexuses of the gastrointestinal system is transported in a retrograde fashion up the vagus nerve to brain regions such as the substantia nigra pars compacta in the midbrain, the primary site of dopaminergic neurons' destruction in Parkinson's [45]. In neural tissue, misfolded α-synuclein contributes to a chronic neuroinflammatory response. Neuropathological post-mortem analysis has demonstrated increased levels of pro-inflammatory factors, microglia activation, and T-cell activation. The pro-inflammatory state, driven in part by α-synuclein, is believed to be both directly cytotoxic to dopaminergic neurons and catalytic for neurodegeneration. Inflammatory signaling increases blood-brain barrier permeability, which allows for increased α-synuclein transport into the brain. The neuroinflammatory response within the brain then drives α-synuclein aggregation, further perpetuating activation of the innate and adaptive immune responses and repeating the cycle. Thus, the neuroinflammatory response seen in Parkinson's disease, which is largely driven by microglial cells, essentially acts as a closed-loop system for neurodegeneration [46]. Animal modeling and retrospective cohort analysis in humans have substantiated the Braak hypothesis, demonstrating that vagotomy is associated with decreased incidence of Parkinson's disease [47]. As suggested by the previous modeling of the Braak hypothesis, the likely mechanism for this finding is that vagotomy prevents α-synuclein transport to the brain; therein limiting the neuroinflammatory response that drives neurodegeneration [46].

Microglial cells are the immune cells primarily responsible for neuronal cell death in Parkinson's disease. As outlined above, evidence suggests in part that a-synuclein activates microglial cells triggering the CNS neuroinflammatory response [46]. Interestingly, α-synuclein structure corresponds with the functional role

of microglial cells in this neuroinflammatory response, and thus disease progression. The more classical mutant structures of α-synuclein, like A30P or A53T, lead to a proinflammatory activation state with subsequent neurodegeneration. However, monomeric a-synuclein results in activated microglial cells that serve a neuroprotective role [46]. The functional diversity demonstrated through the Braak mechanism for Parkinson's illustrates why there is hope to 1 day clinically manipulate the relationship between the CAP and microglial cells to counter neurodegeneration via VNS.

The excitement surrounding the functional diversity of microglial cells and the potential therapeutic implications it offers are not limited to neurodegeneration seen in Parkinson's disease. Microglial cells have been implicated across multiple disease states. One proposed mechanism for neurodegeneration in other pathologies is the re-activation of complement-mediated developmental synapse pruning by microglial cells. During normal neural development, microglia rely on C1q tagging of synapses to identify phagocytic targets to allow trimming of the neuronal network. As the fetus approaches term, such activity is down-regulated [48]. However, mice models for pathologies such as Alzheimer's, Pick's disease (frontal-temporal dementia), acute West Nile encephalopathy, and aging have all demonstrated aberrant interactions between the classical complement system and activated microglial cells leading to synaptic destruction. The functional impact of unregulated synaptic destruction by microglia presents as the cognitive decline that hallmarks these disease processes [15].

The growing body of evidence makes clear that there is still much to be learned about the functional diversity of microglial cells and their role in many CNS pathologies. Data indicate that microglial cells have the capacity to serve a multitude of roles, and which role they take on can be guided by cues from their microenvironment. Additionally, the vagus nerve is more involved in the regulation of the innate immune system than previously thought. In fact, it may provide a mechanism for the modulation of microglial cell microenvironments; a potential pathway toward a clinical intervention for neurodegeneration and other conditions where neuroinflammation is a factor.

4.6 Implications of VNS in Studies of Perinatal Brain Development and Function: New Insights and Opportunities

There remain many open questions. On the systems level, we need to understand the relationship between the vagal activity and microglial phenotype. The expression of α7nAChRs on microglia appears to be dynamic in relation to the vagal activity [2]. How does this dynamics drive the microglial phenotype and can we leverage VNS to modulate microglial properties for salutory purposes? Vagal activity also appears to modulate the systemic metabolic state, at least as far as we can observe under conditions of mild to moderate inflammation as may occur during chorioamnionitis [22].

On the cellular level, the in vitro α7nAChR stimulation modulates the immunometabolic phenotypes of microglia and astrocytes in the perinatal brain [9, 28, 49].

Together, these findings have led to the neuroimmunometabolic hypothesis of the etiology of autism spectrum disorder (ASD), a condition whose origins have been associated with intrauterine adversities such as neuroinflammation and chronic stress [50].

We discuss the implications for the developmental origins of neurodegenerative disorders in a separate subsection.

Methodologically, it is important to emphasize that we propose an approach whereby not only mean intensity levels of Iba-1 positive microglia are quantified in relation to an intervention, but also their complex soma-processes' morphology is accounted for. We suggest that a particular attention be paid in future work to the cell morphometric assessments of microglial plasticity, especially in relation to VNS. That is also likely true for understanding the VNS effects on the plasticity of other glial cell populations, notably the astrocytes.

VNS is usually thought of as an *exogenous* process, but we also observed a dynamic relationship between microglia and CAP activity under *endogenous* "VNS" conditions. These are the types of VNS that arise from physiological or pathophysiological states such as fetal acidemia or inflammation which directly stimulate the vagus nerve [2].

VNS can therefore be thought of as being exogenous or endogenous in origin, with its own set of effects on systems and cellular scales of physiological organization.

This view raises the question how the exogenous or endogenous VNS effects can be decoded indirectly from readily available signal sources such as electrocardiogram (ECG)-derived heart rate variability (HRV), a notion we refer to as the HRV code [51]. In the related contribution, on the systems level, we present a direct (vagus electroneurogram, VENG) approach to decoding vagus nerve activity and demonstrate its relationship to the indirect, HRV-based approach [52, 53]. The ECG-derived HRV inflammatory index tracks the rise of the systemic inflammatory response and the changes in the VENG using the same underlying machine learning algorithm.

An important limitation of the presented work and inferences is the lack of vagotomy-only group as a sham to Vx+LPS groups in the presented experiments. That remains subject of future work.

4.7 Significance

In summary, VNS modulates microglial phenotype in hippocampus of the developing brain. This may be leveraged for acute modulation of perinatal brain injury [54]. Moreover, vagus nerve activity appears to have a long-term impact on the neurodevelopment and

lifetime risk for neurodegenerative disorders. Future research needs to seek for optimal VNS treatment regimes for various adversities to ensure that these do not alter the long-term microglial phenotype unfavorably.

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Chapter 5

Neonatal Sepsis Is Diminished by Cervical Vagus Nerve Stimulation and Tracked Noninvasively by ECG: A Pilot Report and Dataset in the Piglet Model

Aude Castel, Patrick Burns, Colin Wakefield, Kevin J. Jean, Yael S. Frank, Mingju Cao, Andre Desrochers, Gilles Fecteau, Christophe Faure, Christophe L. Herry, and Martin G. Frasch

Abstract

Background: Vagus nerve stimulation (VNS) reduced inflammation induced by lipopolysaccharide (LPS) in an adult rat sepsis model. A multi-dimensional heart rate variability (HRV) index reliably tracked the inflammatory profile in near-term sheep fetuses. The effects of VNS on neonates are not known. First, in a neonatal piglet model of sepsis, we present an approach to evaluate the effect of VNS on systemic inflammatory response induced by a high dose of LPS to mimic late-onset neonatal sepsis. Second, we present an analytical pipeline to validate our fetal-sheep-derived HRV inflammatory index in neonatal piglets to test its performance in different species, older developmental stages, and more robust septic responses.

Methods: Three neonatal piglets of 7–14 days of age with 2.4–4 kg in body weight were used in this proof-of-principle study. Following anesthesia, electrodes were attached bilaterally to the cervical portion of the vagus nerve to allow for stimulation of the left vagus (VNS) and bilateral vagus electroneurogram (VENG). Electrocardiogram (ECG), blood pressure (BP), and VENG were recorded for the duration of the experiment. After baseline recording, the piglets were administered LPS at 2 mg/kg IV bolus. In the VNS-treated piglet, the vagus nerve was stimulated for 10 min prior to and 10 min after the injection of LPS. In both groups, each 15 min post LPS, an arterial blood sample was drawn for blood gas, lactate, and glucose as well as the inflammatory cytokines measured by a quantitative ELISA multiplex panel. At the end of the experiment, the piglets were euthanized. BP and ECG-derived HRV were calculated and VENG was analyzed.

Results: The piglets developed a potent inflammatory response to the LPS injection with TNF-α, IL-1β, IL-6 and IL-8 peaking between 45 and 90-min post-injection. VNS diminished the LPS-induced systemic inflammatory response, with a decrease in measured cytokines levels ranging from two to ten-fold. We present a low-cost, easy-to-implement design of a VNS/VENG probe and a framework to analyze VENG in response to LPS. Furthermore, the HRV index accurately tracked cytokine temporal profiles' which was reflected in the power-spectral and complex properties of VENG when applying the machine learning model derived from HRV.

Discussion: We present a novel method to model, manipulate, and track neonatal sepsis using VNS/VENG. Our supportive findings suggest that (1) the HRV index of the systemic inflammatory

Aude Castel and Patrick Burns contributed equally with all other contributors.

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response applies across species pre- and postnatally, (2) the HRV index performs well at different degrees of sepsis (i.e., nanogram and milligram doses of LPS), and (3) the present VNS paradigm effectively suppresses LPS-induced inflammation, even with high doses of LPS; an effect that is reflected by changes in the shared mathematical properties of both VENG and HRV. These findings suggest that the HRV inflammatory index reflects underlying changes in the VENG activity. The presented method lays a foundation for larger studies to investigate the mechanisms and therapeutic potential of early postnatal VNS intervention to counteract sepsis progression. Moreover, the presented experimental and analytical frameworks highlight the potential for HRV monitoring to serve as an early biomarker for tracking the systemic inflammatory response.

Key words VNS, Sepsis, Neonate, HRV, Piglet

1 Introduction

Sepsis is a life-threatening yet treatable—when detected in time—condition of global significance [1].There are an estimated 30 million episodes of sepsis annually, with neonates having the highest incidence of any age group (~three million babies annually). The burden of neonatal sepsis is profound, carrying an associated mortality rate of 11–19% [2]. Although the majority of children do recover, many suffer lifelong sequelae following the inflammatory response. Given the particularly vulnerable nature of neonates, early detection and prevention of sepsis will have a profound impact on reducing overall morbidity and mortality.

Lipopolysaccharide (LPS) is commonly used to elicit and study septic inflammatory responses as it induces endotoxemia mimicking an infection of the blood by Gram-negative bacteria. The pig is considered an excellent model for septicemia, necrotizing enterocolitis, and neonatal brain injury due to its anatomical and physiological similarities with humans. As a means of modulating the septic inflammatory response, vagus nerve stimulation (VNS) has been shown to exert anti-inflammatory and anticoagulant responses induced by LPS in an adult rat sepsis model [3].

As a primary objective, we aimed at evaluating the effect of stimulating the cholinergic anti-inflammatory pathway (via stimulation of the vagus nerve) on the systemic (plasma cytokines) inflammatory response induced by an IV injection of a high dose of LPS.

We have shown that multi-dimensional heart rate variability (HRV) analysis can reliably track the inflammatory profile in near-term sheep fetuses (*see* Fig. 1). As a secondary objective, we aimed at validating the derived HRV inflammatory index in a different setting of sepsis and at comparing its performance and temporal profile with the direct analysis of the underlying vagus nerve electrical activity (vagus nerve electroneurogram, VENG).

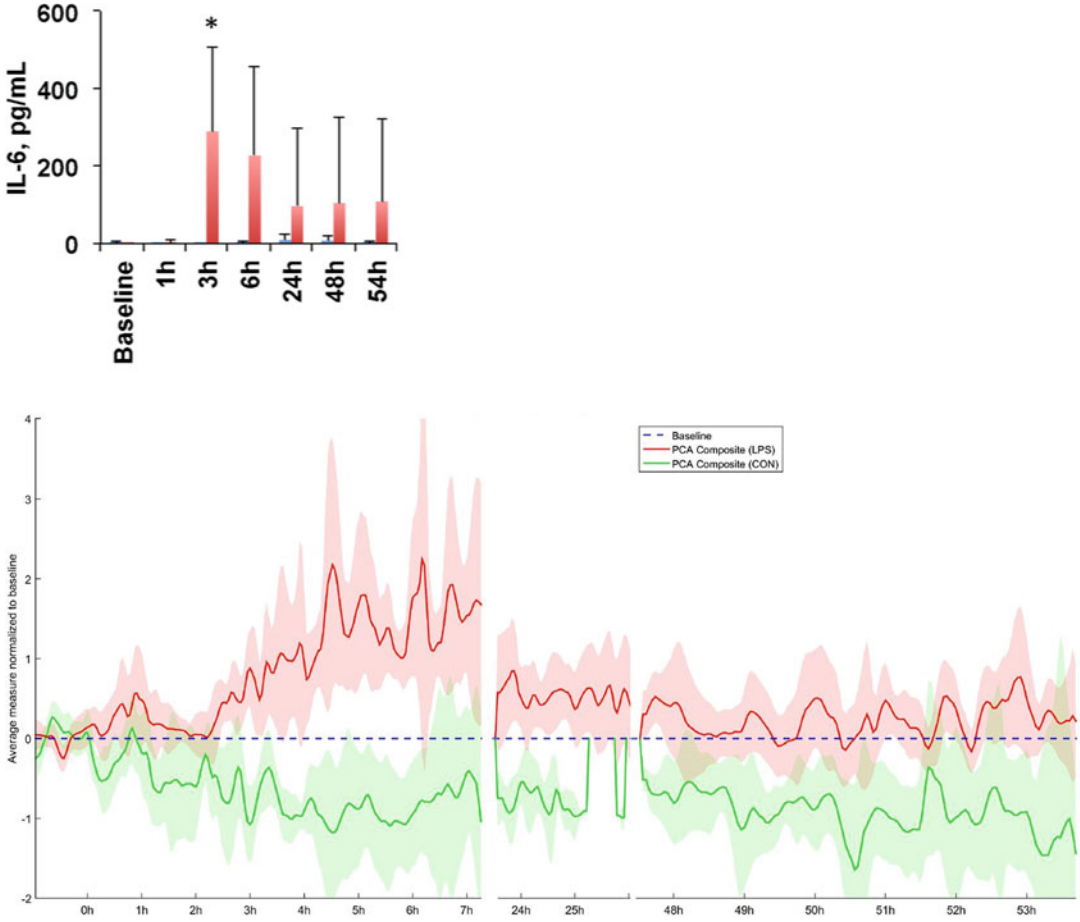

Fig. 1 Heart rate variability (HRV) provides a signature of the fetal systemic inflammatory response in a fetal sheep model of lipopolysaccharide-induced sepsis. *Left*: Temporal profile of LPS-induced fetal sheep inflammatory response measured by plasma levels of IL-6 with a peak at 3 hours post fetal i.v. LPS injection—also reflected in the fHRV inflammatory signatures. Right: Principal component analysis (PCA) approach to identify fHRV signatures that track IL-6 responses. (From Tosato et al. [4] with permission)

2 Materials and Methods

2.1 Ethics Statement

This pilot study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The respective in vivo protocol was approved by the Committee on the Ethics of Animal Experiments of the Université de Montréal (Permit Number: 13-Rech-1695).

Table 1
Animal age, gender and body weight, and treatment

Animal ID	Age (days)	Gender	Bodyweight (kg)	Treatment
Piglet 1[a]	7	Male	2.5	Control
Piglet 2	7	Male	2.4	LPS + VNS
Piglet 3	14	Male	4	LPS

[a]Lost during surgical instrumentation due to a complication with intubation

2.2 Anesthesia

Three neonatal piglets of 7–14 days of age with 2.4–4 kg body weight were used in this pilot study (*see* Table 1). Each piglet was pre-medicated with butorphanol (analgesic) (0.1 mg/kg) and diazepam or midazolam (sedative) (0.2 mg/kg) intramuscularly, approximately 15 min prior to anesthesia. Anesthesia was induced using isoflurane in oxygen via a mask (expired isoflurane 1 to 2.5%; ETISO%). The piglet was then intubated and mechanically ventilated (expired CO_2 = 35–45 mm Hg; $ETCO_2$). Monitoring included capnography, direct arterial blood pressure (ABP), central venous pressure, pulse oximetry, electrocardiography (ECG) and temperature. Body temperature was maintained using warm water blankets. Observations were taken every 5 min.

2.3 Surgery

Once the piglet was anesthetized, the catheter insertion sites (neck and groin) were surgically prepared. A 20–22 G catheter was inserted into the femoral artery in the groin area via a cut-down to measure the direct ABP. An introducer catheter was inserted into the right carotid via a cut-down. A second catheter was then inserted through the introducer catheter and fed into the left ventricle. The correct placement of the catheter was verified using the arterial pressure trace. Lidocaine 2% was instilled (splash block) into the surgical wounds prior to these first two procedures. Another catheter (8–10 Fr G) was inserted into the left jugular via a cut-down to measure the central venous pressure and to administer intravenous fluids (3–5 mL/kg/h). Via an incision on each side of the neck, electrodes were attached to the left and right vagus nerves to allow for stimulation of the left vagus nerve and bilateral recording (VENG). Left VNS has been shown to result in less to no cardiovascular side effects, i.e., no bradycardia [4, 5]. In the control animal (LPS only), the electrodes were also placed on both vagus nerves in the same way to record VENG (but no VNS). Local anesthesia was not used in this surgical incision because of the risk of desensitizing the vagus nerve. A suprapubic urinary catheter was inserted into the urinary bladder to measure the urine output and to minimize the discomfort associated with a distended bladder during the experiment (~3 h postoperatively). A small incision (3–4 cm) was made to facilitate the insertion of this urinary catheter. A purse-string suture was placed to secure the urinary catheter in place.

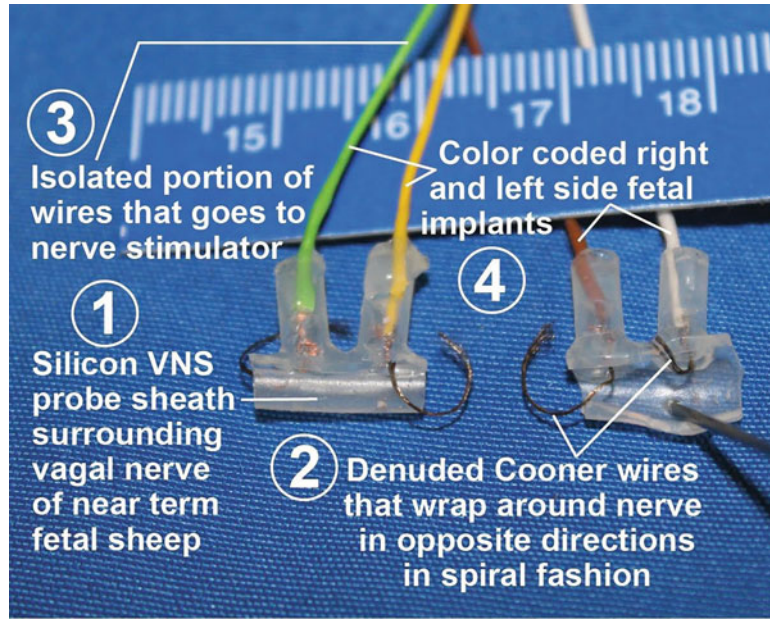

Fig. 2 Design of the fetal sheep cervical bilateral VNS/VENG probe.[8] The scale is metric. This design was also used successfully in the present approach in the neonatal piglet

2.4 Data Acquisition

ECG, heart rate (HR), and ABP were monitored continuously (1902 amplifier and micro3 1401 ADC by CED, Cambridge, U.K., and NL108A, NeuroLog, Digitimer, Hertfordshire, U.K) and sampled at 1000 and 256 Hz, respectively. VNS was applied via NeuroLog's NL512/NL800A using a pulse sequence pre-programmed in Spike 2. The VNS settings were as follows: DC rectangular 5 V, 100 μA, 2 ms, 1 Hz according to Borovikova et al. [6]. VENG was recorded at 20,000 Hz. See Fig. 2 for VNS/VENG electrode design [7].

2.5 Experimental Protocol

2.5.1 Baseline

After a period of stabilization (15 min), 60 min of "baseline" recording was allowed during which ABP, ECG and VENG were recorded. In the VNS+LPS piglet, only the left VENG was recorded. In the LPS piglet, bilateral VENG was recorded. Subsequently, a baseline blood sample was taken for blood gases (Radiometer, 0.8 mL), complete blood count (hematology) (1 cc) and cytokines ELISA (3 cc, spun down at 4 °C, 4000 rpm for 4 min and frozen at −80 °C for plasma) (total of 5 mL blood).

2.5.2 Induction of Endotoxemia with LPS

Next, the piglets were administered LPS 2 mg/kg IV bolus (Sigma L2880) [8]. In the treatment group (VNS), the vagus nerve was stimulated for 10 min prior to and 10 min after the injection of LPS. The control group was observed over the course of 3 h as it developed sepsis. In both groups, every 15 min post LPS

administration, a 0.8 mL arterial blood sample was drawn for blood gas, lactate, and glucose (Radiometer ABL800 Flex). At baseline, 15, 45, 90 and 135 min, these measurements were done as part of the larger blood sample (3–4 mL) together with inflammatory cytokines and for hematology. At the end of the experiment, the piglets were euthanized with an overdose of pentobarbital and tissues were collected. Prior to the injection of pentobarbital, the level of anesthesia was deepened.

2.5.3 Cytokines Assay

Plasma cytokines were measured using the commercial service provided by Eve Technologies (Calgary, AB, Canada). Porcine Cytokine 13-plex Discovery Assay kits were used (Cat.# PCYTMAG-23K-13PX) to examine 13 cytokines including GM-CSF, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18, and TNF-α, Here we report the results of IL-1β, IL-6, IL-8, IL-10 and TNFα, the cytokines of most interest mediated by the cholinergic anti-inflammatory pathway [9]; their assay sensitivities are 42 pg/mL, 9 pg/mL, 5 pg/mL, 9 pg/mL and 6 pg/mL, respectively. The intra-assay and inter-assay variations for all the cytokines were <10% and <20%, respectively.

2.5.4 Hematology

The extent of inflammation was further evaluated by using a complete blood count (CBC) to compare the hematological changes associated with sepsis in both groups.

2.5.5 Data Analysis

Evaluation of piglets' physiology was done based on the literature [10, 11]. Mean (mBP), diastolic (dBP) and systolic (sBP) ABP, as well as HR, were calculated for each animal, at each time point, as an average of the artifact-free 10 preceding minutes (60 preceding minutes for the baseline) using Spike 2 (Version 7.13, CED, Cambridge, U.K.). We reported the CIMVA approach and derivation of the HRV composite measure elsewhere [12, 13]. The VENG analysis was conducted with the open-source EEGLAB package v2019_1 within the Matlab environment (Matlab 2013b for Linux, MathWorks, Natick, MA). The power spectral analysis was done in Python. The dataset and all code scripts are available on FigShare [14].

3 Results

The animals' characteristics are summarized in Table 1. LPS injection had a rapid and profound effect on piglets' acid-base status characterized by rising lactic acidosis which was antagonized by VNS treatment (*see* Fig. 3).

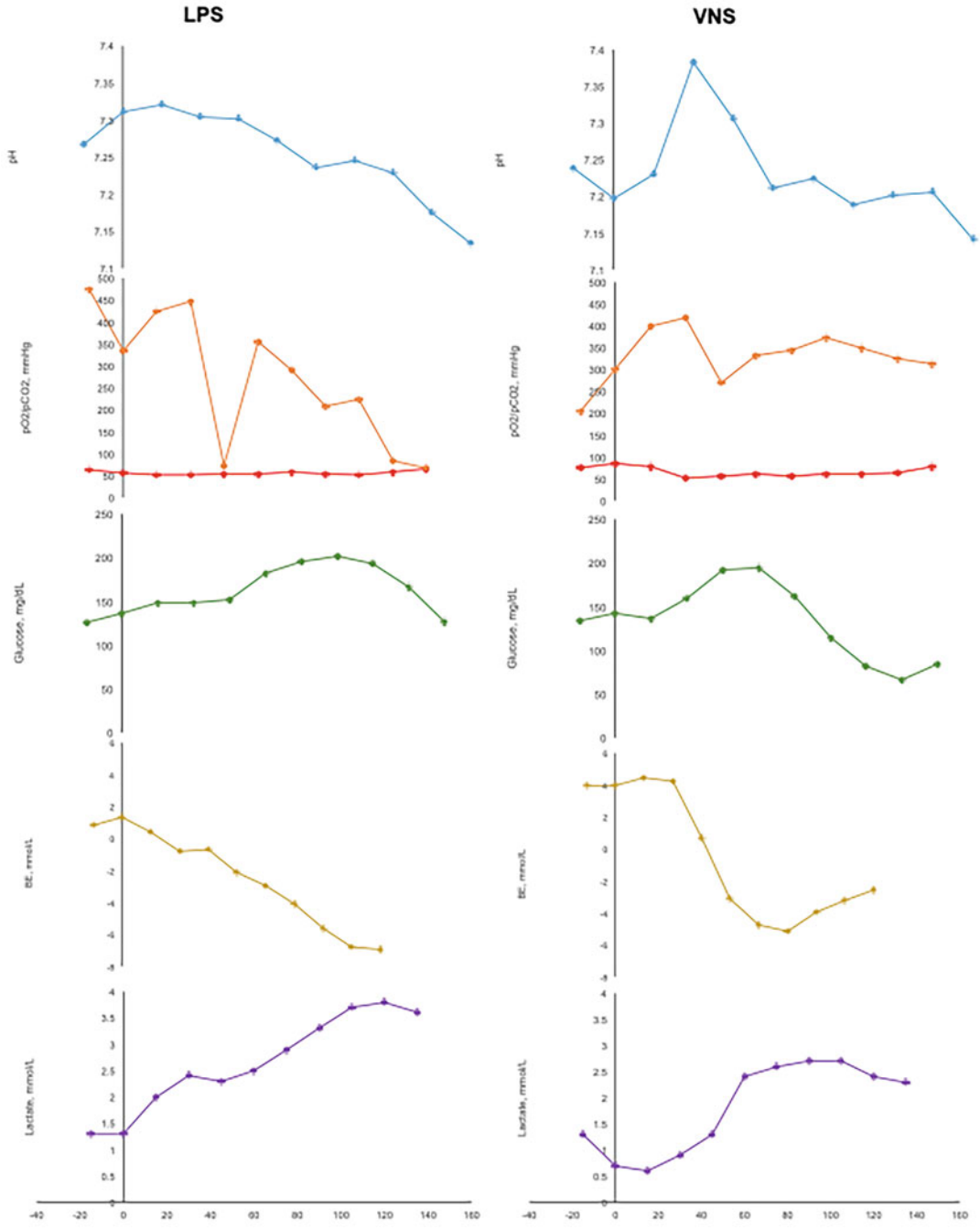

Fig. 3 Blood gas, glucose, and lactate measurements during the experiment

Piglet 3 (LPS) developed profound and progressive leukopenia followed by the appearance of toxic neutrophils toward the end of the experiment at 135 min. Piglet 2 (LPS+VNS) developed toxic neutrophils after 135 min.

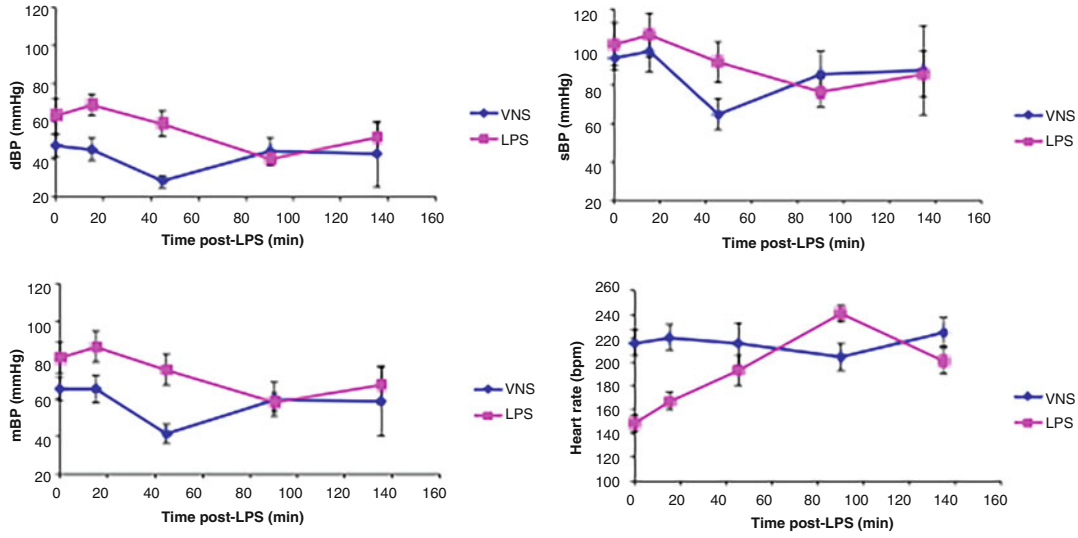

Fig. 4 Cardiovascular responses: diastolic, systolic, and mean blood pressure as well as heart rate

3.1 Cardiovascular Effects

In piglets, HR of less than 120 is considered bradycardia, whereas between 121 and 160 is normal, and more than 161 represents tachycardia. The VNS-treated animal showed a more stable, albeit tachycardic HR of around 220 bpm, while LPS alone resulted in a rapid rise in HR to 240 bpm, following the temporal profile of the cytokine increases toward 100 min post LPS (*see* Figs. 4 and 5). The VNS-treated piglet started out with normal sBP but lower than physiological levels of mBP and dBP, immediately following LPS injection and maintained sub-physiological mBP and dBP even at the time of TNF-α peak around 45 min post LPS injection. This difference disappeared toward 80 min post LPS and in the further course of the experiment.

3.2 Cytokine Responses

At 45 min post LPS injection, TNF-α levels peaked and were eight-fold lower in the VNS-treated animal (*see* Fig. 5). IL-10 and IL-6 followed the same trend reflecting the physiological compensatory rise of the anti-inflammatory IL-10. Surprisingly, VNS also limited the rise of IL-10 at this time point, delaying its increase by ~90 min. IL-6 level responded with a characteristic slight delay in rising at ~90 min and VNS reduced the magnitude of this response by two-fold [15, 16]. There was no effect of VNS on the LPS-induced increases of IL-1β and IL-8. It is possible that the suppression of the IL-10 raised by VNS secondarily abolished its effect on IL-1β. Not shown in Fig. 5, we also observed an increase of IL-4, IL-12, and IL-18 following LPS administration. VNS treatment accelerated the rise of IL-4 and IL-12 by 45 min to a similar peak magnitude of 200 pg/mL and 1200 pg/mL at 135 min, respectively. The IL-18 level showed a biphasic temporal profile rising with a 45 min delay to a similar level of 425 pg/mL in the VNS-treated animal.

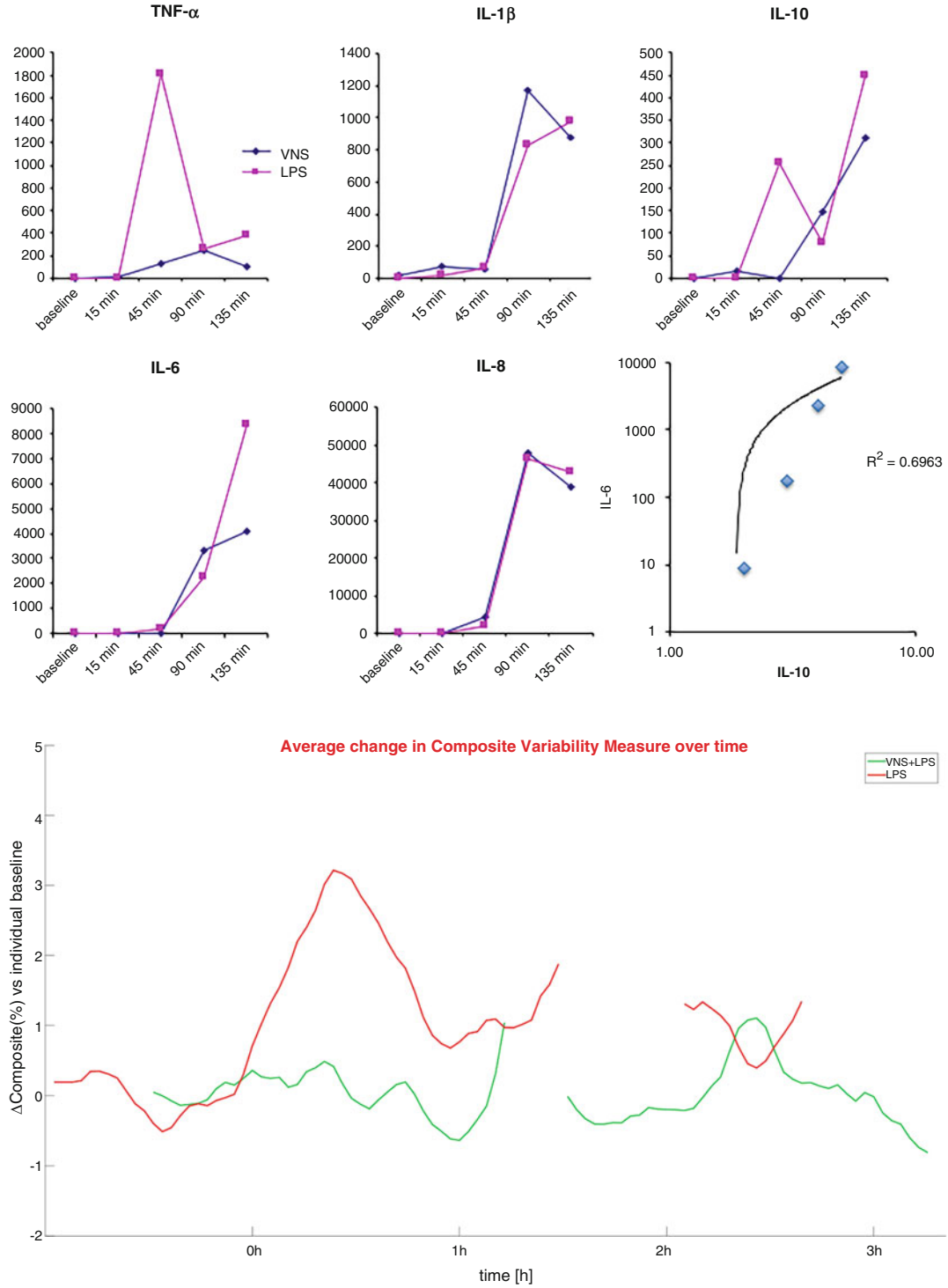

Fig. 5 Cytokines and HRV. Validation of fHRV signature of inflammation (derived from the near-term fetal sheep model of low-dose LPS-induced fetal inflammatory response) in a neonatal piglet model of sepsis at a higher LPS dose. *Top*: Inflammatory response to LPS injection in two piglets receiving each 2 mg/kg intravenous LPS after baseline with or without vagus nerve stimulation (VNS). VNS diminished LPS-induced systemic cytokine production (pg/mL). *Bottom*: The HRV composite measure derived from the fetal sheep model of low-dose

3.3 HRV Behavior

HRV inflammatory index tracked the inflammatory response and its change secondary to VNS closely over time, particularly following the trend of the temporal profile of TNF-α at its primary peak around 45 min and showing a smaller, secondary peak at ~90 min which corresponded to the delayed peaking levels of IL-6, again reduced by VNS (*see* Fig. 5). Due to some missing ECG data in this segment of the experiment, there were corresponding missing time points in the HRV index.

3.4 VENG Patterns in Response to LPS and VNS

VENG power spectra over the course of the experiment are presented in Fig. 6. Since most changes are visible above 2 kHz, we visualized the data in several ways to demonstrate this behavior more clearly. To achieve this, we determined spectral power in low (1–1000 Hz), medium (1000–2000 Hz), and high frequency (2000–10,000 Hz) bands of VENG activity, in addition to the whole-band power spectral analyses.

We observed several immediate differences between the VENG of the LPS+VNS piglet compared to the LPS-only piglet. First, the VENG of the LPS+VNS piglet showed three-fold higher average levels of VENG spectral power density over the course of the experiment compared to the piglet exposed to LPS alone.

Second, there was a change in the frequency peaks distribution across the spectrum. VENG showed several common as well as VNS-treatment-specific spectral frequency patterns. The common patterns included the recurring and time-specific peaks in the ranges of 4.1–4.2 and 8.2–8.3 kHz. VNS moved these peaks to the right of the power spectrum by about 200 Hz.

Third, the slope of the rise of the power spectral density changed with the time course of the experiment following LPS administration. The rise of the slope began earlier in time and started at a lower frequency in the LPS-only piglet's VENG compared to VNS-treated piglet (2500 Hz at ~30–45 min versus 4000 Hz at 60–75 min).

Overall, these patterns are aligned with the changes seen in the VENG inflammatory index profile reported in the next subsection.

Time-frequency representations of VENG, such as the ones shown here or, for example, the synchrosqueezed transform (SST) [17], can be useful in future studies when dissecting the complex patterns in VENG and relating the VENG changes to the experimental states. Future studies will explore the physiological foundations of the observed high-frequency oscillations in VENG.

Fig. 5 (continued) intravenous LPS exposure for tracking the inflammatory response is applied to this piglet model following high-dose intravenous LPS exposure. Note that the HRV composite measure tracked accurately the cytokines' change over time as seen by comparing the peaks and troughs with the timing on the *X*-axes in each diagram)

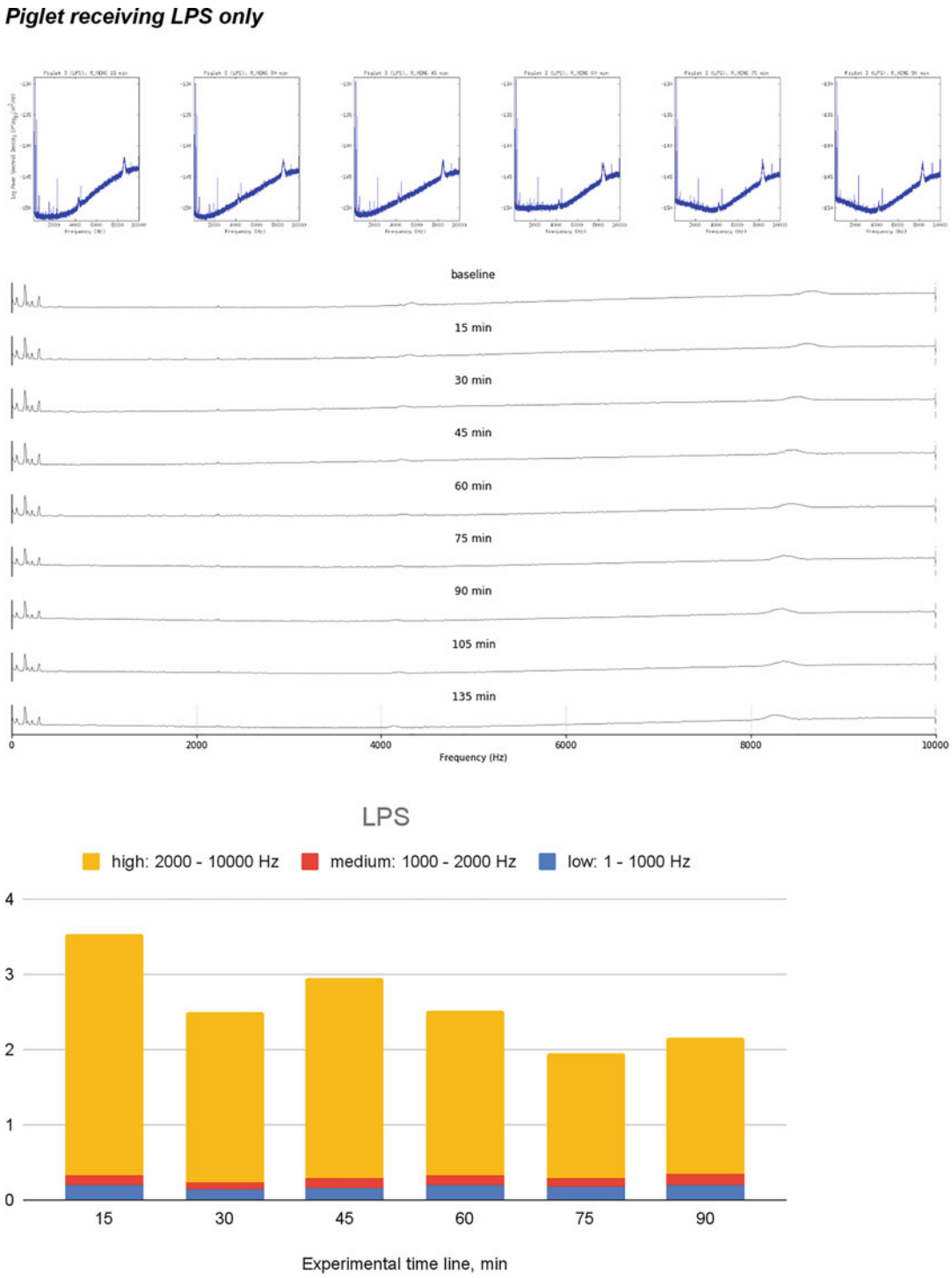

Fig. 6 Temporal frequency dynamics of VENG displayed as power spectral density and summated within sub-frequency bands. Note that most changes occur above 2000 Hz. Data is shown from one neonatal piglet exposed to LPS without VNS (TOP) or another piglet with VNS treatment (BOTTOM). *Y*-axis (mV^2/Hz) is optimized for each series to highlight the frequency peaks

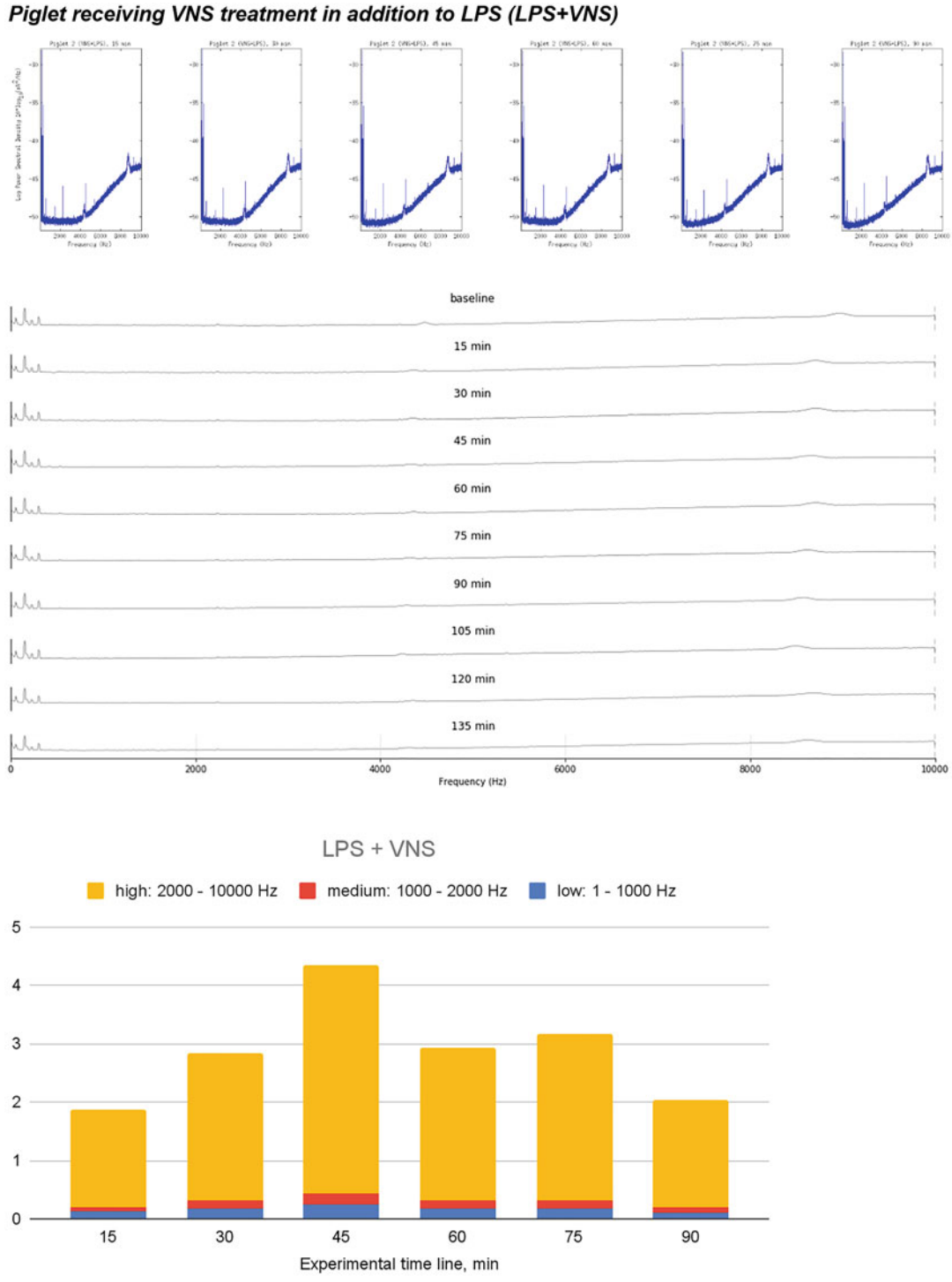

Fig. 6 (continued)

3.5 HRV and VENG Inflammatory Index: Machine Learning Approach

The vagus nerve is a key homeostatic regulator of the inflammatory response. To confirm the hypothesis that the HRV inflammatory index encodes changes in VENG properties we tested whether the HRV-derived inflammatory index applied to VENG also correctly profiled the inflammatory response.

Using the same CIMVA approach that yielded the HRV inflammatory index (*see* Fig. 5), we computed the variability of select mathematical metrics (mean rate, eScalE, sgridTau, AsymI, Multifractal_c1) for both filtered and unfiltered VENG recordings. The mathematical metrics are defined as follows: ScalE: embedding scaling exponent; sgridTau: Grid transformation feature: Time delay similarity index; AsymI: Multiscale time irreversibility asymmetry index; Multifractal_c1: MultiFractal spectrum cumulant of the first order.

We present the resulting dynamics of the computed VENG variability metrics in 5 min intervals for each experimental stage (*see* Fig. 7).

For both LPS-exposed piglets, with and without VNS treatment, there is a temporal profile of VENG-derived inflammatory index that follows that of the HRV inflammatory index and the inflammatory response almost exactly. Interestingly, in the piglet where both nerves were recorded and which received no VNS, we see side-specific differences reflecting the known functional vagus nerve asymmetry.

4 Discussion

HRV inflammatory index accurately tracked the cytokines' temporal profiles, and this was reflected in the power-spectral properties of VENG in this swine model of neonatal sepsis. This approach demonstrates that the HRV inflammatory index (1) applies across two large mammalian species (sheep and swine) with strong similarities with human physiology, pre- and postnatally and (2) performs well at different degrees of sepsis (i.e., nanogram and milligram doses of LPS). Moreover, the VNS paradigm based on Borovikova et al. [6] suppresses LPS-induced inflammation in neonatal piglets, even at high doses of LPS. Notably, the effects of VNS were also reflected by temporally concordant changes in the HRV inflammatory index. This suggests a certain degree of species independence in regards to the performance of the HRV inflammatory index which can be seen as a hallmark of the HRV code, a concept reviewed elsewhere in more details [18–21].

We cannot say with certainty at this stage (having just one animal in each group) that VNS reversed leukopenia seen in the LPS piglet, but the changes are in favor of this conclusion. LPS injection triggered acid-base status changes, as well as cardiovascular and cytokine responses which were all altered by VNS treatment,

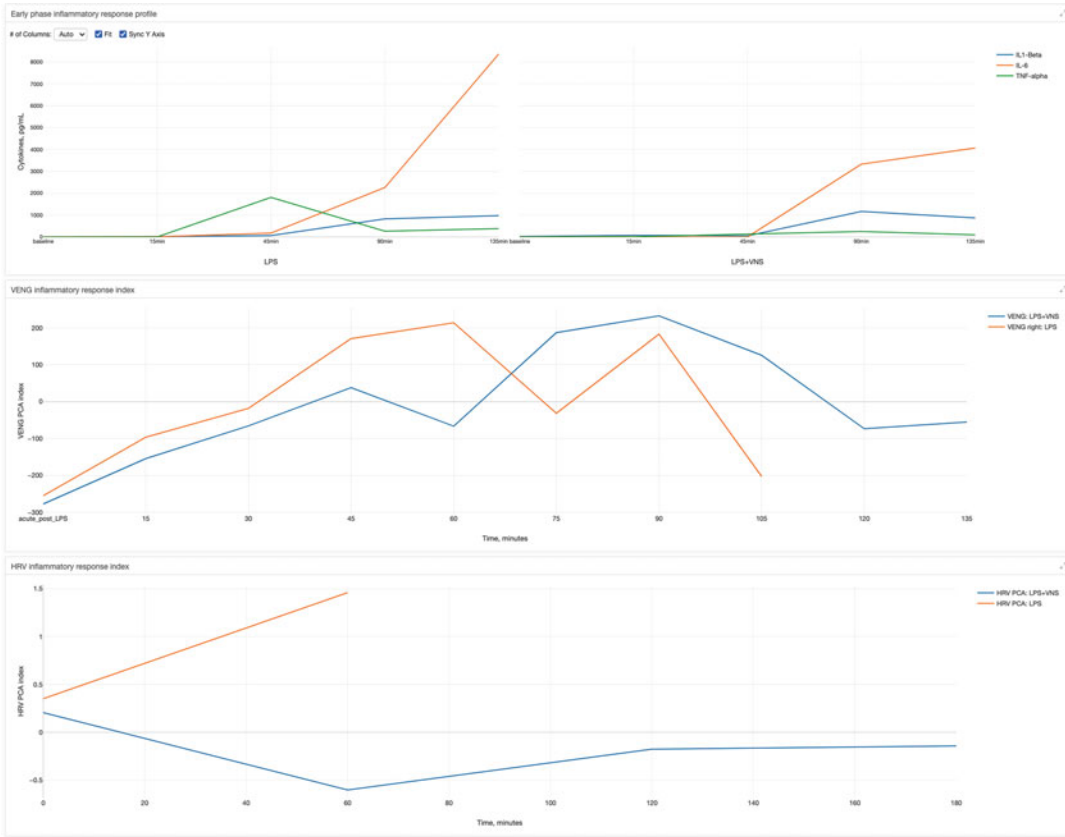

Fig. 7 Temporal profile of the fetal inflammatory index applied to VENG and HRV together with the early stage cytokine inflammatory response. (The interactive version is accessible https://exploratory.io/dashboard/ivC1vEA9IX/Inflammatory-index-indirect-and-direct-computation-JLo5sFg9Pd and https://exploratory.io/dashboard/ivC1vEA9IX/Inflammatory-index-indirect-and-direct-computation-Zuc0uHh7SH)

albeit we did not observe an overt cardiovascular shock. The improved acid-base status is in line with a reduced inflammatory response secondary to VNS treatment. This may also explain the relatively lower, closer to physiological, blood pressure measurements under steady tachycardia in the VNS-treated piglet. The lower initial dBP levels in the VNS-treated piglet may be directly due to early VNS effects. Prolonged, but not acute, VNS has been shown to reduce dBP and mBP levels [22–24]. However, differences in VNS installation, parameter settings and species make comparison difficult requiring further systematic studies, particularly to verify whether the early reduction of dBP is clinically relevant under conditions of neonatal sepsis [19]. In addition, the reported cardiovascular effects of VNS should be seen with restraint, since piglets can show a considerable range of blood pressure and heart rate values at this age [10, 11].

The effects of VNS on LPS-induced cytokine responses were not homogenous and showed some cytokine specificities and differences in temporal response profiles. This is in line with recent findings in rodents showing that specific VNS settings induce specific cytokine responses and, vice versa, that VENG properties encode for specific cytokine sensing in the vagus nerve [16, 25]. Consequently, the selected VNS settings may have been specific to TNF-α and IL-6, but not IL-1β and IL-8: an interesting finding considering that we based the VNS settings on the work by Borovikova et al. which was done in adult rats and reduced TNF-α production [6]. In contrast to their study, we observed a transient early reduction of IL-10 in the VNS-treated piglet.

In the present study, we demonstrated that encoding the multidimensional properties of HRV allows for tracking the inflammatory response in real time and in parallel with the evolving cytokine release. The information we captured this way from HRV is also contained in the vagus nerve electrical activity (VENG) itself, further strengthening the notion that this experiment allows tracking of afferent/efferent brain-body neuroimmunological communication.

5 Limitations

This pilot study has limitations. Only the left VENG was recorded in the VNS+LPS piglet. In the LPS piglet, as well as in future studies, bilateral VENG should be recorded to study the side differences in VENG patterns which are most likely to reflect the underlying side-specific brain-body communication. Furthermore, the control animal died in the beginning of the experiment preventing us from collecting any data on this animal. Additionally, we did not compare the effect of VNS stimulation alone (without LPS administration) on the different variables.

6 Future Directions

Future studies using this model could focus on recording and analyzing the VENG in the setting of septicemia. Do interventions such as VNS alter VENG properties? A similar analysis could be conducted for concomitant HRV dynamics. Next, the correlations between VENG and HRV signals and their features can be gauged. Once the number and types of different states for each signal and the correlations between them have been characterized, it could be investigated whether interventions on one signal (e.g., VNS) can have the desired effect on another signal (HRV). Previous work suggests that this is possible for VENG and HRV [26, 27]. Recently, promising results have been obtained in decoding VENG activity

using a density-based clustering approach implemented in DBSCAN which is readily available in R [25, 28, 29].

Future studies should further direct this approach to derive the mathematical properties of the VENG activity reflecting the inflammatory response which maps onto the respective mathematical properties of HRV captured by the inflammatory index. Specifically, one could ask if the properties can be predicted from VENG, which represents vagus nerve activity, i.e., a direct problem (as opposed to using HRV to predict vagus nerve activity, an inverse problem). Deep Learning approaches could be tractable. For example, one could train an LSTM network on VENG baseline data to predict the corresponding HRV features (VENG -> HRV {features set from our inflammatory index}). Future directions include also tracking image complexity changes in wavelet transform representation of the VENG, e.g., using the approach by Zhao [30].

This research direction has the potential to expand our understanding of the HRV code through the direct study of VENG properties and direct manipulation of VENG properties by VNS. Ultimately, this will lead to closed-loop biocybernetic stimulation/monitoring systems of vagus nerve activity and its surveillance and control of the inflammatory milieu.

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Chapter 6

Cognitive Enhancement Through Vagus Nerve Stimulation: Methodological Considerations for Behavioral Studies in Rats

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Abstract

Optimal cognition is maintained by a balance of excitatory and inhibitory signaling in brain regions such as the prefrontal cortex and hippocampus. Alterations in excitatory/inhibitory balance are linked to cognitive deficits in aging and a variety of neuropsychiatric disorders. Thus, therapeutic interventions such as electrical vagus nerve stimulation (VNS), which has been shown to modulate excitability and effectively regulate seizure activity in intractable epilepsy, may have benefits for improving cognition. In both humans and rodents, VNS can enhance multiple forms of neuroplasticity and cognition, and recent work from our labs in rats has shown that VNS can reliably enhance cognitive flexibility. In this chapter, we present experimental guidelines and considerations for performing studies of VNS effects on cognition in young and aged rats. Our goal is to provide the reader with examples of specific parameters and methods for conducting such experiments.

Key words Vagus nerve stimulation, Rat, Animal models, Reversal learning, Prefrontal cortex, Hippocampus

1 Introduction

Electrical vagus nerve stimulation (VNS) has been used for 30 years as an FDA-approved treatment for refractory epilepsy and several neuropsychiatric disorders, including pharmacoresistant depression [1, 2]. Although the mechanisms by which VNS reduces seizures are poorly understood, its efficacy suggests that VNS can ameliorate excitatory/inhibitory (E/I) dysregulation [3]. Some individuals receiving VNS therapy report cognitive benefits, particularly after long-term use [4–6]. Moreover, a year-long trial of chronic VNS in Alzheimer's disease patients reported improved cognitive outcomes [7].

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Preclinical studies in rodent models further support the efficacy of VNS for enhancing cognition. Acute VNS enhances performance in novel object recognition and extinction learning tasks in rodents [8, 9]. Moreover, VNS facilitates learning to extinguish fear-related responses to a cue previously predictive of electrical shock, which depends critically upon the medial prefrontal cortex (PFC).

1.1 VNS as a Potential Treatment for Cognitive Impairment

Optimal cognition is supported by a balance of excitation and inhibition in brain structures such as the hippocampus and prefrontal cortex, and dysregulation of E/I signaling across these and other brain regions is a key contributor to age-related cognitive decline [10–12]. Critically, however, not all E/I changes in the aged brain are consistent with global hyperexcitability. Particularly in PFC, the data suggest a complex dysregulation of E/I signaling, which likely reflects both primary consequences of aging and secondary compensatory processes recruited in an effort to maintain E/I homeostasis [10]. These primary and secondary effects create challenges for pharmacotherapies that globally increase or decrease excitability. Furthermore, current pharmacotherapies for cognitive decline in aging and AD offer only modest benefits and do not markedly delay disease progression [13, 14]. Thus, alternative therapeutic strategies such as VNS may hold promise.

Vagus nerve efferents modulate the heart, lungs, and viscera (*see* Fig. 1). Efferent fibers comprise only 20% of the nerve, however, whereas vagus afferents (which comprise the other 80%) project to the nucleus of the solitary tract, which in turn directly innervates the locus coeruleus (LC). The LC provides noradrenergic innervation to several brain regions, including the prefrontal cortex and hippocampus (*see* Fig. 1) [15–18]. Notably, preclinical studies show that VNS-induced enhancement of cognitive function and neuroplasticity involves signaling through modulatory neurotransmitters, including norepinephrine [19–23].

In this chapter, we present experimental guidelines and considerations for performing preclinical studies to assess the cognitive effects of VNS. We focus on the rat as an animal model for behavioral studies and provide specific guidelines to consider when working with aged rats.

2 Materials

2.1 Subjects

Rats are widely used laboratory animal models for assessing behavior and cognition. Specific strains used in our labs include the inbred Brown Norway (BN) and Fischer 344 x BN F1 hybrid (FBN) strains, as well as the outbred Long-Evans strain. These pigmented rats exhibit better visual acuity compared to albino rat strains [24], making them well-suited for behavioral experiments

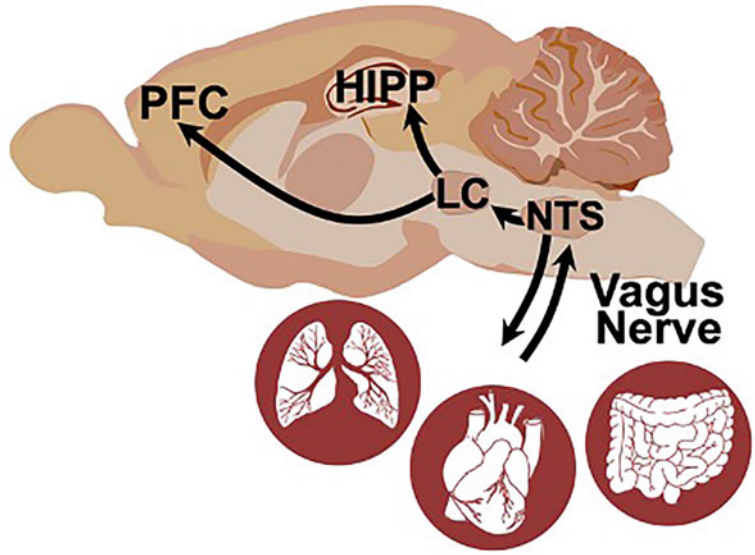

Fig. 1 Schematic of vagus nerve connectivity, showing connectivity of the nucleus of the solitary tract (NTS) with visceral organs, as well as projections from the NTS to the locus coeruleus (LC), which in turn sends noradrenergic projections to the hippocampus (HIPP) and prefrontal cortex (PFC), which are critical for various aspects of cognitive function

conducted in tasks such as touchscreen operant chambers that rely on visual stimuli. Male adult (4–6 months old) BN rats obtained from Charles River Laboratories were used for the experiments described below. Rats of this strain tend to be less active than Long Evans, and thus less likely to tangle or damage the tether used to deliver VNS.

Rats were housed individually in an AAALAC-accredited vivarium facility at the University of Florida McKnight Brain Institute. The vivarium was maintained at 25 °C with a 12 h reversed light/dark cycle (lights on at 1900). Upon arrival in the animal facility, rats were allowed to acclimate for a minimum of seven days prior to experimental procedures. All animal procedures were approved by the University of Florida Institutional Animal Care and Use Committee and followed National Institutes of Health guidelines.

2.2 Vagus Nerve Cuff Electrodes

Custom VNS cuffs (Microprobes Inc, Gaithersburg MD) consist of four 0.39 mm^2 titanium nitride-coated gold electrodes positioned on a planar thiolene/acrylate shape memory polymer substrate (Model 2600, Qualia Labs Inc., Dallas TX) (*see* Fig. 2a1). Although many preclinical studies employ two-electrode designs, we have found that a four-electrode design is better suited for long-lasting behavioral studies that can span several months. This design increases the likelihood of having a functional stimulating electrode pair over time (that is, stimulation can be performed using six

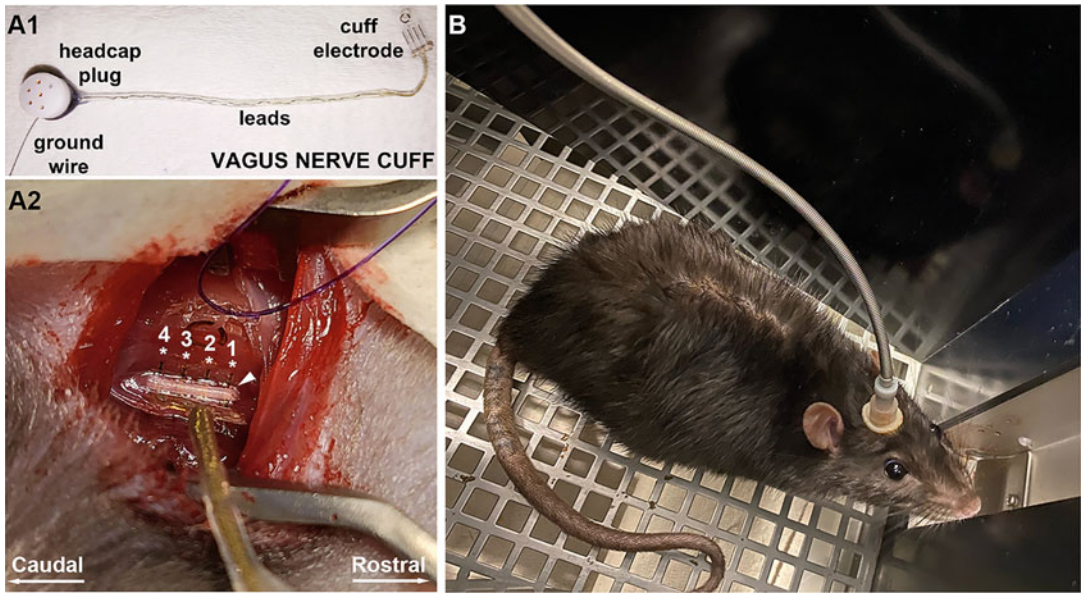

Fig. 2 Vagus nerve cuff implant. (**a1**) Image of custom vagus nerve cuff stimulating electrode. (**a2**) Surgical preparation showing vagus nerve cuff positioned under an isolated section of the left cervical branch of the vagus nerve (arrowhead). Note the use of suture material (purple) to assist placement of the nerve cuff. The vagus nerve cuff consists of 4 stimulating electrode contacts (asterisks) that are secured around the isolated section of the nerve (arrowhead). (**b**) Tethered animal interacting with food trough in a touchscreen operant chamber. Note that although rats are connected to a tether, a commutator permits free movement throughout the operant chamber

different possible pairs of electrodes, should one or two individual electrodes malfunction). Four insulated wires (6.5 cm in length) connected to the four electrode contacts on the cuff are soldered to gold-plated female sockets and fixed in a 6-channel Delrin pedestal (P1 Technologies, Roanoke, VA), which is secured on the rat's skull using dental cement. The insulated wires are contained within a single flexible silicon tube to facilitate subcutaneous threading of the pedestal assembly and wires during surgery (procedures described below). A fifth gold-plated socket inside the Delrin pedestal is soldered to a separate non-insulated stainless-steel wire that can be wrapped around a skull anchor screw to function as an electrical ground. For between-subjects studies employing chronic VNS, sham control cuffs consist of the same rectangular polymer substrate (the cuff) attached to an empty 6-channel pedestal. These sham cuffs are similar in design to the actual electrode cuffs, but do not contain electrode contacts, wires, or sockets. For control procedures, the sham cuff can be surgically implanted around the vagus nerve but does not permit electrical stimulation. Nerve integrity has been reported to decrease in some cases due to long-term placement of cuffs [25], and mechanical manipulation of the vagus nerve, even in the absence of electrical stimulation, has

been shown to activate cholinergic pathways [26]. Thus, to account for the possibility of variables related to cuff implantation influencing study outcomes, a control cuff that closely mimics the experimental condition is ideal for between-subjects experimental designs [27].

2.3 Behavioral Apparatus (Touchscreen Operant Chambers)

Behavioral experiments can be performed in any brand of operant chamber, but the experiments described in this chapter were performed in eight identical "touchscreen" operant chambers housed in sound-attenuating cubicles (LaFayette Instruments, Lafayette, IN) [28]. Touchscreens (touch-sensitive video screens) offer several advantages over traditional operant chambers, which contain relatively few stimuli such as lights, response levers, and a few auditory cues. In the touchscreen chambers, a wider range of visual stimuli can be used for visual discrimination tasks, which can facilitate within-subject experimental designs that allow the same rats to be tested across multiple conditions [29]. Depending on the research question, this can speed the progress of experiments and reduce the overall number of animals needed.

2.3.1 Touchscreen Chamber Design

The touchscreen chamber consists of black plastic walls that are connected to form an isosceles trapezoid-shaped chamber, designed to help direct the animal's attention toward the screen. A 30.7 cm video screen (resolution: 800 × 600 px) forms the wall at the wide end of the chamber. During behavioral testing, a black plastic mask is placed over the touchscreen, with square cutouts that form response windows that allow rats to interact with specific areas of the screen. Touchscreen masks can have different numbers of response windows depending on the task design. For the experiments described in this chapter, we used a screen mask that had 2 response windows (10 × 10 cm squares). A food delivery trough, which could be illuminated by a small lamp, is located at the narrow end of the chamber. A single 45 mg pellet (AIN-76A, Test Diet, Richmond, IN) food reward is delivered into the food trough following correct responses.

Infrared beams cross areas of the chamber in several locations, allowing for recording of movement in the chamber and entries into the food trough as "breaks" of the beams crossing the locations of interest. An overhead house light that can illuminate each chamber is used as a mild aversive stimulus delivered during "time out" periods following incorrect responses. Stimulus presentation and data collection are controlled by computers running ABET II Touch software (Campden Instruments Ltd) and Whisker Server [30].

2.4 Stimulation Apparatus

Electrical stimulation is performed in the touchscreen operant chambers. A commutator (Dragonfly Inc., Ridgeley WV) is mounted in the ceilings of the chambers, and a spring-covered tether (Plastics One, Roanoke VA) attached to the commutator connects directly to the headcap on the rat (*see* Fig. 2b). The low-torque swivel on the commutator allows rats to move freely in the test chamber while still being tethered. The commutators are connected to an 8-channel programmable constant current stimulator (STG 4000, Multi-Channel Systems, Reutlingen, Germany), which in turn is controlled by ABET II Touch software (Campden Instruments Ltd). This software (which also controls stimulus delivery and records behavioral events in the touchscreen chambers) allows triggering of the stimulator so that VNS delivery can be time-locked to specific behavioral and task events.

3 Methods

3.1 Surgical Implantation of VNS Cuffs

VNS cuffs are sterilized prior to surgery with ethylene oxide gas and allowed at least 48 h de-gassing time to ensure that toxic gases are desorbed from the cuff's polymer components. Aseptic technique and sterile procedures are used for all surgical protocols. An antibiotic (Enrofloxacin, 10 mg/kg, s.c.) is administered 30 min prior to the first surgical incision. Rats are anaesthetized with 2–5% isoflurane, and the surgical areas on the head and neck are shaved and disinfected with saline and chlorhexidine. The local anesthetic bupivacaine (0.25 mg/mL) is injected subcutaneously at the surgical sites on the head and neck prior to incisions. Body temperature is monitored and maintained at ~37 °C throughout surgery using heating pads.

3.1.1 Placement of Skull Anchor Screws

1. Rats are secured in a stereotaxic apparatus and a midline incision made to expose the skull.
2. Four holes are drilled in the skull using a hand-held drill (two on either side of the sagittal suture anterior to bregma and two anterior to the interaural line).
3. Four self-tapping stainless steel anchor screws are inserted into the skull to stabilize the head stage. Extra care should be taken to ensure that there is sufficient distance between the rostral and caudal screws such that the Delrin pedestal can sit flat on the surface of the skull. Care should be taken to minimize cortical damage when drilling and securing screws into the skull.
4. Once anchor screws are in place, a damp gauze soaked in warm sterile saline is placed on the skull incision site to maintain tissue hydration, and the rat is removed from the stereotaxic

frame to prepare for cuff implantation on the cervical branch of the left vagus nerve.

3.1.2 Implantation of Cuff Around the Left Vagus Nerve

1. Surgical placement of the vagus nerve cuff is modeled after procedures performed in patients. Given the anatomical differences in innervation between the right and left vagus nerves, the left vagus nerve is used to minimize the chance of unwanted cardiovascular effects [27]. The rat is positioned in dorsal recumbency and a ~1.0 cm skin incision is made on the ventral neck, slightly left of midline, using the carotid pulse as reference. The skin is retracted to expose the underlying muscles.
2. Blunt-tipped Mayo scissors are used to gently separate the muscle fibers within the incision site. Note that special care is required to avoid damage to lymph nodes and blood vessels. Once the carotid artery is exposed, a small retractor is placed in the incision site to allow unimpeded access to the vagus nerve. The vagus nerve is anatomically located along the cervical branch of the carotid artery and contained within the carotid sheath. Warm sterile saline is used as necessary to keep tissue within the surgical site hydrated.
3. Pointed, atraumatic micro-forceps are used to gently separate the fascia and connective tissue around the carotid and vagus nerve. A smooth, pointed-tipped glass micro-probe is used to carefully separate a ~5 mm section of the vagus nerve from the carotid artery to allow for placement of the cuff electrode. Extra care must be taken to avoid puncturing the carotid artery and damaging the nerve.
4. The cuff electrode is inserted under and wrapped around the isolated section of the vagus nerve (*see* Fig. 2a2). Suture material looped through holes in the corners of the cuff can be used to assist in feeding the cuff underneath the exposed portion of the nerve.
5. To verify cuff functionality and proper placement around the nerve once it is in place, a 1 s train of electrical pulses (400 μA, 100 μs/phase biphasic pulse width, 50 Hz) is delivered to the electrode with the intent of eliciting a visually confirmed transient respiratory pause (at least 1 s) induced by activating the Hering-Breuer reflex [31]. Once the cuff is confirmed to be functional, it is secured in place with cyanoacrylate adhesive (Gluture, World Precision Instruments, Sarasota, FL). A bio-compatible silicone adhesive (Kwik-Sil, World Precision Instruments, Sarasota, FL) is used to insulate the edges of the cuff to minimize irritation to surrounding tissue.
6. After placement of the cuff around the nerve, the muscle tissue around the cuff is sutured closed to contain the cuff in the cavity created. Large blunt thumb forceps are used to create a

subcutaneous tract leading from the neck incision site to the incision site on the head. The electrode wire and Delrin pedestal are then tunneled subcutaneously from the neck incision site to the incision over the skull.

7. The pedestal containing the female socket contacts is positioned in the center of the skull and the exposed stainless-steel wire is wrapped around the anterior left anchor screw to form a stable electrical ground. Surgical glue is placed on the bottom of the pedestal to temporarily adhere the pedestal to the skull surface, and dental acrylic (Absolute Dentin dual-cure core composite, Parkell, Edgewood NY) is placed around the anchor screws and pedestal assembly to form a stable headcap. Extra care is taken to ensure that the dental cement does not cover the screw threads on the pedestal, which are used to secure the tether in place during VNS.
8. The skin incisions are closed around the headcap using non-absorbable 4/0 suture. Rats are monitored and temperature maintained with recovery cages placed on a heating pad until they are observed to be ambulatory and eating or drinking. In addition, we have found it beneficial to maintain rats overnight (24 h) in these cages to help them regulate their body temperature.
9. Post-operative care. Specific post-operative procedures should be performed in accordance with institutional guidelines. In our lab, post-operative weights are recorded daily to monitor recovery from surgery, and saline is administered subcutaneously as necessary to avoid dehydration. Rats are provided with soft Transgenic Dough Diet (Bio-Serv, Flemington, NJ) and breeder food pellets (Teklad 2919 Standard Rodent Breeding Diet, Envigo, Tampa, FL) soaked in warm water to provide additional hydration. Single doses of Meloxicam (5 mg/kg s.c.) are administered for 72 h post-surgery. Antibiotic (Enrofloxacin, 10 mg/kg s.c.) is administered for seven days after surgery. Sutures are removed 10–14 days post-surgery. Rats are allowed two weeks of post-operative recovery before experimental testing begins.

3.1.3 Special Considerations for Working with Aged Rats

Pre-clinical studies on aging are commonly conducted using rats as a model system [32–34]. In our labs, we work with aged (24–-26 months old) FBN rats obtained from the colony maintained by the National Institute on Aging (part of the US National Institutes of Health). This rat strain has a longer lifespan (up to 34 months) than other commonly-used strains, as well as a lower incidence of common age-related pathologies [35], rendering them well-suited for long-term studies of cognition at advanced ages. That said, all aged rats are prone to development of malignant and benign tumors, decreased physical performance, and other health issues

[36]; thus, selecting aged rats in good health prior to surgical manipulation and behavioral testing ensures a higher success rate. Upon arrival in our labs, rats are assessed for body weight and assigned a body condition score (BCS) [37]. Ideal subjects will have an initial BCS score of 3–5 upon assessment and maintain a stable body weight prior to surgery. Rats are also assessed for the presence of any observable masses or tumors, possible signs of dehydration, obvious ocular disorders, and general activity. Rats selected for experiments should appear bright and alert when handled.

3.1.4 Surgical Protocols Specific to Aged Rats

Vagus nerve cuff surgery can be particularly stressful for older animals, which typically require longer periods of post-surgical recovery. To help minimize distress during the recovery period, aged rats are acclimated to their post-surgery cage environment for at least three days before surgery. During this acclimation phase, rats are provided daily access to "rodent mash" as a food source, which consists of breeder chow pellets moistened in warm water and mixed to an oatmeal-like consistency.

Buprenorphine is an opioid analgesic commonly used in laboratory animals for pain management after surgery. One undesirable side effect of buprenorphine in rats, however, is that it increases the likelihood that rats will engage in a behavior known as pica, in which they consume nonnutritive substances [38, 39]. We have found that aged rats given buprenorphine are prone to this adverse behavior, which can lead to ingestion of bedding and obstruction of respiratory and gastrointestinal tracts. Thus, in accordance with local IACUC and veterinary approval, we recommend avoiding the use of buprenorphine in aged animals. We have found that aged animals recover sufficiently from VNS cuff surgeries with local anesthetic agents such as bupivacaine administered at the surgical site during surgery, followed by a post-surgery regimen of NSAIDs (e.g., meloxicam, 5 mg/kg s.c.) for pain management and antibiotics (Enrofloxacin, 10 mg/kg s.c.) to minimize infection.

After surgery, rats are monitored daily, and body weight and BCS recorded to assess overall health and recovery [37]. Although it is not uncommon for aged rats to lose up to 100 g of body weight following VNS cuff surgery, a BCS of 3 is required as a marker of good health. To ensure better post-surgical outcomes, saline (8–12 mL, s.c.) is administered as necessary to avoid dehydration. Furthermore, we have found that rats typically prefer "rodent mash" and softer food options following VNS surgery. Access to liquid nutritional "meal replacement" supplements (Ensure, Abbott Laboratories) is also provided post-surgery to provide an additional source of calories for recovery. Rats are slowly weaned off

special post-surgery food sources and standard food pellets are reintroduced starting 10 days after surgery.

3.2 In Vivo Assessment of VNS Cuff Viability

3.2.1 Electrochemical Impedance Spectroscopy Measurements

Prior to surgical implantation, all stimulating electrode cuffs are tested in buffered saline to assess their integrity and obtain baseline impedance values for each electrode contact. Starting 2 weeks after implantation, in vivo electrochemical impedance spectroscopy (EIS) measurements are performed weekly to evaluate electrode integrity, potential development of scar tissue around the electrode-tissue interface, and the state of the surrounding tissue environment [40–42]. Measurements are obtained using an Autolab potentiostat/galvanostat (Metrohm, Herisau, Switzerland). Rats are connected to the potentiostat via a tether, and each electrode contact in the cuff tested in a two-electrode configuration, referenced to ground. Measurements are taken by applying an extremely small (20 mV) voltage, which does not elicit an obvious behavioral reaction. The frequency of a small sinusoidal wave is swept logarithmically over 20 points between 100 kHz to 10 Hz, using a 10-mV sinusoidal perturbation. The potential difference between the working electrode and the reference electrode is recorded and the impedance is determined by performing a Fourier analysis. Nyquist plots are generated from impedance measurements obtained from each electrode [40, 43].

3.2.2 Using a Vagal-Mediated Autonomic Reflex to Assess Electrode Function

Although EIS can provide valuable information regarding the state of the electrode-tissue interface, we also verify electrode functionality over time through a protocol based on the respiratory pause test used to verify proper cuff placement during surgery. VNS induces a brief respiratory pause elicited by activation of afferent fibers from slowly adapting pulmonary stretch receptors [44]. This vagal-mediated Hering-Breuer reflex can be used to confirm functional stimulating electrodes in vivo [31, 45, 46]. Rats are assessed one day before the start of behavioral procedures involving VNS. For this test, a rat is anesthetized with 1.5–3% isoflurane and placed on its back such that a steady rate of respiration can be readily observed. The stimulator is connected to the pedestal on the headcap, and a single stimulus train (biphasic, charge balanced, 400 μA, 100 μs pulse width, 50 Hz, 1.0 s train duration) is delivered during the peak of the exhalation phase. If the cuff electrode pair is functional and maintains good contact with the nerve, there is a transient (~1 s) pause in respiratory rhythm, but no muscle twitches (which would be indicative of current leakage). Increasing stimulus pulse width allows for more directed activation of afferent fibers [47, 48] and, thus, if a respiratory pause is not observed at 100 μs, the pulse width is increased to 260 μs and the electrode combo is tested again. Note that a rest interval of at least 30 s is provided between respiratory pause attempts. If a respiratory pause is not observed at a pulse width of 260 μs, the pulse width is increased to

500 μs, and the test is repeated. In rare circumstances in which a respiratory pause is not observed at these parameters, the stimulus amplitude is increased by 200 μA and the pulse width is reset to 100 μs. This pattern is repeated until a maximum parameter set of 1 mA, 500 μs pulse width, 50 Hz is reached. If a respiratory pause is not observed at any of these parameters, then that electrode lead combination is considered to be nonfunctional and not used further. Different electrode lead combinations are tested until either all possible permutations of electrode lead combinations are exhausted, or a working combination is found. This test is repeated as needed throughout the post-surgery period, and particularly following completion of a series of VNS deliveries (e.g., at the end of an experiment). If a respiratory pause is not observed with the lead combinations used in the preceding experiments, then behavioral data between the last successful test and the failure session are discarded. While conservative, this testing procedure provides confidence that final data reported are from rats with working electrodes. Importantly, in experiments involving sham cuffs in addition to VNS electrodes, the sham control rats are subjected to the same procedures used for electrode evaluation in the experimental rats (impedance testing and anesthesia as described above), although of course no current is delivered.

3.3 Assessing Effects of VNS on Cognition

3.3.1 Configuring Electrodes for Cathodal Stimulation

Bipolar stimulation is ideally performed on a pair of adjacent cuff contacts (although failures of individual electrode contacts may require the use of more distal contacts). Cathodal (negative leading) stimulation and anode block permits directionality of stimulation and primary activation of afferent fibers [49]. Before starting VNS experiments, the polarity of the biphasic electrical stimulus is verified using an oscilloscope. To reduce the likelihood of off-target effects and maximize the likelihood of afferent fiber activation, the cathodal-first stimulus is applied to the rostral electrode contact (closest to the animal's head).

3.3.2 Cognitive Flexibility/Reversal Learning

In the experiments described below, a reversal learning task was used to assess the effects of VNS on cognitive flexibility. This type of learning depends on neural circuits that include the orbitofrontal area of prefrontal cortex as well as the dorsomedial striatum, and is sensitive to cholinergic, noradrenergic, and GABAergic modulation [32, 50, 51]. The basic task design involves initial learning of a pairwise visual discrimination problem in which the S+ is rewarded and the S- is not. Once this problem is learned, the reward contingencies are reversed, such that the S+ becomes the S−, and vice versa. To better test the temporal specificity of the effects of VNS on reversal learning, we developed a more complex task design that allows within-session comparison of the effects of VNS on both a new reversal learning problem and a well-learned discrimination problem (*see* Fig. 3). In this design, rats are first trained on two

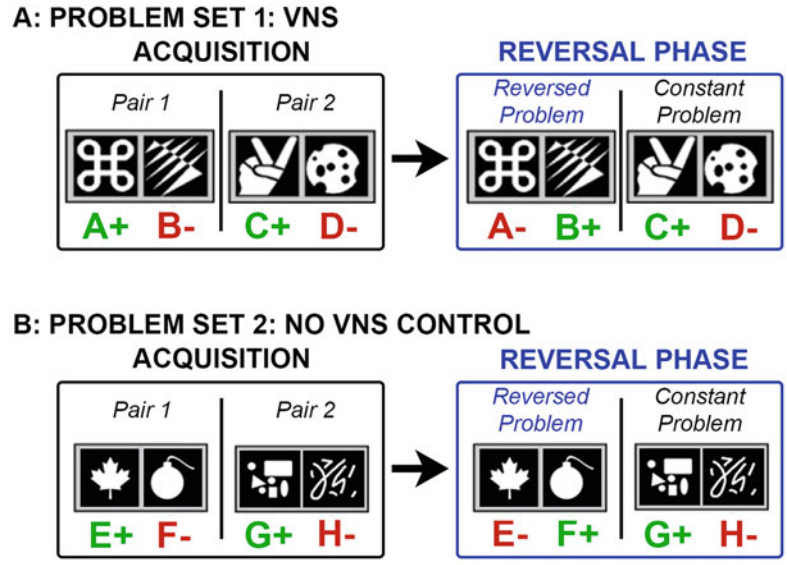

Fig. 3 Experimental design of a visual discrimination reversal learning task performed in touchscreen operant chambers. This task consisted of an initial Acquisition phase and a Reversal phase. Rats initially learned to discriminate between two distinct pairs of visual stimuli (Pair 1: A+, B−, and Pair 2: C+, D−). On each trial, touching the correct stimulus (+) in each pair yielded a food reward, whereas touching the incorrect stimulus (−) activated the house light and resulted in a 5 s "time out". After successfully learning each discrimination problem to criterion, the reward contingencies of one stimulus pair were reversed (e.g., Reversed Problem A−, B+), whereas the contingencies on the other stimulus pair remained unchanged (Constant problem: C+, D−). This specific experiment involved two rounds of acquisition and reversal: one in which rats received VNS (panel A), and one in which rats were tethered but no VNS was delivered (panel B)

pairwise visual discrimination problems simultaneously (A+, B− and C+, D−, in which the "+" indicates the rewarded stimulus in each pair). Once these problems are acquired, the contingencies in one discrimination problem are reversed (reversed set: A−, B+) whereas the contingencies in the other problem remain unchanged (constant set: C+, D−). This design allows us to compare the effects of VNS on cognitive flexibility when it is paired with learning of the reversed problem versus when it is paired with simple recall of the constant problem. The wide range of visual stimuli possible in the touchscreen operant chambers allows the use of a within-subjects design, such that rats can be tested in multiple VNS and control conditions. This provides for more rigorous statistical analyses, more rapid progression of experiments, and more efficient use of animal resources.

3.3.3 VNS Enhances Reversal Learning

Results of an experiment in which this design was used to test rats under both VNS and no stimulation control conditions are shown in Fig. 4 [52]. The VNS parameters (120 μs pulse width/700 μA/30 Hz/0.8 s train duration) were based on those shown by Kilgard and colleagues to enhance cortical plasticity and behavior across multiple contexts (e.g., [53–55]). Using a within-subjects design, rats were tested when VNS was delivered in conjunction with presentation of either the stimuli in the reversed problem or the stimuli in the constant problem. Compared to no-VNS control conditions, rats were significantly more accurate on the reversed problem when VNS was delivered upon presentation of the "to-be-learned" reversed stimuli (*see* Fig. 4a). In contrast, there was no effect of VNS on performance of the reversed problem when stimulation was delivered upon presentation of the "well learned" constant stimuli (unpaired, Fig. 4b). Taken together, these data show that 30 Hz VNS can enhance reversal learning in the absence of adverse effects on previously established discrimination performance (not shown). Moreover, these data demonstrate the importance of stimulus time-locking, as VNS appears to only improve reversal learning when delivered in conjunction with the to-be-learned information.

3.3.4 Stimulus Frequency Parameters

Prior studies have found VNS frequency to be a critical factor in determining its efficacy for enhancing neuroplasticity and cognition (e.g., [53]). The VNS parameters used in our study were modeled on those previously shown to enhance cortical plasticity and extinction learning in rats [31, 54, 56]. To investigate the effects of stimulus frequency on reversal learning, frequencies of 10 and 50 Hz were also tested. Neither of these frequencies affected reversal learning (*see* Fig. 4c and d), consistent with the idea that acute VNS at 30 Hz is most efficacious for enhancing cognitive performance.

3.3.5 Evaluating "Off-Target" Effects of VNS

Additional measures can be evaluated alongside measures of cognitive performance to determine whether cognitive-enhancing effects of VNS are accompanied by "off-target" effects. Although a detailed report of results is beyond the scope of this chapter, here we highlight variables that should be monitored as there is potential for VNS to alter these parameters [57–60]. Importantly, evaluation of physiological and behavioral measures in addition to cognitive measures of interest is critical in any experiment evaluating potential therapeutic approaches, to determine whether changes in cognitive performance measures are secondary to non-cognitive effects (e.g., changes in motivation).

Body weight and food intake measures can be used as non-invasive indicators of an animal's health and well-being. Since rats needed to be food restricted to increase motivation for many cognitive tasks, it is important to monitor them daily to ensure that

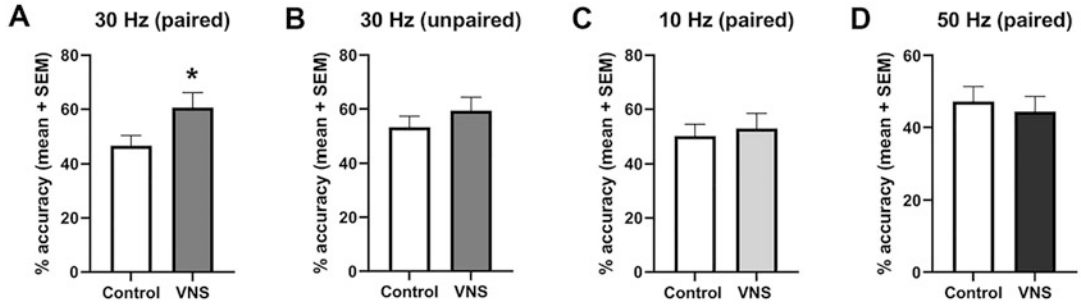

Fig. 4 Effects of specific VNS parameters on reversal learning. (**a**) 30 Hz VNS delivered during presentation of the reversed problem set ("paired") significantly enhanced reversal learning accuracy compared to no stimulation control conditions. (**b**) 30 Hz VNS delivered in conjunction with the constant problem set ("unpaired") did not affect accuracy on the reversal learning task compared to no stimulation control conditions. (**c**) 10 Hz VNS delivered during presentation of the reversed problem set did not affect performance compared to no stimulation control conditions. (**d**) 50 Hz VNS delivered during presentation of the reversed problem set did not affect performance compared to no stimulation control conditions. Error bars represent the standard error of the mean (SEM)

weight and BCS scores fall within an acceptable range throughout the experimental timeline. In our experiments, daily VNS at 30 Hz has no effects on body weight and food intake. In rats tested with VNS in the reversal learning task, we also monitored task performance variables aside from accuracy, including the total number of trials completed per session, locomotor activity within the chamber (measured by beam breaks), and latency to initiate trials, all of which can serve as indices of general motor function and motivation to perform the task. In contrast to several pharmacological manipulations that enhance reversal learning performance, we found that the VNS conditions that enhanced reversal learning (*see* Fig. 4a) had no effects on these off-target measures, suggesting minimal side effects of VNS using the cognition-enhancing parameters employed here [52].

4 Conclusions

There is growing interest in the use of VNS to address a range of neuropsychiatric conditions, including cognitive impairments. The procedures described in this chapter have proven effective for evaluating the effects of VNS on cognitive performance in rats, but much work still needs to be done in this space. Particularly, although published data from young rodents show that VNS can enhance multiple forms of cognitive function that are compromised in aging [8, 61–63], convincing demonstrations that VNS can remediate age-related cognitive impairments are as of yet largely lacking. Moreover, the mechanisms by which VNS might exert pro-cognitive effects in aging are still unclear, although there are

several possibilities including normalization of E/I signaling in the brain, reductions in age-associated increases in pro-inflammatory factors, enhancement of norepinephrine release, and even possibly reductions in markers of age-related neurodegenerative pathology [4–7, 64]. It is hoped that the information provided here will facilitate further research in this area that will allow these and other hypotheses to be addressed, and ultimately help to develop the use of VNS as a tool to promote healthy cognitive functioning across the lifespan.

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Chapter 7

Influence of Vagus Nerve Stimulation on Mood and Associated Disorders

Sarah A. Bottari, Alex Rodriguez, and John B. Williamson

Abstract

There is growing interest in the application of neuromodulatory therapies to the treatment of psychiatric conditions, such as mood and anxiety disorders. Vagus nerve stimulation (VNS) was approved by the United States Food and Drug Administration in 2005 as an adjunctive, long-term therapeutic option for adults with treatment-resistant depression. Clinical studies have demonstrated that augmenting standard antidepressant treatments with VNS is associated with greater reductions in depressive symptoms and improvements in remission rates compared to using standard treatments alone in this population. Moreover, the clinical benefits of VNS appear to increase over time with long-term treatment. Potential mechanisms underlying the antidepressant effects of VNS include effects on neurotransmitter systems, inflammation, and neurogenesis. Despite these demonstrated benefits, the invasive nature of surgically-implanted VNS has limited its clinical application. Noninvasive forms of VNS have recently been developed, including transcutaneous vagus nerve stimulation (tVNS). Although research on clinical applications of tVNS is in its early stages, there is some evidence to suggest that tVNS may improve symptoms of depression, anxiety, and related conditions, such as posttraumatic stress disorder and insomnia. This chapter provides a review of the clinical and preclinical literature exploring the effects of VNS and tVNS on mood and anxiety symptoms. In addition, it discusses potential mechanisms underlying these effects and highlights limitations of available research and important avenues for continued investigation.

Key words Vagus nerve stimulation, Depression, Anxiety, PTSD, Mood disorders, Sleep

1 The Vagus Nerve

The vagus nerve is involved with a myriad of both sensory and motor processes in the body relevant to the emotional experience. Particularly, the afferent pathway has been targeted in clinical interventions, with vagus nerve stimulation receiving United States Food and Drug Administration (FDA) approval for the treatment of epilepsy and depression [1, 2]. Treatments targeting the vagus have gained attention and popularity over the past few decades due to technological advancements and experimental studies that show value for further exploration.

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2 Vagal Nerve Stimulation

Vagus nerve stimulation (VNS) has been shown to impact the function of the brain. The concept of VNS has been traced back to circa 1885, when a neurologist, James Leonard Corning, was working to develop epilepsy treatments in humans. He posited that VNS might decrease cerebral blood flow and therefore reduce "cerebral hyperemia," which he believed to be the cause of seizures at the time [3]. Despite inconsistent results and a limited grasp of the effects of VNS on the individuals tested, Corning provided a method and rationale for targeting the vagus through transcutaneous stimulation. Following Corning's experimentation, research on VNS lapsed for the better part of the following century, only to resurface between the late 1980s and the early 1990s with a number of animal studies [3, 4]. Notably, at that time, Dr. Jake Zabara discovered that repeated electrical stimulation to the cervical vagus was able to interrupt or abolish seizures in dogs [5]. These findings propelled further research into VNS as a potential epilepsy treatment, eventually leading to FDA approval of implanted VNS for refractory epilepsy in 1997. Since receiving FDA approval, VNS has been shown to be effective in modulating the frequency of seizures in adults and children [6–8]. Notably, its beneficial effects have extended beyond seizure reduction.

In 1997, a nurse reported on psychosocial improvements observed in children with VNS for seizure control. Specifically, she noted improvements in socialization, temperament, and the ability to cooperate with others [9]. These observations, in combination with the fact that anti-epileptic drugs are often effective in treating mood disorders, led to the execution of a pilot study investigating the effects of VNS on mood in epileptic patients [10]. Patients receiving VNS showed a significant decrease in depressive symptoms after 3 months of treatment. Moreover, the change in depressive symptoms did not differ significantly between patients who responded to VNS (defined as a >50% reduction in seizure frequency) compared to those who did not respond to VNS, suggesting that VNS exerted a separate and distinct effect on mood symptoms. An independent study conducted in Europe reported similar results within the same timeframe. They found that seven out of nine patients meeting criteria for depression at baseline no longer met criteria after 6 months of VNS treatment. In contrast, only 2 out of 11 total subjects were classified as responders in terms of seizure reduction over the 6 months [11]. These early studies launched further investigation into the effects of VNS on mood, ultimately culminating in FDA approval for VNS as a treatment for treatment-resistant major depressive disorder in 2005.

3 VNS and Depression

Major depressive disorder (MDD) is one of the most common and debilitating mental health disorders around the world. The lifetime prevalence of MDD among U.S. adults is over 20%, and the World Health Organization has ranked depression as the single largest contributor to disability worldwide [12, 13]. MDD is characterized by a combination of five or more depressive symptoms that last for at least 1 week, including depressed mood and/or a loss of interest in daily activities. These symptoms are associated with significant distress as well as impairment in domains such as social and occupational functioning [14]. The first-line treatment for MDD is often antidepressant medications, including selective serotonin reuptake inhibitors (SSRIs) and serotonin and norepinephrine reuptake inhibitors (SNRIs). However, results from the National Institute of Mental Health's Sequenced Treatment Alternatives to Relieve Depression (STAR*D) program have shown that only approximately one-third of patients achieve remission following an initial antidepressant trial, and chances of remission decrease significantly following two failed antidepressant trials [15]. Estimates suggest that as many as 30% of patients with MDD may have treatment-resistant depression (TRD) [16], which is defined as MDD with poor or unsatisfactory response to two adequate (optimal dosage and duration) trials of two different classes of antidepressants [17–19]. Many treatment options still exist for these patients. Common approaches include psychotherapy, switching to a different class of antidepressants, and combination therapies. Alternative treatments are also available, including electroconvulsive therapy, repetitive transcranial magnetic stimulation, and, most relevant to this chapter, VNS [20].

In 2005, the FDA approved cervical VNS as an adjunctive, long-term treatment for adult TRD patients who have had at least four failed medication trials. VNS involves the surgical implantation of a pulse generator into the left chest wall. The generator is programmed and controlled noninvasively by a physician, and several stimulation parameters, such as current (mA), frequency (Hz), and pulse width (μs), can be adjusted to set the "dose" of VNS. The generator is connected to bipolar electrodes that are wrapped around the left vagus nerve in the neck which deliver low-frequency, chronic intermittent electrical signals to the left vagus nerve [21, 22]. The afferent fibers of the vagus nerve project primarily to the nucleus tractus solitarius (NTS) in the medulla, which in turn has projections to various structures involved in emotion production and regulation. For instance, the NTS projects to the locus coeruleus, one of the brain's key producers of norepinephrine, the dorsal raphe nuclei, one of the brain's key producers of serotonin, and the amygdala, insula, cingulate cortex, and

hypothalamic-pituitary circuitry important in regulating emotional experience [23–26]. Therefore, through stimulation of vagal afferents, VNS is able to influence areas of the brain relevant to depression.

3.1 Clinical Studies of VNS in Treatment-Resistant Depression

A number of studies have attempted to assess the efficacy of VNS in treating TRD. In these studies, the primary outcome measures are treatment response (defined as a ≥50% reduction in depressive symptoms from baseline) and remission (defined as a score on the severity rating scale indicating minimal residual symptoms). Studies assessing short-term (10 weeks) VNS treatments have yielded mixed results depending on their methodology. For instance, two studies found treatment response rates of 30–40%; however, there was not a control group receiving an alternative treatment in either of these studies [27, 28]. Therefore, the observed improvements may have been due to placebo effects, which can produce response rates as high as 40% in clinical trials of antidepressants [29]. To account for the placebo effect, one randomized controlled trial (RCT) has been conducted comparing the efficacy of VNS and sham stimulation in TRD patients. This RCT found that 15% of patients in the VNS group and 10% of patients in the sham group responded to treatment [30]. The difference in response rates between groups was not significant, suggesting that there is not strong evidence for the efficacy of VNS compared to sham stimulation as a short-term treatment. It should be noted that sham treatment is a significant challenge in stimulation studies.

Instead, evidence from studies with longer follow-up periods suggests that the therapeutic effect of VNS increases with time. For instance, one study found that 42% of patients with TRD responded and 25% remitted after 1 year of VNS treatment [31], while another showed that 53% of patients responded and 33% remitted after 1 year of VNS treatment [32]. In both these studies, response and remission rates increased slightly following a second year of treatment [31, 33]. These findings provide substantial support for the efficacy of long-term VNS treatment in this population, especially when compared to outcomes found with treatment as usual (any therapeutic regimen including medications, electroconvulsive therapy, and/or psychotherapy).

In one study of 124 TRD patients who received treatment as usual, 12% of patients responded and only 7.8% remitted after 2 years of treatment [34]. Therefore, VNS has produced remission rates over three times as high as treatment as usual in the same timeframe, indicating its clear benefit to TRD patients. Some studies have directly compared VNS and treatment as usual by following participants that were either receiving treatment as usual or treatment as usual and VNS for 1 year. Results demonstrated that participants receiving VNS + treatment as usual showed a significantly greater reduction of depressive symptoms, which remained

for about 55% of patients after a one-year follow-up. In comparison, only 11.5% of participants receiving treatment as usual showed sustained improvements over the one-year follow-up [35]. Therefore, these findings demonstrate that not only does the efficacy of VNS increase with time, but the therapeutic effect of VNS is sustainable over time in most patients who respond. Recently, these results have been extended further, with Aaronson et al. finding that those receiving VNS and treatment as usual had a cumulative response rate of 67.6% after 5 years, compared to 40.9% in the treatment as usual group, and a remission rate of 43.3% after 5 years, compared to 25.7% in the treatment as usual group [36]. Retrospective studies have also shown a benefit of VNS compared to treatment as usual, with one study of Medicare patients showing that those with TRD who received VNS had lower yearly medical costs and reduced annual mortality rates than those who received treatment as usual [37]. Thus, there is substantial evidence that VNS is an effective long-term treatment for TRD that can produce sustainable results, especially when compared to outcomes seen with treatment as usual.

Looking beyond treatment response and remission rates, clinical neuroimaging studies using functional magnetic resonance imaging (fMRI), positron emission tomography (PET), and single-photon emission computerized tomography (SPECT) techniques have also suggested the efficacy of VNS by illustrating the impact of VNS on brain areas relevant to depression. One study by Conway et al. examined cerebral metabolic activity changes following acute (3-month) and chronic (12-month) VNS treatment in 13 patients with TRD [38]. In patients that responded to VNS at 12 months, there was a significant trend of decreasing metabolic activity in the right dorsolateral prefrontal cortex over time. This finding was consistent with previous fMRI and SPECT studies, which also found a decrease in right DLPFC activity following VNS treatments ranging from 10 to 100 weeks [39–41]. Several lines of evidence suggest an inter-hemispheric imbalance in depression, with right hemispheric hyperactivation and left hemispheric hypoactivation of brain structures including the DLPFC [42]. In fact, depression severity and antidepressant treatment resistance have both been correlated with right hemispheric hyperactivity [43–45]. Therefore, decreasing right DLPFC activity may be one mechanism by which VNS alleviates depressive symptoms. The same study by Conway et al. also found a VNS-responder-specific increase in metabolic activity of the ventral tegmental region of the left substantia nigra [38]. This region plays a key role in reward processing, providing dopaminergic projections to several limbic-related brain regions, such as the nucleus accumbens, striatum, and prefrontal cortex [46]. Other brain regions that VNS has been shown to cause immediate or longer-term changes in include the

thalamus, cerebellum, orbitofrontal cortex, limbic system, hypothalamus, and medulla [47].

While clinical neuroimaging studies have helped to provide greater insight into the brain circuitry behind the activity of VNS, they have also produced a number of inconsistent results, which may be due to limitations such as small sample sizes, differences in stimulation parameters, and the influence of the varied pharmacological treatments that participants were undergoing in addition to VNS therapy. Thus, preclinical studies are critical to understanding the effects of VNS on brain circuitry, as they allow for investigation into these effects in a controlled environment, without the confounds inherent to clinical neuroimaging studies in this population.

3.2 Preclinical Studies of VNS

Studies of VNS in rodent models have investigated brain areas activated by VNS using immunohistochemistry for c-fos or ΔFosB, which are indicators of neuronal activation. C-fos expression is a marker of acute neuronal activation, as it is induced rapidly and transiently in specific brain regions following neuronal activation [48, 49]. On the other hand, ΔFosB is a marker of sustained neuronal activation and long-term neural plasticity, as there is a lag in its expression following activation, and its expression persists for a longer period of time [50]. Acute VNS treatment in rats induces c-fos expression in the NTS, paraventricular nucleus of the hypothalamus, parabrachial nucleus, bed nucleus of the stria terminalis, and LC [51]. Two weeks of VNS treatment was associated with significant increases in ΔFosB immunoreactivity in NTS, parabrachial nucleus, LC, peripeduncular nucleus, frontal cortex, cingulate cortex, hippocampus, basolateral amygdala, nucleus accumbens, bed nucleus of the stria terminalis, and dorsal raphe nucleus (DRN) [51, 52]. These results demonstrate that both acute and long-term VNS treatment induce a complex pattern of central nervous system effects that include, but are not limited to, areas important to emotion regulation and depression.

Preclinical studies have also examined the effects of VNS on depressive-like behavior in rodents. For instance, the most widely used paradigm for studying depression in animal models is the forced swimming test (FST). During this test, the animal is placed in a container of water from which it cannot escape. Animals tend to initially make motor efforts to escape; however, they eventually exhibit immobility (i.e., floating without any movement), which is thought to reflect either behavioral despair or learned helplessness, which are parallels of human depressive symptoms [53, 54]. Treating the animal with antidepressants prior to the FST has been shown to reduce immobility [55, 56]. As a result, the FST is commonly used to screen novel antidepressant compounds, and it has been found to reliably predict the efficacy of clinical antidepressants [57, 58]. Both acute and chronic VNS treatment have been shown to decrease the time animals spend immobile in the FST,

which is consistent with an antidepressant-like effect of VNS [59]. This effect was shown to be mediated through the action of VNS on noradrenergic neurons in the LC [60]. Another behavioral paradigm used to measure depression-like behavior in rodents is the novelty-suppressed feeding test (NSF). In this task, animals who have been food-deprived for 24 h are placed in a novel environment with a food pellet in the center of the open space. Rodents tend to not eat in novel environments (a trait known as hyponeophagia). Therefore, this test induces anxiety due to the conflict between an anxiogenic novel environment and a hunger-induced motivation to eat [61]. Latencies to approach and begin eating the food pellet are a sensitive measure of anxiety-like behavior. Chronic, but not acute, administration of antidepressants reduces these latencies, giving the NSF predictive validity for long-term antidepressant effectiveness [62, 63]. Both acute (5 trains) and chronic (10 days) VNS treatment have been found to reduce latencies in this test, further supporting an antidepressant- and anxiolytic-like effect of VNS [64, 65]. Finally, VNS has also been found to reverse a decrease in saccharin preference, which is a rodent parallel of anhedonia [60]. Thus, these preclinical studies all support the potential of VNS as a treatment for depression.

4 Potential Mechanisms

4.1 *Inflammation*

Multiple lines of evidence support a bidirectional association between inflammation and depression. For instance, activation of the immune system produces sickness behaviors that overlap with depressive symptoms (i.e., fatigue, changes in appetite, anhedonia) [66]. Similarly, treatment of medical conditions with pro-inflammatory agents has been found to induce depression [67]. Pro-inflammatory cytokines activate the hypothalamo-pituitary-adrenocortical (HPA) axis and the serotonergic system, both of which have been implicated in MDD [68, 69]. In addition, patients with inflammatory diseases show increased rates of depression, and treatment with anti-inflammatory drugs is associated with a reduced incidence of depression [70, 71]. Patients with depression also exhibit elevated levels of pro-inflammatory cytokines, and antidepressants have been shown to exert an anti-inflammatory effect [72, 73].

The vagus nerve plays a critical role in communication between the brain and the immune system. Accumulation of peripheral inflammatory molecules, such as cytokines, activates afferent vagus nerve fibers which relay signals to the brain, resulting in sickness behavior. The receipt of these inflammation-induced signals then activates an inflammatory reflex, mediated by the efferent vagus nerve. The efferent vagus projects to the spleen, liver, and gastrointestinal tract. Cholinergic signaling in these tissues then

leads to the suppression of proinflammatory cytokine production and the attenuation of immune system activity. Due to its involvement in this reflex, the efferent vagus has been termed the cholinergic anti-inflammatory pathway [4, 74]. Studies in animal models have demonstrated that VNS can reduce inflammatory response by attenuating the synthesis of inflammatory cytokines [75–77]. Therefore, reducing inflammation may be one mechanism by which VNS may improve depressive symptoms.

Clinical studies investigating the effect of VNS on inflammation have yielded mixed results. One study found significant increases in both pro- and anti-inflammatory cytokines in ten TRD patients following three months of VNS treatment [78]. The observed increase in pro-inflammatory cytokines was surprising, given the anti-inflammatory effect of VNS seen in preclinical studies; however, this study was limited by a small sample size and the lack of a control group. A subsequent study of VNS treatment (28 weeks) in 11 patients with epilepsy and 11 controls also found an increase in pro-inflammatory cytokines following treatment. However, a follow-up analysis including only epilepsy patients that responded to treatment (≥50% reduction in seizures per month) revealed a decrease in pro-inflammatory cytokine levels and an increase in anti-inflammatory cytokine levels, which was more consistent with preclinical findings [79]. These results suggest that the effect of VNS on the immune system may be tied to treatment response. Another recent study examined this anti-inflammatory mechanism further in seven patients with epilepsy [80]. The researchers collected blood samples from these patients at three different time points: before the surgery to implant the VNS generator, after putting the patient under general anesthesia for the surgery, and 4 h after a single pulse of VNS was applied to the left cervical vagus during surgery. They then incubated the blood samples with lipopolysaccharide (LPS), a pro-inflammatory stimulating factor, and found that blood samples collected after the VNS pulse contained significantly lower levels of pro-inflammatory cytokines than samples collected before the surgery or after anesthesia. Thus, they concluded that stimulation of the vagus inhibited LPS-induced pro-inflammatory cytokine release. The researchers note that these results were not likely due to placebo, given that patients were under anesthesia at the time of treatment. In the next phase of their study, the researchers implanted VNS devices in 18 patients with rheumatoid arthritis (RA). The patients underwent five weeks of VNS treatment, followed by two weeks without treatment, and then treatment resumed for four more weeks. They found significant reductions in tumor necrosis factor (TNF), a pro-inflammatory cytokine, in blood samples taken after VNS treatment periods, and a significant elevation in TNF after the two weeks without VNS treatment. Patients also demonstrated clinical improvements during the periods of VNS treatment and a

worsening of RA symptoms during the treatment withdrawal [80]. Thus, these preliminary findings show a promising anti-inflammatory effect of VNS treatment, which correlated with clinical improvements. Overall, future studies with larger sample sizes are needed to continue to investigate this potential therapeutic mechanism. However, it is possible that the anti-inflammatory effects of VNS may contribute to its efficacy in treating TRD.

4.2 Neurogenesis

In healthy adults, hundreds of new neurons are added to the hippocampal dentate gyrus every day in a process known as neurogenesis [81]. The ventral dentate gyrus plays a key role in stress and emotion regulation, as it is thought to regulate the HPA axis [82, 83]. Neurogenesis in this region is hypothesized to contribute to limbic functions and influence anxiety and depression through modulation of the stress response [84]. In fact, the neurogenic theory of depression posits that stress-induced decreases in adult hippocampal neurogenesis (AHN) cause episodes of depression, and reciprocally, that restoration of AHN can promote recovery from depression [85]. This theory is supported by a number of experimental studies showing impaired AHN in rodent and non-human primate chronic stress models of MDD [86–92]. Animal studies have also shown that antidepressants increase the rate of AHN, and selectively ablating AHN suppresses the antidepressant effect of the drug [93–95]. However, these findings are complicated by the fact that ablation of AHN does not consistently induce depressive symptoms [96]. There are no validated methods to directly measure AHN in living MDD patients; however, post-mortem and MRI volumetric studies have consistently found reduced dentate gyrus size in patients with MDD, which is suggestive of impaired AHN but may be due to other forms of structural plasticity [97–99].

In animal models, acute and chronic VNS treatment has been found to produce persistent changes in AHN that may play a role in the therapeutic efficacy of VNS. For instance, 48 h of VNS treatment was associated with increased hippocampal cell proliferation in rats, and the relationship between stimulus intensity and proliferation was found to be an inverted U-shape [100]. Another study showed that acute (3 h) and chronic (one month) VNS treatment increased the expression of neurotrophic factors, such as doublecortin (DCX) and brain-derived neurotrophic factor (BDNF), and that these changes persisted for at least three weeks after the cessation of VNS treatment. However, these changes were not correlated with alterations in depressive-like symptoms [101]. More recently, a study by Gebhardt et al. examined the effects of VNS on neurogenesis following bilateral removal of the olfactory bulbs. This surgery is known to decrease cell proliferation in the dentate gyrus, and neurogenesis can be restored with antidepressant treatment [102]. Gebhardt et al. found that VNS treatment (eight

weeks) was able to restore cell proliferation in rats who had undergone bulbectomy surgery, such that there were no differences in cell proliferation between these rats and controls who had undergone a sham operation [103]. Thus, VNS produced a similar restorative effect to antidepressant treatments, suggesting that effects on AHN may underlie the clinical efficacy of VNS in treating depression. Further research into this mechanism in human patients has been limited due to the lack of validated methods of measuring AHN directly in clinical samples. However, the development of such techniques using SPECT or MRI is in progress, and future studies using these methods may be able to improve our understanding of the effects of VNS on AHN in depressed patients [99].

4.3 Effects on Neurotransmitters

The monoamine hypothesis of depression posits that MDD is caused by a deficiency of monoamines (serotonin and/or norepinephrine) and that antidepressants exert their therapeutic effect by restoring monoamine levels. Although many findings have emerged that challenge this hypothesis, it has remained a prevalent and influential theory in the field, guiding the development of antidepressants from the 1980s to the 2000s [104]. Most antidepressant treatments today still act on monoamine transporters or receptors. Therefore, it is not surprising that the antidepressant effects of VNS may also relate to monoaminergic signaling.

Studies of acute VNS in rodent models revealed that VNS increases the firing activity of LC noradrenergic neurons, elevating norepinephrine (NE) levels in the cortex and hippocampus [105–107]. Long-term (2 weeks) VNS treatment also leads to a significant increase in NE levels in the prefrontal cortex and hippocampus in rodents, as well as increased tonic activation of α2-adrenoceptors, which is similar to effects seen following long-term administration of the atypical antidepressant bupropion [108, 109]. Thus, this action of VNS on NE levels and neurotransmission may be involved in the antidepressant effect of VNS. Interestingly, this mechanism may be related to the efficacy of VNS in treatment-resistant populations, as this action on the NE system is different than that of SSRIs, which decrease extracellular NE levels and NE neuronal firing rate [110, 111]. Long-term VNS has also been shown to increase extracellular serotonin levels in the rat brain, particularly in the DRN [105, 109]. Manta et al. hypothesize that the increase in DRN serotonergic neuronal firing rate may be due to the activation of α1-adrenergic receptors on serotonergic neurons, rather than to desensitization of 5-HT_{1A} autoreceptors, which is common in antidepressant treatment [112]. Finally, long-term VNS also increased extracellular dopamine (DA) levels in areas such as the nucleus accumbens, which plays a critical role in reward and hedonia [109]. These findings overall suggest that VNS increases monoaminergic signaling, which likely contributes to its

efficacy in treating depression. Furthermore, the differential actions of VNS on monoaminergic signaling compared to typical antidepressant treatments may help to explain the efficacy of VNS in treatment-resistant patients.

4.4 Safety and Side Effects

Although VNS is generally a well-tolerated treatment, there are a number of surgical risks and potential side effects that limit its application [28]. For instance, following surgery to implant the pulse generator, patients may experience pain or a localized incision around the wound site [113]. There have also been reports of bradycardia and asystole occurring during VNS device placement due to unintentional stimulation of the cardiac branches of the vagus nerve [114]. During active treatment, the most common side effect is voice alteration or hoarseness, as well as coughing and shortness of breath. These symptoms can be reduced by adjusting the stimulation intensity. Other common side effects include headache, neck pain, dysphagia, nausea, sleep-related breathing pattern changes, and worsening of depressive symptoms [28, 113, 115–117]. A recent systematic literature review found that approximately 5.5% of patients receiving VNS experience at least one serious adverse event over the course of a 12-month treatment [118].

4.5 Transcutaneous VNS

To overcome some of these barriers to the application of VNS, noninvasive transcutaneous VNS (tVNS) methods have been developed. Currently, there are two main methods for applying tVNS. The first is using a specialized device, such as GammaCore, which can be held against the side of the neck to deliver stimulation to the cervical branch of the vagus transcutaneously. The second method is applying transcutaneous electrical nerve stimulation electrodes to specific parts of the external ear to stimulate the auricular branch of the vagus. Different sites on the ear have been used in tVNS, including the cymba concha and the inner tragus. These sites have been identified as suitable locations for vagal modulation because they are densely innervated by the auricular branch of the vagus [119], although it is not yet clear which location and which parameters produce the strongest effects.

The first RCT evaluating tVNS as a treatment for depression was conducted in 2013 [120]. Unlike with surgically implanted VNS, patients did not need to be treatment-resistant to be included. Thirty-seven patients with depression were randomized to receive either active tVNS stimulation or sham stimulation of the outer ear as an add-on to standard treatment for two weeks. Compared to the sham group, the tVNS group showed a significant reduction in self-reported symptoms as measured by the Beck Depression Inventory. These results suggest an antidepressant effect of tVNS. This antidepressant effect was again demonstrated in a longitudinal, non-randomized study which showed that MDD

patients treated with tVNS for four weeks showed significant reductions in depressive symptoms as measured by the Hamilton Depression Rating Scale (HAMD) compared to patients who received sham stimulation [121]. Furthermore, patients treated with tVNS continued to show clinical improvements throughout the 12 weeks of treatment. In addition, another study of 12 MDD patients found that two weeks of tVNS treatment significantly reduced depressive symptoms by ≥50% for every patient, and these clinical improvements remained one month after treatment ended [122]. Specifically, tVNS has been shown to reduce symptoms of anxiety, psychomotor retardation, sleep disturbance, and helplessness in patients with depression [123].

Thus, much like VNS, tVNS appears to exert an antidepressant effect, making it a promising potential treatment approach for MDD. tVNS may exert its therapeutic effect through many of the same mechanisms as surgically implanted VNS, although there are likely differences in the distribution of afferent and efferent fibers cervically compared to auricular placements, as well as disadvantages regarding the specificity of stimulation (a cuff electrode is less likely to have off-target effects). Being a noninvasive method, tVNS is able to offer these therapeutic effects without the surgical risks that the invasive treatment involves. tVNS has been shown to be safe and well-tolerated, with reported side effects including tinnitus and pain, prickling, or itchiness at the site of stimulation during or after treatment [121, 124]. In addition to not requiring surgery, noninvasive stimulation of the auricular branch of the vagus may improve the safety of VNS treatment by reducing the possibility of cardiac side effects which sometimes occur with invasive VNS [125]. As interest in this potential treatment grows, future studies will need to address key questions such as the optimal stimulation parameters and dosage, the effect of tVNS on symptoms of comorbid disorders, and the efficacy of tVNS in individuals of different ages and depression severities [123].

5 VNS and Anxiety

VNS may also have potential as a treatment for anxiety-related disorders, a common comorbidity with depression. Anxiety disorders are the most prevalent psychiatric disorders, affecting nearly one-third of U.S. adults during their lifetime [126]. They are characterized by excessive fear and worry that interferes with normal daily activities. Common types of anxiety disorders include panic disorder (PD), generalized anxiety disorder (GAD), social anxiety disorder (SAD), specific phobias, and separation anxiety disorder. Historically, obsessive-compulsive disorder (OCD) and posttraumatic stress disorder (PTSD) were also included as anxiety disorders, but they have been reclassified in the latest edition of the

Diagnostic and Statistical Manual [14]. First-line treatment for anxiety disorders typically consists of either psychotherapy (cognitive behavioral therapy [CBT], exposure therapy) or pharmacotherapy (SSRIs, SNRIs), both of which have shown greater efficacy than placebo [127]. However, globally, less than 15% of patients with anxiety disorders receive treatment that conforms with evidence-based recommendations [128]. Therefore, there is a need for increased access to effective and empirically based treatments.

Clinical support for using VNS in the treatment of anxiety-related disorders initially came from subjective observations that anxiety symptoms improved in patients with epilepsy or depression that were receiving VNS treatment. In an early study of VNS in patients with epilepsy, those who responded to VNS treatment also reported significant reductions in anxiety [129]. Similarly, in a study of 30 patients with depression, VNS was associated with self-reported improvements in agitation and anxiety [27]. These findings led to the approval of a pilot study directly assessing the effect of VNS on anxiety symptoms. The pilot study included 11 patients with a range of anxiety-related disorders, including OCD, PD, and PTSD. Results were modest yet favorable, with 30% of patients showing a greater than 50% improvement from baseline in clinician-rated anxiety symptoms after 12 weeks of VNS treatment [130]. Notably, four out of the 11 patients were still receiving VNS 4 years after the initial implantation, and they showed continued and sustained improvement in anxiety scores. More recently, a small study evaluated the use of tVNS in patients with generalized anxiety disorder and found a trend for a reduction in self-reported anxiety after 4 weeks of treatment which was maintained at the 2-month follow-up [131]. These findings warrant continued investigation into the use of VNS as a potential treatment for anxiety-related disorders.

Preclinical studies have also provided support for an anxiolytic effect of VNS. Across a variety of tests sensitive to detecting anxiolytic effects in rodents, VNS has produced greater anxiety reduction than sham stimulation [64]. For instance, the elevated plus maze (EPM) is a reliable tool for the measurement of general anxiety state in rodents. In this paradigm, rats are placed in the center of a maze facing one of the open arms, and time spent in the open versus enclosed arms is recorded for 5 min. Increased exploration of open arms is indicative of an anxiolytic effect [132]. One train of VNS was found to reduce anxiety in non-stressed rats submitted to the EPM, suggesting an anxiolytic effect [133]. Moreover, there is evidence for a dose-response relationship such that increasing the number of trains of VNS produces a more robust anxiolytic effect [64]. These findings are in line with earlier studies in rats showing that 10 days of VNS treatment reduces anxiety in the NSF test [65]; however, they also expand upon these previous findings by demonstrating that the anxiolytic effects can occur within a shorter

timeframe (i.e., within 10 min following stimulation), highlighting the potential therapeutic utility of VNS.

Relevant to the treatment of anxiety disorders, translational research has also explored the effect of VNS on fear conditioning and extinction learning. Fear conditioning is an associative learning task in which subjects learn to associate a neutral stimulus (conditioned stimulus; CS) with an aversive stimulus (unconditioned stimulus; US) which elicits a fear response. Over time, presentation of the CS alone begins to elicit a fear response. Conversely, in extinction learning, the CS is repeatedly presented without the US, causing the fear response to gradually diminish. Exposure therapy, one of the first-line treatments for anxiety related disorders, is thought to exert its therapeutic effect through promoting extinction learning [134]. During this therapy, individuals with anxiety disorders repeatedly approach a feared situation, stimulus, or memory (i.e., a CS) which allows them to learn that their feared consequence (the US) is unlikely to occur, ultimately leading to a reduction in fear of the CS. VNS has been shown to enhance memory consolidation in both rodent models and humans [135, 136]. Given that extinction learning requires memory consolidation [137], VNS has also been hypothesized to enhance extinction learning.

In one preclinical study, male rats were trained on an auditory fear conditioning task followed by extinction training. During extinction training, one group of rats received VNS during the CS presentation while the other group did not. VNS paired with exposure to the CS was found to enhance extinction learning after one CS presentation, as indicated by reduced freezing response in the rats who received VNS compared to controls. In addition, across 10 extinction training trials, the VNS group showed accelerated extinction of the fear response [138]. The same research team demonstrated that enhanced plasticity between the medial prefrontal cortex and basolateral complex of the amygdala mediated the improvements observed in extinction of conditioned fear with VNS [139]. Extending this line of work, a recent study also examined the effect of VNS on conditioned fear in a rodent model of PTSD. The rats were subjected to a single prolonged stress protocol, which produces impairments in extinction of conditioned fear similar to those seen in PTSD [140]. The rats then completed auditory fear conditioning one week later, with half the rats receiving VNS during the extinction training. Despite 11 days of extinction training, the rats who did not receive VNS failed to extinguish the fear response to the CS. On the other hand, rats who had received VNS showed attenuated fear responses and elimination of other PTSD-like symptoms, such as anxiety, hyperarousal, and social avoidance [141]. Moreover, VNS treatment during extinction learning prevented relapse of the conditioned fear response following subsequent presentation of the CS, whereas

a single trial of the CS was sufficient to fully reinstate conditioned fear in the control group. In sum, preclinical studies have provided evidence that VNS may facilitate extinction of conditioned fear and prevention of fear reinstatement, which could make it a promising adjunct to exposure therapy for treatment of anxiety disorders.

Clinical studies, however, have yielded mixed results. In human participants, tVNS reduces U.S. expectancy ratings, which some researchers interpret as an acceleration of extinction learning [142]. However, other researchers have shown that, despite promising effects on U.S. expectancy ratings, tVNS does not enhance retention of extinction memory in healthy adults without psychiatric diagnoses [143]. Moreover, tVNS was not found to produce significant differences in physiological markers of the conditioned fear response in this healthy population, such as skin conductance response, heart rate response, and fear potentiated startle response, relative to sham stimulation [143, 144]. Thus, the effect of tVNS on extinction learning in humans is still unclear. Given the lack of physiological changes, some have proposed that stimulation may not have been delivered effectively in these studies, which could help to explain the discrepancy in findings compared to preclinical results [145]. Further, results may be different in clinical populations.

Besides effects on extinction learning, there are a number of plausible pathways by which VNS may exert an effect on anxiety symptoms. VNS activates several brain regions involved in mood regulation and arousal, including the amygdala, insula, and brainstem [47, 146]. The vagus also directly influences the hypothalamic-pituitary-adrenal (HPA) axis, which regulates the body's neuroendocrine response to stress [147]. Moreover, the vagus is a principle component of the parasympathetic nervous system, which functions in opposition to the "fight or flight" sympathetic activation that is often associated with symptoms of anxiety. Through increasing parasympathetic activity and reducing sympathetic activity, VNS may be able to improve symptoms of hyperarousal associated with anxiety-related disorders, as has been shown in patients with PTSD [148]. In this study, moderate to high effect sizes were shown in sympathetic nervous system (electrodermal activity) response to acoustic startle as well as increase in resting high frequency heart rate variability, an index of vagal tone. Finally, VNS affects neurotransmitter systems relevant to anxiety, such as NE and 5-HT, which could alter symptom expression [109, 149]. Thus, while the precise mechanism has not been fully elucidated, there are several potential pathways by which VNS could affect anxiety symptoms.

6 VNS and Sleep

While changes in mood are often viewed as the hallmark feature of mood or anxiety disorders, sleep disturbances are also very common in these disorders and are among the most distressing symptoms experienced [150]. The relationship between mood and sleep is bidirectional, with sleep quality having a significant impact on next-day mood and vice-versa [151]. As a result, treating sleep disturbances may be one way to positively influence an individual's mood.

The afferent branch of the vagus projects to several regions relevant to sleep-wake regulation, including the thalamus, hypothalamus, locus coeruleus, and dorsal raphe nuclei. Accordingly, changes in sleep have been noted in patients undergoing VNS. Positively, VNS was associated with a reduction in daytime sleepiness in patients with epilepsy [152, 153]. It has also been linked to decreased sleep latency and greater slow wave sleep time [154, 155]. Furthermore, objective evaluation of sleep using EEG parameters found that VNS enhanced spectral composition of sleep, as indicated by an increase in delta and theta power during non-REM sleep and an increase in alpha power during REM sleep and wakefulness [156]. On the other hand, VNS has also been associated with sleep-disordered breathing, including an increase in the apnea-hypopnea index and number of awakenings during sleep [157–159]. In the majority of these cases, respiratory symptoms were adequately controlled through the adjustment of stimulation parameters [160, 161].

Building off the findings of some beneficial effects of VNS on sleep, recent studies have examined tVNS as a potential treatment for insomnia. For instance, one study of 35 patients with primary insomnia found a significant improvement in self-reported sleep quality after 2 weeks of tVNS treatment [162]. Of note, tVNS was also associated with a reduction in self-reported depressive and anxiety symptoms in these patients, reflecting the relationship between sleep and mood. However, the lack of a control group in this study limited interpretation regarding the effect of tVNS on insomnia. More recently, a randomized clinical trial was conducted in which 63 participants with insomnia were randomized to receive 4 weeks of tVNS targeting the auricular branch of the vagus or 4 weeks of sham stimulation targeting the lesser occipital nerve [163]. Similar to the previous study, the tVNS group showed a significant improvement in self-reported sleep quality as well as depressive and anxiety symptoms. However, the control group showed an improvement as well, and there was no significant difference in the improvement of sleep quality or mood between the tVNS and control groups. It should be noted that stimulation of the occipital nerve has been shown to affect central arousal and is

linked to a reduction in trigeminal nerve pain. The afferent path of the trigeminal also targets the NTS, which is the primary conduit via which the vagus accesses structures important in mediating mood. Thus, further research with carefully designed control groups will be necessary to determine if tVNS impacts sleep quality and mood above and beyond placebo effects in patients with insomnia.

In summary, VNS and its noninvasive forms show substantial promise in the treatment of depression, anxiety, and related conditions (i.e., insomnia, PTSD). Better controlled studies are necessary to establish optimal effectiveness both in acute stimulation and longitudinal stimulation protocols. Factors such as stimulation site, dosing parameters (i.e., stimulation frequency, intensity, and pulse width), length of administration, and type of electrodes and stimulation devices have varied between existing studies and require standardization to clarify findings and guide clinical recommendations. Another important consideration to advance research on tVNS is the design of the sham approach in controlled trials. Most published reports use the earlobe as a sham stimulation site for tVNS because it is thought to be relatively free of vagal afferents [119, 164]; however, stimulation of the earlobe is not physiologically inert and produces similar fMRI patterns to stimulation of the vagus [165]. Moreover, stimulation locations for tVNS are now easily searchable on the internet, and use of the earlobe may compromise study blinding. Thus, further research is needed to develop a better sham approach to ensure that accurate conclusions can be drawn from observed effects in tVNS studies.

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Chapter 8

Transcutaneous Vagal Nerve Stimulation in Trauma Spectrum Psychiatric Disorders

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Abstract

Post-traumatic stress disorder (PTSD) and related trauma spectrum psychiatric disorders, including major depression, borderline personality disorder, and the dissociative disorders, are associated with considerable morbidity and loss of function. Treatment of these disorders, which include medications and psychotherapy, have limitations. New neuromodulation approaches targeting the underlying neurobiology of these disorders—including elevated inflammation, impaired autonomic nervous system activity, and alterations in brain areas that mediate emotion and the stress response—could improve the treatment and management of these patients. Vagal nerve stimulation (VNS) blocks sympathetic and inflammatory responses, and modulates brain areas involved in stress. Implantable VNS devices are approved for treatment refractory depression. New generation transcutaneous VNS devices that stimulate branches of the vagus nerve via the neck (cervical) or ear (auricular) are potentially applicable to trauma spectrum psychiatric disorders. This chapter reviews the effects of VNS on neurobiology and applications to patients with these psychiatric disorders.

Key words Neuromodulation, Stress disorders, Post-traumatic stress disorder (PTSD), Depression, Vagus nerve, Vagus nerve stimulation, Depressive disorder

1 Introduction

Post-traumatic stress disorder (PTSD) and other stress-related or trauma spectrum psychiatric disorders, including major depression, borderline personality disorder (BPD), and dissociative disorders, are disabling and affect the quality of life and productivity of millions of Americans [1–3]. In the general population, PTSD has a lifetime prevalence of 10–12% in women and 5–6% in men [4]. Intrusive thoughts, emotional blunting, avoidance, negative cognitions, sleep disturbance, and hyperarousal are the characteristics of PTSD, with the standard of care including psychotherapy and/or medication [5, 6]. Psychotherapies demonstrated to be effective for PTSD include prolonged exposure, cognitive

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processing therapy, stress inoculation, and eye movement desensitization and reprocessing [7–11]. These treatments, independent of exposure to traumatic memories, can have dropout rates approaching 50% [8, 12]. First-line medication treatment for PTSD includes selective serotonin reuptake inhibitor (SSRI) antidepressants [13, 14]. These medications have limited or no efficacy in some patients, while other patients discontinue treatment due to side effects. Despite that SSRIs are the best studied medication in PTSD, a report from the Institute of Medicine deemed the evidence insufficient to conclude that they are effective for PTSD [15]. For these and other reasons, only one third or less of those affected by PTSD can achieve total remission with the present standard of care [13], and many other patients avoid treatment altogether due to resistance to taking medications and/or avoidance and reluctance to discuss traumatic memories. Similar limitations exist for the treatments of other trauma spectrum psychiatric disorders, including major depression, BPD, and dissociative disorders [3]. Based on this evidence, new approaches are needed for the treatment of these conditions. The trauma spectrum disorders were originally described as a group of psychiatric disorders that had their roots in psychological trauma, typically early childhood trauma, and therefore, a common neurobiological basis related to the effects of stress on the brain [2, 16]. Subsequent research validated this approach and identified a network of brain regions, including the hippocampus, that tied these disorders together [3, 17]. Treatments that target the psychobiology of these disorders, involving core changes in brain and autonomic nervous system (ANS) [18–20] and immune function [21–41], may have promise for modulating the underlying basis for these disorders, and may lead to greater understanding of the physiological underpinnings of successful response to treatment [1, 20].

2 The Vagus Nerve as a Target of Treatment for Trauma Spectrum Disorders

Electrical stimulation stands as a promising new approach to the treatment of mental disorders that may target the adverse changes in underlying neurobiology [42–49]. Electrical stimulation is a form of neuromodulation which has been used to successfully treat chronic pain, epilepsy, and other disorders, whether applied at the level of the spinal cord, peripheral nerves, or directly to the scalp [50–53]. Neuromodulation side-steps issues related to pharmacological treatments outlined earlier, including side effects, lack of efficacy, or potential toxicities [54]. Vagal nerve stimulation (VNS) is a form of neuromodulation targeting the vagus nerve, which is the primary cranial nerve regulating ANS activity. VNS has shown efficacy for epilepsy [55–60] and treatment-refractory major depression [54, 61–74]; the Food and Drug Administration (FDA)

has since approved implantable VNS for direct stimulation in the treatment of these conditions [67, 75, 76]. VNS has effects that may be beneficial for neurophysiological alterations associated with PTSD and other stress-related psychiatric disorders [77]. VNS has been shown to improve autonomic balance, which leads to lower arrhythmia risk in patients with epilepsy [78]. VNS also enhances heart rate variability (HRV), vagal tone, and parasympathetic activity, and a reduction in sympathetic activity, suggesting it might be useful for the characteristic hyperarousal symptoms of PTSD [79]. Moreover, VNS elicits additional effects, including reduction of the immune response, accelerating fear extinction, induction of neural plasticity, and enhancement of cognition [18, 79–84]. The requirement for surgical implantation in the brainstem, however, has limited the wide-spread implementation of VNS to psychiatry due to cost, inconvenience [68, 74], questions about efficacy related to the inability to perform true sham-controlled trials due to ethical considerations [85], and the fact that the treatments are not reimbursed by Medicare or other insurance companies [86]. Furthermore, the FDA approved VNS only for patients with major depression who had failed multiple trials of antidepressants, limiting their wide-spread use. Additionally, the patients involved in these studies are atypical in comparison to patients typically seen in clinical psychiatry practices, potentially explaining why VNS failed to achieve complete remission in all patients despite improving symptom severity [87].

New devices that stimulate the vagus nerve *noninvasively* have the potential to be widely applicable to psychiatry due to lower cost and better tolerability [85]. The vagus nerve has efferent fibers that innervate most of the internal organs of the body and afferent fibers that pass through the carotid sheath in the neck to the brain. Noninvasive VNS (nVNS) devices stimulate a branch of the vagus nerve in the ear (transcutaneous auricular VNS (taVNS)) or vagal nerve fibers as they pass through the carotid sheath (transcutaneous cervical VNS (tcVNS) [85, 88–90]. In our research, we have used measurement of autonomic function and blood biomarkers during stressful tasks paired with tcVNS, as well as brain imaging using high resolution positron emission tomography (HR-PET) and radiolabeled water ($^{15}O[H_2O]$) made by an on-site cyclotron for measurement of brain blood flow in conjunction with exposure to personalized traumatic scripts, applied to patients with a history of exposure to psychological trauma with and without PTSD. In ongoing work, we have assessed a tcVNS device—recently FDA approved to treat intermittent cluster headaches [91, 92]—in traumatized healthy human subjects and patients with PTSD [93–98]. HR-PET has advantages over functional magnetic resonance imaging (fMRI) for this work given the allowance to concurrently measure peripheral physiological outputs during the scan without risk from challenges associated with the magnetic field in fMRI.

2.1 Physiological Correlates of Vagal Nerve Stimulation

The vagus nerve is mostly afferent, relaying sensory information from the visceral organs to the brain through the nucleus tractus solitarius (NTS) in the medulla oblongata (*see* Fig. 1). Efferent branches of the vagus, however, also have important effects, including modulation of inflammatory function [99, 100] and autonomic tone [101]. The pro-parasympathetic, anti-sympathetic, and anti-inflammatory, as well as pro-cognitive and pro-neuroplasticity effects of the vagus, may be beneficial in targeting neurobiological changes incurred with stress-related psychiatric disorders [36, 81]. Projections of the vagus through the NTS extend to the locus coeruleus (LC) and hypothalamus, key areas involved in sympathetic hyperarousal in PTSD, as well as brain areas such as the amygdala that are involved in the fear response [102, 103]. The vagus also projects to the medial prefrontal cortex / anterior cingulate [104–106], and hippocampus, which are involved in fear extinction (the hippocampus is involved in modulation of contextual aspects of fear as well as declarative memory) [107]. Lastly, it projects to the insula, which together with the anterior cingulate modulates peripheral neurohormonal responses to stress [108–111]. The vagus nerve travels through the carotid sheath in the neck, just medial to the sternocleidomastoid muscle [112] with another branch in the ear (auricular).

One of the primary effects of VNS on the brain is the activation of norepinephrine (NE) in the LC. The NTS has inputs to the LC, and VNS acts through the LC to increase NE release in the medial prefrontal cortex, amygdala, and hippocampus [113–116]. Increased NE has a secondary effect on the serotonin (5HT) system through excitatory alpha-1 adrenoreceptors on serotonergic neurons to increase 5HT in the dorsal raphe, the major site of serotonin cell bodies in the brainstem, with secondary effects on the same target brain regions modulated by NE [113, 117–119]. Chronic VNS in animal studies increased the firing rates of NE neurons in the LC and 5HT neurons in the dorsal raphe [120]. Furthermore, it increased extracellular NE in the hippocampus and prefrontal cortex, and 5HT in the dorsal raphe [118], with an increase in both firing rate and burst pattern of NE neurons [113], an effect blocked by scopolamine, indicating VNS acts through myelinated A afferent fibers of the vagus and not through unmyelinated C fibers projecting to the peripheral nervous system [121]. Chronic VNS decreased dopamine (DA) neuronal firing in the ventral tegmental area but led to increases in extracellular DA in the nucleus accumbens (ventral striatum) and medial prefrontal cortex [118, 122], with an enhancement of mesolimbic DA transmission [122]. Chronic VNS treatment for 2 months resulted in an increase in metabolites of DA and 5HT in the cerebrospinal fluid (CSF) in patients with epilepsy [123]. VNS thus has similar effects to antidepressants [113] however without the autoreceptor desensitization seen with chronic use of these medications

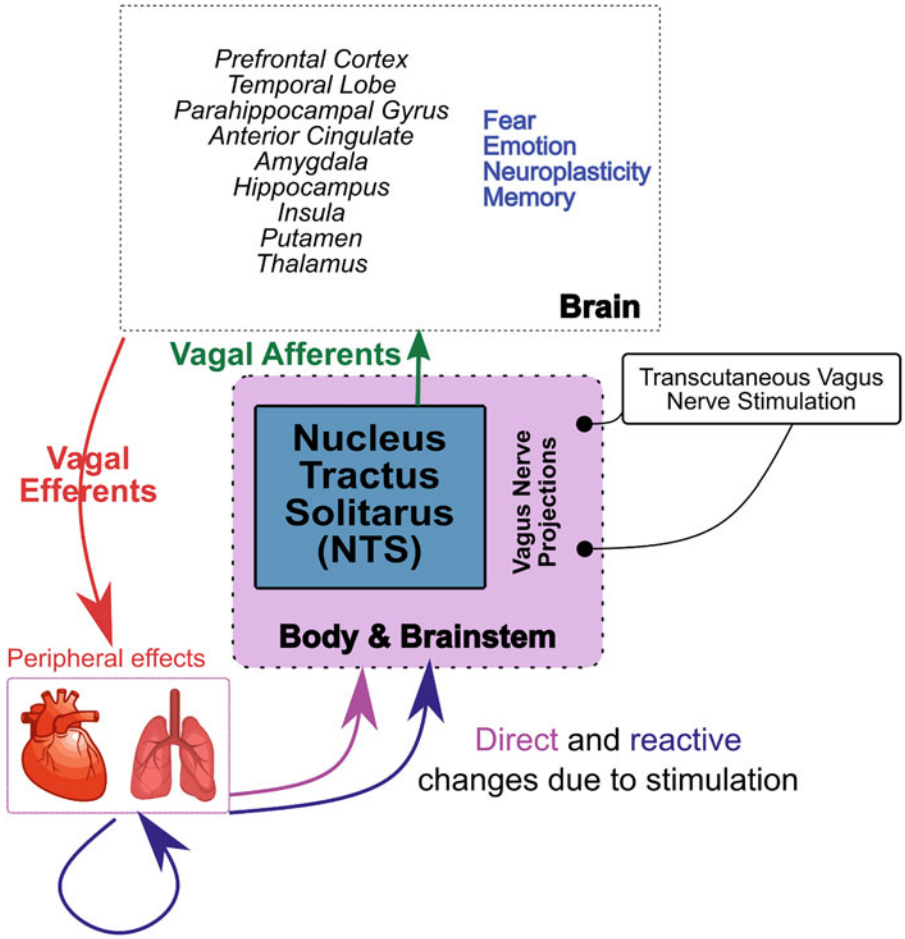

Fig. 1 Model of effects of nVNS based on our findings till date. Stimulation of the vagus nerve in the neck as it passes through the carotid sheath activates the Nucleus Tractus Solitarius (NTS) which has projections to multiple brain areas involved in modulation of fear and emotion as well as memory and neuroplasticity including the anterior cingulate, hippocampus and amygdala. Vagal efferents project to peripheral cardiovascular, autonomic, and inflammatory pathways. Stimulation results in both direct and reactive effects in peripheral organs

[113, 118]. VNS acts through these central brain areas in ways that are incompletely understood to decrease peripheral sympathetic function and enhance parasympathetic function [101, 123–127]. In our studies, we used tcVNS applied to the neck in traumatized subjects with and without PTSD. These studies are particularly relevant to patients with PTSD, given the known central role of NE in both VNS action, and the neurobiology and symptoms of PTSD (e.g., hyperarousal) [128–131]. Our data showed tcVNS blocks peripheral sympathetic activation and enhances parasympathetic tone [93, 94, 97, 98] as well as reducing inflammatory responses to stress and modulating brain areas involved in stress and emotion [96].

Anti-inflammatory effects of VNS may counteract neurobiological alterations in PTSD and other stress-related psychiatric disorders [80, 81, 99, 132–136]. Interleukin 1B (IL1B), IL-6, interferon gamma (IFNg), tumor necrosis factor (TNF) and C-reactive protein (CRP) are elevated in PTSD [21–41, 137], and several of these immune mediators are increased after acute stress [138, 139]. Our studies showed that patients with a recent myocardial infarction (MI) and the diagnosis of PTSD had an enhanced IL-6 response to mental stress compared to MI patients without PTSD [41]. High mobility group protein B 1 (HMGPB1), a proinflammatory master mediator, is increased in PTSD and inhibited by VNS [140, 141]. T helper cell differentiation is partly controlled by cholinergic neurotransmission [142]. Dysregulation of TH cells differentiation and function has been proposed in PTSD [143, 144]. TH1 cytokines include proinflammatory mediators (IFNg) as well as IL-2 and IL-3. TH2 cytokines are IL-4, IL-5, and IL-13. IL10 is stimulated by catecholamines, is broadly anti-inflammatory, and induces a shift in the TH1/TH2 balance toward TH2 dominance. The TH17 subset is generally proinflammatory and secretes IL-17 and IL-22. Activation of the a7 nAChR decreases TH17 responses [142]. Several studies have suggested that PTSD is linked to a dysregulation of the TH1/TH2/TH17 balance [144]. The close links of these inflammatory mediators with the HPA axis affirms their role in acute stress pathways [138, 139, 145]. Although both afferent and efferent arms of the vagus may contribute to the reduction in these proinflammatory cytokines, selective unidirectional stimulation of the cervical vagus has been demonstrated to dampen TNF production [146].

Other cytokines are relevant to stress and may be modifiable by VNS therapy. RANTES (CCL5) is a chemokine elevated in PTSD and decreased by nicotinic receptor activation [144]. Macrophage migration inhibitory factor (MIF) is a novel neuroimmune modulator that overrides the action of glucocorticoids on multiple cell types. It is released from the pituitary in acute stress in animal models and regulates neurogenesis in the hippocampus, which plays a vital role in PTSD [147]. MIF could influence glucocorticoid sensitivity in PTSD. MIF is inhibited by the efferent cholinergic anti-inflammatory pathway [148]. The kynurenine pathway is also relevant to the effects of VNS on physiological function. Activation of the IDO pathway increases Kynurenine (KYN), which crosses BBB (high KYN is linked to depression and suicide) [149]. VNS in humans resulted in a tendency to a reduction in kynurenine [136], and an increase in anthranilic acid (AA), which is neuroprotective [150]. Kynurenic acid (KYNA) is an antagonist of alpha-7 Nicotinic receptors, which are key mediators of the efferent cholinergic anti-inflammatory loop [142, 146, 149]. The hypothalamic-pituitary-adrenal (HPA) axis modulates the stress response, and dysregulation is commonly observed with PTSD

[151–154]. Studies suggest VNS may facilitate HPA axis homeostasis [155–157]. Abnormal sensitization of the noradrenergic system is also associated with PTSD [128–130, 158], which VNS may improve [131].

2.2 Vagal Nerve Stimulation as a Modulator of Neuroplasticity

VNS appears to improve neuroplasticity, fear circuits in the brain, learning and memory, and autonomic function that may be beneficial in the treatment of stress-related psychiatric disorders [79, 90, 101, 123–127, 159–174]. VNS inhibits cortical spreading depression [175], and has a variety of effects on brain amino acids, metabolites, and neurotransmitters [115, 176, 177]. VNS also has effects on neuroplasticity and on learning and memory systems relevant to both impaired extinction and deficits in declarative memory in PTSD [162, 167–174, 178]. The vagus mediates signals from peripheral neurohormones such as cortisol and epinephrine to brain areas involved in memory such as the hippocampus and amygdala [114, 119, 179–186], solving the puzzle of how stress-responsive hormones that do not cross the blood-brain barrier modulate memory for emotional events [179, 180, 183, 187–191]. VNS enhances memory retention [186, 192–198] with a U-shaped curve [195], acting through afferent fibers of the vagus [114, 196] to the LC and beta-adrenergic receptors on the perforant pathway-CA3 region of the hippocampus to enhance synaptic transmission [194, 199], long-term potentiation (LTP) [193] and neurogenesis [192–194]. These effects are relevant to declarative memory dysfunction seen in PTSD and depression [200–202] and findings that stress inhibits neurogenesis and hippocampal LTP, the molecular underpinning of memory formation [203, 204]. VNS, when paired with conditioned cues, facilitates extinction in animal models of classic fear conditioning [164–166, 205] and reduces fear-like behaviors [164, 205] an effect mediated through connections between the medial prefrontal cortex and amygdala [166]. Effects of VNS on fear circuits indicate a potentially beneficial role in modulation of conditioned responses and promotion of extinction to fear-related memories [79, 90, 162–166, 205, 206]. Other animal studies show beneficial effects of VNS on neural plasticity when paired with an auditory tone in animal models of tinnitus [168–171], with training after stroke for recovery of cognitive function [172] and motor movement [207–210] for cerebral hemorrhage. VNS also promotes recovery from traumatic brain injury (TBI), acting through the LC to enhance learning, memory, synaptic plasticity and motor recovery [197, 198] and facilitates recovery from congestive heart failure [211] and other cardiovascular events based on animal studies [159, 212, 213]. VNS sped motor recovery when paired with rehabilitation in patients with ischemic stroke [214], reduced tinnitus when paired with musical tones in patients with tinnitus [215], promoted learning and memory function in patients with Alzheimer's disease

[82, 83], enhanced memory when paired with a face-name pairing task in older healthy individuals [216], and reduced symptoms in patients with a variety of neurological and psychiatric disorders including headaches [91, 217–219], epilepsy [55–60], and major depression [61–74]. Clinical studies show VNS also promotes recovery following cerebral hemorrhage [220] and cardiovascular events [101, 159, 211–213]. Transcutaneous VNS has shown promising results for depression [221], PTSD [93, 95, 222] and mild traumatic brain injury (mTBI) [222].

2.3 Effects of Vagal Nerve Stimulation on Brain Function: Relevance to Stress-Related Psychiatric Disorders

VNS elicits changes in neural activations which are potentially beneficial for PTSD [223–225]. A network of brain areas, including the hippocampus, insula, amygdala, and medial prefrontal cortex (including anterior cingulate) have been associated in the pathophysiology of stress-related psychiatric disorders, including PTSD, BPD, depression, and dissociative disorders [3, 226–228]. The hippocampus, which plays a critical role in declarative (or explicit) memory, is very sensitive to stress. Studies in both stressed animals and patients with stress-related psychiatric disorders show alterations in declarative memory and hippocampal volume/structure [3, 226–233] that reverse with treatments involving antidepressants or behavioral interventions such as aerobic exercise [200, 204, 233, 234]. It also mediates the contextual appraisal of fear [147]. The medial prefrontal cortex/anterior cingulate is associated with emotional regulation and appraisal along with inhibiting the amygdala (site of fear memory) [235], representing the mechanism of extinction of fear responses [236]. The insula (with the anterior cingulate) controls peripheral responses to stress [109, 111] and shows an increased function in anxiety-prone individuals [237]. Brain imaging studies in PTSD showed reduction in MRI-based hippocampal volume in adult patients with both combat and abuse-related PTSD, which were associated with deficits in hippocampal-based verbal declarative memory [147, 238–243] and a failure of hippocampal activation during declarative memory tasks [244–247]. Other studies showed smaller hippocampal volume in BPD [248], depression [249] and dissociative disorders [228]. In PTSD, previous literature suggests both, the anterior cingulate [250–254] and insula may undergo shrinkage [250, 255, 256]. Brain imaging studies in which patients were exposed to traumatic reminders in the form of traumatic slides and/or sounds, traumatic scripts resulted in an increase in PTSD symptoms along with decreased blood flow, or failure of activation, in the medial prefrontal cortex/anterior cingulate [257–273], thalamus [268, 269] visual association cortex [260, 261, 268, 269, 273], parietal cortex [259, 260, 273–275] and inferior frontal gyrus [259, 260, 265, 268, 273–275], and increased function in the insula [262, 266, 276–279] and posterior cingulate cortex [257, 260, 269, 273]. There was increased function in the

amygdala with a variety of fear, negative emotion, or working memory tasks [262, 270, 272, 274, 280–296]. During emotional tasks, including an emotional Stroop task or recall of emotional words (such as "rape"), PTSD patients showed decreased function in medial prefrontal cortex/anterior cingulate [190, 265, 297]. These brain alterations respond to successful treatments of PTSD [226, 298]. Significant improvements in verbal declarative memory and/or an increase in hippocampal volume followed treatment with paroxetine [234], sertraline [299], or phenytoin [300]. Both verbal declarative memory and medial prefrontal (anterior cingulate) function in response to traumatic scripts increased with paroxetine treatment in PTSD [301, 302].

Brain imaging studies in human subjects have begun to map neural correlates of VNS [223, 224, 303, 304]. Functional imaging studies in human subjects using PET [305–308] and fMRI [309] showed that VNS, as predicted, resulted in effects on brain regions connected to the NTS, including the medial prefrontal cortex (anterior cingulate), insula, hippocampus and amygdala [305, 306, 309]. Successful treatment of depression with VNS also resulted in changes in brain regions implicated in that disorder [304, 310], as well as beneficial effects for cardiovascular function/atrial fibrillation [311, 312] and cognition [73]. These findings with implanted VNS devices have been replicated in fMRI studies with noninvasive VNS applied both through the ear in a sample of healthy adults [225, 313, 314] and patients with tinnitus [315], and depression [316–320] and the neck in healthy adults [321], traumatized subjects [96] and those with PTSD [95], showing effects on the insula, anterior cingulate and hippocampus [313, 316–318, 321, 322].

3 Studies of Transcutaneous Vagal Nerve Stimulation in PTSD and Trauma

We have studied traumatized subjects with and without PTSD comparing the effects of tcVNS or sham paired with stress stimuli [93, 94, 97, 98]. tcVNS is delivered in conjunction with behavioral assessments, cardiovascular /biomarker measurements, and brain imaging using HR-PET and radiolabeled water [93, 95], as illustrated in Fig. 2. Participants undergo a three-day protocol with personalized traumatic script stress, public speaking, and mental arithmetic stress. Miniature and inexpensive sensors collect information on autonomic nervous system function by monitoring electrical activity of the heart (through electrocardiogram, ECG), mechanical activity of the heart (through seismocardiogram, SCG), blood volume pulse (through peripheral photoplethysmogram, PPG), sweat gland activity (through electrodermal activity, EDA), and respiratory effort (through thoracic expansion, RSP) (*see* Fig. 3). In trauma-exposed individuals without PTSD, we have

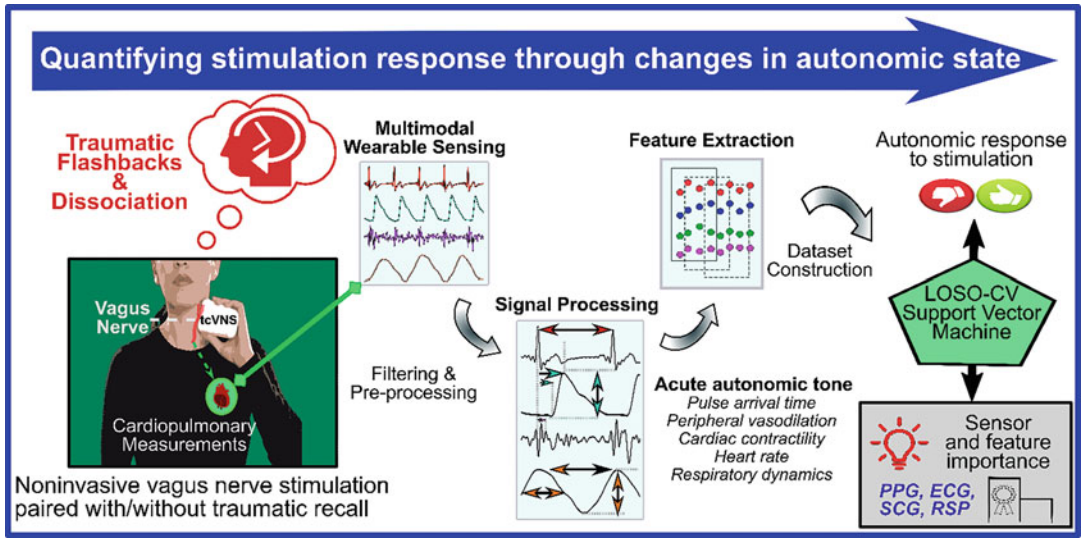

Fig. 2 Illustration depicting the daily use of tcVNS upon traumatic flashbacks and/or dissociation. In our work [98], we replicated this scenario by pairing stimulation with trauma recall, and designed methodologies to understand whether the stimulation resulted in expected changes in the autonomic tone by the use of wearable sensors and machine learning. As noninvasive VNS devices could be used by lay individuals who could mislocate the proper electrode location, the question of whether administration created desired effects is important to provide instant feedback to the user

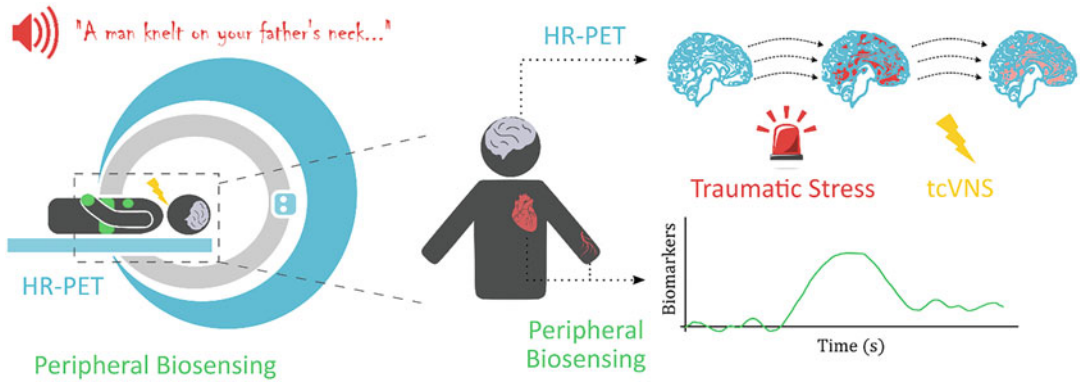

Fig. 3 Simplified illustration of the concurrent neuroimaging and autonomic sensing approach unique to our work. On the first day of the protocol, subjects listen to personalized traumatic scripts followed by transcutaneous cervical vagus nerve stimulation (tcVNS); all the while, functional images of the brain are collected using high-resolution positron emission tomography (HR-PET), and peripheral biosignals are collected using a respiration belt, electrodermal activity sensing, electrocardiography, photoplethysmography, and seismocardiography

observed better recovery in measures related to cardiac contractility (pre-ejection period, PEP), peripheral vasoconstriction and an indicator of peripheral sympathetic activity (amplitude of the PPG waveform), EDA slope which is another measure of sympathetic activity, and respiratory effort.

Our studies show a consistent effect of tcVNS inducing sympathetic reduction, as measured via PEP and PPG amplitude, and enhancing parasympathetic function as measured by respiratory rate. Effects on PPG amplitude and respiratory effects were consistent for both tcVNS paired to personalized traumatic scripts (but not sham stimulation), and tcVNS paired to mental stress tasks (public speaking, mental arithmetic) over multiple days [94, 97, 98]. In the absence of any task (i.e., at rest), tcVNS blocked sympathetic function as measured by an increase in PEP and PPG amplitude, and decrease in EDA slope. Similar effects were seen in PTSD patients for tcVNS paired with traumatic scripts and mental stress. tcVNS also reduced heart rate (HR) and increased pulse arrival time (PAT), or the time from depolarization of the left ventricle to the arrival of peak blood volume in the periphery, which is sensitive to sympathetic tone, arterial stiffness, and other factors. We also found that tcVNS blocked hippocampal and insular responses to traumatic scripts stress [96] (*see* Fig. 4) and blocked stress-induced increases in inflammatory marker IL-6 in PTSD, reduced anger responses to traumatic script in PTSD symptoms, enhanced memory encoding, and blocked the stress neuropeptide pituitary adenylate cyclase activating peptide (PACAP) over the three-day stress protocol. Using a machine learning paradigm, we found that the physiological signals HR, PAT, and PPG amplitude correctly classified subjects as having received tcVNS or sham with 96% accuracy, although the use of other signals have also been useful in the classification task as we detailed in our work. Since HR, PAT, and PPG amplitude computation requires two sensors (ECG and PPG) that are highly amenable to be incorporated in wearable settings, we detailed our analyses based on the features obtained from them. Figure 3 shows a potential application of tcVNS for an individual undergoing traumatic flashbacks and/or dissociation, and our methodology on assessing the effects of tcVNS on the effects of stimulation in daily settings.

4 Conclusions

VNS has promising applications to patients with trauma spectrum psychiatric disorders that are related to stress. An extensive literature supports the use of implantable devices for patients with refractory depression, and given the overlap of symptoms, neurobiologies, and etiologies, it is likely that patients with other mental disorders related to stress, including PTSD, BPD, and the dissociative disorders, will benefit from this intervention. These disorders share alterations in stress-responsive systems, including the sympathetic nervous system, inflammatory biomarkers, and brain areas, including the medial prefrontal cortex, insula, and hippocampus, that mediate symptoms of these disorders. New technologies

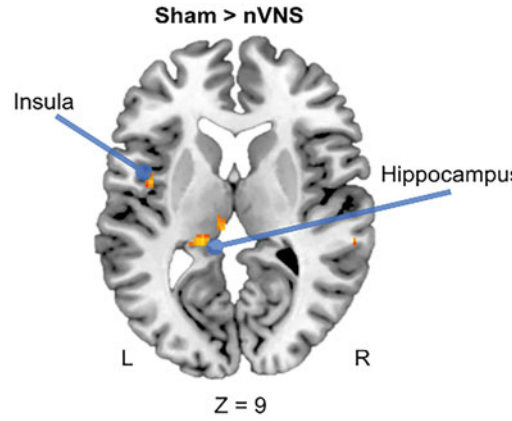

Fig. 4 Effects of tcVNS on the brain. Pairing of tcVNS with trauma scripts in traumatized subjects resulted in a blocking of hippocampal and insula activation normally seen with re-experiencing of traumatic memories induced by personalized scripts of traumatic events [96]. Yellow areas show greater activation with sham versus active tcVNS ($p < 0.005$)

utilizing noninvasive transcutaneous VNS, which can be applied to the ear or the neck, offer the potential to allow more wide-spread utilization of these interventions. Future studies are needed to further identify the effects of transcutaneous VNS on the neurobiology and symptoms of trauma spectrum disorders, including studies of patients in daily life where real world triggers of symptomatology occur.

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Chapter 9

Vagus Nerve Stimulation for Migraine and Cluster Headaches

Matthew Bloch and Alexander Mauskop

Abstract

Vagus nerve stimulation is a promising alternative to medication for the treatment of both migraine and cluster headaches. Over the past decade, there has been a growth in our understanding how vagus nerve stimulation can affect the pathophysiology of migraine and cluster headaches. Moreover, there is compelling evidence emerging that vagus nerve stimulators are effective at both aborting and preventing both migraine and episodic cluster headache.

Key words VNS, Migraine, Cluster headache, Mechanisms, Clinical studies

1 Introduction

Despite recent advancement in pharmacologic treatment, migraines and other primary headache disorders remain highly prevalent and disabling. Migraine has a global prevalence of 14.7% according to a 2010 World Health Organization estimate. The Global Burden of Disease Study lists migraine as the second most common disabling condition worldwide. For patients with primary headache disorders refractory to medical treatment or who experience side effects that make medication impractical or impossible, neurostimulation represents a potential solution. This is particularly important as studies have shown that 80% of headache patients do not continue their preventive headache treatment after the first year. Cluster headache is a comparatively rare disease. It has been estimated that 124 people out of 100,000 people will develop cluster headache over their lifetime. The rarity of this condition and its features that overlap with migraine and sinus headaches result in years of delay before a correct diagnosis is made [1]. There is a pronounced lack of evidence-based treatment options for patients suffering from cluster headache. Only galcanezumab, an anti-CGRP monoclonal antibody is approved by the

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FDA with occipital nerve block with steroid listed by the American Headache Society as having level 1 A evidence for cluster prevention [2, 3].

Vagus nerve stimulation (VNS) represents one of many promising therapeutic advances for the treatment of migraine and cluster headaches [4]. This chapter outlines both the evidence for and pathophysiologic explanation of its effect for these disorders. VNS is a safe alternative for cluster and migraine patients that have been refractory to medical treatment or who are unable to tolerate medication side effects.

Much like value of botulinum toxin for migraineurs was discovered serendipitously when patients receiving Botox for cosmetic purposes reported a reduction in their migraine symptoms [5], early interest in VNS stimulation for headache treatment arose after epilepsy patients being treated with implanted VNS for drug-resistant epilepsy with comorbid migraines reported an incidental decrease in their headache symptoms. Early case reports showed promising reductions in headache symptoms and duration in both patients with migraine and cluster headaches using an implanted stimulator [6]. Early open label trials also suggested that noninvasive VNS (nVNS) stimulation could abort acute migraine attacks [7].

Randomized controlled studies have continued to show the value of nVNS stimulation for both migraine and cluster relief. Moreover, VNS stimulation appears to be a safe alternative to medication. Several randomized controlled trials have shown a side effect profile comparable to placebo [8].

2 Mechanism of Action

Before describing the mechanism of action in VNS in migraine and cluster headache it is helpful to review the basic pathophysiology of both diseases.

At its most basic, migraine is a disease characterized by a set of changes in the brainstem, hypothalamus, thalamus, and cortex with the involvement of the peripheral branches of the trigeminal nerves. Laboratory models have demonstrated that migraine pain may be related to plasma extravasation of pro-nociceptive molecules in the dura. Dural pain is relayed centrally by a plexus of unmyelinated fibers coming from the ophthalmic division of the trigeminal ganglion. Imaging models have shown contralateral activation of the thalamus as well as activation of the dorsal mid brain and dorsal pons. Migraine aura, a transient focal neurologic phenomenon which can be visual, sensory, or motor, occurs in 30% of migraine patients. Animal models have demonstrated that these phenomena are likely caused by cortical spreading depression. This cortical

spreading depression has been shown to be accompanied by hyperemia [9].

The pathophysiology of cluster headache is less well understood than migraine. PET imaging has shown significant activation of the posterior hypothalamus in patients with cluster headache. There is activation of the trigeminal vascular complex during acute cluster attacks. Many of the clinical features of cluster headache are attributed to activation of the trigeminal autonomic reflex including rhinorrhea, lacrimation, and nasal congestion. Many of the same pro-nociceptive molecules that are implicated in migraine pain are also released in cluster headache. These molecules include Calcitonin Gene Related Peptide, Substance P, Vasoactive Intestinal Peptide, Neurokinin A, Neuropeptide Y, Pituitary Adenylate Cyclase-activating Peptide, Nitric Oxide Synthase and ATP [10].

The trigeminal nerve and trigeminal ganglion play a key role in both cluster and migraine pathophysiology. Afferent vagus nerve fibers terminate in the trigeminal nucleus caudalis and the nucleus tractus solitarius, a nucleus of the vagus nerve that receives dural nociceptor fibers [11].

In migraine, VNS may inhibit cortical spreading depression. Animal models have demonstrated VNS leads to activation of the locus coeruleus, nucleus tractus solitarius and dorsal raphe nuclei. Activation of these structures may lead to inhibition of cortical spreading depression. In one rat model, VNS increased the electrical stimulation needed to provoke slow way depression and decreased the speed of cortical spreading depression. This effect persisted up to 3 h after stimulus [12].

In another rat model, rats either received VNS stimulation or no treatment prior to having noxious stimuli applied directly to their dura. The rats with VNS stimulation prior to noxious stimulation had a significantly smaller increase in glutamate levels compared with rats that received no treatment [13]. VNS stimulation has also been associated with a decrease in pro-inflammatory Il-10 levels [8]. A rat model has demonstrated reduced spontaneous and nociceptive-induced firing of trigeminocervical neurons following VNS stimulation [14]. While it has been hypothesized from animal models that there are connections between vagus nerve nuclei and trigeminal nuclei, no human models have shown the existence of a trigeminovagal complex [15].

2.1 Cluster Studies

In an open-label study, 19 cluster patients were given nVNS stimulators. Over 12 months, 15 of 19 patients reported broadly defined improvement in their condition, with a mean improvement of 48%. Patients reported attacks were aborted after a mean duration of 11 min. Mean attack frequency decreased from 4.5 attacks per 24 h to 2.6 attacks per 24 h ($p < 0.001$) post treatment [16].

3 PREVA Study

In this randomized, prospective, multicenter, European study, cluster patients were randomized into groups receiving either twice daily nVNS with medical treatment versus medical care alone. The patients were observed for two weeks prior to nVNS treatment. The randomization phase lasted four weeks after which patients in both arms had the option to continue and receive nVNS therapy for four weeks. The primary endpoint of the study was mean reduction of the number of cluster attacks per week. 97 patients were enrolled. During the randomization phase the nVNS group had a mean reduction of 5.6 cluster attacks per week, compared with a reduction of 2.1 in the control group ($p = 0.02$). During the extension phase, the nVNS group had a mean reduction of 2 headaches a week ($p \leq 0.001$) and the control group had a reduction of 3.3 cluster attacks per week ($p < 0.001$). While 48.6% of patients reported a greater than 50% reduction in cluster attacks in the nVNS group, only 8.5% of patients experienced a similar reduction in the control group. Additionally, while there was a 57% decrease in the use of abortive medications in the nVNS group ($p < 0.001$) there was no significant change in abortive use in the control group [17].

4 ACT-1

In this randomized, double-blind, multicenter, sham-controlled prospective study, 150 cluster patients were randomized into groups receiving either nVNS treatment or sham treatment during an acute cluster attack. The primary endpoint of the study was response rate, defined as participants who achieved pain relief (0 or 1 out of 5) 15 min after they began treatment and did not use additional rescue medication for 60 min. Secondary endpoints included sustained relief at 15–60 min. Patients received nVNS or sham treatment for up to one month or until 5 cluster headache attacks were treated. After this phase was completed, patients had an option to enroll in an open label study for three months where patients would receive nVNS at each attack. During the double-blind phase, 26.7% of cluster headaches achieved pain relief at 15 min in the nVNS group compared with 15.1% in the sham group. This did not meet at statistical significance. However, in subgroup analysis, patients with episodic cluster diagnosis, but not chronic cluster diagnosis had significant response to nVNS vs sham treatment. Similarly, sustained response at 15–60 min was significantly higher in the nVNS than sham group in episodic, but not chronic cluster headache patients. During the open label phase there was no significant difference in response rate between chronic

and episodic cluster patients. Of the group that received sham treatment in the randomized phase, a significant percentage reported treatment response in the open label phase ($p = 0.007$) [18].

5 ACT 2 Study

In this randomized, double-blind, sham-controlled prospective, multicentered study, after a one-week run-in phase, 102 patients were randomized to receive either nVNS therapy or sham nVNS therapy during acute cluster attacks. The primary endpoint of the study was 0/5 pain 15 min after treatment. Episodic and chronic cluster subgroups were analyzed. For all study participants there was no significant difference in 0/5 pain at 15 min in the nVNS and sham group. However, on subgroup analysis for patients with episodic cluster headache 48% of patients achieved 0/5 pain at 15 min compared with 6% in the sham group ($p < 0.01$). Adverse events did not differ significantly between the sham and the nVNS group [19].

5.1 Migraine Studies

5.1.1 The PRESTO Study

In this multicenter, double-blind, randomized, sham-controlled trial, 248 patients with episodic migraine both with and without aura were randomized into nVNS and sham study arms. Patients received nVNS stimulation or sham stimulation after 20 min of headache pain and again after 35 min of headache pain if pain had not improved. All patients were under 50 years of age and had less than 15 days of headache a month. Exclusion criteria included Botulinum injections within six months and nerve blocks within two months. Migraine medications were required to be stable for two months prior to intervention. While nVNS stimulation did not produce a statistically significant increase in the number of patients that were pain free after 2 h, it did show a statistically significant increase in the number of patients that were pain free at 30 ($p = 0.012$) and 60 ($p = 0.023$) min. Moreover, the percentage of patients that had pain relief at 2 h was statistically significant ($p = 0.03$) [20].

5.1.2 The EVENT Study

In this 2015 prospective, multi-center, randomized, double-blind, sham-controlled study of patients with chronic migraine headache, 59 patients were randomized into groups receiving prophylactic nVNS or sham treatment. For two months participants either received sham or real nVNS stimulation. There was a subsequent six month open-label phase where all participants received nVNS treatment. Safety and tolerability were the primary endpoints of this study. Efficacy was primarily assessed by measuring number of headache days. Only 27 patients completed the trial. There were no serious adverse events. During the nVNS and sham stimulation

part of the study there was not a significant change in the number of headache days experienced by each group ($p = 0.56$). However, during the open-label phase of the trial there was a significant change compared to baseline in the number of headache days experienced in the group initially receiving nVNS treatment (mean decrease in 3.6 HA days per 28 days $p = 0.02$) though not the sham treatment (mean decrease in 2.5 HA days per 28 days $p = 0.06$) [21].

Optimal stimulation of the vagus nerve is still a question of active debate. In a 2015 randomized controlled double-blind study by Straube et al., 46 chronic migraine patients were randomized to receive either 4 h of 25 Hz stimulation or 1 Hz stimulation per day to the auricular branch of the vagus nerve for three months. The patients were observed without intervention for one month prior to receiving auricular transcutaneous VNS therapy. The primary outcome of the study was headache free days per 28 days. Secondary outcomes included percentage of people having 50% reduction in headache days a month, mean headachy intensity, days with abortive migraine treatment and changes in migraine disability. The 1 Hz group experienced an average decrease in seven days headache compared with 3.3 days in the 25 Hz group ($p = 0.035$). Secondary outcomes were not significant in the 95% confidence interval [22].

6 Conclusion

nVNS stimulation represents a promising, if understudied, solution for refractory cluster and migraine headache. At present the European Headache Foundation states that neuromodulation should be tried only in medically refractory patients evaluated at a tertiary headache center [23]. Insurance reimbursement, despite of the mounting evidence for the efficacy of device-based treatment of migraine and cluster headaches, remains a major barrier. The monthly cost of nVNS treatment in the US can be as high as 700 dollars month [24]. Other barriers include the convenience factor or rather, inconvenience factor and the perceived low efficacy when compared to oral, intranasal, and injectable triptans, and preventive medications, including botulinum toxin, and CGRP antagonists.

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Chapter 10

Vagus Nerve Stimulation and Language Learning

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Abstract

This chapter discusses methodological issues when applying transcutaneous stimulation of the aural branch of the vagus nerve (taVNS) to language research. We review a handful of studies that have investigated language learning using taVNS. Subheading 1 provides a general introduction and describes the potential impact of taVNS on language research. Subheading 2 discusses methodological considerations of applying the taVNS technique to language research, including the preparation of linguistic materials, the eligibility of participants, experimental design, types of language training, assessment of language learning, and parameter settings of taVNS. Subheading 3 gives examples and summarizes results from previous taVNS language studies that differ from one another with respect to the methods used. Subheading 4 concludes this chapter with future directions for applying the taVNS technique to language research.

Key words Language learning, Language training, taVNS, Peri-stimulus stimulation, Priming

1 Introduction

Speaking and understanding human language are seemingly effortless actions for most adults; however, these processes involve mapping and combining information across many domains of representation (phonological form, orthographical form, meaning, syntactic information, event representation, situation model) and engage many cognitive systems (e.g., long term memory, attention, cognitive control) [1, 2]. For instance, while listening to sentences, incoming speech sounds need to be matched to representations of words in long-term memory. Stored information associated with these words is incrementally combined into larger linear and hierarchical chunks of information. This is not done in isolation, but rather is both integrated in and driven by recent context and past experience. The unfolding signal can be massively ambiguous, and attention and cognitive control are needed to deal with competing representations and to focus on new and relevant information [3, 4].

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Once a language processing system is in place, as it is in adults, learning another language becomes challenging, especially if it involves the learning of new speech sounds and/or the mapping between new (sound, word) sequences and meaning. Learning an additional language also involves language control—that is, the ability to inhibit or activate the language(s) one already knows and switch between languages when needed [5].

Vagus nerve stimulation (VNS) has been shown to affect cognitive and perceptual mechanisms such as attention [6], cognitive control [7], associative memory [8], short term memory for words [9] (but *see* Mertens et al. [10]), and perceptual plasticity [11]. These mechanisms may also be involved in language processing and learning. Therefore, a valid question to ask is in what respect and to what extent can VNS affect language processing and learning. Potential impactful applications of these research outcomes are the development of effective and efficient language learning methods (e.g., for military purposes), and intervention and treatment methods for people with speech or language disorders (e.g., [12]). VNS studies may also provide additional theoretical insight into, for instance, the role of cognitive control and attention in language learning and (multi-lingual) language processing. Below, we will discuss the most important methodological considerations when using VNS to study language learning and review some of the language learning studies published thus far using this technique.

2 Materials and Other Methodological Considerations

The obvious limitation of studying language processing and language learning is that this research is mostly restricted to human participants (but *see* Engineer et al. [11]). Consequently, these studies are primarily constrained to noninvasive methods. In this chapter, we focus on noninvasive transcutaneous stimulation of the aural branch of the vagus nerve (taVNS) as a moderator of language processing. This stimulation is accomplished by placing electrodes on the left outer ear and passing small currents through external ear structures such as the tragus [13]. The left ear is deliberately stimulated to avoid cardiac effects, as the right branch of the vagus provides the majority of the parasympathetic innervation of the sinoatrial node [14]. Another challenge within language learning studies is that human research participants come into the laboratory with a lifetime of unique experience with language and languages. This has major consequences for experimental design and methodology.

2.1 Participant Selection and Experimental Design

Language learning in the lab is heavily influenced by language experience factors such as bilingualism [15], the specific language (s) a participant knows [16], and whether one has been passively exposed to different languages in daily life [17]. Language learning is also affected by language aptitude [18], motivation [19], perceptual abilities [20], musical experience [21], and genetics [22], among other things. In studies that use between-participant designs, wherein one group receives sham stimulation and the other taVNS, it is therefore essential to match the groups on parameters known to affect the aspect of language learning that one is investigating. Given the numerous factors that may affect performance on language learning tasks, it may be best to use a within-participant design, in which one participant receives both sham and taVNS, hence serving as their own control. This can either be done by having different sessions or blocks per participant, each with a different type of stimulation (with order counterbalanced across participants, [23]), or by linking stimulation type to different language stimuli within one session [13]. Within-participant designs, however, are not ideal. Habituation to the task over sessions and general learning effects may confound the effect of stimulation type [23]. Exposing the participant to both sham and taVNS may also complicate the ability to test effects of stimulation type on the generalizability and long-term retention of what has been learned (*see* Subheading 2.4).

2.2 Linguistic Training Materials

When designing the materials for a language learning task, it is also important to take the participants' language background into consideration. Take, for instance, a task in which participants associate new words with familiar concepts (e.g., *grop* refers to a table). These new words need to be constructed very carefully to avoid confounds and reduce noise in the data. For example, if one is only interested in the formation of word-meaning associations and not in the learning of new speech sounds (e.g., lexical tones in the case of English speakers), the new words should be pronounceable and follow orthographical patterns in the participants' native language to avoid confounds [24, 25]. Second, the new words need to control for phonological or orthographical overlap with existing words in the participants' language or languages, in addition to the frequency of sound sequences or orthographical patterns. This is because new words that overlap with existing words will lead to activation of known words, which in turn may either hinder or facilitate the processing and learning of the new words [26, 27]. Also, form overlap among the new words [28], as well as the number of new words [29], can affect their processing and learning. Controlling for these factors becomes especially important if the type of stimulation (taVNS or sham) is varied with respect to the type of linguistic material. The items presented with one type of stimulation versus the other need to be carefully

matched for the factors just described. Similar considerations are needed in experiments investigating the learning of grammar. For instance, speakers of languages that use grammatical gender are better at learning grammatical gender in a second language [30]. It is therefore critical that participants in the study are rather homogeneous as to their native language(s) and language experience, and that language learning materials are designed with the specific participant population in mind.

One objection to the use of carefully constructed artificial language materials is that such studies do not reflect natural language and typical language learning situations. On that account, several recent studies have used widely available language learning software such as Duolingo and Rosetta Stone to investigate natural language learning and, for example, its effect on brain function [31]. It can be expected that the results from such tasks will be much noisier compared to those in which the language materials are tightly controlled. When applying such natural language learning paradigms to VNS studies, it is critical that the participants are extremely well-matched across stimulation groups in between-participant designs. On the other hand, for within-participant design studies, where stimulation type varies depending on the language materials, one should carefully match the materials across stimulation types based on factors such as frequency of occurrence (in the training and in general) and overlap with the participants' languages, as for the artificial language learning paradigms described above.

2.3 Type of Training

Within the second-language acquisition literature, a distinction is made between implicit and explicit learning. Explicit learning implies that the learner is aware of what they have learned; implicit learning means that the learner is not able to verbalize what they have learned. The terms implicit and explicit learning can also pertain to training techniques used. In explicit learning paradigms, learners receive instructions (e.g., about grammar rules) and get feedback as to what the correct form, meaning or usage is; in implicit learning paradigms, the learner is not informed about what needs to be learned and does not receive corrective feedback. These two forms of learning have been linked to different memory systems (declarative and procedural, respectively [32, 33]) and have different learning outcomes. For instance, implicit learning may lead to better retention over the long run than explicit learning [34].

Most taVNS studies on language learning have used intentional training paradigms in which participants are explicitly instructed to learn or memorize speech sounds or words [35]. Training tasks either provide explicit feedback to the participants' responses [13, 36, 37] or use tasks that do not require behavioral responses, but in which the presentation of the correct associate can serve as

implicit feedback [36–38]. The effect of taVNS and of the type of stimulation may vary depending on the type of training [37].

2.4 Assessment of Learning

Learning is typically assessed by comparing performance on a task without feedback before and shortly after the training, or by looking at improvement over the course of training. Critical aspects pertaining to language learning are first, that the new knowledge is retained over a long(er) period, and second, that the knowledge can be generalized to new materials or situations. In addition to learning new words, another aspect of learning is that new words become integrated into a knowledge network and interact with other words rather than being retained as separate items [27, 39]. It is therefore important to assess these aspects when evaluating the effects of VNS on language learning.

Longer term retention can be tested by having participants return for another post-test after a week, a month, or longer, depending on one's goal and resources. Generalization can be assessed by testing participants on materials or tasks that are different from the training materials in one or more respects. For instance, if the task was about discriminating or classifying new speech sounds, the generalization test can consist of stimuli spoken by a person different from the one who produced the training stimuli, or by varying the vowels or consonants of the words. Formation of connections with other items can be tested by investigating the degree of competition among the new items. If newly learned words have formed connections with other words, it will take longer to recognize a newly learned word when its form overlaps with that of many other words than when its form is unique [39]. To our knowledge, the effects of VNS on word learning have not been investigated with relation to the latter.

Traditional learning assessment tasks are based on behavioral responses. Learning is said to occur if performance after training is faster or more accurate than performance before training or at the beginning of training, and if performance generalizes to new stimuli. In VNS studies, one expects such learning effects (e.g., the difference in performance after versus before training) to be different depending on the type of stimulation (taVNS vs. sham or other control). Based on the few existing studies, behavioral effects of VNS on language learning tend to be rather small in healthy young adults [37]. It is therefore recommended to also collect EEG and/or pupillometry data [38]. These methods can uncover differences in processes, cognitive effort, or degree of integration of newly learned items into a network, in spite of an absence of behavioral differences related to type of stimulation [38].

2.5 Stimulation Parameters

taVNS has only very recently been used in relation to language learning, and as such there are no standards for stimulation parameter settings. Studies differ with respect to the site used for taVNS stimulation (cymba conchae [13, 36], superior and inferior walls of the outer ear canal [37, 38], tragus [23]), and with respect to the intensity of stimulation (stimulation can either be above the perceptual threshold but below the level of any discomfort [23, 36] or below the perceptual threshold where the participant does not feel it [13, 37, 38]). Sub-perceptual threshold stimulation has been found to work more effectively in the modulation of neural plasticity in animal models [40]. Furthermore, there is controversy over what constitutes a good control or sham stimulation: stimulation of the earlobe ([23, 36]; but *see* Rangon [41]) or no stimulation [13, 36, 37]. No stimulation is a good control, since there are no differences in the positioning of the apparatus or in sensation between the stimulation and sham conditions—especially when using sub-threshold stimulation in the taVNS condition. Another variable to consider is the moment during language training stimulation wherein occurs. Some studies apply stimulation continuously [23] while others stimulate over a short period (e.g., 10 min) before training or testing (priming, [38]). A handful apply stimulation briefly (500 ms) around the presentation of critical stimulus types (peri-stimulus, [13, 38]) or paired with corrective feedback [36].

3 Methods: Illustrative Examples of taVNS Studies on Language Learning

Thus far, there have only been a handful of taVNS studies on language learning. In this section, we will summarize five studies that illustrate the methodological issues discussed above. Llanos et al. [13] conducted a study that examined whether peri-stimulus taVNS could enhance native English speakers' performance on non-native tone discrimination in Mandarin Chinese. Mandarin, as opposed to English, uses pitch differences (tones) to distinguish among word meaning. Native English-speaking participants underwent 6 training blocks and 1 generalization block. They were asked to respond to five different Mandarin monosyllabic sounds pronounced in four different tones via a button press. Participants were divided into three training groups: one group received sub-threshold peri-stimulus taVNS on two tone types that were considered easy to learn (tones 1 and 3), but not on tones that were considered hard to learn (tones 2 and 4); the second group received taVNS on tones 2 and 4 but not on tones 1 and 3; the third group served as a control and did not receive stimulation. None of the groups received stimulation in the generalization block. The results of the 6 training blocks revealed that the peri-stimulus taVNS increased accuracy of discrimination for the easy-to-learn tones

1 and 3, but not for the harder-to-learn tones 2 and 4. This improvement was also found in the generalization block. This suggests that enhancement of learning induced by peri-stimulus taVNS was limited to perceptually salient categories, but could be sustained and generalized.

Phillips et al. and Pandža et al. [37, 38] investigated whether the taVNS could strengthen native English speakers' learning of associations between Mandarin tones and word meaning. Three monosyllabic Mandarin pseudo-words were paired with tone 1, tone 2 and tone 4 in the study, yielding 9 target Mandarin tone-word stimuli. These 9 target tone-word stimuli were paired with 9 different English nouns that were matched in word frequency, concreteness, word length, and animacy. Participants in the study had to learn these 9 target Mandarin tone-word stimuli and their associated meanings in English. They underwent two training sessions on two consecutive days and were assigned to one of the three stimulation conditions during their training sessions: a sham, peri-stimulus, or priming taVNS condition. The relevant tone-word training tasks in the training sessions included a passive paired-associates word learning task, a match/mismatch lexical recognition task, and a learned-word lexical recall task. In the passive paired-associates word learning task, each Mandarin tone-word stimulus was presented with its paired English word on the screen. Participants had to memorize each stimulus and its corresponding meaning in English. In the match/mismatch lexical recognition task, two English words were presented side by side on the screen, followed by the auditory presentation of a target Mandarin tone-word stimulus. Participants had to choose the correct translation of the Mandarin tone-word stimulus and received feedback to their responses. In the learned-word lexical recall task, an English word was presented on the screen, followed by an auditory presentation of a target Mandarin tone-word stimulus. Upon hearing the auditory stimulus, participants had to respond to whether the meaning of the auditory stimulus matched the English translation. No feedback was received in this task. Pandža et al. [38] found that both peri-stimulus and priming taVNS overall strengthened the learning of Mandarin tone-word associations, as opposed to the sham stimulation condition. This improvement was reflected by either higher accuracy or faster responses in matched/mismatched trials across the lexical recognition and lexical recall tasks. They also found that learning effort was less sustained with peri-stimulus taVNS, based on pupillometric results [38]. In the same study, Phillips et al. [37] also found that both peri-stimulus and priming taVNS led to better performance on Mandarin tone-word associations, reflected by either higher accuracy or faster responses, as compared with sham taVNS. Additionally, their event-related brain potential (ERP) results of the N400 component revealed that both peri-stimulus and priming taVNS facilitated the

development of Mandarin tone-word associations, with fine-grained distinctions of the modulations in different ways. To summarize, both peri-stimulus and priming taVNS, as opposed to the sham condition, enhanced the learning of non-native Mandarin tone-word associations [37, 38].

Thakkar et al. [36] investigated whether taVNS could strengthen the learning of novel orthography (Hebrew) in young adults. Participants were randomly assigned to one of four experimental groups: control, sham, earlobe stimulation, and taVNS. Participants in the control group were not fitted with the stimulator, but the other groups were. Participants in the sham group received no stimulation while those in the earlobe and taVNS groups received sub-perceptual threshold stimulation on the earlobe and the outer ear (cymba conchae), respectively. Participants underwent training sessions for five separate days. In each training session, participants learned to sound out new letters and pronounce letter sequences. After each trial, the letter sequences were displayed again and correct responses were presented auditorily. The earlobe and taVNS groups received stimulation during this feedback phase. Learning was assessed by means of a letter identification task, a timed letter identification task (assessing automaticity), and a pseudo word reading task (decoding task). Participants in the taVNS stimulation group outperformed the other groups in the automaticity and decoding tasks, reflected by higher accuracy or faster responses.

Finally, Kaan et al. [35] investigated the effect of taVNS on implicit learning of word segmentation [42]. Participants were exposed to 11 min of an auditory stream without pauses (*kibudulatibilomarimodipalatibi*. . .). Unbeknownst to the participant, this stream consisted of six of 3-syllable nonce words randomly strung together (e.g., *kibudu, latibi, modipa,* . . .). After the exposure, implicit learning of the six words was tested by means of a discrimination task—in which participants had to say which of two strings sounded more familiar—in addition to a repetition task, wherein participants had to repeat 6-syllable strings that either consisted of two "words", or were random syllables [43]. Continuous taVNS or sham stimulation (earlobe) was applied during the exposure and test phases at a supra-threshold level. Each participant received both types of stimulation, with the order of stimulation type counterbalanced over two sessions, 3 weeks apart. Echoing other studies, participants responded more quickly and accurately to the "words" than to strings of three random syllables. However, learning was not affected by the type of stimulation. In the same study, small effects of stimulation were found in another task that explicitly probed memory for sequences of English words [23]. A speculative explanation is that taVNS affects intentional but not implicit learning.

In sum, previous language learning studies using taVNS have found that taVNS can facilitate aspects of language learning. However, the outcomes of these studies differ depending on the materials and tasks used. Learning effects may be limited to perceptually salient information [13] or intentional learning [35] and may affect automaticity rather than the formation of associations [36, 38]. Furthermore, effects of stimulation on learning tend to be rather small for healthy young adults and may not always apparent in behavioral responses [37]. It is therefore recommended to also collect EEG or pupillometry data [13, 38] and/or to expand to elderly populations and other demographics (but *see* Mertens et al. [10]).

4 Notes

Research investigating effects of noninvasive VNS on language learning has only just begun. At this point, there are no agreed-upon standards as to the many parameters that may affect the outcome of these studies. Several parameters have been discussed in the previous sections.

Interesting questions that might advance our knowledge of taVNS application on language learning, are, for instance, what is the effect of the duration and stimulation intensity of the taVNS on language learning? That is: does longer and more taVNS stimulation lead to better performance? Relatedly, what is the effect of tVNS on language learning depending on the duration of training? How long does the effect of taVNS on learning last? In addition, taVNS seems to facilitate relatively simple letter-sound associations in language learning. One can continue to ask whether more complex aspects of language learning (syntax, pragmatics) can also be facilitated by taVNS.

Another avenue of research is to specify the mechanisms through which taVNS affects language learning. For instance, is it attributable to enhancement of inhibition and attention? If taVNS facilitates inhibition [7], could taVNS help inhibit the native language and lead to faster language learning [44]? Would taVNS lead to less cross-language interference and facilitate language switching in bilinguals [45]?

Despite the current methodological controversies and limitations, noninvasive VNS is a very promising technique to investigate language processing and learning. Outcomes of such studies will lead to further insight in language processing and learning, and can inform the development of effective and efficient language learning intervention and treatment methods.

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