Hitting the right nerve: effects of transcutaneous vagus nerve stimulation on symptoms of anxiety

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Table of Contents

List of Commonly Used Abbreviations

AB	Attentional Blink
ABVN	Auricular Branch of the Vagus Nerve
BFT	Breathing Focus Task
CS	Conditioned Stimulus
EMG	Electromyography
FPS	Fear-Potentiated Startle
HRV	Heart Rate Variability
LC	Locus Coeruleus
NA/NE	Noradrenaline/Norepinephrine
NTS	Nucleus of the Solitary Tract
SCR	Skin Conductance Response
tVNS	transcutaneous Vagus Nerve Stimulation
RMSSD	Root Mean Square of Successive Differences of heart beats
US	Unconditioned Stimulus
VNS	Vagus Nerve Stimulation

Chapter 1

General Introduction

Anxiety disorders constitute the most prevalent class of mental disorders in Western society, affecting close to 30% of the population at some point in their lifetime [1,2]. As such, anxiety does not only place a large burden of disease on individuals and their immediate social environments [3], but also places a large economic burden on society (ie. direct and indirect costs of anxiety disorders in Europe totaled 74 billion euros in 2010 [4]). Cognitive-behavioral therapy (CBT) is the first-line intervention for anxiety disorders, with exposure therapy being its cardinal component. Despite CBT being a 'gold standard' treatment for anxiety disorders, a substantial proportion of patients do not remit after therapy (between 20 and 67%, [5]). Additionally, as exposure therapy relies on the inherently stressful process of testing one's appraisal of what situations are dangerous, dropout is a very commonly occurring problem (up to 50% drop out of treatment, [5,6]). As such, there is a clear need to either improve currently existing treatment protocols, or develop alternative treatments that may tackle these problems.

Meta-analyses have provided mixed evidence for an augmentation of treatment efficacy when CBT is combined with pharmacotherapy [7–9]. Moreover, the interactions, contra-indications, and side-effects of these pharmacological agents should be considered when using medications as add-on therapy. Alternatively, recent technological advances, as well as increased understanding in the neurobiological underpinnings of anxiety, have sparked an interest in findings ways to augment the effects of CBT by using neurostimulation (e.g. through the use of deep brain stimulation or transcranial magnetic stimulation, [10]). One of the most recent developments in the area of neurostimulation is that of transcutaneous vagus nerve stimulation, a non-invasive technique designed to stimulate the auricular branch of the vagus nerve. In this thesis, we aim to assess whether this technique has the potential to ameliorate symptoms of anxiety, either as an add-on treatment for exposure therapy, or as a stand-alone treatment.

The Vagus Nerve

The vagus nerve is the tenth cranial nerve and owes its name - vagus is Latin for 'wandering' - to its length and complexity: travelling from the brain stem to the abdomen and branching out to innervate most visceral organs, the vagus nerve is the largest cranial nerve in the body. Broadly speaking, the vagus nerve sprout from four nuclei in the medulla and form two nerve trunks (left and right) before entering the jugular foramen and passing through the superior and inferior ganglia. In the superior ganglion, the auricular branch of the vagus nerve arises and innervates the external auditory canal and parts of the external ear. After exiting the cranium via the jugular foramen, the main branch of the vagus nerve travels down and branches out to innervate the lungs, heart, spleen, kidneys, liver, stomach, small intestines and colon [11–14]. The distinction between the left and right vagus nerves is of critical importance for the stimulation of the nerve, because of differential innervation of either

nerve with the sinoatrial node of the heart. Specifically, the right vagus nerve is known to innervate the sinoatrial node – the heart's pacemaker – more strongly than the left vagus nerve, which primarily terminates on the atrioventricular node [15]. For this reason, invasive vagus nerve stimulation is preferably applied to the left vagus nerve to avoid severe bradycardia [16,17].

The vagus nerve consists of different fibers that can be broadly categorized into A-, B- and C fibers, ranging from large, myelinated, fast-conducting A fibers to small, unmyelinated and slow-conducting C fibers. The majority of all fibers within the vagus nerve are part of this last category of afferent unmyelinated C-fibers. These fiber types each have a specific physiological role: large A fibers carry somatic afferent or efferent information, small A fibers carry visceral afferent information, B fibers carry efferent sympathetic and parasympathetic information, and small unmyelinated C fibers carry afferent visceral information. Recent studies conducted in anesthetized dogs revealed different activation thresholds for these fiber types; 0.4mA for A-fiber, 3.8mA for B-fiber and 17mA for C-fibers. Given the similarities in fiber thickness between dog and human vagal nerves, the human vagus nerves may follow the same pattern.

Due to the central role of the vagus nerve in the parasympathetic activation of peripheral organs, the vagus nerve is often mistakenly described as a parasympathetic nerve. However, the vagus nerve also has a sympathetic function via the peripheral chemoreceptors, which trigger a vasoconstrictor response and increase blood pressure. Additionally, approximately 80% of the fibers of the vagus nerve are afferent fibers that transfer sensory information from peripheral organs to the brainstem [18,19], a process that is neither parasympathetic nor sympathetic [20].

The Auricular Branch of the Vagus Nerve

The auricular branch of the vagus nerve (ABVN) – the target for most tVNS interventions – appears to be a phylogenic remnant of nerves that supply the lateral line organs in amphibians and fish to sense vibrations and movements in the surrounding water [21,22]. In mammals, the ABVN is an exclusively afferent sensory nerve that innervates part of the skin of the outer ear as well as the ear canal. Research on the anatomical distribution of the auricular branch of the vagus nerve is scarce. In an anatomical study performed on macaques in 1897 [23], Sherrington noted that the ABVN innervates the cavum and cymba conchae and the antitragus, and also part of the tragus and the antihelix. In 1927, a case study was published concerning a patient suffering from severe pain in the ear and throat. In an initial procedure, Fay performed a resection of the trigeminal nerve supplying the area of the tongue and throat. This initial surgical procedure resulted in complete analgesia of the tragus, indicating that although the ABVN may also innervate this part of the ear, it is not the only nerve to provide sensory feedback from this part of the ear. In a subsequent procedure, the ABVN was sectioned, resulting in complete anesthesia in the cymba concha, and to a lesser degree the antihelix

and antitragus [24] (see figure 1). Finally, a study performed on human cadavers showed that the ABVN is the only nerve to stimulate the cymba concha [25]. Additionally, the ABVN may innervate the tragus, antihelix and cavum concha, although the study reports contradictory innervation percentages in the main text and the corresponding table, making it impossible to assess whether and to what extent the ABVN innervates these areas of the outer ear [26].

The afferent fibers of the ABVN terminate in the nucleus of the solitary tract – similarly to the thoracic vagus nerve – as well as the spinal trigeminal nucleus [27,28]. The ABVN seems to be innervated predominantly by the large, myelinated A-fibers [29]. Given that stimulation intensities of VNS and tVNS typically vary between 0.3-3mA, it seems likely that these techniques almost exclusively recruit A-fibers of the vagus nerve, and thus it seems unlikely that tVNS will have efferent cardiac effects.

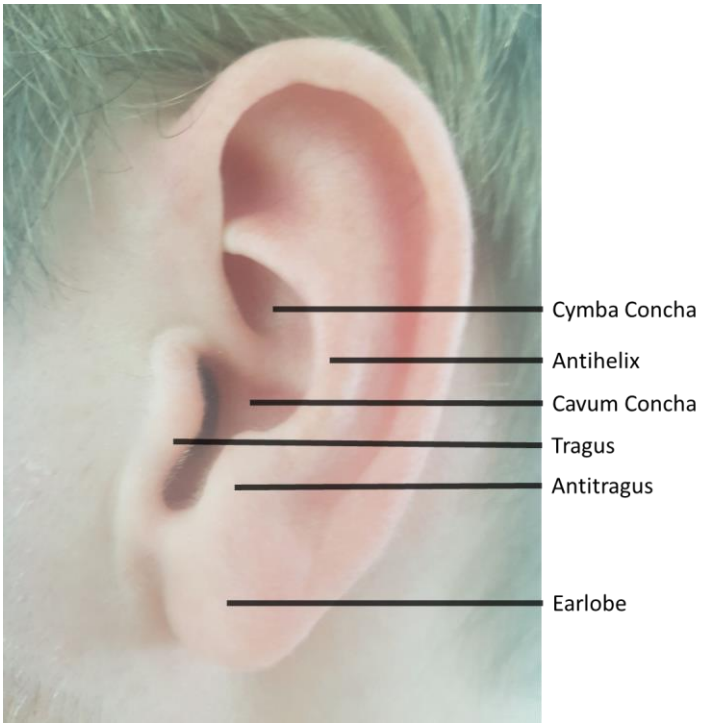


Figure 1. Picture of the author's ear. The ABVN innervates the cymba concha of the ear, and to a lesser degree may also innervate the antihelix, antitragus and tragus.

Stimulating the vagus nerve

Although vagus nerve can be activated through chemical [30,31], mechanical [31], and electric means [32], research on vagus nerve stimulation has mainly focused on the therapeutic effects of electrical stimulation of the vagus nerve. This may have been due to the relative ease of controlling the frequency and the intensity of stimulation through electric means compared to chemical or mechanical means. Additionally, prolonged chemical or mechanical stimulation may lead to fiber damage and

increased fibrotic tissue compared to electric stimulation. As such, electric stimulation provides a relatively safe and controllable means of activating the vagus nerve.

The first reports of electrical vagus nerve stimulation are as early as 1884, and describe a technique of transcutaneous vagus nerve stimulation (tVNS) in the neck as a treatment for seizures [33]. This initial 'electrocompressor' combined compression of both carotid arteries with simultaneous electrical stimulation of the vagus nerve and the cervical sympathetic nerves. This technique was not widely adopted by Corning's contemporaries due to a lack of consistent positive results, and the technique was abandoned for over a century [34]. In 1990, Penry and Dean first described a technique of invasive vagus nerve stimulation (VNS) in humans, again as a treatment for intractable seizures in epilepsy patients [35]. During invasive VNS, the vagus nerve is electrically stimulated by two electrodes that have been surgically implanted and sutured around the vagus nerve at the level of the neck. The electrodes are connected to a battery that is also surgically implanted, and the vagus nerve is typically stimulated with a '30 seconds on, 5 minutes off' duty cycle. Due to the invasiveness of the procedure as well as its costs, VNS is not a commonly used intervention, and not a lot of research has been done on VNS in humans.

Ten years after the development of invasive VNS, the concept of a non-invasive, transcutaneous vagus nerve stimulation (tVNS) method of the ABVN was proposed by Ventureyra [36]. During tVNS, two electrodes are attached to the surface of the outer ear at a location that is believed to be innervated by the ABVN. Typically, tVNS aims to stimulate either the cymba concha or the tragus of the ear, based on the anatomical distribution of the ABVN [25]. Unfortunately, parametric research on tVNS is scarce, and stimulation parameters are mainly based on preclinical and clinical research on invasive VNS (e.g. [37–44]). Specifically, electrical stimulation during tVNS typically consists of an alternating current (usually 250-500μs stimulation wavelength delivered at 25Hz) delivered intermittently (30 seconds on, 30 seconds off). To stimulate the vagus nerve, electrical pulses must penetrate the skin and the nerve's epineurium, and exceed the excitation threshold of fibers in the vagus nerve [29]. The stimulated fibers fire action potentials that propagate through the vagus nerve to the nucleus of the solitary tract in the brain stem, which in turn affects other cortical and subcortical brain structures.

tVNS as an add-on for exposure therapy

The central afferent projections of the vagus nerve suggest that tVNS could play an important role in the treatment of anxiety disorder. Notably, the indirect projections to the locus coeruleus-noradrenaline (LC-NA) network, reflect its integral role in the encoding and consolidation of memory traces. Indeed, during stressful or threatening situations, peripheral adrenaline binds to beta-adrenergic receptors of the vagus nerve, which triggers action potentials in the afferent vagus nerve

and subsequently increases memory encoding and consolidation through enhanced activity in brain areas including the LC-NA system [45]. Preclinical studies show that when the peripheral beta-adrenergic receptors of the vagus nerve are blocked, or when the afferent fibers of the vagus nerve are cut entirely, encoding and consolidation of emotional memory is strongly attenuated [46–48]. These findings suggest that direct stimulation of the vagus nerve may strengthen learning and memory through activation of the LC-NA network. Moreover, since stimulation of the vagus nerve circumvents the necessity of peripheral adrenergic receptor binding, VNS may also strengthen non-emotional learning and memory [49]. This suggests that tVNS could be used as an adjunct to exposure-based therapy, a learning-dependent psychological treatment that is currently considered the gold-standard for anxiety disorders.

A series of preclinical fear conditioning studies repeatedly and consistently demonstrated that invasive VNS strengthened the extinction of auditory conditioned fear [50–53]. Although these animal studies confirm the importance of vagal nerve activity during extinction learning and highlight a potential role for VNS in augmenting exposure therapy, it remains unclear to what extent these preclinical findings can be translated to humans [54–56]. Apart from this translational issue, it also remains unclear whether the effects of VNS can be achieved through non-invasive, transcutaneous means of stimulating the vagus nerve. Therefore, a main goal of this dissertation is to assess whether tVNS accelerates extinction learning and strengthens the consolidation of extinction memories in humans. This could have clear implications for the utility of tVNS as an add-on for exposure therapy.

Learning and Memory in Anxiety Disorders

To understand the importance of learning and memory in the treatment of anxiety disorders, we should first discuss their roles in the etiology and maintenance of anxiety disorders. Specifically, according to the Learning Theory of anxiety [57,58], individuals are thought to acquire fear of a certain stimulus or context through a Pavlovian associative learning process. For example, when an aversive event unfolds (e.g. a traffic accident) in a neutral context (e.g. when driving a car), the initially neutral context may come to elicit fear or anxiety due to its association with the aversive event. Apart from this direct learning, fear can also arise vicariously through verbal instructions or visual observations of responses of others [59,60]. Exposure-based treatments are reliant on a similar associative learning process: patients undergo prolonged and repeated exposure to the feared stimulus or context (e.g. driving a car) in absence of the expected aversive event (e.g. a traffic accident). During this process, the propositional expectancies of the original fear memory are repeatedly violated, since the feared stimulus or context is not followed by an aversive event. As such, a new, inhibitory memory is created, which competes with the fear memory for activation upon being presented with the feared stimulus. Successful exposure therapy relies on this expectancy violation of the original fear memory, leading to

the creation of a strong inhibitory memory capable of being preferentially activated upon presentation of the once-feared stimulus or context.

Fear learning and exposure therapy have been studied extensively using the fear conditioning framework, which provides a valid experimental analogue for a range of processes relevant to studying the etiology, maintenance, generalization, treatment, and reinstatement of fear [61–63]. Typically, during experimental fear acquisition, participants are presented with a conditioned stimulus (the CS+, often a geometrical shape or a tone) that is repeatedly paired with an aversive unconditioned stimulus (the US, often a loud noise or an electric shock), and a different conditioned stimulus (the CS-), which is never followed by the US and serves as a safety cue. Participants learn that the presentation of the CS+ predicts the occurrence of a US, and thus the presentation of the CS+, even in absence of the US, will elicit a fear response. During subsequent extinction learning, as an analog for exposure therapy, participants are presented repeatedly with the CS in absence of a US, which will eventually extinguish the fear response to the CS+.

An alternative approach: targeting worry

Alongside preclinical studies that point towards tVNS as a potential add-on treatment for exposure therapy, there are also indications that tVNS may be used as a stand-alone treatment. Specifically, stimulating the vagus nerve may affect anxiety disorders by targeting one of their cardinal symptoms: perseverative cognition.

According to the neurovisceral integration model, individual differences in vagus nerve activity at rest – as indexed by heart rate variability (HRV) - underlie differences in worrying [64–67]. HRV is a reliable indicator of efferent vagal tone [68] and is predominantly affected by the inhibitory control of the vagus nerve on the heart's sinoatrial node. High HRV (i.e., greater vagal tone) at rest reflects prefrontal inhibitory control over subcortical emotional areas in the brain, allowing the organism to respond to environmental challenges in a controlled and adaptive manner [69]. In contrast, a chronically low HRV represents a breakdown of these inhibitory influences, allowing subcortical brain areas to become hyperactive. This facilitates an excitatory positive feedback loop, reflected at the psychological level by hypervigilance and worry (cf. [67]).

In support of this model, many studies have found lower baseline HRV in GAD patients compared to healthy participants (for a meta-analysis, see [70]). The severity of worry – and not the diagnosis of an anxiety disorder – was associated with the most robust negative correlations with HRV [71]. These results also extend to non-clinical samples, where high dispositional worry has been found to be related to lower average HRV [72]. Experimental inductions of worry lead to a strong reduction in HRV (for a meta-analysis see [73]), and this reduction is more pronounced and slower to recover in chronic worriers [72,74–78]. Together, these studies indicate that low vagal activity could be a

vulnerability factor for chronic worrying. In summary, both the chronically low HRV of dispositional worriers, and the acute decrease of HRV during induced worry episodes are well established. Studies that have examined the relation between HRV and worrying have only examined this association on a cross-sectional basis or through worry inductions, but have never experimentally manipulated HRV to test its effects on worry. It remains unknown whether low vagus nerve activity is a mere reflection of the breakdown of prefrontal inhibitory control on subcortical areas, or is playing a causal, role in maintaining worry.

Although it remains unclear to what extent these associations between efferent vagus nerve activity and perseverative cognitions are predictive of the effects of tVNS – which is believed to activate primarily afferent fibers of the vagus nerve –, there are indications that tVNS may strongly affect central processes and cognitive functions thought to underlie perseverative cognition and stress-related cognition. Crucially, fMRI studies have shown that tVNS directly promotes activity in brain areas that reduce worry, including the prefrontal cortex and the anterior cingulate (for a review, see [79]). Furthermore, tVNS increases the functional connectivity between the amygdala and the prefrontal cortex in depressed patients [80]. Decreased functional connectivity between the amygdala and the prefrontal cortex has repeatedly and robustly been demonstrated as a function of anxiety [81], and has also been linked to self-reported worry intensity in patients suffering from GAD [82–84].

Previous studies have indicated that invasive VNS affects cognitive functions that rely on prefrontal activity, e.g. cognitive flexibility, decision making, and memory formation and consolidation (for a review, see [85]). Similarly, tVNS affects cognitive functions that rely on prefrontal activity, e.g. enhanced associative memory formation and consolidation [86] and action control [87]. Critically, tVNS promotes the ability to inhibit task-irrelevant processing [88,89], a process which is strongly compromised in patients suffering from GAD [90]. Finally, the potential effects of tVNS on worrying are further illustrated in a non-randomized study that showed positive effects of tVNS on symptoms of depression and anxiety in patients suffering from a major depressive disorder [80,91,92], a condition that is characterized by perseverative cognitions including worrying and rumination [93]. Thus, it could be worthwhile to assess whether tVNS could affect perseverative cognition, and thereby could also ameliorate anxiety symptoms by targeting one of the core symptoms of stress related psychopathology. Thus, a second aim of this thesis is to test whether tVNS affects perseverative cognition, one of the cardinal symptoms underlying anxiety disorders.

Working Mechanisms

The working mechanisms underlying tVNS are currently poorly understood. One of the most important working mechanisms hypothesized to underlie the effects of tVNS on psychological and neurological disorders is an increased activity of the locus coeruleus – norepinephrine (LC-NE) network. Specifically,

afferent fibers of the vagus nerve are known to terminate in the nucleus of the solitary tract, from which there are direct and indirect routes that can both activate and inhibit neurons in the LC [94]. Indeed, animal studies that tested the effects of invasive VNS have repeatedly found that rats receiving VNS, compared to those that had undergone sham surgery, show increased firing rates in LC neurons both acutely [45,95–98] as well as over a longer timespan (after a period of 90 days: [95]; after 14 and 90 days: [99]). In line with these findings, several studies found increased concentrations of NE in brain areas that the LC projects to, including the hippocampus [11,12], basolateral amygdala [13], and medial PFC [14].

Although the effects of VNS on LC-NE activity is well established in animals, studies on the noradrenergic effects of (t)VNS in humans are lacking. Direct measurement of NE in humans requires an invasive procedure and suffers from poor reliability and sensitivity [100]. Therefore, measuring NE in humans relies on assessing biomarkers that are related to LC – NE activity. Common biomarkers used as a proxy of NE include pupil diameter, the P300 component of event related potentials, and salivary alpha amylase. Several studies performed in small samples of patients wearing invasive vagus nerve stimulators provided some preliminary indications that LC-NE activity is increased when the stimulator is turned on compared to when it is turned off. Specifically, patients showed increased P300 amplitudes to visual cues [101], and increased resting pupil diameters [102], when VNS was turned on compared to when it was turned off. However, other studies were unable to find effects of VNS and auditory P300 [101,103], resting pupil diameter [104] and pupillary light reflexes [102]. As such, the working mechanisms underlying invasive and transcutaneous VNS remain unclear, and there's a clear need to assess whether the effects of VNS on LC-NE activity found in animals can be replicated using tVNS in humans. Thus, a final aim of this thesis is to assess whether tVNS increases LC-NE activity in humans, thereby testing the central working mechanism underlying the effects of tVNS.

Aims and Outline

To summarize, this thesis aims to study the potential for tVNS as a stand-alone or add-on treatment for stress-related disorders through a series of experimental studies focusing on stress-related cognition. Specifically, we examine how tVNS affects extinction learning, the experimental surrogate of exposure therapy. Additionally, we assess the effects of tVNS on worry frequency in a population of high worriers, since worrying is thought to underlie most stress-related psychopathology. Finally, we assess the hypothesized working mechanisms of tVNS in a series of studies that measure the effects of tVNS on noradrenergic activity.

In **Part I**, *Extinction of Fear*, we examine whether tVNS facilitates the extinction of fear. In **chapter 2**, we test whether tVNS accelerates the extinction of fear and strengthens the consolidation of immediate extinction memories (i.e. extinction occurring on the same day as acquisition). In **chapter**

3, we attempt to replicate these findings using a delayed extinction protocol (i.e. extinction occurring one day after fear acquisition). **Chapter 4** describes a conceptual replication attempt of these studies, where we studied the effects of tVNS on the immediate extinction of prepared fear in a highly arousing environment. Finally, **chapter 5** describes an experiment where we tested whether tVNS decreases the generalization of fear memories, which is believed to be one of the main contributing factors in the development and maintenance of anxiety disorders.

In **Part II**, *Negative Thought Intrusions*, we assess whether tVNS can decrease the number of negative thought intrusions in a population of high worriers (**chapter 6**).

In **Part III**, *Working Mechanisms*, we point out several critical inconsistencies in a cornerstone anatomical publication on the nerve supply in the human ear, thereby questioning the validity of the tragus as a target site for tVNS (**chapter 7**). Furthermore, in **chapter 8**, we describe a series of three studies are described, wherein the main working mechanism hypothesized to underlie the effects of tVNS is tested. Specifically, we tested whether tVNS increases activity in the LC-NE network by measuring the effects of tVNS on resting pupil diameter, task-related pupil dilation, and task performance on an Attentional Blink task.

To conclude, an overview of the results found in chapters 2 to 7 is provided in a general discussion (**chapter 9**). The theoretical and practical implications of the results, as well as directions for future research, are discussed.

Part I

Extinction of Fear

Chapter 2

The effects of transcutaneous vagus nerve stimulation on conditioned fear extinction in humans

Burger AM, Verkuil B, Van Diest I, Van der Does W, Thayer JF, Brosschot JF (2016). *Neurobiology of Learning and Memory, 132*, 49–56. doi:10.1016/j.nlm.2016.05.007.

Abstract

A critical component of the treatment for anxiety disorders is the extinction of fear via repeated exposure to the feared stimulus. This process is strongly dependent on successful memory formation and consolidation. Stimulation of the vagus nerve enhances memory formation in both animals and humans. The objective of this study was to assess whether transcutaneous stimulation of the vagus nerve (tVNS) can accelerate extinction memory formation and retention in fear 0 were randomly assigned to receive tVNS or sham stimulation during the extinction phase. Retention of extinction memory was tested 24 hours later. tVNS accelerated explicit fear extinction learning (US expectancy ratings), but did not lead to better retention of extinction memory 24 hours later. We did not find a differential physiological conditioning response during the acquisition of fear and thus were unable to assess potential effects of tVNS on the extinction of physiological indices of fear. These findings complement recent studies that suggest vagus nerve stimulation could be a promising tool to improve memory consolidation and fear extinction.

Introduction

Anxiety disorders are among the most prevalent mental disorders, with a point prevalence of 7.3% and a lifetime prevalence as high as 28.8% [1,2]. A critical component of the treatment of anxiety disorders is the extinction of fear via repeated exposure to the feared stimulus. Although repeated exposure combined with cognitive therapy is the treatment of choice, roughly 22% of patients do not respond to this type of treatment [105]. This may be due to the fact that patients with anxiety disorders have more difficulties forming extinction memories [106–109]. Understanding the neurobiological mechanisms by which full extinction of fear is achieved may improve currently available extinction-based treatments for anxiety disorders, as shown by existing augmentation strategies of exposure therapy using for example MDMA or D-cycloserine [110].

Successful extinction of conditioned behavior is strongly dependent on successful memory formation and consolidation. During extinction, a new memory is formed wherein the conditioned stimulus is re-appraised as safe. Critically, fear extinction is not a process of unlearning the conditioned memory or behavior. Instead, a new memory (so called extinction memory) has to be created and consolidated to compete with the conditioned fear memory and reduce conditioned responding [111]. Patients suffering from anxiety disorders create strong fear memories, and therefore have more difficulties creating and consolidating extinction memories that can contest these fear memories [108].

Most neurobiological studies have focused on the role of the central nervous system in fear extinction and show that increasing central norepinephrine through the use of norepinephrine agonists improves extinction memory (e.g. [112,113]). In contrast, relatively little is known about the role of the peripheral nervous system. Yet, several studies suggest a critical function of the vagus nerve in memory formation and consolidation [114–116]. Memory consolidation is often facilitated in arousing circumstances, when excitatory effects of peripheral epinephrine on the vagus nerve lead to the release of norepinephrine in limbic brain structures (for a review, see [94]). Direct stimulation of the vagus nerve during extinction learning may also increase the release of norepinephrine in these learning-relevant brain structures (i.e. hippocampus, amygdala, prefrontal cortex), thereby strengthening the consolidation of extinction memory [95,117].

Manipulating vagus nerve activity indeed affects the rate of fear extinction in rats. For instance, cutting the afferent (but not efferent) vagal nerve fibers attenuated extinction learning [48], whereas stimulating the vagus nerve accelerated extinction learning [50,51]. In humans, chronically low vagal tone may be a risk factor for the onset and maintenance of emotional disorders [64,67,70,118]. Yet, the effects of vagus nerve stimulation on extinction learning have not yet been studied in humans, although positive effects have been found on cognition and memory [85]. Furthermore, surgically implanted VN stimulators have been approved by the FDA for treatment-resistant depression since

2005 and are also being investigated for treatment-resistant anxiety disorders [119,120]. Still, the mechanism of vagus nerve stimulation therapy is not well understood [94,119].

Using VNS to attenuate fear responses in humans has been relatively understudied because until recently it required surgical implantation of a neurostimulator. However, recent technological developments allow transcutaneous stimulation of the vagus nerve (tVNS) via a vagally innervated part of the outer ear (i.e., the concha; [25]). tVNS has been shown to be a safe method to stimulate this auricular branch of the vagus nerve [121]. Short periods of tVNS immediately modulate the activation of brain areas related to extinction learning (eg. the hippocampus, amygdala and prefrontal cortex [122,123], and increase performance in memory tasks and other cognitive tasks that are dependent on norepinephrine activity [86,88]. tVNS is therefore suited to examine the role of the vagus nerve in extinction learning in humans.

The aim of the present study was to test the effects of tVNS on fear-extinction rate in previously fear-conditioned healthy participants. We conducted a randomized controlled trial comparing tVNS versus sham stimulation. Fear learning was operationalized in multiple ways, consistent with the idea that memory formation occurs in different memory systems [124,125]. At the explicit level we measured US expectancy ratings, which may be largely dependent on hippocampal activation [126]. At the physiological level, we examined the startle blink response and heart rate acceleration, that are not only dependent on the hippocampus, but also on amygdala and prefrontal activation [127]. We hypothesized that tVNS would have an effect on both explicit and implicit indices of extinction learning. We also explored whether any effects of tVNS would be maintained the following day by testing the retention and reinstatement of fear and extinction memory.

Methods

Participants

Thirty-eight participants were recruited from the Leiden University student population (for a breakdown of demographics, see table 1). Eligible participants were healthy college students between the ages 18 and 25. Participants with epilepsy, bradycardia, cardiac arrhythmia, cardiac diseases, significant head trauma, pregnancy, drug use, neurological or psychiatric disorders were excluded from participating in this study. Participants received either course credits or 12 euro as compensation for participating in the study. The study was approved by the Institutional Ethical Board of Leiden University, Institute of Psychology (CEP #9394209653). All participants gave their written informed consent prior to the start of the experiment.

Stimuli and Materials

Stimuli

Two geometrical shapes (one blue triangle, one blue square) served as conditioned stimuli (CS; [128]). The slides were 400 mm high and 400 mm wide and were presented on a 17-inch CRT monitor in the middle of the screen on a grey background. Conditioned stimuli were assigned as CS+ and CS- in a counterbalanced order. Both CS+ and CS- were presented for 8 seconds. During the acquisition phase, the CS+ co-terminated with the US in 75% of the trials. The CS- never co-terminated with the US. The US was a 95dB loud scream presented for 2000ms, 6 seconds after CS onset. The scream that was used as US was a shorter version of the IADS sound number 275 [129]. Additionally, a 50ms, 100dB burst of white noise was administered to both ears via headphones, 5 seconds after the onset of every CS presentation and every intertrial interval (ITI). During the ITI, participants were presented with a blank screen. The ITI duration varied randomly between 15 and 25 seconds.

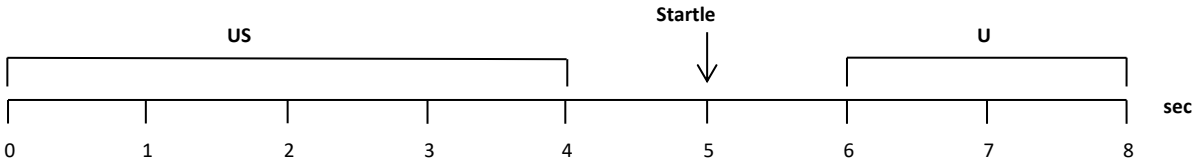


Figure 1. Schematic overview of the timing in a CS+ conditioning trial during the acquisition phase. CS+ trials during the extinction, retention and reinstatement phases were never followed by a US. CS- trials were never followed by a US. CS, conditioned stimulus; US, unconditioned stimulus.

tVNS and sham stimulation

Transcutaneous vagus nerve stimulation (tVNS) is a non-invasive method of electrically stimulating the afferent auricular branch of the vagus nerve located at the cymba conchae [121].

In this study, we used a tVNS instrument that provides electrical stimulation using two titanium electrodes, positioned on top of a silicon earplug, which are connected by a wire to a portable neurostimulator (Nemos®, Cerbomed, Erlangen, Germany). The electrodes deliver 30-second waves of electrical stimulation (0.5mA, 25Hz) to the concha of the left outer ear [25], alternated by 30-second breaks. In the sham condition, the electrodes are connected to the center of the earlobe instead of the concha. In contrast to the concha, the earlobe is not innervated by the vagus nerve [25]. We stimulated the left ear to avoid potential cardiac effects that have been related to efferent vagal fibers of the right ear [119] but not the left [121].

Explicit Fear Rating

Participants were asked to rate the extent to which they expected a scream to occur during every CS presentation using a visual analogue scale that ranged from 0 ('not at all') to 100 ('certainly'). Participants were instructed to give these ratings quickly since the rating scale would disappear from the screen 4 seconds after CS onset, before the startle probe. The scale was presented at the bottom of the screen so as not to draw too much attention away from the stimuli. At the beginning of every new CS presentation, the slide would reappear and the cursor would return to the 'uncertain' middle position (cf. [130]).

Implicit Fear Rating

We measured the potentiation of the eyeblink startle reflex to an acoustic startle probe by using electromyography (EMG) of the left orbicularis oculi muscle. The startle probe was a 100dB, 50ms burst of white noise with a near instantaneous rise time. The 100dB sound burst was administered to both ears via headphones, 5 seconds after the onset of every CS presentation and every intertrial interval (ITI). To measure the eyeblink reflex, we used two 4 mm Ag-AgCl Biopac electrodes, one placed below the lower left eyelid in line with the pupil in forward gaze, and the second one placed approximately 1cm lateral to the first (in accordance with the guidelines specified by [131]). EMG was measured using a Biopac system, and filtered by 500Hz low-pass and 10Hz high-pass hardware filters. To offset delays in startle probe presentations, we used a broad response window of 20 – 400 ms following probe onset. The EMG response was calculated by subtracting the mean EMG signal in the 20 ms period preceding the startle probe presentation from the maximum EMG amplitude within the response window [131].

Cardiac activity

Heart rate (HR) and heart rate variability (HRV) were derived from the raw ECG signal, which was measured continuously using a three-lead set-up of the Biopac system. The raw ECG signal was measured at 1000Hz and subsequently filtered using 2Hz low-pass and 50Hz high-pass software filters. The signal was subsequently visually inspected and artifacts were manually corrected. Interbeat intervals were extracted from the filtered signal, from which HR and the root mean square of the successive differences (RMSSD) between heart rates were calculated.

A five-minute baseline recording of every participant's RMSSD level was used to assess participants' vagally-mediated HRV and to check for possible differences in baseline vagal tone.

As an exploratory measure, we examined HR acceleration in reaction to the presentation of the CS+ and CS-. Phasic HR responses to threat and safety can grant us insight into participants' conditioned preparation for defensive action [132]. These phasic HR responses were captured by assessing the

interpolated HR in the first 5 seconds after CS onset and segmenting that signal into 0.5 second epochs. HR acceleration was measured by subtracting the mean HR of the second prior to CS onset from every 0.5s epoch. To assess the conditioned preparation for defensive action, we captured the maximum HR acceleration within a time window of 3-5 seconds after CS onset (cf. [133,134]).

Questionnaires

Participants completed several questionnaires between the acquisition and the extinction phases to check for possible differences between the groups in terms of levels of trait worrying, trait and state anxiety and current mood, without having received the experimental manipulation.

The Penn State Worry Questionnaire *(PSWQ*; [135]*)* is a 16-item self-report questionnaire that assesses the duration and uncontrollability of worry. The PSWQ has demonstrated high reliability, high temporal stability and substantial validity in the assessment of trait-worry [135,136].

The State Trait Anxiety Inventory (*STAI*; [137,138]) is a self-report questionnaire consisting of 2 versions with 20 questions each, measuring both state and trait anxiety. The STAI has shown acceptable internal consistency and validity [137,139].

Participants rated their current mood (happiness, anxiety, irritableness, sadness) on a visual analogue scale ranging from (0) 'not at all' to (100) 'completely'. The scores on these scales were converted into two comprehensive scores, 'positive affect' (score on the happiness subscale) and 'negative affect' (mean score on anxiety, irritableness and sadness subscales).

At the end of the first day, participants rated whether they experienced any negative side-effects as a result of the stimulation on a scale of 1 ("applies not at all") to 5 ("completely applies to me"). Side-effects included in the list were headache, pain in the neck, nausea, muscle contractions in the face or neck, prickling sensation under the electrodes, burning sensation under the electrodes and a general feeling of discomfort. Both the number of side effects (scores above 1 were counted as a side effect) and the mean intensity of the side effects were compared between the groups.

Experimental Procedure

Day 1

EMG-, skin conductance-[1] and ECG electrodes were attached to the participants' skin. Participants sat in front of a computer and were instructed to start the computer task.

Prior to the acquisition phase, a 5 minute baseline measurement of HR(V) was obtained. After this baseline period, a habituation phase followed, wherein participants viewed one unreinforced

[1] Although electrodermal activity has been measured as part of this project, mechanical errors strongly decreased the signal-to noise ratio in this signal. Therefore, skin conductance responses will not be reported in this study.

presentation of the to-be conditioned stimuli. Additionally, participants heard one presentation of the US and received six startle pulses in the absence of any other stimuli, to habituate startle responses.

In the acquisition phase, both to-be conditioned stimuli were presented 12 times. The CS+ was followed by the US, a 2000ms human scream, using a 75% partial reinforcement paradigm (resulting in 9 CS-US pairings; [140]. The CS- was never followed by a scream. Conditioned stimuli were assigned in a counterbalanced order across participants.

At the end of the acquisition phase, participants were asked to rate the unpleasantness of the US on a scale from 0 (not unpleasant at all) to 100 (very unpleasant).

After the acquisition phase, participants were asked to complete the PSWQ and STAI. Subsequently, we attached the tVNS device to the ear of the participant and we started either tVNS or sham stimulation. Participants were sequentially assigned to receive either tVNS or sham stimulation to reduce the odds of unbalanced group sizes. Regardless of experimental allocation, participants were told that stimulation was expected to affect physiological processes during the tasks. Participants wore the nerve stimulator throughout the rest of the session on Day 1. With the tVNS device in place and active, participants completed a short demographics questionnaire and the VAS scales. Prior studies have noted a temporal latency in the neurological effects of tVNS (e.g. [122]), which is why we decided to start the stimulation while participants completed the demographics form instead of 10 minutes later at the start of the extinction phase.

At the start of the extinction phase, participants were instructed that the same geometrical shapes would be presented as in the previous task, and they would again have to predict when the scream would occur. The extinction phase consisted of 20 presentations of both CS+ and CS- trials (without the US). At the end of the extinction phase, participants reported any potential side-effects from the nerve stimulation procedure.

Day 2

On the day after the acquisition and extinction sessions, we assessed the retention of extinction memory and the reinstatement of fear memory. We did not administer tVNS or sham stimulation during day 2.

During the retention phase, we presented three unreinforced presentations of the CS+ and the CS- in a randomized order. Then, to assess the reinstatement of fear memory, participants received three unsignaled and unpaired presentations of the US, followed by five unreinforced presentations of both the CS+ and the CS- in a randomized order.

Statistical Analyses

All analyses were conducted using SPSS 21.0. The questionnaire data were analysed using independent samples *t*-tests. Baseline HRV levels between experimental conditions were compared using independent samples *t*-tests.

Visual inspection of the raw physiological data was used to exclude artefacts from the HR and startle EMG data. To reduce the variability in the responses on the physiological measurements, startle responses and phasic HR responses on day one were averaged into blocks of four trials prior to analyses (cf. [134]). Subsequently, startle EMG responses were standardized as z-scores over all trial blocks on day one. These z-scores were used in all subsequent analyses [141].

We used multilevel mixed model analyses to assess whether the conditioning procedure resulted in successful fear learning in our participants in terms of both self-reports and physiological outcomes. When we found significant response differentiation between CS- and CS+ trials on a measurement modality during acquisition, we continued to use multilevel mixed model analyses to analyze the effects of tVNS.

All multilevel mixed models were created using maximum likelihood modelling. We allowed intercepts to vary randomly across participants. Independent variables were left uncentered, because all covariates were either dummy variables or already possessed clearly interpretable zero points. In our results section, we report only the fixed effects from our models.

We performed additional analyses, also using multilevel mixed model analyses, to assess the long-term effects of tVNS on the retention of extinction memory and the reinstatement of fear on the second day of testing.

Results

Participants, demographics and baseline measurements

Thirty-eight participants participated in this study (30 female, 8 male, M_{age} = 21.50). Visual inspection of the expectancy ratings showed that seven participants (five from the sham condition, two from the tVNS condition) did not show discriminative US expectancy learning for CS+ and CS- presentations during the acquisition phase. Six participants reported similar expectancy ratings for the last three CS+ and CS- trials and one showed extreme shifts in US expectancies between trials up to the last trial. Because the absence of contingency learning during fear acquisition precludes subsequent extinction learning, these participants were excluded from further analyses.

Of the remaining 31 participants, 18 had been randomized to the tVNS condition (14 female, 4 male, M_{age} = 20.72) and 13 to the sham condition (10 female, 3 male, M_{age} = 22.08). The participants did not differ on the baseline questionnaire scores, as displayed in table 1. Additionally, there was no

significant difference in the unpleasantness rating of the US between conditions (M_{tVNS} = 68.83, M_{Sham} = 66.69), $t(29) = -.24$, $p = .82$. Participants in both conditions scored average on the PSWQ compared to the general Dutch population (range 39-48; [142]). Similarly, the scores on the STAI state and trait scales correspond to average norm scores in both experimental conditions [143].

There was no difference in resting RMSSD between participants in the tVNS and sham condition (M_{tVNS} = 44.31, M_{Sham} = 46.52), $t(26) = -.16$, $p = .88$, indicating that there were no significant differences in cardiac vagal tone between conditions prior to the experimental allocation. There was also no difference in resting HR between participants in the tVNS and sham condition (M_{tVNS} = 76.74, M_{Sham} = 74.61), $t(26) = -.37$, $p = .71$, indicating that there were no significant differences in autonomic nervous system activity.

Table 1. Descriptive statistics.

	tVNS	Sham	
	M (SD)	*M (SD)*	*p*
RMSSD	44.31 (22.57)	46.52 (41.48)	.88
HR	76.74 (15.62)	74.61 (13.23)	.71
Age	20.72 (1.74)	22.08(2.32)	.07
US Unpleasantness Rating	68.83(21.96)	66.69(28.59)	.82
PSWQ	40.06 (8.49)	43.62 (9.97)	.31
STAI State	39.94 (6.46)	38.62 (6.87)	.60
STAI Trait	36.81 (7.41)	37.23 (7.96)	.89
Positive affect	61.44 (12.78)	60.76 (10.76)	.87
Negative affect	26.85 (14.73)	24.26 (13.47)	.60

Note : RMSSD = root mean square of the successive differences between heart rates, HR = Heart Rate, US = Unconditioned Stimulus, PSWQ: Penn State Worry Questionnaire, STAI: State Trait Anxiety Inventory.

tVNS and the extinction of explicit fear

Figure 2A shows the changes in US expectancy ratings as a function of time during the acquisition phase. A multilevel analysis with CS-Type and Trial Number as independent variables showed that participants showed strong overall discriminatory effects in their expectancy ratings towards CS+ and CS- during the acquisition phase, $F(1, 713) = 98.21$, $p < .001$. Additionally, a significant interaction between CS-Type and Trial Number was observed, $F(1, 713) = 139.03$, $p < .001$. That is, the discrepancy between expectancy ratings for CS+ and CS- became more distinct as a function of trial number. Importantly, when Condition was entered into the model, there was no difference in expectancy ratings between the group that received tVNS and the group that received sham stimulation, $F(1, 713) = .82$, $p = .37$.

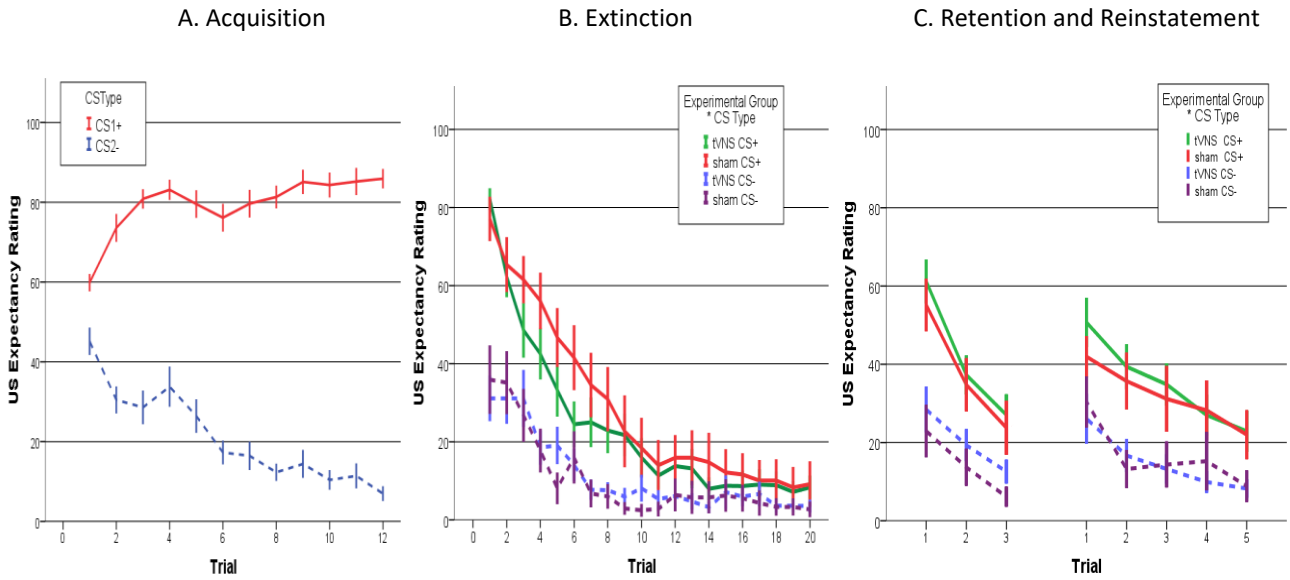


Figure 2. US expectancy ratings of the CS+ and CS- stimuli during acquisition for all participants (panel A). Panels B and C show US expectancy ratings of the CS+ and CS- stimuli during extinction (panel B), retention and reinstatement (panel C) for both experimental conditions. A significantly faster extinction of US expectancies to the CS+ was observed for the tVNS condition (green line), compared to the sham condition (red line) during the extinction phase (panel B). Error bars represent ± 1 Standard Error.

Figure 2B depicts the extinction rates of US expectancy ratings for both CS+ and CS- stimuli in the tVNS condition and the sham condition. Participants in both experimental conditions showed a decline in US expectancy ratings during the first 10 trials, after which a floor effect occurred where expectancy ratings stabilized on a level similar to that of CS- trials. To account for this non-linear learning rate, we added both a linear and a log linear component of time to our model. Participants in both experimental conditions showed a distinct decline in US expectancies for CS+ trials, reflected in a negative linear time component, $F(1, 1209) = 6.87$, $p < .01$, as well as a log linear component, $F(1, 1209) = 35.76$, $p < .001$. Participants who received tVNS showed a faster decline in US expectancy ratings for CS+ trials compared to participants receiving sham stimulation, which is again reflected in a significant interaction between Condition and Trial Number, $F(1, 1209) = 8.48$, $p < .01$, as well as the significant interaction between Condition and log-linear transformed Trial Number, $F(1, 1209) = 6.87$, $p < .01$.

Prolonged effects of tVNS on extinction memory

On day 2, we assessed the prolonged effects of tVNS by looking at the retention and reinstatement of extinction memory (see figure 2C). In both the tVNS and the sham condition, there was a strong increase of US expectancy for the first trials of both CS+ and CS- compared to the final trials of the extinction trials the day before (for the tVNS condition: ΔCS+ = 52.00(25.58), ΔCS- = 20.89(22.19), for the sham condition: ΔCS+ = 46.00 (20.98), ΔCS- = 20.23(22.41)). Both conditions, however, displayed a rapid decrease in US expectancy over time, $F(1, 170) = 5.76$, $p < .05$. There was a marked difference in US expectancy between CS types, $F(1, 170)$ 12.84, $p < .001$, but there was no significant interaction between CS type and trial number, $F(1, 170) = 2.187$, $p = .14$, indicating a parallel decrease in US expectancy irrespective of CS type. Similarly, there was no overall effect of tVNS on US expectancy, $F(1, 199) = .221$, $p = .64$, nor was there an interaction between experimental condition, CS type and time, $F(1, 170) = .09$, $p = .77$.

Compared to the final trial of the retention procedure, the first trial of the reinstatement procedure showed a strong return of US expectancy, irrespective of experimental condition (for the tVNS condition: ΔCS+ = 18.15(22.81), ΔCS- = 24.23(23.88), for the sham condition: ΔCS+ = 24.17 (39.66), ΔCS- = 11.72 (26.96)). Again, participants showed a subsequent decline in overall US expectancy ratings as a function of Trial Number, $F(1, 300) = 7.318$, $p < .001$, regardless of an interaction with CS type, $F(1, 300) = .08$, $p = .77$ or experimental condition, $F(1, 300) = .50$, $p = .48$. Overall, participants reported higher US expectancies to the CS+ compared to the CS-, $F(1, 300) = 5.75$, $p < .05$. There was no significant main effect of experimental condition on US expectancy ratings during the retention procedure, $F(1, 300) = 1.33$, $p = .25$, nor was there an interaction between trial number, CS type and experimental condition, $F(1, 300) = .49$, $p = .49$.

Physiological Outcomes

There was a significant negative effect of Trial Block on startle responses during the acquisition phase, $F(1, 983) = 128.71$, $p < .001$, indicating a strong habituation to the startle probe. Participants did not show discriminative fear conditioning to the CSs, $F(1,980) = .02$, $p = .98$.

Similarly, there was no significant differentiation in phasic HR responses to the CSs during acquisition, as indicated by a non-significant main effect of CS type, $F(1,641) = .23$, $p = .63$, and a non-significant interaction between CS Type and Trial Block, $F(1,641) = .64$, $p = .42$.

Since the acquisition of conditioned fear responses is a necessary condition for extinction of fear responses to take place, we could not assess whether tVNS affects the extinction of physiological fear responses and their retention the next day.

Side-effects

Out of the 31 participants who displayed acquisition of fear, 23 have filled in the side effects questionnaire (n_{sham} = 10, $_{ntVNS}$ = 13). There was no significant difference in the intensity of side-effects reported by experimental groups, $Mean_{tVNS}$ = 1.74 (.34), $Mean_{Sham}$ = 1.46(.52), t(21) = -1.56, p = .14. There was also no significant difference in the number of side-effects reported by experimental groups, $Mean_{tVNS}$ = 2.77 (1.01), $Mean_{sham}$ = 1.90 (2.08) , t(21) = -1.32, p = .20.

Discussion

In this study we present the first preliminary evidence for the facilitating effects of non-invasive vagal nerve stimulation in the formation of explicit extinction memory in humans. Compared to sham, tVNS improved extinction learning, reflected in an accelerated decrease in US expectancy ratings in response to repeated presentations of the CS+. However, we were not able to test whether tVNS also facilitates the extinction of the physiological fear responses (startle as well as cardiac responses), as acquisition of the physiological fear responses was unsuccessful. Furthermore, US expectancy ratings were only affected by tVNS during the stimulation period, and an equally strong return of fear was observed for both conditions on the next day.

The improved associative explicit extinction learning is in line with prior animal studies showing accelerated extinction of fear in rats [50,51,115]Increases in norepinephrine in the PFC and limbic areas such as the amygdala and hippocampus could be a possible working mechanism for the memory enhancing effects of VNS [50,115]. Previous research has demonstrated that norepinephrine levels are critically involved in the formation and consolidation of new memory, possibly by altering the excitability and synaptic plasticity of target neurons in the aforementioned brain areas (for a review, see [113]). Increased norepinephrine levels have indeed been found repeatedly as a result of VNS in animal literature (e.g. [95,144,145]. Yet, evidence for this pathway in humans is still restricted to fMRI studies showing increased activity in the locus coeruleus after tVNS [122].

One could wonder whether US expectancy ratings are a good representation of actual fear responses, especially in the absence of clear physiological reactions to the CS+. US expectancy is a good representation of anxious behavior in terms of face validity, diagnostic validity and construct validity [146]. Specifically, US expectancies represent heightened danger expectancy, which is an important symptom of fear and anxiety (e.g. [106,147,148]). As such, increased US expectancies are an important aspect of fear and anxiety disorders, and an accelerated decrease in US expectancy ratings as a result of tVNS signifies an accelerated extinction of fear, even in the absence of physiological outcomes.

The lack of physiological responding was unexpected. Despite the high unpleasantness ratings given by participants at the end of the acquisition phase, participants displayed a strong habituation

in their startle blink responses. It is possible that physiological habituation to the US has interfered with physiological conditioning to the CS+. This strong physiological habituation may have been due to the use of a scream as a US used in the current study. The scream is an ecologically and evolutionarily valid threat cue with a high survival relevance. However, previous research has also indicated that participants show less fear potentiated startle responses on human screams when compared to shock US [149]. Indeed, in a recent conditioning study by Guhn et al. [150] where a scream was used as US, close to half of the included participants were excluded from the analyses because of failed physiological conditioning. Possibly, this is due to the fact that in contrast to a shock US, a scream is a social stressor that does not pose an immediate threat to oneself and might even divert attention away from the task at hand by enhancing environmental monitoring [151].

In this study, tVNS was not associated with improved retention rates of extinction learning, indicating that although extinction memory encoding was accelerated, subsequent extinction memory consolidation was not affected by tVNS. This is likely due to the timing of tVNS used in this experiment: recent fMRI findings indicate that the activation patterns found in limbic and prefrontal brain areas revert back to baseline levels within minutes after discontinuing tVNS [122]. In our study, tVNS was applied only during extinction memory encoding, and was discontinued afterwards during memory consolidation. Animal studies that applied VNS subsequent to memory encoding (i.e. during the consolidation phase) show contrasting findings. Whereas post-training VNS has been found to affect both emotional and non-emotional memory encoding [45,116], a more recent study by Peña and colleagues [51] showed that rats receiving VNS after extinction training did not outperform rats receiving sham stimulation. Interestingly, Peña and co\lleagues [50,51] did find strong effects of VNS on memory consolidation when stimulation patterns were exactly aligned to the presentation of CSs, although these effects have not been contrasted to a less precise stimulation alignment, such as the one used in the current study. Clearly, more research needs to be done on how changes in timing and patterns of VNS affect its effect on memory and retrieval.

An alternative interpretation of the results from this study that cannot be ruled out in this experiment is that the sensation of the tVNS device served as a safety cue during the extinction phase. This could also explain the strong return of fear during the second testing day, when participants did not wear the tVNS device. In the current study, we cannot rule out that the accelerated extinction rates in the tVNS group occurred partly because of differences in sensory side-effects between experimental groups. However, given the small, non-significant differences in sensory side effects between experimental groups, it seems unlikely that these differences between groups occurred solely because of differences in potency of these safety signals. Clearly, the interpretations of these results would be facilitated if there would have been a reliable measure to assess whether the vagus nerve was truly affected by tVNS. Unfortunately, no reliable, non-invasive manipulation check of tVNS currently exists.

In the current study, participants received tVNS or sham stimulation in between the acquisition and the extinction phase. The administration of tVNS prior to the start of the extinction phase may have enhanced the consolidation of fear memories, possibly leading us to underestimate the effects of tVNS on fear extinction. However, previous trials on vagosectomized rats indicated that vagal nerve activity affect extinction learning while having no effect on initial fear learning [48]. Thus, it seems unlikely that tVNS has enhanced the consolidation of fear memories in this trial. Future studies are warranted that more specifically examine these specific aspects of tVNS.

A limitation of the current study is the small sample size, which increases the Type II error rate of the statistical tests that were used (due to lack of statistical power). Additionally, in the current study we did not measure possible demand characteristics of the tVNS and control conditions. Thus, although participants were blinded to their experimental allocation, we cannot entirely rule out that participants in the control condition had different treatment expectancies than participants in the tVNS condition, even though there were only small, non-significant differences in reported side effects or sensations due to the stimulation. In addition, only one current level of stimulation (0.5mA) was used, even though a more tailored approach could potentially lead to better outcomes in learning. Although previous VNS studies in rats and humans have found an intensity-dependent memory performance effect that follows an inverted U-shape function, with stimulation intensities around 0.5mA producing the strongest memory-enhancing effects (for an overview, see[85]), it is important to note that these studies have all been performed using invasive VNS. Studies have yet to find whether tVNS follows the same inverted U-shape function or whether other stimulation intensities would have stronger effects. Finally, the current study has also focused solely on the effects of tVNS on the extinction of cue conditioning. In their most recent animal study on tVNS and extinction, Peña et al. [50] reported that rats showed reduced freezing behavior outside of the CS presentations (ie, in the intertrial intervals), which could indicate that tVNS facilitates the generalization of extinction learning to the context. It would therefore also be pivotal to examine whether tVNS affects the extinction of context conditioned fear responses.

Notwithstanding these limitations, we here present the first preliminary evidence for the direct memory facilitating effects of tVNS in explicit fear extinction learning in humans. To further address the specific role of the vagus nerve in fear-related learning and memory, and to be able to evaluate the possible therapeutic value of tVNS, future studies are clearly warranted.

Chapter 3

Mixed evidence for the potential of non-invasive transcutaneous vagal nerve stimulation to improve the extinction and retention of fear

Burger AM, Verkuil B, Fenlon H, Thijs L, Cools L, Miller HC, Vervliet B, Van Diest I (2017). *Behaviour, Research and Therapy, 97*, 64–74. doi:10.1016/j.brat.2017.07.005.

Abstract

Extinction memories are fragile and their formation has been proposed to partially rely on vagus nerve activity. We tested whether stimulating the auricular branch of the vagus (transcutaneous VNS; tVNS) accelerates extinction and reduces spontaneous recovery of fear. Forty-two healthy students participated in a 3-day fear conditioning study, where we tested fear acquisition (day 1), fear extinction (day 2) and the retention of the extinction memory (day 3). During extinction, participants were randomly allocated to receive tVNS or sham stimulation concurrently with each CS presentation. During the acquisition and retention phases, all participants received sham stimulation. Indexes of fear included US-expectancy, startle blink EMG and skin conductance responses. Results showed successful acquisition and extinction of fear in all measures. tVNS facilitated the extinction of declarative fear (US expectancy ratings), but did not promote a stronger retention of the declarative extinction memory. No clear effects of tVNS on extinction and retention of extinction were found for the psychophysiological indexes. The present findings provide tentative indications that tVNS could be a promising tool to improve fear extinction and call for larger scale studies to replicate these effects.

Introduction

Fear is an evolutionarily adaptive response to actual or potential harm that predisposes the body towards a defensive reaction [152]. The acquisition of fear is strongly dependent on the process of Pavlovian conditioning [57,108,153]: When a neutral stimulus (conditioned stimulus, CS) is contingently paired with an inherently aversive stimulus (unconditioned stimulus, US), the CS will start to elicit a learned or conditioned fear response (CR). This Pavlovian conditioning of fear is most often adaptive as it allows an individual to learn from aversive experiences. However, it can also lead to pathological anxiety. For example, in recent years it has become clear that patients with anxiety disorders and stress-related disorders including post-traumatic stress disorder have difficulties extinguishing the learned fear response (for a recent meta-analysis, see [109]). That is, when the CS is no longer followed by a US, anxiety patients show prolonged fear responses in the absence of clear threat. This finding is in line with studies showing that exposure therapy, the treatment of choice for most anxiety and trauma-related disorders [63,154], is only moderately effective [105]. Understanding the neurobiological mechanisms behind fear and safety learning is therefore crucial in order to improve the treatment of anxiety and trauma-related disorders.

Knowledge about the neurobiological underpinnings of fear learning is accumulating. During situations of imminent threat, the body initiates a fight-flight-response, consisting of a cascade of bodily reactions that allow appropriate responding to the stressor. Of particular importance to fear learning, the appraisal of danger or threat leads to the release of peripheral epinephrine [155], which activates beta-adrenergic receptors on the afferent vagus nerve. When this afferent information reaches the nucleus of the solitary tract, noradrenergic projection neurons in the locus coeruleus (LC) are activated and release norepinephrine (NE) in several cortical and subcortical brain regions that support memory formation [156]. Due to this increased release of NE, fear memories are more strongly consolidated and subsequently more easily remembered than neutral memories [157].

Meta-analyses have indicated that this system of learning new and emotional memories is thwarted during extinction learning [108,109]. Experimental studies have found that the consolidation of extinction memory could be enhanced by utilizing the same mechanism through which a fear memory attains its privileged position in memory storage. For example, promoting NE release in cortical and limbic structures through the use of yohimbine, an alpha2-adrenoreceptor, has the potential to facilitate fear extinction [113]. Unfortunately, yohimbine increases the release of central NE by increasing peripheral adrenal activity. Therefore, the use of yohimbine in patients is not warranted, as it may increase arousal which may have anxiety-provoking effects in anxiety patients [158]. Especially in patients with panic disorder, increased peripheral arousal during exposure therapy

may have iatrogenic effects and strengthen the fear memory instead of establishing an extinction memory.

More recently, stimulation of the vagus nerve (VNS) has been proposed as a non-pharmacological alternative to enhance extinction memory through the increase of noradrenergic transmission [51]. Low levels of vagus nerve activity – as measured by vagally-mediated heart rate variability - have been observed in anxiety patients [70,159]. Furthermore, higher levels of vagus nerve activity have been associated with increased ability for safety learning and inhibition of conditioned fear responses [141,160]. Contrary to yohimbine, VNS is unrelated to peripheral adrenergic activity [115] and has repeatedly been found to have anxiolytic effects (e.g., [91,120,161]). Electrical stimulation of the vagus nerve leads to activation of the noradrenergic projection neurons in the LC, which causes NE to be released in the brain [94,162]. In line with this, several studies have reported on memory-enhancing effects of VNS in animals as well as in humans (for a review, see [85]). Specifically, studies in rats have repeatedly underlined the importance of the vagus nerve on the extinction of fear. For instance, cutting the afferent vagal nerve fibers attenuated extinction learning in rats [48]. By contrast, stimulating the vagus nerve in rats led to enhanced extinction learning [50,51,53], but only when VNS was conducted during and not after the extinction phase [51]. Due to the invasive nature of VNS, research on potential effects of vagus nerve stimulation on fear extinction in humans has been limited.

In the past decade, non-invasive ways of stimulating the vagus nerve in humans have been developed, commercialized and approved for clinical use in epileptic and depressive patients [163]. Evidence indicates that implanted VNS and transcutaneous stimulation of the auricular branch of the vagus nerve stimulate similar brain structures [122]. In line with this, recent studies have documented a range of effects of tVNS in humans, including an enhancement of associative memory and memory of emotional events [86]. Critically, tVNS has been found to promote inhibitory processes, which might be compromised in anxiety patients, such as inhibitory control [89,164] and – at the neural level – the functional connectivity between the right amygdala and the dorsolateral prefrontal cortex [80]. We have previously examined the effects of tVNS on fear extinction and retention. These preliminary findings suggested that tVNS accelerates the formation of declarative extinction memories in healthy humans [165], although we found no evidence for an enhanced consolidation of the extinction memory, as reflected by the lack of significant differences in explicit fear on the retention test 24 hours later. The paradigm that was used failed to elicit differential fear conditioning on psychophysiological indices of fear, and thus we were unable to assess potential effects tVNS may have on psychophysiological fear responses.

The present study therefore aimed to further investigate effects of tVNS during extinction training in healthy humans with another type of paradigm. First, to ensure fear learning, the present

study used an electrocutaneous stimulus as US, as opposed to the auditory US used in our previous study. Second, acquisition, extinction, and retention of extinction were tested on three separate days, ensuring sufficient time for both the acquisition and extinction memories to consolidate. Furthermore, in contrast to Burger and colleagues [165], we now specifically paired the extinction learning trials with tVNS, which yielded the strongest effects in the animal studies by Pena et al [51]. Our main hypotheses were that tVNS would accelerate the extinction of both declarative and psychophysiological fear responses. Additionally, we hypothesized that tVNS would increase the consolidation of extinction memories, contrary to what was found in our previous study [165] but in line with animal studies on the effects of VNS on fear extinction [51].

Methods

Participants

Forty-two healthy students from the University of Leuven (16 men and 26 women; age range: 20 – 36 years) participated in the experiment.[2] In return they received a financial compensation of 70 euros and a one in three chance to win a cinema ticket after completion of the entire experiment. Participants between the ages of 18 and 50 could participate in this study. Exclusion criteria included self-reported current or past psychiatric, cardiac or neurological disorders, use of psychopharmacology or any medication that affects autonomic nervous functioning (e.g., beta-blockers) and pregnancy.
The study was approved by the Medical Ethical Committee of the University of Leuven. Additionally, this study has been preregistered at ClinicalTrials.gov under NCT02113306.

Experimental Design

The experiment consisted of three sessions, run on separate days: acquisition on day 1, extinction training on day 2, and a test of retention of extinction on day 3[3]. The time in between sessions was 24 hours.

[2] The current study was part of a larger study. Halfway through data collection, a second control group was added that included a context shift during day 2, comparable to the tVNS condition. In contrast to the first control condition, participants in this condition received sham stimulation to their right ear on the second day. However, participants in this second control group reported significantly lower US expectancy ratings to the CS+ during the acquisition phase compared to both the tVNS group and the first control group. For this reason, we concluded that the participants in this second control group were not comparable to the participants who were recruited from the beginning of the study. The data of the second control group is not included in this manuscript but can be requested alongside the data for the other two experimental groups from the first author.

[3] After the retention phase, participants were also subjected to a reacquisition phase and a generalization phase. These phases were added to the experimental paradigm for exploratory reasons and are beyond the scope of the current study. Exploratory analyses of these experimental phases are added as supplementary

In the tVNS condition (*N* = 21, 11 women and 10 men), participants received tVNS stimulation on the concha of the left ear during extinction training (day 2), and sham stimulation on the left ear on day 1 (acquisition) and 3 (retention of extinction). The 'sham' condition (*N* = 21, 15 woman and 6 men) received sham stimulation on the left ear on all 3 days.

Stimuli and materials

Stimuli

Two geometrical figures presented on a computer screen with a black background, served as the CSs: a blue-colored triangle (width: 27.5 cm, height: 20.5 cm) and a yellow-colored circle (width: 22 cm). CS allocation was counterbalanced so that half of the participants received the blue triangle as the CS+ and half received the yellow circle as the CS+.

The order of CS+ and CS- presentations was semi-randomized; the restriction used implied that no more than 3 trials of the same type in a row were allowed. Each CS was presented for 30 seconds, followed by a 40 second inter trial interval (ITI). Stimulation (sham) with the tVNS device occurred concurrently with each CS for 30 seconds.

An electrocutaneous stimulus served as the unconditional stimulus (US). Two electrodes were placed on the inside of the non-dominant leg, right underneath the knee, about 2,5 cm apart. An electrical stimulus generator, producing a bipolar constant current (DS5 Isolated Bipolar Constant Current Stimulator), generated a 500 ms stimulus that was individually tailored with a calibration procedure on day 1 (see procedure section). The mean stimulation given was 6.3 mA (range 2.0 mA - 9.9 mA).

Acoustic startle probes (95 dB, 50 ms with near instantaneous rise time) were presented binaurally through headphones. Two acoustic startle probes were presented during each CS and each ITI. Startle probes occurred at a random time within the following two time windows: 4-8, and 16-23 after CS and ITI onset.

tVNS and sham stimulation

Transcutaneous vagus nerve stimulation was conducted using the NEMOS® stimulator unit (Cerbomed, Erlangen, Germany). Stimulation was programmed to coincide with the presentation of every CS. Active stimulation consisted of 250µs monophasic square wave pulses at 25Hz. During tVNS, the stimulator is fitted on the concha of the left ear, an area of the ear that is 100% innervated by the vagus nerve [25]. During sham stimulation, the tVNS device was fitted on the earlobe of the left ear.

material to this manuscript. All data related to these exploratory analyses can be requested from the first author.

However, the sham position of the electrode did not fit in the ear of 3 participants in the sham group, and so for these participants the electrode was placed on the concha of the left ear and set at an intensity of 0.1 mA to avoid having any effects on the vagus nerve.

Stimulation intensity was set at 0.5 mA, but was lowered if the participant experienced the stimulation to be painful. 7 out of 21 participants in the tVNS condition and 8 out of 21 in the sham condition received an adjusted intensity. Specifically, in the tVNS condition 1 participant received 0.2mA, 3 received 0.3mA and 3 received 0.4mA. One participant in the tVNS condition considered intensities higher than 0.1mA to be painful and was excluded from analyses, as an intensity of 0.1mA has been found to not affect vagus nerve activity [166,167]. In the sham condition, 2 participants received 0.1mA, 1 received 0.3mA and 2 received 0.4mA.

Measurements

US expectancy

A custom-made dial knob allowed participants to continuously rate how much they expected the painful US to occur (US-expectancy) during the experiment. Participants were instructed to continuously indicate their US-expectancy using the dial knob. The dial's scale ranged from 0 (“I am positive that the electric shock is not coming now”) to 100 (“I am positive that the electric shock is coming now”). The analogue output signal was digitized and stored at 10 Hz. US-expectancy ratings were averaged across the 30 s for each CS and each trial.

Skin Conductance Response

The skin conductance response (SCR) was measured using standard Ag/AgCl electrodes (1 cm diameter) filled with K-Y gel lubricant on the palm of the non-dominant hand [168]. The skin on the palm of the non-dominant hand was cleaned with a disposable hypo-allergenic wipe before the start of the procedure. Afterwards, the electrodes were placed 2 cm apart. A constant 0.5 V was maintained by a Coulbourn skin conductance coupler (LabLinc v71-23). This signal was digitized and stored at 100 Hz. SCR were calculated by subtracting the mean skin conductance level (SCL) during 2 s prior to stimulus onset from the maximum SCL during 6 s following CS onset. SCRs with a value below 0.01 µSiemens were set at 0, as such low values are generally accepted to reflect a non-response [169]. Skin conductance responses were log transformed to reduce skewness of the data.

Eye blink startle response

Activity of the *orbicularis oculi* electromyographic activity (EMG) in response to the acoustic startle probe was measured using three Ag/AgCl Sensormedics electrodes (0.25 cm diameter) filled with Microlyte gel. After the skin was cleaned with a disposable hypo-allergenic wipe to reduce any potential inter-electrode resistance, two electrodes were placed just below the left eye, and one electrode was placed at the center of the forehead. A Coulbourn isolated bioamplifier with bandpass filter (Lablinc v75-04; 13 Hz-500Hz) was used to amplify the signal. This was then rectified online and smoothed out using a Coulbourn multifunction integrator (LabLinc v76–23 A) with a time constant of 50 ms. The EMG signal was digitized at 1000 Hz from 500ms before the onset of the auditory startle probe until 1000 ms after probe onset.

Eye blink startle EMG responses were calculated by subtracting the mean baseline (0 to 20 ms after probe onset) from the peak value found in the 21-175ms time window after probe onset. EMG signals with artifacts (e.g., excessive noise from muscular activity prior to the startle probe) were rejected from analysis and defined as missing. The average percentage of rejected responses per participant was 10%. Non-rejected startle responses were averaged per trial and subsequently standardized into T-scores for every individual [131].

Electrocardiogram

The electrocardiogram (ECG) was obtained using three standard Ag/AgCl electrodes (1 cm diameter) filled with electrolyte and placed on the thorax across the heart: two electrodes were placed below the left and right clavicle, one electrode was placed on the left lower rib cage. The signal was sampled at 1000 Hz and transduced, amplified and filtered through a Coulbourn S75-04 Isolated Bioamplifier. Low frequencies were cut off at 10 Hz, high frequencies at 1 kHz.

The signal was visually inspected and artifacts were manually corrected. Interbeat intervals were extracted from the filtered signal, from which HR and the root mean square of the successive differences (RMSSD) between heart rates were calculated.

A seven-minute baseline recording of every participant's RMSSD level was conducted at the start of every testing day to assess participants' vagally-mediated HRV and to check for possible differences in baseline vagal tone.

Self-reports

Prior to the first session, participants were asked to fill in several questionnaires to check for possible differences between the groups in terms of sensitivity to fear and pain prior to having received the experimental manipulation.

The Pain Catastrophizing Scale (PCS) [170,171]was administered to assess how individuals experience pain. The PCS consists of 13 items scored on a 5-point scale.

Anxiety sensitivity, or the fear of anxiety-related bodily sensations, was measured using a Dutch translation of the Anxiety Sensitivity Index – 3 (ASI-3; [172]). The ASI-3 consists of 18 items that are scored on a 5-point scale, with higher scores indicating more anxiety sensitivity.

The Fear of Pain Questionnaire (FPQ-III; [173]) consists of 30 items that are scored on a 5-point scale ranging from “no fear at all” to “extreme fear”.

After each session, the participants had to fill in the Positive And Negative Affect Schedule (PANAS; [174,175]). The PANAS consists of two mood scales, one that measures positive affect (PA scale) and one that measures negative affect (NA scale).

Procedure

The first session started with participants providing informed consent and completing the questionnaires. Following this, participants, seated in front of a computer, were fitted with all electrodes (psychophysiological measures, tVNS, and electrodes for electrocutaneous stimulation). The intensity of the electric shock was individually calibrated in the first session only, to an intensity that was “moderately painful and demanding some effort to tolerate”. Participants were informed that this level of intensity, could be used in all three sessions. Then, the intensity of the tVNS was determined. The starting intensity was at 0.1 mA and stopped until 0.5 mA was reached. If an intensity was considered painful by the participant, the intensity was lowered until it was no longer regarded as painful. On each day, prior to the experimental procedure, participants sat quietly for a 7 min ECG-baseline measurement. Following this, headphones were put on and participants were exposed to twelve acoustic probes in order to habituate them to the probe.

Day 1: Fear Acquisition. Participants received twelve CS+ and twelve CS- trials. The CS+ was reinforced with the US (electrocutaneous stimulus) in 75% of the trials. The US occurred unpredictably in the first 8- 12 second time interval of the CS+ trial, and 67% of these reinforced trials also had a second US in the last 23 - 27 second time interval of the CS+ trial. Thus, participants received a total number of fifteen electrocutaneous stimuli during the acquisition phase. All participants received sham stimulation during this phase.

Day 2: Fear Extinction. The extinction phase consisted of twenty unreinforced presentations of the CS+ and CS-. Participants were randomized to receive either tVNS or sham stimulation during this phase.

Day 3: Retention of Extinction. Participants received six unreinforced presentations of the CS+ and CS-. All participants received sham stimulation during the retention tests.

Statistical analyses

Differences between experimental conditions on the questionnaire and baseline HRV data were analyzed using independent samples *t*-tests.

We used multilevel mixed model analyses to assess whether the conditioning procedure resulted in successful fear learning in our participants in terms of both self-reports and psychophysiological outcomes. When we found significant response differentiation between CS- and CS+ trials on a measurement modality during acquisition, we continued to use multilevel mixed model analyses to analyze the effects of tVNS during the extinction and retention phases.

All multilevel mixed models were created using maximum likelihood modelling. We allowed intercepts to vary randomly across participants, but did not add random slopes to avoid overfitting our models. We did, however, model the heterogeneous AR1 autoregressive covariance structure of trials for within each experimental phase by specifying the nestedness of every trial within CStypes (or ITI, for startle probe responses) within individual participants.

Mixed model analyses are a similar but more flexible approach to data analysis than repeated measures analyses, which seem to have become the standard for fear conditioning research. Specifically, mixed model analyses can incorporate a more detailed clustering of the data and therefore provide a more accurate fit of the covariance structure of the data [176]. Additionally, mixed model analyses do not use list wise deletion when encountering missing data, which means there is no more need to aggregate trials to avoid the risk of missing data.

The independent variable Time, signifying trial number within each session, was group mean centered around the first trial of every phase. Experimental Condition was dummy coded (0 = Sham, 1 = tVNS) and treated as an interval variable. CStype was also dummy coded (0 = CS-, 1 = CS+) and treated as an interval variable when the dependent variable was either US expectancy rating or SCR. When EMG responses were the dependent variable, CStype had three levels: CS-, CS+ and ITIs. For these analyses, CStype was coded so that the CS+ was the reference variable to enable the comparisons between CS+ and CS- and CS+ and ITI.

In all models, the learning curve was fitted using a linear and a loglinear curve to account for non-linear learning rates [165]. Either component was removed when this would result in a higher model fit as indexed by the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC). AIC and BIC are methods of estimating model fit that can both be interpreted using similar methods of model fit approximations, only differing in the extent to which they penalize models with more parameters (generally, BIC favors parsimonious models more strongly than the AIC; [177]). For both AIC and BIC, smaller criterion values indicate a better model fit, and thus the models with the smallest values for AIC and BIC were selected. In some cases, AIC and BIC showed discordant results in model fit preferences when selecting between a model using both time effects or just one. In these

cases, the more parsimonious model was selected to improve interpretability of the main variables of interest.

All analyses concerning the effects of tVNS on extinction and retention learning are reported as one-tailed tests as the hypotheses we tested were directional and based on previous studies [51,165]. Specifically, we aim to test whether tVNS accelerates the extinction of fear memories and strengthens the retention of these extinction memories. Analyses that were not focused on the effects of tVNS but assess the fear conditioning process were tested using two-tailed tests.

Results

Participants

Data of one participant was excluded from analyses because the participant considered tVNS intensities higher than 0.1mA to be painful. Data from two other participants were excluded because they were unable to learn the CS-US contingency – specifically, they had higher US-expectancies for the CS- than for the CS+ in the last two trials of the acquisition phase. Furthermore, SCR data of two participants were missing because an electrode broke. Startle EMG data from four participants were excluded from analyses, because three participants were defined as non-responders (these participants showed startle responses after fewer than 33% of the startle probes), and data from yet one other participant was extremely noisy. As such, the final datasets were n = 39 for US-expectancy (n_{tVNS} = 19, n_{sham} = 20), n = 37 for SCR (n_{tVNS} = 18, n_{sham} = 19), and n = 35 for startle EMG (n_{tVNS} = 18, n_{sham} = 17).

As displayed in table 1, participants in the tVNS and sham condition did not differ significantly on background variables that may affect fear conditioning and fear extinction. Participants' scores on the PCS, ASI-III and FPQ-III were comparable to norm scores from healthy college students or community samples [178–180].

Table 1. Descriptive statistics. Mean scores on baseline variables with standard deviations presented between brackets.

		Sham	tVNS	*p*
Day 1	PCS	18.05(8.13)	18.29(8.71)	.93
	ASI-III	15.20(9.56)	14.57(8.61)	.83
	FPQ-III	59.55(16.77)	66.10(16.77)	.19
	PA	31.93(6.43)	33.06(6.21)	.62
	NA	18.87(5.36)	17.56(4.46)	.47
	RMSSD	34.72(26.41)	45.00(30.36)	.27
	HR	83.29(10.77)	77.47(13.20)	.15
Day 2	PA	31.33(6.60)	32.75(6.50)	.55
	NA	16.93(3.97)	17.06(4.67)	.94
	RMSSD	30.90(21.50)	43.27(28.17)	.14
	HR	81.83(11.77)	78.85(9.67)	.40
Day 3	PA	31.87(5.13)	33.00(4.75)	.63
	NA	17.00(4.75)	16.94(5.08)	.97
	RMSSD	37.42(31.17)	40.05(25.53)	.78
	HR	81.50(12.85)	81.66(12.90)	.97

Note: *N* = 39. PCS = Pain Catastrophizing Scale, ASI-III = Anxiety Sensitivity Index III, FPQ-III = Fear of Pain Questionnaire III, PA = Positive Affect, NA = Negative Affect, RMSSD = Root mean square of successive differences between heart rates, HR = Heart Rate.

Acquisition

US Expectancy

As depicted in figure 1A (left column), participants showed clear signs of differential fear learning on US expectancy ratings during the acquisition phase. Importantly, participants showed a clear differential acquisition of fear, as indicated by a significant interaction between LogTrial*CStype (*b* = 23.06, *t*(92.06) = 10.41, *p* < .001; see also Table 2). There was a significant decrease in US expectancy ratings for CS- trials (LogTrial, *b* = -13.70, *t*(92.06) = -8.75, *p* < .001).

We found no significant main or interaction effects of Condition on US expectancy ratings (all *p*s > .05). Thus, there were no significant between group differences in the declarative acquisition of fear prior to the experimental manipulation.

Startle EMG

A significant main effect of time showed that startle responses decreased overall during the acquisition phase, which is indicative of a general habituation to the startle probe, *b* = -3.85, *t*(276.64) = -4.24, *p* < .001 (see Table 3). Yet, participants' startle responses during the acquisition phase reflected a clear differential fear learning, as depicted in figure 1B. The significant interaction between LogTrial*$CStype_{CS-}$, indeed indicated that there was a stronger decline in startle response for CS- trials compared to CS+ trials, *b* = -4.36, *t*(274.02) = -3.42, *p* = .001.

Similarly to the US expectancy ratings, there were no main or interaction effects of Condition (all *p*s > .05).

SCR

The differential learning curve for SCR is depicted in figure 1C (left column). The model that provided the strongest model fit for SCR did not include either a Trial or a LogTrial effect, indicating that there was no distinct *learning curve* present in the SCR data during the acquisition phase. However, participants did show a significant differentiation in SCR between CS+ and CS- trials, which is indicative of differential fear conditioning (main effect of CStype, $b = .09$, $t(227.90) = 4.80$, $p < .001$; see Table 4).

In accordance with our expectations, we found no significant effects of Condition for SCR on either CS- or CS+ trials during the acquisition phase (both $p > .05$).

Extinction

US Expectancy

As depicted in figure 1A (middle column), participants in both conditions had initially higher US expectancies during CS+ trials than during CS- trials (main effect of CStype, $b = 47.33$, $t(161.82) = 10.27$, $p < .001$; see Table 2), indicating successful retrieval of the fear memory at the start of the extinction phase. Extinction of fear was also successful for all participants: participants showed a stronger decrease in expectancy ratings for CS+ trials than for CS- trials over the course of the extinction phase, CStype*LogTrial, $b = -15.59$, $t(181.22) = -8.54$, $p < .001$.

To test whether tVNS facilitated extinction learning, the CStype*LogTrial*Condition interaction was examined. This interaction was not significant ($p = .94$), indicating that there was no overall difference between the experimental conditions in differential learning rates during the extinction phase. However, the Condition*CStype interaction was significant, $b = -12.60$, $t(181.22) = -1.91$, $p = .03$. The latter interaction indicates that participants in the tVNS condition reported significantly lower differentiation between CS+ and CS- trials during the extinction phase, compared to sham (see Table 2 – an initial difference of 12.60).

As can be observed in Figure 1A, successful extinction had already occurred halfway during the extinction phase, in both conditions. We therefore tested more specifically whether any differences in the learning curves could be observed during these trials during which learning actually took place. To do so, we selected only the first 10 CS+ and CS- trials of the extinction phase and ran the mixed model analyses again. There was no significant CStype*LogTrial*Condition effect ($p = .22$), nor was there a Condition*CStype interaction ($p = .07$). By contrast, if we included only the first CS+ 10 trials, we did

find a significant Condition*Logtrial effect, b = -11.73, t(83.18) = -1.93, p = .03, indicating a faster deceleration of US expectancy ratings for CS+ trials in the tVNS condition.

Startle EMG

As shown in figure 1B (middle column), participants in both conditions had higher startle responses for CS+ trials than for CS- trials at the start of the extinction phase (main effect of $CStype_{CS-}$, b = -9.06, t(441.28) = -4.67, p < .001; see Table 3), indicating successful retrieval of the fear memory acquired during the acquisition phase. Subsequently, there was a faster decline in startle EMG for CS+ trials compared to CS- trials ($CStype_{CS-}$*LogTrial, b = 2.69, t(500.27) = 3.31, p < .001), which indicates a successful fear extinction. This is also clearly visible in figure 1B: at the end of the extinction procedure, both the tVNS and the sham condition no longer show an elevated startle response to the CS+ compared to the CS-.

In contrast to our expectations, there were no differences between the conditions in the differential learning rates during the extinction phase, as indicated by the non-significant $CStype_{CS-}$*LogTrial*Condition (CS+ versus CS- trials) and $CStype_{ITI}$*LogTrial*Condition (CS+ versus ITI trials) interactions (both ps > .05). However, there was a generally accelerated decrease in startle response for both CSs and ITI in the tVNS group compared to the sham group (Condition*LogTrial interaction, b = -2.72, t(495.02) = -3.27, p < .001). As can be seen in figure 1, this accelerated decrease in startle responding is likely driven by the higher startle EMG response displayed by participants in the tVNS condition at the start of the extinction phase. The tVNS indeed started out with higher startle responses, as indicated by the main effect of Condition, b = 7.00, t(441.28) = 3.48, which was in the unexpected direction (p = .99).

SCR

As depicted in figure 1C (middle column), participants' SCR reflected a significant differentiation between CS+ and CS- trials at the start of the extinction phase, indicating a retention of the initial fear memory (main effect of CStype, b = .14, t(251.93) = 2.78, p = .006; see table 4). Subsequently, as indicated by the significant LogTrial * CStype interaction (b = -.05, t(301.44) = -2.39, p = .02), participants showed a stronger decrease in SCR for CS+ trials than for CS- trials over the course of the extinction phase, indicating a successful extinction of fear in both groups.

In contrast to our main hypotheses, there was no main effect of Condition, nor did we find any interaction effects of Condition (all ps > .05), indicating that tVNS did not affect the extinction of fear as reflected by SCR.

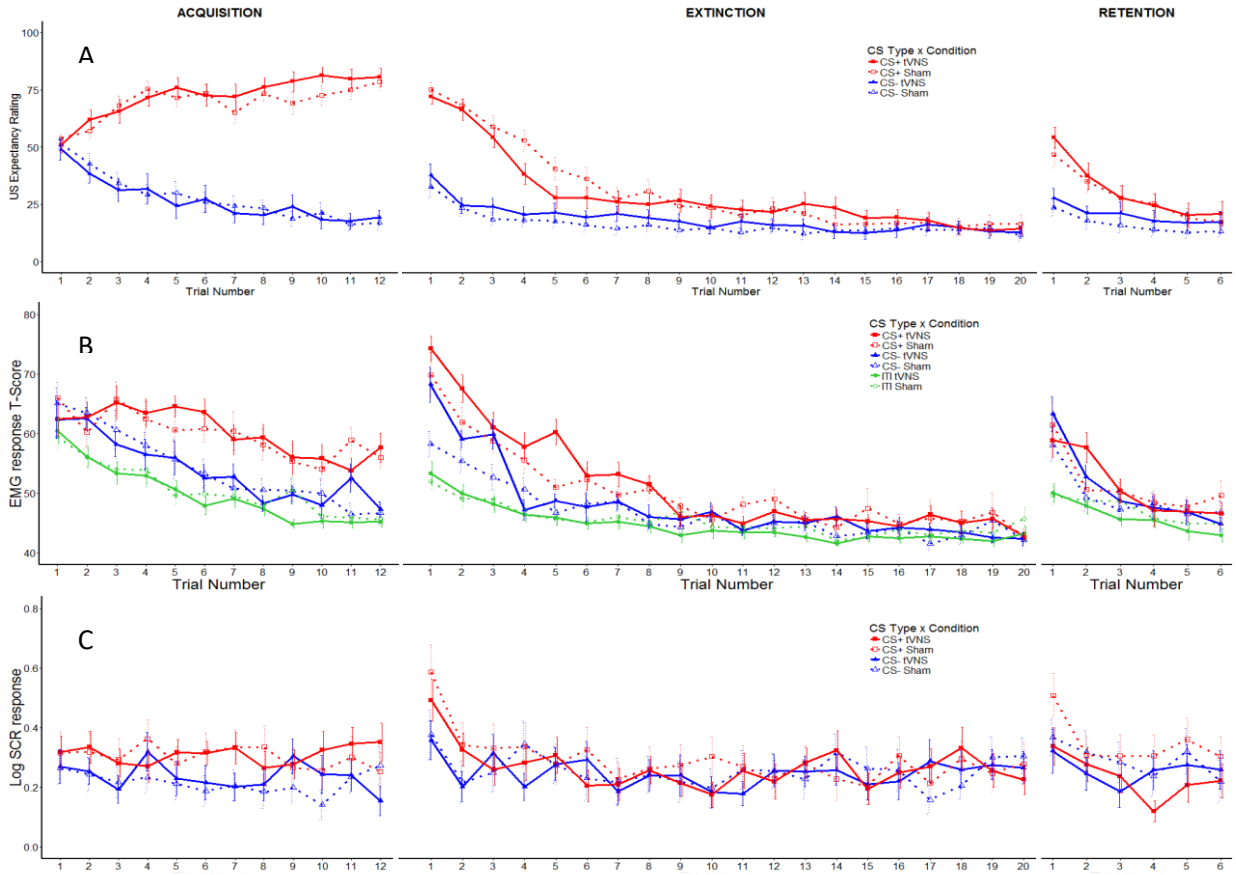


Figure 1. Overview of results for the acquisition (left), extinction (middle) and retention (right) phases of the study. The figure shows mean responses per trial for US expectancy ratings (A), EMG (B) and SCR (C). Error bars indicate ±1 standard error confidence intervals.

Retention

US Expectancy

As shown in figure 1A (right column), participants reported higher US expectancies for CS+ trials than for CS- trials at the start of the retention phase (main effect of CStype, b = 23.97, t(80.75) = 4.30, p < .001; see table 2). Again, there was a clear differential re-extinction curve, where participants had stronger decreases in US expectancy ratings for CS+ trials than for CS- trials as indicated by the CStype*LogTrial interaction, b = -10.84, t(128.42) = -3.29, p = .001. This differential learning indicates that renewed declarative extinction learning took place in both groups during the retention phase.

In contrast to our main hypotheses, Condition did not affect the return of declarative fear, nor did it affect extinction learning rates during the retention phase (all ps > .05).

Startle EMG

As can be seen in figure 1B (right column), participants' EMG responses during CS+ and CS- trials are elevated compared to during ITI at the start of the retention phase. Indeed, although there was no differentiation in EMG responses between CS+ and CS- trials at the start of the retention phase, startle responses during CS+ trials were significantly larger than during ITI ($CStype_{ITI}$, b = -5.54, t(198.02) = -2.28, p = .02; see table 3). The lack of differentiation in startle responses between CS+ and CS- trials could possibly indicate a generalization of the initial fear memory. Over the course of the retention phase, participants displayed a renewed overall decrease in startle responses (LogTrial, b = -5.19, t(240.43) = -4.10, p < .001), possibly reflecting a renewed habituation to the startle probe. There was no renewed differential startle extinction learning ($CStype_{ITI}$*LogTrial and $CStype_{CS-}$*LogTrial both p > .05).

Similarly to the extinction phase, there were no differences between the conditions in the differential learning rates during the retention phase, as indicated by the non-significant $CStype_{CS-}$*LogTrial*Condition and $CStype_{ITI*}$LogTrial*Condition interactions (both ps > .05). However, there was – similar to the startle responses during extinction - an accelerated decrease in startle responding in the tVNS group (Condition*LogTrial interaction, b = -3.34, t(238.06) = -1.82, p = .04), possibly reflecting an accelerated habituation to the startle probe.

SCR

Participants showed a small, non-significant differential return of fear in SCR during the retention test (main effect of CStype, b = .05, t(93.24) = 1.66, p = .10; see table 4). Similarly to the Acquisition phase, the model that provided the strongest model fit for SCR was a model that did not include either a Time or a LogTrial effect, indicating that participants did not show a clear learning curve during the retention phase.

We found no significant differences between the experimental conditions on overall SCR, as indicated by the main effect of Condition (p = .65). There was, however, a significant Condition*CStype interaction, b = -.07, t(93.24) = -1.73, p = .04), indicating that compared to the sham condition, participants in the tVNS condition had lower differential SCRs during the retention test. However, consistent with the absence of a main effect of CStype in the main analysis, when we performed an exploratory mixed model analysis on the effects of LogTrial and CStype for each experimental condition separately, participants in neither condition showed a significant differential fear response as indexed by a main effect of CStype (both p > .05). Thus, although the initial analysis revealed that participants in the tVNS condition showed a smaller differential SCR to the CS+ than participants in the sham condition, this result should be interpreted with caution, as participants in neither group showed a significant differential fear response as reflected by SCR during the retention phase.

Table 2. Regression weights and standard errors for mixed model analyses predicting US expectancy ratings in Acquisition, Extinction and Retention phases.

Predictor	Acquisition	Extinction	Retention
Intercept	50.87(3.29)***	28.01(4.10)***	22.83(5.29)***
CStype	4.14(4.22)	47.33(4.61)***	23.97(5.58)***
LogTrial	-13.70(1.57)***	-5.53(1.29)***	-5.79(2.33)*
LogTrial*CStype	23.06(2.22)***	-15.59(1.83)***	-10.84(3.30)**
Condition	-2.06(4.72)	6.10(5.88)	4.45(7.58)
Condition*LogTrial	.97(2.24)	-1.88(1.85)	-.25(3.30)
Condition*CStype	-2.01(6.04)	-12.60(6.60)*	.48(7.99)
Condition*LogTrial*CStype	2.02(3.17)	4.30(2.62)	-1.51(4.72)

Note. Reference category for CStype is the CS- trial type. All analyses on the effects of tVNS were conducted using one-sided hypothesis tests. *p < .05, **p < .01, ***p < .001.

Table 3. Regression weights and standard errors for mixed model analyses predicting EMG in Acquisition, Extinction and Retention phases.

Predictor	Acquisition	Extinction	Retention
Intercept	66.05(1.89)***	66.19(1.40)***	56.39(1.98)***
$CStype_{CS-}$	1.65(2.48)	-9.06(1.94)***	-2.19(2.44)
$CStype_{ITI}$	-6.06(2.48)*	-16.00(1.93)***	-5.54(2.43)*
LogTrial	-3.85(.90)***	-7.77(.57)***	-5.19(1.27)***
LogTrial*$CStype_{CS-}$	-4.36(1.28)***	2.69(.81)***	.37(1.78)
LogTrial*$CStype_{ITI}$	-1.78(1.28)	5.46(.81)***	1.83(1.78)
Condition	-.44(2.73)	7.00(2.04)^{t}	4.29(2.87)
Condition*$CStype_{CS-}$	-2.41(3.60)	-1.96(2.81)	1.48(3.53)
Condition*$CStype_{ITI}$	1.03(3.60)	-5.75(2.80)^{t}	-4.68(3.53)
Condition*LogTrial	.57(1.31)	-2.72(.83)***	-3.34(1.83)*
Condition*LogTrial*$CStype_{CS-}$	.93(1.85)	.95(1.18)	-.70(2.59)
Condition*LogTrial*$CStype_{ITI}$	-1.35(1.85)	1.76(1.17)	2.59(2.59)

Note. Reference category for CStype is the CS+ trial type. All analyses on the effects of tVNS were conducted using one-sided hypothesis tests. Regression weights denoted by ^{t} reflect variables in the regression model that were significant, but in the non-hypothesized direction. *p < .05, **p < .01, ***p < .001.

Table 4. Regression weights and standard errors for mixed model analyses predicting SCR in Acquisition, Extinction and Retention phases.

Predictor	Acquisition	Extinction	Retention
Intercept	.21(.04)***	.27(.06)***	.29(.05)***
CStype	.09(.02)***	.14(.05)**	.05(.03)
LogTrial	-	-.01(.02)	-
LogTrial*CStype	-	-.05(.02)*	-
Condition	.02(.06)	-.02(.08)	-.03(.07)
Condition*CStype	-.01(.03)	-.05(.07)	-.07(.04)*
Condition*LogTrial	-	-.01(.02)	-
Condition*LogTrial*CStype	-	.02(.02)	-

Note. Reference category for CStype is the CS- trial type. All analyses on the effects of tVNS were conducted using one-sided hypothesis tests. *p < .05, **p < .01, ***p < .001.

Discussion

Non-invasive stimulation of the vagus nerve has been proposed as a promising tool to improve the rate and consolidation of extinction learning [50,51,53,165]. In this study, we found evidence that tVNS accelerated the extinction of declarative fear. This finding is in line with findings from prior animal studies showing accelerated extinction of fear in rats [50,51,53], as well as our previous study where we found that tVNS accelerated declarative fear extinction in humans [165]. However, we found no indication of an enhanced consolidation of declarative extinction memory during the retention test 24 hours later. These results are also in line with findings from our previous study, where we also found no effects of tVNS on declarative fear retention [165]. However, the present study did not find clear effects of tVNS on physiological indices of fear during either the extinction or the retention phase. Taken together, the results of the current study point towards a positive effect of tVNS on the declarative but not the physiological extinction of fear.

The accelerated declarative fear extinction learning found in this study may be caused by the effects of VNS on NE concentration in the PFC as well as limbic areas such as the amygdala and hippocampus [50,115]. Increased NE levels have been found repeatedly as a result of VNS in animal literature (e.g. [95,144]). Similarly, VNS has been associated with increased activity in the LC, the main hub for NE synthesis, in humans [102,122]. NE is involved in the formation and consolidation of new memories by increasing the excitation and synaptic plasticity of the target neurons (for a review, see [113]). A recent study provided additional insights into the molecular mechanisms by which VNS speeds up extinction learning. In this study with rats, VNS promoted plasticity by increasing levels of the protein kinase CMKII and decreasing expression of Arc protein [53]. This amount of change in the level of proteins, induced by stimulating the vagus, was not observed in the sham group, and was only reached in a group of non-stimulated rats that received *extended* extinction training, lasting *five times longer.* This indicates that VNS could indeed speed up extinction learning. From a clinical point of view, an accelerated declarative fear extinction could be very promising, since populations that receive exposure therapy often have difficulties extinguishing fear primarily at the start of the extinction phase due to heightened fear expression – a phenomenon called *fear load* [181,182]. Accelerating extinction learning in this pivotal early phase may have large consequences for the effectiveness of exposure treatments, for example by reducing treatment dropout for patients suffering from PTSD, which occurs frequently during the early stages of treatments [183].

A visual inspection of figure 1A indicated that the effects of tVNS on declarative fear extinction were mainly visible in the first half of the extinction phase. Therefore, we performed exploratory analyses to assess the differential and non-differential fear extinction in this subset of the data. TVNS was only associated with an accelerated declarative extinction curve when non-differential fear

extinction was assessed, that is to say when CS- trials were not included in the statistical model. This discrepancy is unlikely to indicate that the effect of tVNS on CS+ is genuinely non-differential: in line with our hypotheses, figure 1A clearly shows that the US expectancy ratings of participants in the tVNS condition decrease more quickly for CS+ trials and do not show such a pattern for CS- trials. Instead, the difference between these models is likely to reflect a lack of robustness of our statistical models, as a consequence of this study's small sample size and the relatively modest effect that tVNS had on US expectancy ratings. Clearly, although the effects of tVNS on declarative fear extinction seem positive, they call for larger scale studies to replicate these effects.

Despite the accelerated extinction of declarative fear, there was no evidence for an enhanced retrieval of the declarative extinction memory during the retention phase. These results suggest that the encoding or acquisition of the extinction memory was affected by the stimulation, but the subsequent consolidation and retrieval of that memory was not affected by stimulation. In this respect, the findings from the current study differed from the animal studies conducted by Peña and colleagues [51], who repeatedly found effects of tVNS paired with extinction training on the subsequent test day. Although this finding may reflect a true effect where tVNS affects the speed of encoding but not the subsequent consolidation of memories in humans, this contrasting finding may also be due to a characteristic of this experiment itself. Specifically, due to the high number of extinction trials, both groups may have had a chance to create a strongly consolidated extinction memory by the end of the extinction phase. As can be clearly seen in figure 1, participants in both conditions had finished learning the CS-noUS association halfway through the extinction phase, leaving more than half the session to further consolidate the extinction memory. In future studies, researchers could consider including fewer extinction trials to avoid potential ceiling effects in the consolidation of extinction memories. Alternatively, in anxious populations, fear memories are more resistant to extinction and there is a greater risk of a return of fear [108]. Thus, future research may benefit from focusing on populations with subclinical or clinical anxiety to further elucidate the effects of tVNS on the retention of extinction memories.

The current study is the first to report on the effects of tVNS on psychophysiological indices of fear in humans. In contrast to our previous study, participants in this study showed clear differential fear learning during the fear acquisition phase, as indexed by both SCR and startle EMG. However, in contrast to the declarative indices of fear extinction, psychophysiological indices of fear extinction were not affected by tVNS. The accelerated decrease in startle responding during the extinction and retention phases for participants in the tVNS condition likely reflected a general habituation pattern instead of a clear fear extinction curve, since the accelerated curve was not specific to CS+ trials and may have partially been caused by the increased initial startle response during the extinction phase, leaving more 'room for improvement'. Skin conductance responses were not significantly affected

during the extinction phase. Participants in neither condition showed a differential skin conductance response during the retention phase, and thus the small but significant effect of tVNS on differential skin conductance responses is too preliminary to interpret and may be just a chance finding. Clearly, these results are puzzling and call for larger, more highly powered replication studies.

To speculate, the discrepancy in the effects of tVNS on declarative and psychophysiological indices of fear may be in line with the two-factor account of emotional memory proposed by Phelps [184]. In short, this theory proposes that distinct aspects of fear are controlled by at least two independent memory systems: The first is linked to the amygdala and is mainly involved in the processing of the emotional load of the event, whereas the second is linked to the hippocampus and specializes in forming declarative memories of the event [184]. Although these two memory systems often interact with each other, studies in patients with damage to either brain area have revealed that either memory system also operates independently (e.g. [185,186]). Thus, since tVNS mainly affects the declarative extinction of fear, this could possibly indicate that tVNS leads to more prominent changes in activity of the hippocampal complex, and less so in the amygdala. This explanation of increased hippocampal activity after tVNS is strongly in line with animal studies that have shown increased NE activity and increased cellular proliferation in the hippocampus after VNS [95,187,188]. However, recent neuroimaging studies of tVNS in humans stand in contrast to this speculation. These studies suggest that tVNS may lead to a decrease in hippocampal activity while people are resting [122,123]. It is possible that the hypothesized increased activation of the hippocampal complex is only apparent when participants are actively learning and creating new declarative memories. Clearly, more research needs to be done to assess the effects of tVNS on brain activity during emotional learning.

There were several large differences in the experimental designs between this study and our previous study [165]. The current study issued a 24h period between the acquisition and extinction phases to allow for a stronger consolidation of the fear memory. Additionally, the timing of tVNS or sham stimulation was programmed to coincide with the presentation of the CS, similar to previous animal studies [50,51]. Finally, the current study used a painful electrocutaneous shock instead of a loud noise as a US. Despite these differences in experimental designs, the rates of declarative extinction learning in this study and in our previous study are strikingly similar for both the tVNS and the sham stimulation conditions, possibly suggesting that the efficacy of tVNS is not conditional on any of these factors. It also suggests that the timing of the tVNS (unpaired with extinction trials as in [165], or paired as in the current study) might not be of crucial importance. Indeed, animal studies have shown that even short stimulation periods lead to prolonged increases in NE levels [115], and thus tVNS is likely to affect the attentional processing of stimuli regardless of the exact timing of the stimulation.

The current study has several limitations. First, the limited sample size reduces the statistical power of our analyses, thereby increasing the risk of type II errors. Secondly, we decided to decrease the stimulation intensity of the tVNS device if participants felt uncomfortable with the intensity set at 0.5mA. The stimulation intensity was adjusted for 7 out of 19 participants in the tVNS condition. Although this approach towards determining the optimal stimulation intensity has also been used in previous research (e.g. [122]), the decreased stimulation intensities may have also negatively affected the efficacy of the stimulation procedure for these participants. Clearly, too little is currently known about what effects stimulation parameters may have on the efficacy of noninvasive VNS. Parametric studies on basic behavioral outcomes (e.g. associative memory, mood, inhibitory control) are clearly needed. Thirdly, a limitation of tVNS studies in general is that there is currently no reliable, non-invasive measure to assess whether tVNS has successfully increased the activation of the vagus nerve. One possible alternative would be to test the hypothesized working mechanism through which tVNS affects learning and memory. For example, future studies could include assessments of alpha amylase or pupil dilation as indirect measures of NE (e.g. [189,190]), to see whether changes in NE levels predict extinction rates for tVNS.

One final important limitation of this particular study is the potentially confounding role that the context switch has had on extinction learning. One could argue that participants in the tVNS condition received a context switch on the second day due to the stimulator being switched from the 'sham' to the 'active' position (ie. stimulation of the concha instead of the earlobe). By contrast, participants in the sham condition received sham stimulation on all three days. We tried to control for these effects by recruiting an additional control group post-hoc, in which participants received a context switch on the second day by applying sham stimulation to the right ear instead of the left ear. Unfortunately, participants that were recruited for that purpose appeared not to be comparable to the participants in the initial two groups, as reflected in their significantly lower US expectancy ratings during the acquisition phase, prior to the context switch. Because of our inability to compare the tVNS condition to a control group that was also exposed to a context switch, we cannot exclude the possibility that the accelerated extinction in the tVNS condition was confounded by the context switch that occurred on day 2. Although fear memories are generally believed to be context independent [191,192], specific studies on the effects of context switches on the rate of extinction learning (measured using US expectancy ratings) are scarce and less consistent. For example, Effting and Kindt ([193]; experiment 1) reported a marginally faster extinction learning rate in a group that experienced a context switch during extinction, but did not replicate this accelerated extinction learning in a subsequent experiment ([193], experiment 2), although less discrimination between the CSs was observed on the first extinction trial in the context switch groups. In addition,[193,194] reported that a context switch did not facilitate extinction learning when assessed using US expectancy ratings

(facilitation was observed only when assessed using SCRs), although US expectancy ratings to both CS types were reduced after the context switch. Given these preliminary data a careful interpretation of the results in this study is still warranted. More research is clearly needed on the effects of context switches on the rates of extinction learning, which is crucial for a wide range of studies that aim to boost this process. Conversely, one of the major problems facing exposure therapy interventions and their experimental counterparts is the context-dependency of extinction- and safety-memories (for a review, see [195]), indicating that the switch from tVNS back to sham stimulation on day three (retention testing) may have also lead to a stronger return of fear, possibly causing us to underestimate the effects of tVNS on the retention of extinction memories. Future studies may avoid these limitations by applying tVNS or sham stimulation only during extinction, and withholding any stimulation during both the acquisition and retention phases. That way, all participants receive a context switch during extinction, regardless of experimental condition. In fact, this approach was used in our previous study [165], in which effects of tVNS on extinction curves were found that were very similar to the ones reported in the current study. The similarity in results found in this study and in our previous study suggest that it is unlikely that the effects found in the current study are driven solely by an unwanted context switch.

In this study, we found indications that tVNS may positively affect learning and memory in a fear extinction paradigm. These results clearly call for more large scale studies to assess the effects of tVNS on fear-related learning processes, including extinction and retention learning.

Supplementary

Methods

Directly after the retention test, participants were presented with one unreinforced CS- trial and one reinforced CS+ trial. Subsequently, we tested the re-acquisition of fear as a result of the renewed CS-US contingency. We also tested the generalization of the fear memory, by presenting one more reinforced CS+ trial, one unreinforced CS-, three unreinforced generalized stimuli that looked similar to the CS+ (GS+) and three unreinforced trials that were similar to the CS- (GS-), presented in a semi-randomized order.

To test the effects of tVNS on the reacquisition of fear, we will focus on the changes in fear responses between the first and second CS+ and CS- trials. The generalization of fear will be tested in a separate analysis using only the three GS+ and GS- trials.

Results

Reacquisition of fear

US expectancy ratings

Participants' US expectancy ratings for the CS+ were significantly higher than for the CS- at the start of the reacquisition phase, even before the CS+ had been reinforced once again (main effect of CStype, b = 14.16, t(35.46) = 3.44, p = .002). After the first reinforced CS+ trial, US expectancy ratings for the subsequent CS+ trial increased significantly (CStype*Trial, b = 35.36, t(76.00) = 5.52, p < .001), whereas US expectancy ratings for the CS- trial did not (main effect of Trial, b = 5.58, t(76.00) = 1.23, p = .22).

There were no between-group differences in US expectancy ratings at the start of the reacquisition phase prior to the CS+ being reinforced. Experimental condition also did not affect the reacquisition of declarative fear, as indexed by the non-significant CStype*Trial*Condition interaction (p = .64).

EMG

Participants did not show differential startle responding at the start of the reacquisition phase, as reflected by the non-significant main effects of $CStype_{ITI}$ and $CStype_{CS-}$ (both p > .05). Participants showed a clear reacquisition of differential fear, as reflected by the significant increase in startle responding to the second CS+ trial (main effect of Time, b = 10.07, t(105.16) = 4.40, p < .001). By contrast, startle responses to the CS- did not increase after the first trial ($CStype_{CS-}$*Time, b = -8.37, t(103.59) = -2.60, p < .001), nor did the startle response to the ITI ($CStype_{ITI}$*Time, b = -13.49, t(103.59) = -4.20, p < .001).

Participants in the tVNS condition had a lower startle response during the first reinforced CS+ trial compared to the sham condition, as shown by the main effect of Condition, b = -4.57, t(110.56) = -2.04, p = .02. At this point, participants were unaware of the renewed CS-US contingency, and thus this effect could suggest a difference in the extended retention of fear between conditions. The non-significant Condition*Trial interaction indicates that there are no differences between conditions on fear potentiated startle responses during the second reinforced CS+ trial, b = 4.75, t(104.96) = 1.50, p = .94.

SCR

The model that provided the strongest model fit for SCR was a model that did not include a Time effect, indicating that the renewed CS+ reinforcement did not lead to a clear increase in SCR magnitude. Participants did, however, display a larger SCR for CS+ trials than for CS- trials, b = .12, t(37.00) = .02.

This differential responding was not significantly affected by Condition (both Condition and Condition*CStype, $p > .05$).

Fear Generalization

US expectancy ratings

Participants rated GS+ stimuli as being more likely to be followed by a shock than GS- trials (main effect of CStype, $b = 16.03$, $t(56.45) = 3.07$, $p = .003$). Participants' US expectancy ratings decreased in subsequent trials as reflected by the main effect of Trial, $b = -9.45$, $t(135.86) = -4.25$, $p < .001$, and this decrease in US expectancy ratings was irrespective of GStype (GStype*Trial, $p = .76$).

There were no between-group differences in the generalization of declarative fear, nor in the subsequent extinction rate of the generalized fear response (all $p > .05$).

EMG

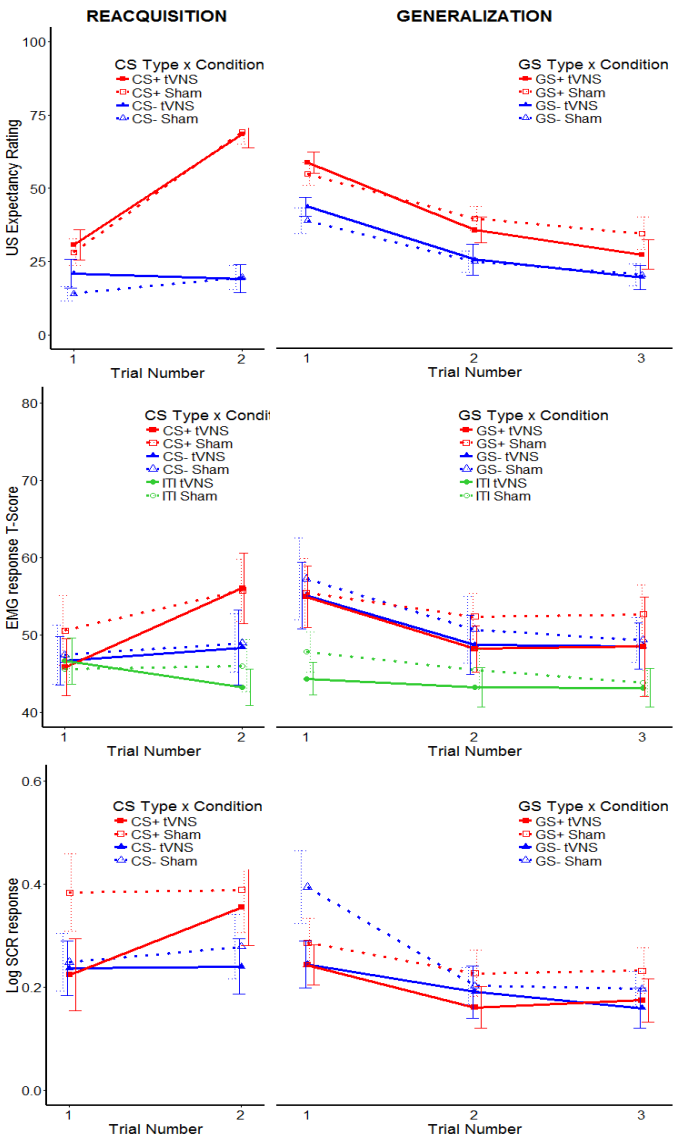
Participants showed differential startle responses to GS+ trials compared to ITIs as reflected by the main effect of $CStype_{ITI}$, $b = -7.11$, $t(122.13) = -3.40$, $p = .001$, but not compared to GS- trials, $p = .52$. Indeed, participants showed overall increased fear responses to both novel stimuli. There was no effect of Time ($p = .22$), and no differential learning curve for GS trials during subsequent trials (both $CStype_{CS-}$*Time and $CStype_{ITI}$*Time, $p > .05$).

Condition did not affect the generalization of the fear potentiated startle response, nor did it affect the extinction rate of startle responses to the generalized stimuli (all ps > .05).

SCR

Participants showed a trend towards increases in SCR magnitude to GS+ trials compared to GS- trials as reflected in the main effect of CStype, $b = .12$, $t(104.22) = 1.84$, $p = .07$. Subsequently, there was a stronger decline in SCR magnitude for GS+ trials as indicated by the significant CStype*Time interaction, $b = -.10$, $t(176.33) = -1.97$, $p = .05$, indicating an extinction of fear for the generalized CS+ trials.

Condition did not affect the generalization of the SCR magnitude, nor did it affect the extinction rate of SCR to the generalized stimuli (all ps > .05).


REACQUISITION
GENERALIZATION
US Expectancy Rating
EMG response T-Score
Log SCR response
Trial Number
CS Type x Condition
GS Type x Condition
CS+ tVNS
CS+ Sham
CS- tVNS
CS- Sham
GS+ tVNS
GS+ Sham
GS- tVNS
GS- Sham
ITI tVNS
ITI Sham

Chapter 4

Transcutaneous vagus nerve stimulation and extinction of prepared fear: A conceptual non-replication.

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Abstract

Transcutaneous stimulation of the auricular branch of the vagus nerve (tVNS) may accelerate fear extinction in healthy humans. Here, we aimed to investigate this hypothesis in healthy young participants in a prepared learning paradigm, using spider pictures as conditioned stimuli. After a fear conditioning phase, participants were randomly allocated to receive tVNS (final *N* = 42) or sham stimulation (final *N* = 43) during an extinction phase. Conditioned fear was assessed using US expectancy ratings, skin conductance and fear potentiated startle responses. After successful fear acquisition, participants in both groups showed a reduction of fear over the course of the extinction phase. There were no between-group differences in extinction rates for physiological indices of fear. Contrary to previous findings, participants in the tVNS condition also did not show accelerated declarative extinction learning. Participants in the tVNS condition did have lower initial US expectancy ratings for the CS- trials than those who received sham stimulation, which may indicate an enhanced processing of safety cues due to tVNS. In conclusion, the expected accelerated extinction due to tVNS was not observed. The results from this study call for more research on the optimal tVNS stimulation intensity settings.

Introduction

Increasing insights into the neurological underpinnings of fear have sparked an interest in neuromodulatory techniques aimed at enhancing fear extinction [110]. Notably, promising extinction-modulating effects have been found for various neurostimulation techniques that specifically target areas of the brain involved in extinction learning [10]. Among these techniques, stimulation of the vagus nerve (VNS) is of particular interest, as preliminary evidence from animal models and human fear conditioning studies point towards treatment-augmenting effects of VNS during exposure therapy [50–53,165,196].

The first studies on the effects of vagus nerve stimulation on fear extinction were performed in rats. In two separate experiments, Peña and colleagues demonstrated that rats who received VNS displayed less freezing after extinction training than rats who had undergone sham surgery [50,51]. Decreased fear responses were also found during fear retention, two weeks after the initial extinction training [51]. These results were later replicated by the same research group, who showed that VNS improved the extinction of fear in rats by increasing the activation of the medial prefrontal cortex – basolateral amygdala pathway [53].

VNS as a neuromodulatory add-on to extinction learning in humans has been an understudied subject up until now, because until recently VNS required surgical implantation of a neurostimulator. Recent studies have indicated that electrical stimulation of the concha of the left outer ear is a safe method to stimulate the auricular branch of the vagus nerve [121]. This transcutaneous VNS (tVNS) has similar effects on brain activation patterns as invasive VNS [122] and increases performance in memory tasks and other cognitive tasks [86,88]. Although the working mechanisms of tVNS are currently still poorly understood [94], invasive VNS is associated with the modulation of several neurotransmitters that could play an integral role in associative learning and memory. Firstly, VNS has been shown to increase levels of gamma-aminobutyric acid (GABA) [197] and is associated with increased GABA receptor density [187] in humans. GABA is the principal inhibitory neurotransmitter in the brain, and is associated with dampening fear learning. Although research on the effects of GABAergic activity on fear extinction is still somewhat limited, preliminary evidence suggests that increased GABAergic signaling would lead to decreased extinction learning and memory consolidation (for a review, see [198]). As such, the effects of invasive and transcutaneous VNS are unlikely to be mediated by GABAergic effects of the stimulation, as this would produce a general slowing in extinction rates, which is opposite of what has been found in previous studies [198]. Instead, a more likely working mechanisms for the effects of tVNS is through its afferent connection to the nucleus tractus solitaries (NTS), which activates the locus coeruleus to secrete norepinephrine (NE) [95–99]. NE is an important determinant of the extent to which salient (eg., threat and safety) memories are first

encoded and subsequently consolidated in long term memory [12,199]. Importantly, the effects of NE on memory have been shown to be associated with activation of peripheral vagal afferents [45,114] and thus provide a physiological basis for the potential effects of VNS on fear extinction in the present study.

The effects of tVNS on fear extinction in humans have been assessed in three previous studies. In the first study (*N* = 31) [165], using a two-day protocol, participants who received tVNS showed accelerated declarative fear extinction learning compared to those who received sham stimulation on day one. No effects on retention of fear memories 24h after extinction training were found. Effects of tVNS on physiological indices of fear could not be assessed due to technical issues and a lack of differential fear conditioning during the fear acquisition phase. A subsequent study (*N* = 39) used a three-day protocol with acquisition, extinction and retention of extinction on day 1, 2 and 3 respectively [196]. Participants who received tVNS again showed accelerated declarative fear extinction and no effects 24h after fear extinction. In this study, no effects of tVNS on physiological indices of fear extinction were found, possibly indicating that tVNS affects fear extinction primarily via hippocampal, declarative pathways. Finally, another study tested the effects of tVNS on contextual fear conditioning in a virtual reality environment (*N* = 75, divided into a sham, tVNS, and no stimulation group) [200]. The study used a three day protocol. Contrary to the cue conditioning studies, no effects of tVNS were found on either declarative or physiological indices of fear, and no effects were found on fear retention. One possible caveat of these studies was the limited sample size, which reduced the statistical power to detect meaningful differences.

In the current study, we aimed to assess the effects of tVNS on both declarative and physiological fear extinction of cue-conditioned fear in a sample large enough to provide us with adequate statistical power to detect meaningful effects. Fear acquisition and extinction phases were conducted on the same day, similarly to one of our previous studies [165].We conducted a randomized single-blinded controlled trial to compare the effects of tVNS and sham stimulation during the extinction of fear. Pictures of spiders were used as CS, as previous studies have indicated that these evolutionarily relevant stimuli lead to more pronounced fear responses and delayed fear extinction [201]. Similarly, other changes to the experimental paradigm were made, including the addition of a background noise and increased startle probe intensity (cf. [202]). High-intensity auditory stimuli are known to increase subjective and physiological arousal [203,204], which in turn strengthens fear conditioning and subsequently slows down fear extinction [205]. These procedural changes were implemented to slow down fear extinction, thus allowing for a stronger differential effect of tVNS compared to sham stimulation. We hypothesized that tVNS would accelerate fear extinction, both on a declarative and a physiological level.

Results

Demographics

Out of the original ninety-seven participants, 1 participant was excluded because she wanted to stop the experiment pre-emptively out of fear for the spider pictures used as conditioned stimuli in the experiment. 4 participants had to be excluded due to mechanical failures with either the computer (n = 1), the shock device (n = 2) or the tVNS device (n = 1). Finally, 7 participants were excluded because they had difficulty understanding the CS-US contingency during the Acquisition phase. Specifically, these participants either did not show higher average US-expectancy ratings for the final two CS+ trials than for the final two CS- trials (n = 4), or they reported US expectancy ratings below 50% for the CS+ trials during the final two trials (n = 3). The analyses described in this article were performed on the data of the remaining 85 participants (N_{tVNS} = 42 (out of which 5 were male), N_{Sham} = 43 (out of which 9 were male), M_{age} = 21.01 (SD = 1.87)).

As displayed in Table 1, there were no significant differences between experimental groups on background variables that may affect fear conditioning and extinction. Although participants in the tVNS group scored higher on the Abbreviated Spider Phobia Questionnaire (A-SPQ, difference not significant), participants in both groups still scored well within the range of a healthy sample [206]. Participants' scores on trait worry (assessed using the Penn State Worry Questionnaire or PSWQ) and trait anxiety (assessed using the State-Trait Anxiety Questionnaire or STAI-Trait) were also comparable to norm scores from healthy college students or community samples [142]. State anxiety (assessed through the STAI-State) were slightly elevated compared to healthy college students or community samples ($M_{healthy\ norm}$ = 35.2, SD = 8.4), but still well below state anxiety scores reported by clinical patient populations ($M_{psychiatry\ patients}$ = 56.4, SD = 13.8) [137]. Since these questionnaires were administered shortly after the acquisition phase, the elevated STAI-State scores in both groups may be a consequence of the fear conditioning procedure. Finally, no between-group differences were found on ratings of positive or negative mood.

No between-group differences were found on resting HR or HRV, which was assessed prior to the acquisition phase. Additionally, no differences were found after the Acquisition phase, when participants were asked to rate the unpleasantness of the US (see table 1).

Table 1. Descriptive statistics.

	tVNS *M*(*SD*)	Sham *M*(*SD*)	*p*
PSWQ	47.07 (8.59)	46.21 (9.98)	.63
STAI state	41.83 (10.21)	41.28 (9.80)	.95
STAI trait	38.44 (7.46)	37.51 (6.35)	.48
A-SPQ	4.44 (3.43)	3.09 (2.78)	.05
Positive mood	57.15 (15.41)	56.92 (19.35)	.95
Negative mood	29.74 (18.25)	27.81 (18.23)	.63
US unpleasantness Rating	65.14 (15.42)	63.54 (13.72)	.61
Resting HRV (RMSSD)	45.61 (23.86)	43.91 (24.70)	.75
Resting HR	75.10 (11.20)	76.64 (13.56)	.57

Note. PSWQ: Penn State Worry Questionnaire, STAI-S: State-Trait Anxiety Questionnaire, A-SPQ: abbreviated Spider Phobia Questionnaire, US: Unconditioned stimulus, HRV: heart rate variability (Root mean square of the successive differences), HR: heart rate. Measurements of resting HR(V) were performed prior to the Acquisition phase. Between-group differences were tested using independent-samples t-tests.

Acquisition

Multilevel mixed model analyses were used to assess fear and extinction learning in our participants in terms of both self-reports and physiological outcomes. For a more detailed description of this statistical procedures, please refer to Statistical Analyses in the Methods section.

Expectancy Ratings

Participants showed clear signs of differential fear learning on US expectancy ratings during the acquisition phase, as reflected by the LogTrial*CStype interaction, $b = 30.80$, $t(1269) = 11.74$, $p < .001$ (see table 2). Participants successfully learned that the CS- was safe, as reflected in the significant decrease in US expectancy ratings, $b = 19.55$, $t(1269) = -10.05$, $p < .001$. The significant main effect of CStype shows that US expectancies for the to-be-conditioned CS+ were already higher from the first trial, $b = 25.06$, $t(1269) = 5.11$, $p < .001$. This apparent 'prior knowledge' of the CS-US contingency can easily be explained by the standardized presentation order of CSs at the start of the acquisition phase: every acquisition phase started with a CS- trial, followed by a non-reinforced CS+ trial. Participants were instructed that one CS trial would never be followed by a shock, and therefore likely deduced that since the first trial was not followed by a shock, the second picture they saw would likely be the CS+. As expected, we found no effects of Condition on US expectancy ratings during acquisition (all *p*s > .05).

Electromyography

Participants' EMG responses reflected successful differential fear conditioning during the acquisition phase, as indicated by the significant differential decrease of CS- trials compared to CS+ trials, $b = -.58$,

t(1853) = -1.96, *p* = .05, as well as the differential decrease of ITIs compared to CS+ trials, *b* = -1.45, *t*(1853) = -4.87, *p* < .001 (see table 3). There were no significant differences in EMG responses between the CS+ and the CS- or the CS+ and the ITI at the start of the acquisition phase (both *p*s > .05). There were no significant between-group differences in EMG during the acquisition phase (all *p*s > .05).

Skin Conductance Responses

Participant's SCR reflected a clear differential learning curve, where SCR habituated over time for both CS+ and CS- trials as reflected by the main effect of LogTime, *b* = -.17, *t*(1224) = -10.21, *p* < .001, but CS+ trials showed a differential increase compared to CS- trials over the course of the acquisition phase, *b* = .08, *t*(1224) = 4.87, *p* < .001 (see table 4). Initial responses to the CS+ were lower than to the CS-, as reflected by the main effect of CStype, *b* = -.08, *t*(1224) = -2.89, *p* =.01. The initial difference between CStypes likely reflects the non-randomized initial order of CS presentations: the first trial of the acquisition phase was always a CS- trial. There were no significant between-group differences in SCR during the acquisition phase (all *ps* > .05).

Extinction

Expectancy Ratings

Participants in both groups showed a clear differential declarative fear response at the start of the extinction phase, as reflected in the significant main effect of CStype, *b* = 29.56, *t*(1949) = 5.36, *p* < .001 (see table 2). US expectancy ratings for both CS types decreased over the course of the extinction phase, *b* = -13.62, *t*(1949) = -8.30, *p* < .001. Expectancy ratings for CS+ trials showed a stronger decline than CS- trials, *b* = -11.61, *t*(1949) = -5.00, *p* < .001, indicating extinction learning.

There were no significant effects of Condition on learning curves for CS+ trials or CS- trials (both *p* > .05). However, there was a significant main effect of Condition on US expectancy ratings, *b* = -9.51, *t*(83) = -1.60, *p* = .05, δ = .36, reflecting lower US expectancy ratings in the tVNS condition. This main effect of Condition should be interpreted with caution, as the regression weights of the non-significant interactions of Condition*LogTrial (*b* = 3.16, *t*(1949) = 1.36, *p* = .91) and Condition*CStype (*b* = 10.09, *t*(1949) = 1.29, *p* = .90) indicate that the significant effect of Condition specifically reflects lower US expectancy ratings in the tVNS condition for CS- trials at the start of the extinction phase (see figure 1).

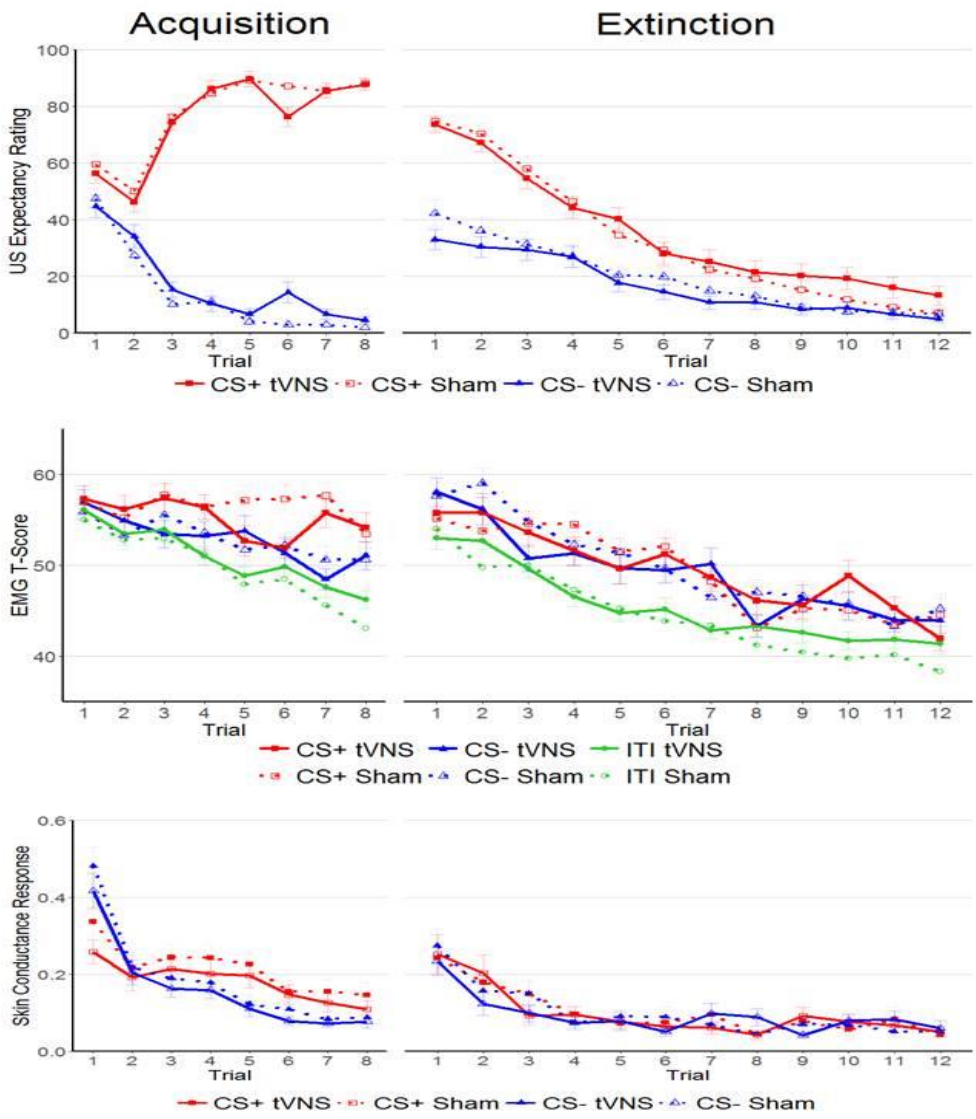


Figure 1. Overview of results for the acquisition (left) and extinction (right) phases of the study. The figure shows mean responses per trial for US expectancy ratings (top), EMG (middle) and SCR (bottom). Error bars indicate ±1 standard error of the mean.

Electromyography

Participants showed strong overall startle responses at the start of the extinction phase, irrespective of CStype, as indicated by the overall intercept, $b = 56.10$, $t(2799) = 70.52$, $p < .001$ (see table 3). They displayed a significant differential fear response to the CS+ compared to the ITI, $b = 4.41$, $t(2799) = 3.92$, $p < .001$. However, they did not show differential responding when comparing the CS+ trial to the CS- trials, $b = 1.12$, $t(2799) = 0.99$, $p = .32$, possibly indicating a generalization of the fear memory. In subsequent trials, we see a significant decrease in startle responses as indicated by the main effect of Trial, $b = -1.24$, $t(2799) = -10.38$, $p < .001$. However, there was no significant differential learning

curve for CS+ trials either in comparison to ITIs or CS- trials (both $p > .05$). Thus, although participants displayed a strong general decrease in fear potentiated startle responses, participants did not show differential extinction learning.

There were no significant effects of Condition on initial EMG or on EMG learning curves over the course of the extinction phase (all $p > .05$, see table 3 for regression weights).

Skin Conductance Responses

Participants did not show significant differential fear responses at the start of the extinction phase (main effect CStype, $p = .67$, see table 4). Specifically, as displayed in figure 1, participants in both conditions had larger SCR at the start of the extinction phase compared to the end of the acquisition phase, irrespective of CStype. Over the course of the extinction phase, SCR decreased significantly, $b = -.01$, $t(1875) = -4.00$, $p < .001$, irrespective of CS type (interaction CStype*Trial, $p = .64$). Although this non-differential reduction in SCR may reflect the extinction of fear, it is difficult to disentangle this effect from a more general habituation response that was also evident during the acquisition phase. There were no significant effects of Condition on initial SCR or on SCR learning curves over the course of the extinction phase (all $p > .05$, see table 4 for regression weights).

Side-effects

Using a short form of seven potential side-effects that we have observed in prior studies, we asked participants to rate their sensations of the neurostimulation at the end of the extinction phase while the stimulation was still active. Although participants in the tVNS condition reported higher side-effect intensity levels on average, it should be noted that average side-effect ratings were relatively low in both groups (overall $M_{tVNS} = 2.20(.65)$, $M_{Sham} = 1.90(.71)$, $t(87) = -2.01$, $p = .05$).

Exploratory Analyses

We conducted additional exploratory analyses to assess possible moderators of the effects of tVNS on US expectancy ratings during the extinction phase. Specifically, the questionnaires that participants had completed in between the acquisition and extinction phases (PSWQ, STAI-S, STAI-T and SPQ), as well as baseline RMSSD, were added to the model described in section 3.2 to see whether they moderate the effects of tVNS on declarative fear extinction. These factors were selected as potential moderators because their underlying constructs (i.e. perseverative cognition, state and trait anxiety, and vagal tone) have been associated with fear and extinction learning in previous studies (e.g. [108,141,153,160]). All factors were added separately to the model, both as continuous and as median-split variables. However, none of these variables improved the overall model fit or resulted in

significant interactions between the moderator and Condition. None of the possible moderators provided main effects for US expectancy ratings, either. Thus, we can conclude that in our current sample, RMSSD nor anxiety at baseline significantly affected the effects of tVNS.

Table 2. Regression weights and standard errors for mixed model analyses predicting US expectancy ratings in Acquisition and Extinction phases.

Predictor	Acquisition	Extinction
Intercept	40.70 (3.67)**	40.62 (4.18)**
CStype	25.06 (4.91)**	29.56 (5.51)**
LogTrial	-19.55 (1.94)**	-13.62 (1.64)**
LogTrial*CStype	30.80 (2.62)**	-11.61 (2.32)**
Condition	-2.32 (5.22)	-9.50 (5.95)*
Condition*CStype	.70 (6.98)	10.08 (7.84)
Condition*LogTrial	2.42 (2.77)	3.16 (2.33)
Condition*LogTrial*CStype	-1.33 (3.73)	- .97 (3.30)

Note. Reference category for CStype is the CS- trial type. All analyses on the effects of tVNS were conducted using one-sided hypothesis tests. *$p < .05$, **$p < .001$.

Table 3. Regression weights and standard errors for mixed model analyses predicting EMG in Acquisition and Extinction phases.

Predictor	Acquisition	Extinction
Intercept	57.03 (.88)**	56.10 (.80)**
$CStype_{CS-}$	-1.76 (1.25)	1.12 (1.12)
$CStype_{ITI}$	-1.51 (1.25)	-4.41 (1.12)
Trial	-.16 (.21)	-1.24 (.12)**
$Trial*CStype_{CS-}$	-1.45 (.30)*	-0.09 (.17)
$Trial*CStype_{ITI}$	-.58 (.30)**	-0.07 (.17)**
Condition	.02 (1.26)	-0.47 (1.13)
$Condition*CStype_{CS-}$	.61 (1.78)	-1.32 (1.60)
$Condition*CStype_{ITI}$	.27 (1.78)	-0.02 (1.59)
Condition*Trial	-.37 (.30)	0.13 (.17)
$Condition*Trial*CStype_{CS-}$	.64 (.42)	0.03 (.24)
$Condition*Trial*CStype_{ITI}$	.18 (.42)	0.13 (.24)

Note. Reference category for CStype is the CS+ trial type. All analyses on the effects of tVNS were conducted using one-sided hypothesis tests. *$p < .05$, **$p < .001$.

Table 4. Regression weights and standard errors for mixed model analyses predicting SCR in Acquisition and Extinction phases.

Predictor	Acquisition	Extinction
Intercept	.41 (.04)**	.13 (.02)**
CStype	-.08 (.05)**	.01 (.02)
Trial[a]	-.17 (.02)**	-.01 (.002)**
Trial[a]*CStype	.08 (.02)**	-.001 (.002)
Condition	-.05 (.05)	-.02 (.03)
Condition*CStype	-.01 (.03)	.01 (.02)
Condition* Trial[a]	.02 (.02)	.004 (.003)
Condition* Trial[a] *CStype	-.002 (.02)	-.002 (.003)

Note. Reference category for CStype is the CS- trial type. All analyses on the effects of tVNS were conducted using one-sided hypothesis tests. *$p < .05$, **$p < .001$.

[a]: trial variable was log transformed in the Acquisition model.

Discussion

We tested the effects of tVNS on fear extinction learning in humans in a single-day fear conditioning procedure. Based on previous research [165,196], we expected accelerated fear extinction after tVNS. The results showed no effect of tVNS on the rate of declarative fear extinction nor on any of the physiological indices of fear. We did find a small effect of tVNS on US expectancy ratings for CS- trials at the start of the extinction phase.

The lack of effects of tVNS on declarative fear extinction learning was unexpected, as this seems to contradict findings from our previous studies [165,196]. There they are in line, however, with a recent study which found no effects of tVNS on contextual fear extinction in a virtual reality environment [200]. The current study was designed to be a more highly powered conceptual replication of our previous studies. However, there were several differences between the paradigm of the current study and the ones used in the previous studies. First, in the current study, we used pictures of spiders instead of geometrical shapes as CSs. Previous studies have indicated that spiders and other evolutionarily relevant threat pictures may lead to stronger acquisition of fear and slower extinction learning [206]. Other changes we made to the paradigm included adding a 70dB background noise and increasing the intensity of the startle probe (104dB instead of 100dB and 95dB). All changes were made to promote a high arousal level in participants, which would lead to a stronger acquisition of fear, and – theoretically - allowing tVNS to make a larger difference.

In line with the expected increased arousal experienced by participants in this study, participants in the sham condition reported higher US expectancy ratings for CS- trials compared to previous studies. Additionally, participants in both groups showed a strong, non-differential increase in SCR and startle responding at the start of the extinction phase compared to the end of the

acquisition phase. These increased non-differential fear responses at the start of the extinction phase clearly reflect the increased apprehensiveness of participants in this study, and may partly explain the discrepancy in the results from this study compared to our previous studies. Considering that the expected working mechanism of tVNS is through the modulation of noradrenergic activity, one possible explanation for the lack of effects of tVNS on fear extinction learning is that the vagus nerve had been activated through adrenergic pathways in both conditions, as a result of the increased arousal experienced by participants in our current conditioning paradigm. Indeed, administration of peripheral adrenaline leads to an increased firing rate of vagus nerve fibers in rats [47]. In turn, administration of peripheral adrenaline prior to the extinction phase has been associated with stronger extinction learning in mice, possibly due to subsequently increased central noradrenergic activity [207]. Clearly, there is a need for more fundamental studies on the working mechanisms of tVNS in humans and its interactions with background levels of arousal, since this could strongly affect the clinical applicability of tVNS.

The lower initial US expectancy rating for CS- trials in the tVNS condition was an unexpected finding, since our previous studies found effects of tVNS on the learning rates of the CS+, not the CS-. This effect of tVNS on CS- ratings at the start of the extinction may simply reflect baseline differences between participants, independent of the experimental manipulation. Alternatively, this effect may reflect an improved ability of participants in the tVNS condition to immediately recognize the CS- as a safety cue. This result would be in line with the Generalized Unsafety Theory of Stress (or GUTS [208,209]), which posits that vagal activity is an important determinant of the maintenance of prefrontal inhibition of the stress response once safety is detected. As such, vagus nerve activation may increase a person's ability to identify and remember that a situation is indeed safe and can prevent a stress response from generalizing from a certain stimulus (e.g. the CS+) to a wider context (e.g. the CS-). Indeed, figure 1 shows a clear increase in US expectancy ratings for CS- trials at the start of the extinction phase compared to the end of the acquisition phase, indicating a generalization of the fear response and an increase in the uncertainty about CS-US contingencies. Even though we did not formally hypothesize this effect to occur based on previous findings, the results found in this study are clearly in line with the GUTS and could point towards an interesting therapeutic effect of tVNS. Further research is clearly warranted to test whether these results can be corroborated.

One could argue that groups may not have been similar on their abbreviated SPQ score, and participants in the tVNS condition reported slightly higher symptoms of spider phobia than the sham condition. However, it's important to note that participants in both conditions scored well within the normal range and should not be classified as spider phobics. As such, we do not believe that differences in spider phobia are likely to explain the lack of effects of tVNS found in this study.

The current study included mainly female participants, which may have possibly limited the generalizability of the findings. Although research on this topic is limited, previous studies in animals [210] and in humans [211,212] have found no consistent differences on vagus nerve morphology between males and females. However, effects of tVNS on LC-NE activity may be different for men and women due to differences in morphology of the LC and CRF1 receptors [213]. Notably, LC dendrites in female compared to male rats are longer and more complex [214], which could lead to a stronger information relay from the NTS (the main terminal of vagal afferents) to the LC [215]. While these intricate differences in LC dendrite morphology have not yet been studied in humans, possible sex differences in the sensitivity of the LC to changes in afferent signaling of the vagus to the NTS clearly warrant additional research. With respect to our current study, we cannot be certain whether the skewed male-to-female participant ratio has affected the results of our analyses. One important argument that we've made before [165], is that not much is known about the optimal stimulation intensity for human auricular tVNS. The stimulation intensity used in this study (0.5mA) is based on invasive VNS studies that found cognitive effects using this stimulation intensity. An important difference between invasive and transcutaneous VNS is that during invasive VNS, the stimulation coil is wrapped directly around the vagus nerve. During tVNS, the stimulation current first has to pass a layer of skin tissue before diffusely reaching the vagus nerve. Thus, the electrical current is impeded by skin, leading to a smaller overall electrical current reaching the vagus nerve and a larger between-participant variability in the amount of electrical current that does reach the nerve, based on inter-individual differences in impedance. These factors may have reduced the effects tVNS may have had on extinction learning. This further highlights the need for more fundamental studies of optimal stimulation intensities but also of biomarkers of afferent vagus nerve activation.

In summary, in this study we found no indications that tVNS accelerated the extinction of conditioned fear. However, participants who received tVNS displayed lower US expectancy ratings to the CS- trials at the start of the extinction phase compared to participants in the sham condition. This effect was not expected beforehand and may reflect a coincidental finding. On the other hand, it is in line with the GUTS model of anxiety, which posits that the vagus nerve plays an integral part in recognizing safety signals in the environment. The results from this study clearly call for more elaborate studies which focus on the ideal tVNS stimulation settings, the comparability of transcutaneous and invasive VNS, and search for possible biomarkers to non-invasively assess vagus nerve activity in humans.

Methods

Participants

We conducted a sample size calculation beforehand to estimate the number of participants required to detect a medium effect size for the main effect of condition in a multilevel analysis. This calculation indicated that given a power of $1 - \beta = .80$, a significance level of $\alpha = .05$, 12 repeated measurements during the extinction phase and a minimum effect size of $\delta = .5$, we needed at least 35 participants in each condition [216].

Eligible participants were healthy college students between the ages of 18 and 25. Participants with spider phobia, epilepsy, bradycardia, cardiac arrhythmia, cardiac diseases, significant head trauma, pregnancy, drug use, neurological or psychiatric disorders were excluded from participating in this study. Participants received either course credits or 10 euro as compensation for participating in the study. The study was approved by the Institutional Ethical Board of Leiden University, Institute of Psychology (CEP #4782302709). The experiment was performed in accordance with relevant guidelines and regulations. All participants gave their written informed consent prior to the start of the experiment.

Stimuli and Materials

Stimuli

CSs were pictures of spiders (IAPS numbers 1200-1201, based on [217]). The slides were 18 cm high and 25 cm wide and were presented on a 17-inch CRT monitor in the middle of the screen on a black background. Both CSs were presented for 8 seconds. During the acquisition phase, one of the CSs was followed by the US in 75% of the trials (CS+), while the other CS was never followed by a US (CS-). To-be conditioned stimuli were assigned as CS+ and CS- in a counterbalanced order. The US occurred 7.5 s after CS+ onset. Intertrial interval durations varied randomly between 15 and 25 seconds. Presentation of CSs was semi-randomized, to ensure that one CS type could not be presented on more than three subsequent trials.

The US was a 20 ms electric shock that was delivered to the wrist of the non-dominant hand. A conductive gel was used between the electrodes and the skin. The shock was delivered using a Grass S48 stimulator. Shock intensity was determined at the start of the experimental procedure. The intensity was individually set at a level that was very uncomfortable, but not painful. Participants received shocks of gradually increasing intensity, starting at 1mA and increasing in 5 mA increments. After every shock, participants were asked to rate what they had felt and whether the shock intensity would have to be increased to reach a level that was 'very uncomfortable, but not painful'. Once participants felt that they had reached a shock intensity that corresponded to this level, the shock intensity was kept stable at this level for the rest of the experiment.

The startle probe consisted of a 50ms, 104dB burst of white noise with near instantaneous rise time that was administered to both ears via headphones. Startle probes were presented 7 seconds after every CS and intertrial interval (ITI) onset. Throughout the acquisition and extinction phases, participants also heard a continuous background noise of 70dB pink noise from their headphones. Both the startle probes and the continuous background noise were created using Audacity 2.0.2 software.

tVNS and sham stimulation

Transcutaneous vagus nerve stimulation (tVNS) is a non-invasive method of electrically stimulating the afferent auricular branch of the vagus nerve located at the cymba conchae [121].

We used a tVNS device that provides electrical stimulation using two titanium electrodes, positioned on top of a silicon earplug, which are connected by a wire to a portable neurostimulator (Nemos®, Cerbomed, Erlangen, Germany). The electrodes deliver 30-second waves of electrical stimulation (0.5mA, 25Hz, 250µs wavelength) to the concha of the left outer ear [25], alternated by 30-second breaks. In the sham condition, the electrodes are connected to the center of the earlobe instead of the concha [25]. The stimulation parameters (current, frequency, on/off cycle) were fixed for all participants. We stimulated the left ear to avoid potential cardiac effects that have been related to efferent vagal fibers of the right ear [119] but not the left [121].

Expectancy Ratings

Participants were asked to rate the extent to which they expected a shock to occur during every CS presentation using a visual analogue scale that ranged from 0 ('not at all') to 100 ('certainly'). Participants were instructed to give these ratings by moving the cursor within 5 seconds after CS onset, after which the scale would disappear from the screen. The scale was presented at the bottom of the screen so as not to draw too much attention away from the stimuli. At the beginning of every new CS presentation, the slide would reappear and the cursor would return to the 'uncertain' middle position (cf. [130]).

Psychophysiological Measures

We measured the potentiation of the eyeblink startle reflex to an acoustic startle probe by using electromyography (EMG) of the left orbicularis oculi muscle. To measure the eyeblink reflex, we used two 4 mm Ag-AgCl Biopac electrodes, one placed below the lower left eyelid in line with the pupil in forward gaze, and the second one placed approximately 1cm lateral to the first (in accordance with the guidelines specified in [131]). EMG was measured using a Biopac system, and filtered by 500Hz low-pass and 10Hz high-pass hardware filters. The EMG signal was grounded by the electrodermal electrodes. The raw response signals were visually checked by the first author in a blinded procedure,

and trials that were affected by movement artifacts or overall poor signal quality were manually removed (0.5% of trials).

EMG responses were calculated by subtracting the mean EMG signal in the 20 ms period directly following the startle probe presentation from the maximum EMG amplitude within the response window between 21-150 ms following startle probe onset [131].

Electrodermal activity was measured using two Ag/AgCl electrodes (Biopac EL507-10). The electrodes were attached to the distal phalanges of the index and middle finger of the nondominant hand [168].

The skin conductance response (SCR) in response to the CS was determined by subtracting the average baseline skin conductance level (2 s before CS onset) from the peak skin conductance level in the first 6 seconds following CS onset. Responses lower than 0.02 micro Siemens were scored as zero and remained in the analyses [218]. SCRs were further log transformed to normalize the data distribution.

Cardiac activity

Heart rate (HR) and heart rate variability (HRV) were derived from the raw ECG signal, which was measured continuously using a two-lead set-up of the Biopac system. The ECG signal was grounded by the electrodermal electrodes. The raw ECG signal was measured at 1000Hz and subsequently filtered using 2Hz low-pass and 50Hz high-pass software filters. The signal was subsequently visually inspected checked by the first author in a blinded procedure and artifacts were manually corrected. Interbeat intervals were extracted from the filtered signal, from which HR and the root mean square of the successive differences (RMSSD) between heart rates were calculated using a custom Matlab script. A five-minute baseline recording of every participant's RMSSD level was used to assess participants' vagally-mediated HRV and to check for possible differences in baseline vagal tone.

Questionnaires

The State Trait Anxiety Inventory (STAI) is a self-report questionnaire consisting of 2 scales with 20 questions each, measuring both state and trait anxiety [137,138]. The STAI has shown high internal consistency and validity [138,139]. The range of both scales of the STAI is between 20 and 80. Norm scores from the general population are 33.16 for the state scale and 36.35 for the trait scale.

The Penn State Worry Questionnaire (PSWQ) is a 16-item self-report questionnaire that assesses the duration and uncontrollability of worry [135]. The PSWQ has demonstrated high reliability, temporal stability and validity in the assessment of trait-worry [135,136]. The range of the PSWQ is between 16 and 80. A PSWQ score of 62 has been validated as a screening tool for generalized anxiety disorders [219].

The Abbreviated Spider Phobia Questionnaire (SPQ) is a self-report questionnaire consisting of 15 yes-or-no questions that assess the subjects fear of spiders [220]. Since pictures of spiders were used as conditioned stimuli, between groups differences in spider phobia severity may affect fear and extinction learning rates. The abbreviated SPQ has shown high internal consistency and strong discriminatory validity [206]. Scores on the abbreviated SPQ range between 0 and 15, with spider phobic participants scoring significantly higher than nonphobics (M_{phobics} = 10.31, $M_{\text{non-phobics}}$ = 2.06) [206].

Participants rated their current mood (happiness, anxiety, irritableness, sadness) on a visual analogue scale ranging from (0) 'not at all' to (100) 'completely'. The scores on these scales were converted into two comprehensive scores, 'positive affect' (score on the happiness subscale) and 'negative affect' (mean score on anxiety, irritableness and sadness subscales). Visual analogue scales are brief and valid measurements of mood state [221].

At the end of the experiment, participants rated potential negative side-effects as a result of the stimulation on a scale of 1 ("applies not at all") to 5 ("completely applies to me") (cf. [165,196]). Side-effects included in the list were headache, pain in the neck, nausea, muscle contractions in the face or neck, prickling sensation under the electrodes, burning sensation under the electrodes and a general feeling of discomfort. Both the number of side effects (scores above 1 were counted as a side effect) and the mean intensity of the side effects were compared between the groups.

Experimental Procedure

At the start of the experimental procedure, the electrodes for EMG, SCR and ECG recordings was attached to the participant's skin. The shock device was then attached to the participant's non-dominant wrist, after which the shock intensity was individually determined.

Participants were told that they would see two pictures, and it was their task to learn to predict which one was often followed by a shock and which one was not. As such, this design included a partial instruction on CS-US contingencies, which leads to a more uniform fear learning compared to a uninstructed fear study, while still leaving enough room for associative learning to take place [222]. Prior to the start of the acquisition phase, a five-minute baseline measurement of every participant's RMSSD level was recorded to assess participants' vagally-mediated HRV, during which time participants watched a muted neutral film clip.

We included a habituation phase prior to the acquisition phase to ensure that participants habituated to the stimuli used in the paradigm prior to differential associative fear learning[222]. Participants were informed that in this phase, they would be introduced to the different stimuli that would be presented in the rest of the task. First, we presented both CS pictures once. Subsequently,

we presented 10 startle probes over a period of 150 seconds to habituate startle blink responses. During this period, participants were also habituated to the background noise, which would stay on for the remainder of the Acquisition and Extinction sessions, although it was temporarily switched off while participants filled in questionnaires and while the tVNS device was attached.During the acquisition phase, both the CS+ and the CS- were presented eight times. The acquisition phase for every participant started with a CS- trial, followed by a CS+ trial. The CS+ was followed by the US in 75% of the trials – specifically, the first and the fifth presentation were always unreinforced (cf. [223,224]). The CS- was never followed by a shock.

At the end of the acquisition phase, participants were asked to rate the unpleasantness of the US on a scale from 0 (not unpleasant at all) to 100 (very unpleasant).

After the acquisition phase, we attached the tVNS device to the ear of the participant and we started either tVNS or sham stimulation. Participants were sequentially assigned to receive either tVNS or sham stimulation to reduce the odds of unbalanced group sizes. Regardless of experimental allocation, participants were told that stimulation was expected to affect physiological processes during the tasks. Participants wore the nerve stimulator throughout the rest of the experimental procedure. Prior fMRI studies have noted a temporal latency in the neurological effects of tVNS [122], which is why we instructed participants to complete a short demographics questionnaire and several other questionnaires with the tVNS device in place and active, before starting the extinction phase.

Completing the questionnaires took roughly 12 minutes. The extinction phase consisted of 12 presentations of both CS+ and CS- trials. Both CS types were unreinforced during the extinction phase. At the end of the extinction phase, participants reported any potential side-effects from the nerve stimulation procedure. Afterwards, the tVNS device was removed from the participant's ear. On average, the experimental session lasted roughly 50 minutes, during which participants received electrical stimulation to their ear for roughly 25 minutes. See figure 2 for an overview of the experimental procedure.

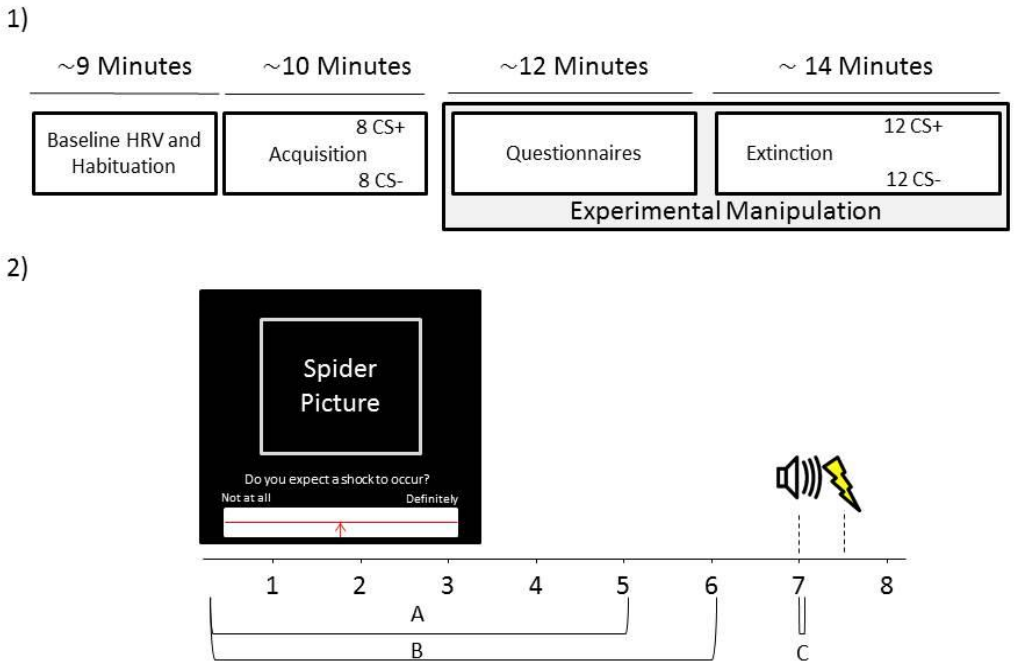


Figure 2. Experimental Overview. 1) The overall experiment lasted approximately 45 minutes and could be broadly subdivided into a baseline phase, an acquisition phase, a phase where participants filled in some questionnaires and finally an extinction phase. Participants received tVNS or sham stimulation only in the last two phases. 2) Every trial lasted 8 seconds in total. Participants were asked to rate to what extent they expected a shock to occur within the first 5 seconds of CS onset (response window A). Maximum skin conductance responses were recorded within the first 6 seconds (response window B) and maximum startle responses were recorded within 21-150ms after startle probe onset (response window C).

Statistical Analyses

Between-group differences on all baseline questionnaires and baseline HRV data were analyzed using independent samples *t*-tests.

Multilevel mixed model analyses were used to assess whether the conditioning procedure resulted in successful fear learning in our participants in terms of both self-reports and physiological outcomes. After we ascertained that participants showed a significant response differentiation between CS- and CS+ trials on an index of fear during acquisition, we continued to use multilevel mixed model analyses to analyze the effects of tVNS during the extinction phase.

All multilevel mixed models were created using maximum likelihood modeling. We allowed intercepts to vary randomly across participants. Adding random slopes did not improve model fit and were thus removed from all models. We modeled the error covariance structure of the repeated

measurements (every trial was nested within CStypes, which were in turn nested within individual participants) by specifying a heterogeneous AR1 autoregressive structure.

The independent variable Trial, signifying trial number within each session, was group mean centered around the first trial of every phase. CStype was dummy-coded, using CS- trials as the reference category for SCR and US expectancy ratings and using CS+ trials as the reference category for EMG, to allow comparisons of CS+ trials with both CS- trials and ITI.

To account for possible non-linear learning rates, we fitted linear and loglinear time curves to all models, as we did previously [165,196], and removed either of these variables if this resulted in better model fit according to BIC estimates.

Cohen's d effect sizes were calculated for significant effects of tVNS using the formula $d = \frac{\mathrm{b}}{\mathrm{pooled\ SD}}$, where b denotes the regression coefficient of the corresponding effect and SD corresponds to the pooled within-group standard deviation [225].

All analyses concerning the effects of tVNS on extinction learning are reported as one-tailed tests to increase our power to detect an effect in the direction we expected. Analyses were conducted using the *nlme* and *lmerTest* packages in *R*.

Additionally, we performed post-hoc Bayesian re-analyses of the effects of tVNS during the extinction phase. The results of these re-analyses support the main analyses and are presented in a supplementary file.

The datasets analyzed during the current study are available on the Open Science Framework, osf.io/p2wfc.

Supplementary

Bayesian re-analysis

In the main manuscript, we tested the effects of tVNS on fear extinction within a null-hypothesis significance testing (NHST) framework. In short, the NHST framework tests the likelihood that one would gather certain data under the assumption that the null hypothesis is true. Significant results allow researchers to reject the null hypothesis, but non-significant results do not allow us to accept the null, as the validity of the null hypothesis is an underlying assumption of the test and not something that is directly tested. Alternatively, Bayesian analyses allow researchers to test the likelihood of either the null hypothesis or the alternative hypothesis, *given the data*. Here, we will re-analyze the effects of tVNS on the extinction of fear using a repeated measures Bayesian analysis in *JASP* (version 0.8.6) [226,227].

In the following sections, we will report the results of the Bayesian re-analyses, focusing on the effects of tVNS on declarative and physiological fear extinction. We will focus on reporting the Bayes Factors, which reflect a ratio of the likelihood that the data fit under the null hypothesis compared to the likelihood that the data fit under the alternative hypothesis. Specifically, we will report the BF_{01}, with higher values reflecting more evidence in support of the null hypothesis.

As these analyses were conducted post-hoc, no changes were made to the default priors given by JASP (*r* scale fixed effects = 0.5, *r* scale random effects = 1, *r* scale covariates = 0.354). These priors constitute a non-informative uniform prior distribution [228].

In all instances, the null model consisted of a model that included the terms CStype, Trial, and Trial*CStype, as well as a random intercept for every subject. This null model was compared with models that included the Condition*CStype*Trial interaction and all lower order interactions and main effects.

Results

<u>Expectancy Ratings</u>

The results of this study strongly support the hypothesis that tVNS did not affect the extinction of declarative fear. When comparing the null model to the 'full' alternative model including Condition and its interactions with CStype and Trial, the Bayes Factor indicated that these data were over 100,000 times more likely to be observed under the null hypothesis (BF_{01} = 122,654, see table 1). Simpler models that did not include higher order interaction terms between Condition and CStype and/or Trial

resulted in smaller BF_{01}, but the data supported no model that contained the Condition term compared to the null model.

Fear Potentiated Startle Responses

The results of this study strongly support the hypothesis that tVNS did not affect the extinction of fear potentiated startle responses. When comparing the null model to the 'full' alternative model including Condition and its interactions with CStype and Trial, the Bayes Factor (BF_{01} = 4.823e^6, see table 1) indicated that these data were over 4,000,000 times more likely to be observed under the null hypothesis. Simpler models that did not include higher order interaction terms between Condition and CStype and/or Trial resulted in smaller BF_{01}, but the data supported no model that contained the Condition term compared to the null model.

Skin Conductance Responses

The results of this study strongly support the hypothesis that tVNS did not affect the extinction of skin conductance responses. When comparing the null model to the 'full' alternative model including Condition and its interactions with CStype and Trial, the Bayes Factor (BF_{01} = 1.624e^6, see table 1) indicated that these data were over 1,000,000 times more likely to be observed under the null hypothesis. Simpler models that did not include higher order interaction terms between Condition and CStype and/or Trial resulted in smaller BF_{01}, but the data supported no model that contained the Condition term compared to the null model.

Discussion

The Bayesian re-analyses provide very strong support for the null model, which corroborates and extends the results from the main analyses. The null model, which posits that tVNS did not affect individuals' ability to learn in this trial, was supported by strong evidence for both physiological and declarative indices of fear.

Chapter 5

The effect of transcutaneous vagus nerve stimulation on fear generalization and subsequent fear extinction

Burger AM, Van Diest I, Van der Does W, Korbee JN, Waziri N, Brosschot JF, Verkuil B. *Currently Under Review.*

Abstract

Fear overgeneralization is thought to be one of the cardinal processes underlying anxiety disorders, and a determinant of the onset, maintenance and recurrence of these disorders. Animal studies have shown that stimulating the vagus nerve (VNS) affects neuronal pathways implicated in pattern separation and completion, suggesting it may reduce the generalization of a fear memory to novel situations. In a one-day study, 58 healthy students were subjected to a fear conditioning, fear generalization, and fear extinction paradigm. Participants were randomly assigned to receive either transcutaneous auricular VNS (tVNS; final *N* = 29) or sham stimulation (final *N* = 29) during the generalization and extinction phases. tVNS did not affect fear generalization, as reflected by US expectancy ratings and fear potentiated startle responses. However, participants who received tVNS reported lower US expectancy ratings to the CS+ during the extinction phase, possibly reflecting a stronger declarative extinction of fear. No effects of tVNS on fear potentiated startle responses during extinction were found. The pattern of findings regarding extinction of declarative fear suggest a facilitating effect of tVNS.

Introduction

Associative learning is fundamental for survival, as it enables us to understand the relationship between stimuli, contexts, or actions, and outcomes. A key aspect of associative learning is the ability to generalize what has been learned to new situations, allowing individuals to discern threatening from non-threatening stimuli or contexts through unconscious inductive processes. The downside of generalized learning appears when individuals overgeneralize fear towards cues or contexts that are typically safe. Indeed, the *over*-generalization of threat towards cues and contexts that are neutral or actually safe has been proposed to be strongly associated with the onset as well as the maintenance of anxiety disorders [108,109,229].

Memory generalization can be conceptualized as a result of interference resolution between novel stimuli or contexts and similar memories. The resolution of this interference is critically dependent on subregions within the hippocampus, where the neuronal pattern of the novel memory trace is compared to the pattern of other memory traces (for reviews on this subject, see [230,231]). In case of a large overlap between these neuronal representations, CA3 neurons in the hippocampus are thought to initiate a process of 'pattern completion', where the representational overlap between the memory patterns is increased, leading to generalization of the previous memory trace [232]. Alternatively, in case of a small overlap between neuronal representations, neurons in the dentate gyrus (DG) and the CA3 of the hippocampus initiate a process of 'pattern separation', inhibiting subsequent memory generalization [232]. The importance of the DG for the inhibition of fear generalization has repeatedly been demonstrated in animal studies. Rats with inactive DG receptors either due to hippocampal lesions or genetic abolition exhibited normal levels of freezing during fear conditioning, but were less able to inhibit freezing responses in similar contexts, indicating that they were unable to separate their neuronal representations of the novel and the previously learned threatening context [233–237]. In line with these findings, high-resolution fMRI in humans showed increased BOLD activity in the CA3/DG during pattern seperation of highly similar cues [238]. Additionally, during a cue fear conditioning study in humans, participants demonstrated stronger BOLD activity in the hippocampus as an inverse function of perceptual similarity between generalized stimuli and the feared stimulus, possibly reflecting stronger pattern separation for stimuli that are more easily discernable from the feared stimulus [239]. Conversely, stronger functional connectivity of the hippocampus with the amygdala and the insula – brain areas that are involved in fear excitation – was found for stimuli that resembled the CS+, confirming a role of the hippocampus in pattern completion [239]. In sum, the hippocampus, and specifically the DG, seems to be an important determinant of fear generalization in both animal and human literature. Interventions aimed at increasing activity in the

DG may affect individuals' ability to discriminate between novel and feared cues or contexts, and could therefore constitute a promising add-on to psychological treatment.

Vagus nerve stimulation (VNS) has recently drawn attention as a potential add-on for exposure therapy. VNS is thought to affect learning and memory by activating the locus coeruleus to secrete norepinephrine (NE) to fear- and learning-related brain areas, including the hippocampus [12]. Studies in rats demonstrated that cutting the afferent fibers of the vagus attenuated cued but not contextual extinction learning [48], whereas invasively stimulating the vagus nerve strengthened the extinction of auditory conditioned fear [50–53]. In humans, transcutaneous, non-invasive stimulation of the auricular branch of the vagus nerve (tVNS) has been found to accelerate declarative fear extinction in a cue conditioning paradigm [165,196], although this effect has not been found consistently [240] and has not been found in a context conditioning paradigm [200]. VNS may potentially also facilitate the inhibition of fear generalization, as secretion of NE is positively correlated to pattern separation in humans [241]. Additionally, animal studies showed that acute VNS increases cell proliferation in the DG of the hippocampus [187,242], while both acute and chronic VNS increased dendrite complexity of neurons in the DG, indicating stronger neuronal plasticity [187]. Finally, one study found that VNS ameliorated the decreases in DG cell count that occurs after the bilateral removal of the olfactory bulbs, which is a validated animal model in depression research [243]. In sum, these studies indicate, at least in animal models, a clear effect of VNS on hippocampal DG activity and morphology, which may indicate that VNS may also strengthen the associated process of pattern seperation and thereby decrease fear generalization.

The effects of tVNS on the generalization of fear have been tested in one controlled study (supplementary in [20]), which found no significant differences between groups on the generalization of fear. However, that study had been primarily designed to assess the effects of tVNS on fear extinction, and the generalization of fear had been tested as a secondary outcome *after* extinction and retention had already taken place. Additionally, as the study was primarily designed to test the effects of tVNS on fear extinction, participants only received tVNS during the extinction phase on day 2, and not during the generalization test 24h later.

In this study, we aim to test the hypothesis that tVNS decreases the generalization of fear. We hypothesize that participants who receive tVNS will show a steeper downwards generalization gradient than participants who receive sham stimulation. Additionally, we aim to perform a conceptual replication of previous studies [165,196] on the effects of tVNS on fear extinction, where we hypothesize that tVNS accelerates the extinction of fear. To test these hypotheses, we conducted a randomized single-blinded controlled trial to compare the effects of tVNS and sham stimulation during the generalization and extinction of fear. Fear was measured on both a declarative level through the use of US expectancy ratings as well as on a physiological level through the use of fear potentiated

startle responses. Startle responses are among the most commonly used physiological indices of fear, because of the critical involvement of the amygdala in the amplitude of the startle expression. Compared to skin conductance responses, they have the added benefit of having high temporal specificity, and return to baseline levels quickly after threat has subsided [244,245]. Participants were subjected to a fear conditioning paradigm based on Lissek and colleagues [239].

Methods

Participants

Sixty-two healthy college students between the ages 18 and 25 participated in the experiment. Exclusion criteria for the study were epilepsy, cardiac diseases, significant head trauma, pregnancy, drug use, or any neurological or psychiatric disorder.

Ethical approval for this study was given by the ethical committee of the Institute of Psychology of Leiden University (CEP#16-1102/339). Participants were rewarded with either 10 euros or course credit.

Procedure

After participants provided informed consent, the electrodes for ECG and EMG measurements were attached. Next, the shock electrode was attached to the forearm of the non-dominant hand and the shock intensity was individually calibrated. Specifically, the shock was individually calibrated to an intensity that was 'highly uncomfortable, but not painful'. Participants received shocks of gradually increasing intensity, starting at 1mA and increasing in 5mA increments. After every shock, participants were asked to rate whether the intensity could be increased, or whether it was already at a level that was 'highly uncomfortable, but not painful'. After participants reported that the shock intensity had reached the desired level, the shock intensity was kept stable for the rest of the experiment.

Participants were told that during the computer tasks they would see different pictures, and it was their task to learn to predict whether they'd receive a shock based on what picture was being presented to them. As such, we gave participants partial instructions on CS-US contingencies. These kinds of partial instructions lead to more uniform fear learning curves compared to uninstructed procedures, while still leaving enough room for associative learning to take place [222].

Prior to the start of the acquisition phase, a five-minute baseline measurement of every participant's RMSSD level was recorded as a measure of vagally-mediated HRV. Afterwards, participants received instructions via the computer monitor about what stimuli would appear on screen during the rest of the experiment and how to rate shock expectancies. Participants were also presented with 10 startle probes over a period of 150 seconds to habituate startle blink responses. During this period, participants were also habituated to background noise (70dB pink noise), which would stay on for the remainder of the Acquisition, Generalization and Extinction phases (cf. [125]).

During the acquisition phase, both the CS+ and the CS- were presented 12 times. The CS+ was followed by the US in 75% of trials, the CS- was never followed by a shock. During the acquisition phase

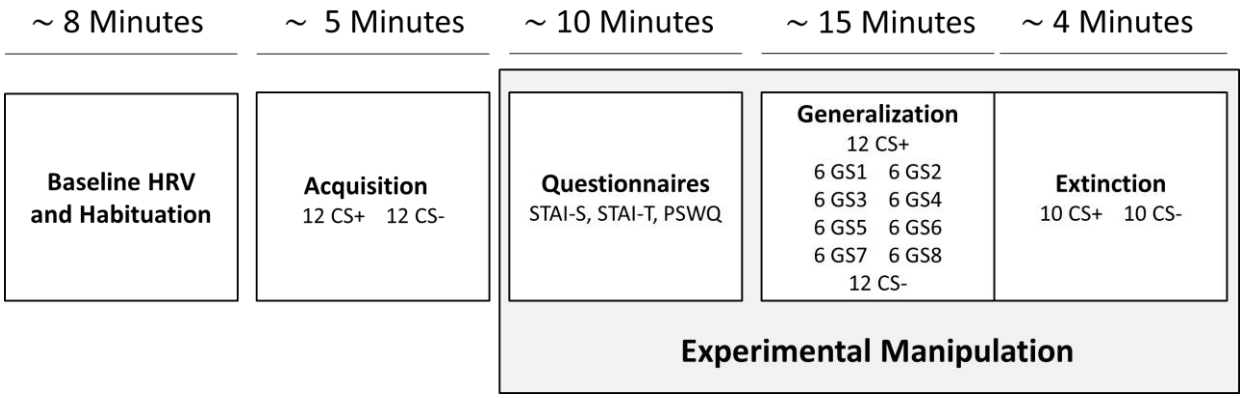
as well as all subsequent phases, trials were presented in a quasi-randomized order, to ensure that one CS type could not be presented on more than 2 subsequent trials.

After the acquisition phase, we attached the tVNS device to the ear of the participant and we started either tVNS or sham stimulation. Participants were randomly assigned to receive either tVNS or sham stimulation. They were told that stimulation was expected to affect physiological processes during the tasks, and wore the nerve stimulator throughout the rest of the experimental procedure. With the tVNS device in place and active, participants completed a short demographics questionnaire and several questionnaires before starting the final task. This period, lasting roughly 10 minutes, was used as a 'ramp up' period for tVNS. Although not much is known about the temporal latency of potential learning effects of tVNS, a recent fMRI study has shown that effects of tVNS on hippompcal activity occur and plateau within roughly 6 minutes after stimulation onset [122]. As such, having a 10 minute period between stimulation onset and the subsequent generalization phase should leave enough time for tVNS to have an effect.

The generalization phase consisted of 12 presentations of the CS+ and CS-, and 6 presentations of every intermediate-sized GS. Thus, participants were presented with 12 CS+, 12 CS-, and 48 intermediate-sized GS circles during the generalization phase. During this phase, the CS+ was still followed by a shock in 50% of trials.

The generalization phase segued into the extinction phase, where the CS+ and CS- were presented another 10 times. No GS were presented during this phase, and the CS+ was no longer followed by a shock. For a graphical overview of the entire experimental procedure, see figure 1.

A)



B)

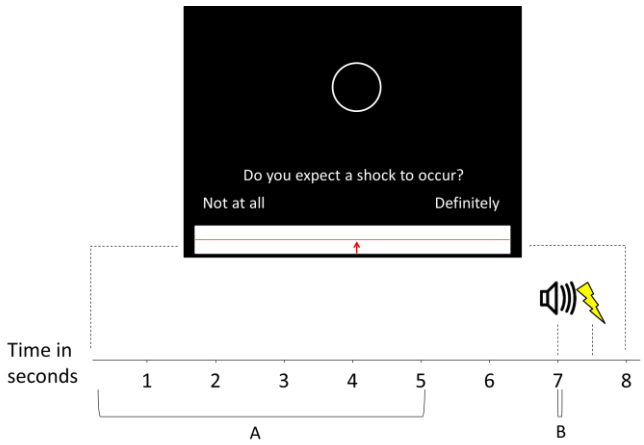


Figure 1. Experimental overview. A) The overall experiment lasted approximately 42 minutes. Participants received active tVNS or sham stimulation during the phases indicated by the grey frame. B) Trials lasted 8 seconds, followed by an intertrial interval lasting between 3.6 and 4.8 seconds. Participants were asked to rate to what extent they expected to receive a shock based on the stimulus that was being presented within the first 5 seconds (response window A). Startle probes presented after 7 seconds, and fear potentiated startle responses were recorded within 21-150ms after startle probe onset (response window B). The US was presented for 20ms, 7.5 seconds after CS onset.

Stimuli and Materials

Conditioned and Unconditioned Stimuli

The fear conditioning procedure was based on the methods used by Lissek and colleagues [239,246]. Specifically, 10 rings of gradually increasing size presented on a computer monitor were used as conditioned stimuli and generalization stimuli. The largest and smallest rings were assigned as CS+ and CS- trials, counterbalanced across participants. The eight intermediately sized rings served as generalization stimuli.

All conditioned and generalization stimuli were presented for 8 seconds, followed by an inter trial interval (ITI) varying in duration between 3.6 and 4.8s. The order of CS and GS presentation was quasi-randomized, allowing no more than 3 trials of the same type to appear in a row.

The unconditioned stimulus (US) was a 100-ms electrocutaneous stimulus delivered to the forearm of the non-dominant hand. The shock was delivered using a Grass S48 stimulator. The US was individually calibrated to an intensity that was 'highly uncomfortable, but not painful'. The US occurred 7.5s after CS+ onset.

Startle probes were presented during every CS and GS, 7s after stimulus onset. Additionally, startle probes were presented halfway through the ITI. Every startle probe consisted of a 40ms loud broadband noise (104dB) with an instantaneous rise and fall time.

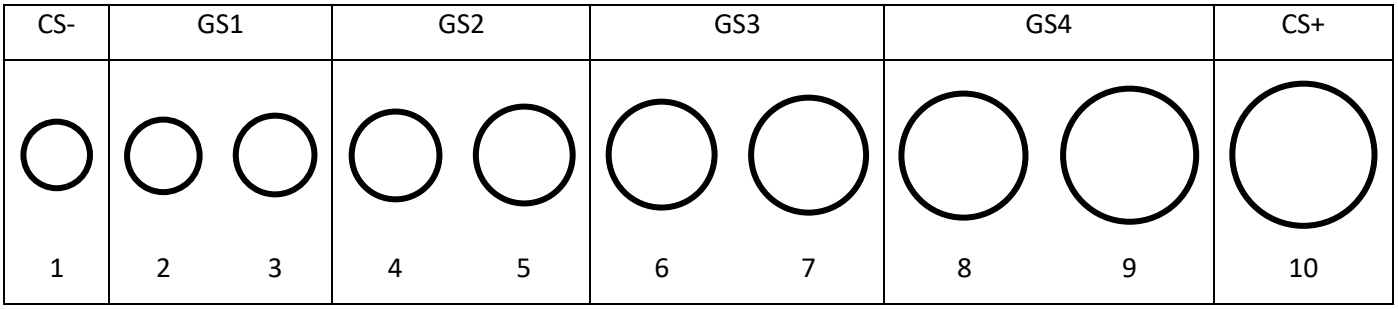


Figure 2. The smallest and largest circle served as CS- and CS+, while 8 circles of intermediate sizes were used as generalization stimuli. CS allocation was counterbalanced so that the smallest circle was the CS- for half of the participants and the CS+ for the other participants. GS categories were created by collapsing the eight intermediate ring sizes into four classes of generalization stimuli (GS1-GS4, GS4 being the category closest to the CS+). The diameter for the smallest ring was 2.1cm, and diameter size increased with 3mm (roughly 15% increase compared to the smallest ring size, cf. [246]) for every subsequent ring up to 4.8cm for the largest ring.

tVNS and Sham Stimulation

The tVNS instrument consisted of two titan electrodes mounted on a gel frame and connected to a wired neurostimulation device (CM02, Cerbomed, Erlangen, Germany). The tVNS® device was programmed to a stimulus intensity of 0.5 mA with a stimulation frequency of 25 Hz and a stimulation wavelength of 250µs. Stimulation was active for 30seconds, followed by a break of 30 seconds. Participants started receiving stimulation after the acquisition phase, and it was continued for the remainder of the experiment.

Depending on their experimental allocation, participants received stimulation of either the cymba concha of the outer ear or the earlobe. The concha of the outer ear is 100% innervated by the

vagus nerve [25]. Participants in the sham condition received stimulation on the earlobe, which is not innervated by the vagus nerve.

Measurements

ECG

Heart rate (HR) and heart rate variability (HRV) were derived from the raw ECG signal, which was measured continuously using a three-lead set-up of the Biopac system. The raw ECG signal was measured at 1000Hz and subsequently filtered using 2Hz low-pass and 50Hz high-pass software filters. The signal was subsequently visually inspected and artifacts were manually corrected. Interbeat intervals were extracted from the filtered signal, from which HR and the root mean square of the successive differences (RMSSD) between heart rates were calculated.

A five-minute baseline recording of every participant's RMSSD level was used to assess participants' vagally-mediated HRV and to check for possible differences in baseline vagal tone.

Electromyography

We measured the potentiation of the eyeblink startle reflex to an acoustic startle probe by using electromyography (EMG) of the left orbicularis oculi muscle. To measure the eyeblink reflex, we used two 4 mm Ag-AgCl Biopac electrodes, one placed below the lower left eyelid in line with the pupil in forward gaze, and the second one placed approximately 1cm lateral to the first (in accordance with the guidelines specified by [131]). EMG was measured using a Biopac system, and filtered by 500Hz low-pass and 10Hz high-pass hardware filters. The EMG signal was grounded by the ECG electrodes.

EMG responses were calculated by subtracting the mean EMG signal in the 20 ms period directly following the startle probe presentation from the maximum EMG amplitude within the response window [131]. The response window was defined as the period between 21-150 ms following startle probe onset.

US Expectancy Ratings

Participants were asked to rate the extent to which they expected a shock to occur during every CS presentation using a visual analogue scale that ranged from 0 ('not at all') to 100 ('certainly'). Participants were instructed to give these ratings quickly since the rating scale would disappear from the screen 5 seconds after CS onset. The scale was presented at the bottom of the screen so as not to draw too much attention away from the stimuli. At the beginning of every new CS presentation, the slide would reappear and the cursor would return to the 'uncertain' middle position (cf. [130]).

Questionnaires

The State Trait Anxiety Inventory (STAI) is a questionnaire containing 2 scales with 20 questions each, measuring both state and trait anxiety [137,138]. The STAI has shown high internal consistency and validity [138,139]. The range of both scales of the STAI is 20 - 80.

The Penn State Worry Questionnaire (PSWQ) is a 16-item self-report questionnaire that assesses the generality and uncontrollability of worry [135]. The PSWQ has demonstrated high reliability, temporal stability and validity in the assessment of trait-worry [135,136]. The range of the PSWQ is 16 - 80.

Statistical Analyses

Between-group differences on all baseline questionnaires and baseline HRV data were analyzed using independent samples *t*-tests.

Multilevel mixed model analyses were used to assess whether the conditioning procedure resulted in successful fear learning in our participants in terms of both self-reports and physiological outcomes. All multilevel mixed models were created using maximum likelihood modeling. We allowed intercepts to vary randomly across participants. Random slopes were fitted to the models but did not improve model fit so were removed from all final models. Additionally, we modeled the nestedness of every trial within Stimulus within individual participants by specifying a heterogeneous AR(1) autoregressive covariance structure of these repeated measurements.

For the analyses of the acquisition and extinction phases, the independent variable Trial, signifying trial number within each session, was group mean centered around the first trial of every phase. The independent variable Stimulus was dummy-coded, using CS- trials as the reference category for US expectancy ratings and using CS+ trials as the reference category for EMG, to allow comparisons of CS+ trials with both CS- trials and ITI.

To account for possible non-linear learning rates, we fitted linear and log transformed (ln) time curves to all models, and removed either of these variables if this resulted in better model fit according to BIC estimates.

For the analyses of the generalization phase, GS categories were created by collapsing the eight intermediate ring sizes into four classes of generalization stimuli (see figure 2, cf. [239]). Trial number was based on the collapsed GS type presentation.

Generalization curve analyses were conducted according to the analysis recommendations detailed by VanBrabant and colleagues [247], analyzing the generalization curve as continuous scale ranging from non-threatening (CS-, 0) to most threatening (CS+, 5), with all intermittent GS types scoring in between (GS1, 1; GS2, 2; GS3, 3; GS4, 4).

All analyses concerning the effects of tVNS on extinction and generalization learning are reported as one-tailed tests to increase our power to detect an effect in the direction we expect. Analyses are conducted using the *nlme* and *lmerTest* packages in R.

Results

Demographics

In total, 61 students participated in this study. Three participants were excluded from all analyses because of unchanging US expectancy ratings throughout the entire experiment. Thus, the final dataset consisted of 29 participants in the tVNS condition (21 female, 8 male, M_{age} = 22.1, SD_{age} = 2.80) and 29 participants in the sham condition (27 female, 2 male, M_{age} = 21.5, SD_{age} = 1.99).

As displayed in table 1, there were no between-group differences on state or trait anxiety as indexed by the STAI or trait worry as indexed by the PSWQ. Mean scores on these questionnaires were similar to the mean scores of our previous study on the effects of tVNS on fear extinction [165]. Additionally, there were no significant baseline differences in heart rate or heart rate variability, as indexed by the natural log of RMSSD.

Table 1. Descriptive statistics. Mean scores on baseline variables with standard deviations presented between brackets.

	tVNS (*n* = 29)	Sham (*n* = 29)	*p*
STAI-S	43.8 (9.9)	45.8 (10.1)	.44
STAI-T	41.0 (9.0)	38.7 (8.6)	.32
PSWQ	46.9 (9.0)	46.0 (8.8)	.70
HR	76.8 (11.8)	79.9 (10.9)	.28
Ln RMSSD	3.73 (.55)	3.51 (.36)	.09

Note: STAI = State-Trait Anxiety Inventory, *PSWQ* = Penn State Worry Questionnaire, HR = Heart Rate, RMSSD = Root Mean Square of Successive Differences between heart rates.

Acquisition phase

US Expectancy

US expectancy ratings of participants in both conditions reflected clear differential fear learning, as reflected by the LogTrial*Stimulus interaction, b = 28.78 (SE = 3.05), t(1328) = 9.44, p < .001. A significant effect of Stimulus indicates that this differential learning already started after the first trial, b = 15.77 (5.92), t(1328) = 2.67, p = .01. US expectancy ratings for CS- trials diminished over the course of the Acquisition phase, as reflected by the main effect of LogTrial, b = -12.96 (2.16), t(1328) = -6.01, p < .001.

There were no differences between the tVNS and Sham condition in US expectancy ratings during the Acquisition phase (all p > .05, see table 2).

EMG

Participants' fear potentiated startle responses reflected clear fear responses to the CS+ compared to the ITI, b = -8.91 (1.47), t(2034) = -6.08, p < .001. However, participants only displayed a small, non-significant differential fear response to the CS+ compared to the CS-, b = -1.74 (1.47), t(2034) = -1.19, p = .24. Regardless of Stimulus, participants showed a significant habituation of startle responses over the course of the Acquisition phase, as reflected by the main effect of Trial, b = -.76 (0.16), t(2034) = -4.63, p < .001.

There were no differences between the tVNS and Sham condition in EMG responses during the Acquisition phase (all p > .05, see table 3).

Generalization phase

US Expectancy

Participants in both conditions showed a significant increase in US expectancy ratings for stimuli as a function of perceptual similarity to the CS+, as reflected by the effect of Stimulus, b = 10.79 (0.84), t(58) = 12.82, p < .001 (see figure 3). There was no main effect of Condition on US expectancy ratings in this phase, b = -2.87 (3.77), t(58) = -.76, p = .45, nor was there an effect of tVNS on the generalization gradient, reflected by the Condition*Stimulus Type interaction, b = 1.63 (1.19), t(58) = 1.37, p = .18.

EMG

Similarly to the US expectancy ratings, participants in both conditions showed a significant increase in EMG for stimuli as a function of perceptual similarity to the CS+, as reflected by the main effect of Stimulus, b = 1.26 (0.16), t(61.46) = 8.09, p < .001. There was no main effect of Condition on EMG, b = 0.32 (.56), t(61.53) = 0.57, p = .57, nor was there an effect of tVNS on the generalization gradient, reflected by the Condition*Stimulus interaction, b = -0.11 (0.22), t(61.33) = -0.51, p = .61.

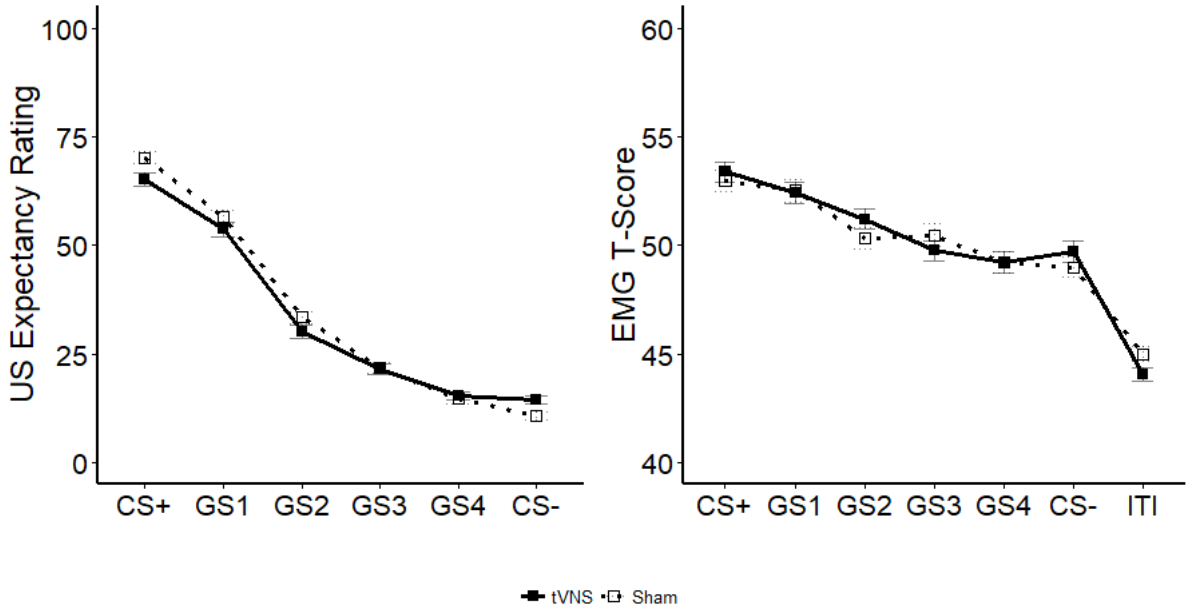


Figure 3. A) US expectancy ratings for CS and GS stimuli during the generalization phase. B) Startle responses during CS and GS stimuli during generalization. Solid lines represent the tVNS condition, dotted lines denote the Sham condition. No significant between-group differences in fear generalization on either US expectancy ratings or EMG. Error bars indicate ± 1 standard error confidence intervals.

Extinction phase

US Expectancy

At the start of the extinction phase, participants reported significantly higher US expectancy ratings for CS+ trials compared to CS- trials, *b* = 68.22 (4.76), *t*(1096) = 14.32, *p* < .001. US expectancy ratings for CS- trials did not significantly change over the course of the extinction phase, as indicated by the main effect of Trial (*b* = -.25 (0.52), t(1096) = -.48, *p* = .63). Similarly, as can be seen in figure 4, the differential decline in US expectancy ratings for CS+ trials is only small and does not result in extinction of fear by the end of the extinction phase. This is also reflected in the non-significant Trial*Stimulus interaction, *b* = -1.32 (0.73), *t*(1096) = -1.81, *p* = .07.

As can be seen in figure 4, participants in the tVNS condition reported lower US expectancy ratings for CS+ trials during the extinction phase, as reflected in the Stimulus*Condition interaction, *b* = -12.02 (6.74), *t*(1096) = -1.78, *p* = .04. There was no significant difference in extinction learning curves between conditions, as reflected by the Trial*Stimulus*Condition interaction, *b* = -.39 (1.03), *t*(1096) = -.37, *p* = .71.

EMG

At the start of the extinction phase, participants displayed stronger EMG responses to the CS+ compared to the CS-, *b* = -6.84 (1.19), *t*(1748) = -5.75, *p* < .001, and compared to the ITI, *b* = -10.34 (1.19), *t*(1748) = -8.70, *p* < .001. Subsequently, participants showed a clear decline in EMG responses

to the CS+, b = -.60 (0.16), t(1748) = -3.88, p < .001. Importantly, this decline in EMG responses reflected a differential decline compared to CS- trials, b =.36 (0.22), t(1748) = 1.65, p = .10, as well as ITI, b = 0.41 (0.22), t(1748) = 1.88, p = .06.

There were no significant between-group differences on fear extinction as indexed by fear potentiated startle responses (all p > .05).

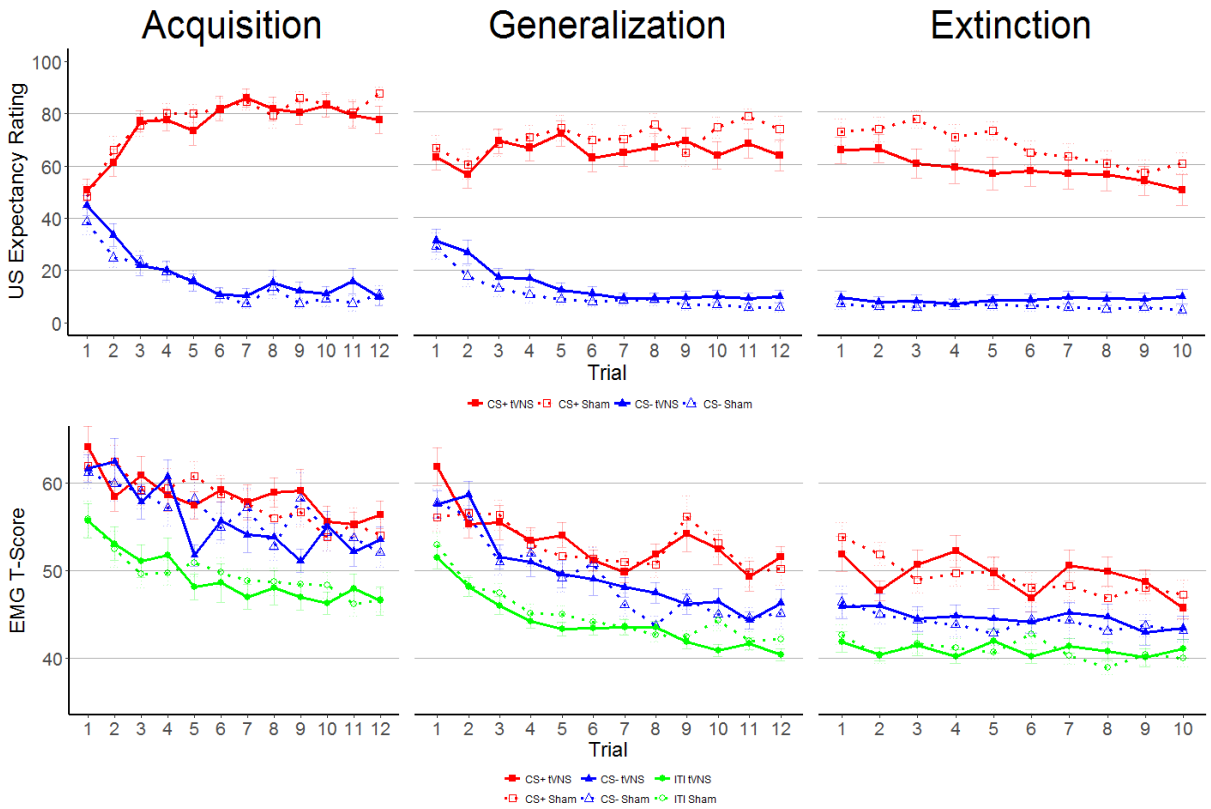


Figure 4. US expectancy ratings (top) and fear potentiated startle responses (bottom) during the fear acquisition (left), generalization (middle), and extinction (right). Only the the CS+ (red), CS- (blue) and ITI (green) are represented in this figure. Solid lines represent the tVNS condition, dotted lines denote the Sham condition. Participants in the tVNS condition report significantly lower US expectancy ratings during the extinction phase compared to participants in the sham condition.
Generalization stimuli, presented only during the generalization phase, were omitted from these graphs to increase readability.
Error bars indicate ± 1 standard error confidence intervals.

Exploratory Analysis

We conducted an additional exploratory analysis to test the effects of tVNS on RMSSD throughout the generalization and extinction phases. Specifically, we calculated the RMSSD of heart beats occurring within the first 7 seconds after every CS or GS onset. RMSSD scores were subsequently log transformed to ensure normal distribution of errors in the mixed model analysis. Visual inspection of the data showed no systematic differences in RMSSD between CStypes. Therefore, CStype was left out of the analysis. The final analysis consisted only of the main effects of Condition and Trial, and a Condition*Trial interaction. In this analysis, Trial is a continuous variable varying from 0 (first trial in the generalization phase) to 91 (last trial in the extinction phase).

There was a significant decrease in RMSSD over time, as indicated by the main effect of Trial, b = -0.001 (0.001), $t(1549) = -2.20$, $p = .03$. There was no main effect of Condition on RMSSD, b = 0.15 (0.19), $t(65) = 0.81$, $p = .42$, nor was there a significant Condition*Trial interaction, b = -0.001 (0.001), $t(1549) = 1.26$, $p = .21$.

Table 2. Regression weights and standard errors for mixed model analyses predicting US expectancy ratings in Acquisition, Generalization, and Extinction phases.

Predictor	Acquisition[a]	Generalization	Extinction
Intercept	36.53 (4.18)***	6.43 (2.67)*	6.96 (3.81)
Stimulus	15.77 (5.92)***	10.79 (0.84)***	68.22 (4.76)***
Trial	-12.96 (2.16)***		-0.25 (0.52)
Trial*Stimulus	28.78 (3.05)***		-1.32 (0.72)
Condition	4.98 (5.92)	-2.87 (3.77)	1.96 (5.39)
Condition*Stimulus	-1.14 (8.37)	1.63 (1.19)	-12.02 (6.74)*
Condition*Trial	-1.41 (3.05)		0.33 (0.73)
Condition*Trial*Stimulus	-2.41 (4.31)		-0.39 (1.03)

Note. Reference category for Stimulus is the CS- trial type. All analyses on the effects of tVNS were conducted using one-sided hypothesis tests. *p < .05, **p < .01, ***p < .001.

[a]Trial variable was log transformed to increase model fit for Acquisition data.

Table 3. Regression weights and standard errors for mixed model analyses predicting EMG in Acquisition, Generalization, and Extinction phases.

Predictor	Acquisition	Generalization	Extinction
Intercept	61.91 (1.37)***	46.17 (.39)***	52.08 (0.92)***
Stimulus		1.26 (.16)***	
Stimulus_{CS-}	-1.62 (1.50)		-6.87 (1.21)***
Stimulus_{ITI}	-8.84 (1.50)***		-10.42 (1.21)***
Trial	-0.71 (0.17)***		-0.59 (0.16)***
$\text{Trial*Stimulus}_{CS-}$	-0.01 (0.22)		0.35 (0.22)
$\text{Trial*Stimulus}_{ITI}$	0.07 (0.22)		0.27 (0.22)
Condition	-0.56 (1.92)	0.32 (0.56)	-1.54 (1.29)
Condition*Stimulus		-0.11 (0.22)	
$\text{Condition*Stimulus}_{CS-}$	0.50 (2.11)		2.02 (1.70)
$\text{Condition*Stimulus}_{ITI}$	0.39 (2.11)		0.91 (1.70)
Condition*Trial	0.20 (0.23)		0.28 (0.22)
$\text{Condition*Trial*Stimulus}_{CS-}$	-0.23 (0.31)		-0.31 (0.31)
$\text{Condition*Trial*Stimulus}_{ITI}$	-0.21 (0.31)		-0.12 (0.31)

Note. Reference category for Stimulus is the CS+ trial type. All analyses on the effects of tVNS were conducted using one-sided hypothesis tests. *p < .05, **p < .01, ***p < .001.

Discussion

The current study aimed to assess whether tVNS affects the generalization of fear in fear conditioned healthy students. The generalization gradient of physiological and declarative indices of fear did not significantly differ between the tVNS and sham conditions. Participants in the tVNS condition reported significantly lower US expectancy ratings for the CS+ stimuli over the course of the extinction phase. However, they did not differ from participants in the sham condition on physiological indices of fear during this phase. These latter results are in line with previous studies that have shown that stimulation of the vagus nerve may facilitate the extinction of declarative, but not physiological expressions of fear.

In the current study, we found no support for the hypothesis that tVNS reduces fear generalization. Given the evidence on the effects of VNS on hippocampal cell proliferation and differentiation from animal literature, it seemed likely that stimulating the vagus nerve in humans could have similar effects. The non-significant results found in this study reflect the difficulty of translating animal studies to humans, and may also reflect differences between invasive and transcutaneous VNS. It should be noted, however, that the non-significant results found in this study may also be due to the relatively short duration of tVNS in this study: indeed,, not much is known about the temporal latency of the effects of VNS on progenitor proliferation or cell differentiation. In rats, acute effects on the increase in cell proliferation and dendritic complexity have only been tested after 3 hours of VNS. It is unclear whether these effects would also occur on a shorter timescale, such as the 30-minute timescale used in this experiment. It would be interesting to test whether possible effects of tVNS on the generalization of fear would appear after stimulating during multiple sessions. Currently, it remains unclear whether and how tVNS can be implemented in clinical practice for exposure therapy.

Participants in both conditions showed only limited extinction of fear during the extinction phase. The lack of complete extinction found in this study can be explained by the sudden change in CS-US contingency that occurred from the generalization phase to the extinction phase. During the generalization phase, the CS+ was followed by an electric shock in 50% of the trials, whereas the CS-US contingency dropped to 0% during the extinction phase. Since the generalization phase seamlessly transitioned into the extinction phase, the change in contingency from partial- to non-reinforcement was probably noticed by participants more slowly than when these phases would have been separated. Indeed, given the critical importance of expectancy violations for new learning to take place [58], lower CS-US contingencies during the generalization phase may have hampered expectancy violations and subsequent belief updating about the CS-US contingencies during the subsequent extinction phase [248]. To illustrate, after two CS-noUS trials, participants would be strongly inclined to update prior

beliefs on CS-US contingencies if their prior belief was that the CS+ was followed by a US 100% of the time, whereas two non-reinforced trials would still fall in their realm of expectations when they held the prior belief that the CS+ was followed by a US 50% of the time. Finally, it is important to note that the extinction phase contained only ten trials. If more trials had been included, it seems likely that participants in both conditions would have eventually shown complete extinction of fear.

Although on average participants in neither condition showed complete extinction of fear in this study, participants in the tVNS condition reported consistently lower US expectancy ratings for the CS+ over the course of the extinction phase. This effect of tVNS on the declarative extinction of fear is in line with previous studies on tVNS during fear extinction [165,196]. In these previous studies, participants who received tVNS showed an accelerated declarative extinction curve, whereas in this study participants who received tVNS reported lower US expectancy ratings already from the start of the extinction phase. The differences between these results may reflect differences between the underlying study designs. Specifically, as discussed in the previous paragraph, the generalization phase that preceded the extinction phase included a reduction in the CS-US contingency and thus already allowed for at least partial inhibitory learning to take place. Figure 4 shows that at the end of the generalization phase, participants in the tVNS condition already reported lower US expectancy ratings than participants who received sham stimulation, although this difference was not statistically significant. During the subsequent extinction phase, participants in the tVNS condition retained these decreased US expectancy ratings over the course of the extinction phase, although extinction was not completed. Nevertheless, the results from this study are in line with our previous findings, which suggest that tVNS may accelerate declarative inhibitory learning in humans.

In contrast to the declarative extinction of fear, participants in the tVNS condition did not differ from sham stimulated participants in their physiological fear responses during the generalization or the extinction phases. This is in line with previous studies on the effects of tVNS on fear extinction in humans [165,196]. Additionally, administration of the β-adrenergic receptor blocker propranolol during fear extinction has been found to significantly reduce the extinction of fear on declarative indices of fear, but did not affect physiological indices including fear potentiated startle responses and SCR [249]. The similarities between the effects of propranolol and tVNS in how they affect fear extinction provide further indications for the hypothesized noradrenergic working mechanism of tVNS.

The discrepant results of tVNS on declarative and physiological indices of fear could provide further support for a two-factor account of fear [184,250,251], which suggests that the declarative and emotional aspects of fear are controlled by two separate memory systems that are driven by separate brain areas (i.e. the hippocampus and the amygdala, respectively) [184]. Alternatively, the effect of tVNS on US expectancy ratings could reflect a non-emotional improvement in associative learning that is accelerated through tVNS, corresponding to earlier studies showing that tVNS strengthens

associative learning of names and faces in older individuals [86]. Future studies should focus on whether the results found in this and in previous fear conditioning studies indeed extend towards non-emotional associative memory performance.

Although the fear generalization paradigm used in the present study has been commonly used in previous studies, there are two concerns with this method that we think should be addressed. Firstly, given that the only physical property distinguishing the CS+ from the CS- is the size of the circle, and no a priori knowledge on GS-US contingencies was available, it can theoretically be questioned if and to what extent the generalization of fear in this particular experimental paradigm can actually be described as maladaptive - especially since the CS+ is still followed by a US 50% of the time, meaning that threat is still imminent. Secondly, it is also not entirely clear whether participants displayed a fear response because they feared the GS as such, that is, despite perceiving it as different from the CS+, or because they were simply unable to discriminate the GS from the CS+. In a recent study, Struyf and colleagues [252] found that misspecification of the novel GS as the CS+ strongly increased fear responses compared to when the GS was categorized as a novel stimulation, and that misspecification of the GS as a CS+ occurs in up to 50% of trials depending on the similarity between stimuli in terms of circle size. These possible shortcomings of the experimental paradigm may explain why tVNS did not affect fear generalization in this trial, as the GSs could have been perceptually misspecified as a CS+ and therefore no pattern completion or separation took place. Future studies could consider alternative paradigms which utilize CSs and GSs which are clearly distinguishable by testing, for example, conceptual generalization (e.g. the generalization of one type of bird to different types of birds).

The current study utilized an immediate extinction paradigm, with generalization and extinction phases occurring on the same day as fear acquisition. Although not much is known on the differences between immediate and delayed extinction, some reports indicate that immediate extinction is associated with delayed extinction learning [253,254]. One could argue that utilizing this immediate extinction paradigm increases the translational gap between animal and human studies, since animal studies studying the effects of VNS on fear extinction have exclusively used delayed learning paradigms. However, previous studies on the effects of tVNS on fear extinction in humans have utilized both immediate [165,196] and delayed extinction protocols [200,240], and have found inconsistent results for both kinds of protocols. This may be due to other differences in the experimental protocol of these studies (e.g. cue vs. context conditioning, neutral vs. prepared learning). Clearly, there is a need for more consistency and more direct replications in this field of neurostimulation in extinction research.

Startle probes presented during CS or GS presentation were presented 7 seconds after stimulus onset. This fixed timing may have increased the predictability of the startle probe, and thereby

decreased startle responses. This diminished startle responding may also partly explain why fear potentiated startle responses to CS+ stimuli were not significantly different from CS- stimuli during the acquisition phase. Indeed, preliminary evidence suggests that attending a startle probe diminishes the emotional modulation of the startle response, although this has only been studied when the startle probe required an active response [255]. The importance of startle probe onset predictability has not yet been systematically studied [222]. Nonetheless, future studies should consider presenting startle probes at random intervals relative to CS onset to circumvent the risk of attenuated startle responses to due stimulus predictability.

In the current study, we used the preprogrammed settings of the Nemos tVNS device of 30 seconds on, 30 seconds off stimulation at 25Hz, 250μs wavelength duration, with a stimulation intensity set to 0.5mA. One could argue that ensuring that stimulation occurs simultaneously with stimulus presentation is an important step towards optimizing treatment efficacy, given that (t)VNS is hypothesized to affect associative learning through phasic activation of the LC-NE network. Indeed, most studies that tested the effects of VNS on neuronal plasticity - as a way of treating tinnitus complaints or enhancing the rehabilitation for stroke patients – ensured that stimulation coincided with stimulus presentation (e.g. [256–260]). However, to our knowledge, there are no studies that have tested whether synchronization of stimulation and stimulus presentation is a determinant of the efficacy of (t)VNS. In our earlier studies, we have found significant effects of tVNS on declarative fear extinction using stimulation paradigm that fixed the stimulation timing based on CS presentation [196] as well as when stimulation timing was purely based on a 30s on, 30s off schedule [165]. However, we have never tested both settings in one study before. Clearly, more research on the optimal stimulation parameters is clearly necessary, both in terms of stimulation intensity and frequency, but also in terms of timing of stimulation presentation.

Finally, we conducted an exploratory analysis to test the efferent cardiac effects of tVNS on RMSSD throughout the generalization and extinction phases. The potential for tVNS to elicit cardiac efferent effects is a contested issue, possibly fueled by the existing literature on HRV as a marker of cardiac vagal tone. In this study, tVNS did not affect participants' RMSSD compared to sham stimulation. Although there are some studies that have reported effects of tVNS on cardiac activity [261–263], the effects that have been reported are quite inconsistent. Additionally, studies that have found cardiac efferent effects of tVNS have used stimulation parameters that are quite different from the ones used in this study: firstly, they stimulated either the right ear or both ears, whereas we stimulated the left ear. Anatomical studies have pointed out that the right branch of the vagus nerve innervates the sinoatrial node of the heart more strongly than the left, which is also the reason why invasive VNS is most often applied to the left branch of the vagus nerve [15]. Secondly, studies that elicited cardiac efferent effects used stimulation intensities that were up to 10 times higher than the

ones used in this study. This is in line with parametric studies performed in dogs, which showed that the stimulation intensities used in this study are sufficient to activate afferent A-fibers, but will not exceed the stimulation thresholds for activating cardiac efferent B-fibers (threshold of 0.4 mA and 3.8 mA, respectively) [11,264].

To summarize, our hypothesis that tVNS affects the fear generalization gradient was not supported in this study. The risk of perceptual misspecification of CS and GS stimuli warrant a conceptual replication of this study using different stimuli. In line with previous studies, we observed an effect of tVNS on the extinction of declarative fear and no effect on physiological fear extinction. These findings support the theory that tVNS affects associative learning performance.

Part II

Negative Thought Intrusions

Chapter 6

Transcutaneous Vagus Nerve Stimulation Reduces Spontaneous but not Induced Negative Thought Intrusions in High Worriers

Burger AM, Van der Does W, Thayer JF, Brosschot JF, Verkuil B (2019). *Biological Psychology, 142,* 80-89. DOI: 10.1016/j.biopsycho.2019.01.014.

Abstract

Worrying is a central component of anxiety disorders. We tested whether non-invasive vagus nerve stimulation reduces negative thought intrusions in high worriers. Worry was assessed with a Breathing Focus Task, which consists of a pre-worry period, a worry induction, and a post-worry period. Ninety-seven high worriers were randomly allocated to receive transcutaneous electrical stimulation of the auricular branch of the vagus nerve at the concha (tVNS), or of the earlobe (sham stimulation) throughout the lab session. Participants who received tVNS reported significantly fewer negative thought intrusions during the pre-worry period, but the effects of tVNS after the worry induction were mixed. An exploratory analysis indicated that participants in the tVNS condition were more likely to report negative thought intrusions shortly after the worry induction, but became less likely to do so as the post-worry period went on. No effects of tVNS on RMSSD were observed. These findings provide preliminary indications that tVNS may decrease the occurrence of worrisome thoughts.

Introduction

Perseverative cognition such as worry and rumination is observed in a wide range of stress-related disorders including depression, anxiety, and burnout [265,266]. Perseverative cognition is a symptom of generalized anxiety disorder (GAD) in particular. GAD is a highly prevalent condition that is mainly characterized by excessive and uncontrollable worrying. Roughly 5% of the population suffer from GAD at some point in their life, and another 12% suffer from subthreshold GAD characterized by excessive worrying [2,267]. People suffering from GAD, as well as high worriers in general, are extremely occupied with stress-related thoughts and continuously prioritize threat-related information at the expense of safety information. Given the high prevalence of GAD and high worriers, it is crucial to understand what factors maintain worrying and to develop interventions that reduce it. Current psychological and pharmacological interventions are moderately effective, with 46% of patients assigned to cognitive behavioral therapy responding to treatment [268]. Furthermore, worrisome thoughts can be easily re-activated after successful treatment, resulting in high relapse rates [269]. Thus, it seems critical to test new interventions that might reduce worry. Besides the currently available psychological and pharmacological interventions, recent years have seen an increase in neuromodulation techniques, One such method is the non-invasive stimulation of the auricular branch of the vagus nerve, which is the method under scrutiny in this study.

In this study, we aimed to examine whether experimentally enhancing vagus nerve activity will acutely decrease worrying in a group of high worriers. Recent technological advances allow us to test this hypothesis using a non-invasive approach, by transcutaneously stimulating the auricular branch of the vagus nerve (ABVN) via the concha of the outer ear. This procedure is called transcutaneous vagus nerve stimulation or tVNS. Crucially, fMRI studies have shown that tVNS directly promotes activity in brain areas that reduce worry, including the prefrontal cortex and the anterior cingulate (for a review, see [270]). Furthermore, tVNS increases the functional connectivity between the amygdala and the prefrontal cortex in depressed patients [80]. Functional connectivity between the amygdala and the prefrontal cortex has repeatedly and robustly been demonstrated as a function of anxiety [81], and has also been linked to self-reported worry intensity in patients suffering from GAD [82–84].

Previous studies have also indicated that tVNS affects cognitive functions that rely on prefrontal activity, e.g. enhanced associative memory formation and consolidation [86,165,196] and action control [87]. Critically, tVNS promotes the ability to inhibit task-irrelevant information processing [88,89,271], a process which is strongly compromised in patients suffering from GAD [90]. Finally, the potential effect of tVNS on worrying is further illustrated by a non-randomized study that showed medium to large effect sizes of tVNS on symptoms of depression and anxiety in patients suffering from depressive disorders [91]. However, knowledge of the effects of tVNS on worry, a core

pathological component of mental disorders, is still lacking. In this study we tested if tVNS acutely decreases worry in high worriers, compared to sham – using the same stimulation procedure as in our previous studies (e.g. [88,165,196,272]).

In summary, previous studies suggest that enhancing vagus nerve activity via tVNS produces neural, cognitive and emotional effects that indicate a possible effect on worry. In this experiment, we compared the effects of tVNS to sham stimulation on worry in high trait worriers. Worry was assessed by measuring the frequency of negative thought intrusions before and after a worry induction during a Breathing Focus Task [273–275] as measurements of both spontaneous and induced worry behavior. We tested the hypotheses that high worriers who received tVNS have fewer negative thought intrusions than those who received sham stimulation, both before and after the worry induction part of the BFT. As an additional exploratory analysis, we tested the effects of tVNS on both resting levels and worry-induced reductions in heart rate variability . Heart rate variability is often used as an index of efferent vagal tone, and although tVNS is unlikely to lead to cardiac effects - due to the lateralization of cardiac input of the vagus nerve, and low intensity of stimulation [264,276] - we will still report these findings to contribute to the literature on efferent effects of stimulating the left ABVN.

Methods

Participants

Ninety-seven students (78 female, 19 male), between the ages 18-25 (M_{age} = 21.04, SD_{age} = 2.08), were included. Participants were recruited from Leiden University through pamphlets and a designated university website, specifically targeting participants who worry frequently. Participants could participate if they scored at least 45 on the Penn State Worry Questionnaire (PSWQ). The cut-off score of 45 was suggested as a highly sensitive and specific cut-off score for clinical GAD in an advertised-for population [219]. Participants suffering from epilepsy, cardiac arrhythmia or bradycardia, alcoholism, or migraines requiring medication use were excluded from the study.

Ethical approval for this study was given by the ethical committee of the Institute of Psychology of Leiden University (CEP #8988381492). Participants were rewarded with either 10 euros or course credit.

Instruments and Questionnaires

Breathing Focus Task

The breathing focus task (BFT) was developed by Borkovec et al. [275] and later adapted by Hirsch et al. [273,274] as a measure of spontaneous and induced worry. For a graphical overview of the BFT, see figure 1. The BFT consisted of three periods: a 5 minute pre-worry breathing focus period, followed by

a 5 minute worry induction period, and finally a 5 minute post-worry breathing focus period. During the breathing focus periods, participants were instructed to close their eyes and simply focus their attention on their breathing. Importantly, participants were not given any instructions on how to breathe in terms of technique, depth or pace. An auditory cue was presented through their headphones every 20-30 seconds for a total of 12 cues per breathing focus period. Participants were instructed to open their eyes after hearing the cue, at which time a question appeared on the computer screen, asking them whether their attention was focused on their breathing or whether they were thinking of something else at the moment they heard the tone. If participants reported that their attention was focused on 'something else', they were asked whether this was something positive, neutral or negative. Contrary to previous studies that have used the BFT [273,274], participants were not asked to provide a short description of the intrusion (e.g. "worrying about whether I'll pass my exams"), but were simply instructed to once again close their eyes and focus on their breathing.

At the end of each breathing focus period, three questions were asked to check whether the breathing focus prompts were truly representative of worrying: ''Estimate the percentage of time you were able to focus on your breathing (0% not at all – 100% all of the time)''; ''Rate how difficult you found it to focus on your breathing (0 not at all difficult – 100 extremely difficult)''; and ''Estimate the percentage of time you worried during the last 5 minutes (0% none of the time – 100% all of the time)''.

During the worry induction period, participants were instructed to worry "as they normally do" about the topic they specified beforehand. No auditory cues were presented during this period.

After the worry induction period, participants were asked to answer several questions concerning their worry behavior: ''Estimate the percentage of time that you were able to spend worrying (0% not at all – 100% all of the time)'', ''Rate how intensely you were worrying (0 not intensely at all – 100 extremely intensely)", "To what extent could you control your thoughts? (0 not at all – 100 all of the time)" [274].

Transcutaneous vagus nerve stimulation

The tVNS instrument provided electrical stimulation using two titanium electrodes, positioned on top of a silicon earplug, which was connected by a wire to a portable neurostimulator (Nemos®, Cerbomed, Erlangen, Germany). The electrodes delivered 30-second waves of electrical stimulation (0.5mA, 25Hz, 250µs), alternated by 30-second breaks. The electrodes were attached to either the cymba concha (tVNS condition) or the center of the earlobe (sham stimulation condition) of the left ear. In contrast to the cymba concha, the earlobe is not innervated by the vagus nerve [25]. We stimulated the left ear to avoid potential cardiac effects that have been related to efferent vagal fibers of the right ear [119] but not the left [121].

Questionnaires

Prior to the lab session, participants were asked to complete the Penn State Worry Questionnaire (*PSWQ*; [135,277]) at home. The PSWQ is a 16-item self-report questionnaire that assesses the duration and uncontrollability of worry. The PSWQ has demonstrated high reliability, high temporal stability and substantial validity in the assessment of trait-worry [135,136].

To ascertain that there were no between-group differences prior to the experimental manipulation that could have affected responses on the BFT, participants were asked to complete four questionnaires at the start of the lab session. The questionnaires were used to assess worrying, state and trait anxiety, attentional control and ruminative thoughts.

The Generalized Anxiety Disorder-7 scale (GAD-7; [278]) is a 7-item clinical measure assessing the severity of GAD symptoms with good reliability and validity in the general as well as clinical population [279].

The State Trait Anxiety Inventory (*STAI*; [138])is a self-report questionnaire consisting of 2 versions with 20 questions each, measuring both state and trait anxiety. The STAI has shown acceptable internal consistency and validity [137,139].

The Attentional Control Scale (ACS; [280]) is a 20-item self-report measure consisting of a 9-item measure of attentional focusing and an 11-item measure of attentional shifting with good internal and predictive validity [281].

The Ruminative Response Scale (RRS; [282]) is a 22-item questionnaire assessing the engagement of participants in response to feeling sad or depressed. The RRS has been shown to have good internal consistency and moderate to high validity for predicting depression.

Side Effects

At the end of the experimental paradigm, participants were presented with a list of seven side-effects commonly reported during tVNS (ie. headache, neck pain, nausea, muscle contractions, pricking sensations, burning sensations, and general feelings of uncomfortableness). Participants were asked to rate to what extent they had experienced each of these side-effects on a scale of 1 (‘applies not at all’) to 5 (‘strongly applies to me’). As this side-effects form was only added to the experimental procedure after data acquisition had already started, 91 out of 97 participants completed this questionnaire.

Heart Rate Variability

Over the course of the experimental procedure, participants were asked to wear a chest strap with a sensor at the base of the sternum to measure cardiovascular activity through two electrodes connected to the belt (Movisens, GmbH, Karlsruhe, Germany). Raw ECG was measured at 1024Hz and

was automatically cleaned for outliers and measurements artifacts by the Movisens Data-Analyzer software, after which 60-s averages of the root of the mean square of successive differences in interbeat intervals (RMSSD) were further aggregated to 5 minute means to separate the different periods of the BFT. Contrary to high frequency HRV – an alternative measure of vagally mediated HRV -, some studies appear to show that RMSSD is hardly affected by changes in breathing patterns or movement [283,284]. LF/HF ratio was not included as a measurement of vagally mediated HRV because it remains difficult to interpret this index, based on studies that showed involvement of the vagus nerve in both high and low frequency components of heart rate variability [285].

Procedure

This study was part of a larger project on the effects of tVNS on worry behavior and stress-related attentional biases. This larger study was preregistered on the Open Science Framework, https://osf.io/za9mu.

Students who showed interest in the study received a link via email asking them to fill in the PSWQ online. Participants scoring 45 or higher were invited to the lab. Potential participants who scored lower than 45 were informed that they did not fulfill the criteria for participating and the questionnaire was locked for that particular IP address.

For a graphical overview of the experimental procedure, see figure 1. At the start of the experiment, after having signed an informed consent form for the experiment, participants were instructed to write down a personally relevant worry topic. Participants were instructed to wear an ECG chest strap throughout the remainder of the study. Subsequently, a 2-minute pupillometry measurement was conducted. During this baseline recording, participants were instructed to simply look at a fixation cross in the middle of a screen. Afterwards, the tVNS device was attached to the participant's left ear, stimulating either the concha (tVNS) or the earlobe (sham stimulation).

Since not much is known about the temporal latency of the effects of tVNS [115,122,144,187], a short build-up period of the effects of tVNS and sham stimulation was used during which participants were instructed to sit and relax for five minutes. During this time, a baseline recording of their heart rate was conducted. Subsequently, participants were instructed to complete several questionnaires. The questionnaires included a short demographics form, the STAI, the GAD-7, the ACS and the RRS. On average, completing the questionnaires took 15 minutes.

After completing the questionnaires, participants were instructed to complete the BFT, which consisted of two breathing focus periods separated by a worry induction, as described below. Subsequent to the BFT, participants completed a second pupillometry measurement, followed by two cognitive tasks and one final pupillometry measurement, which are not reported here.

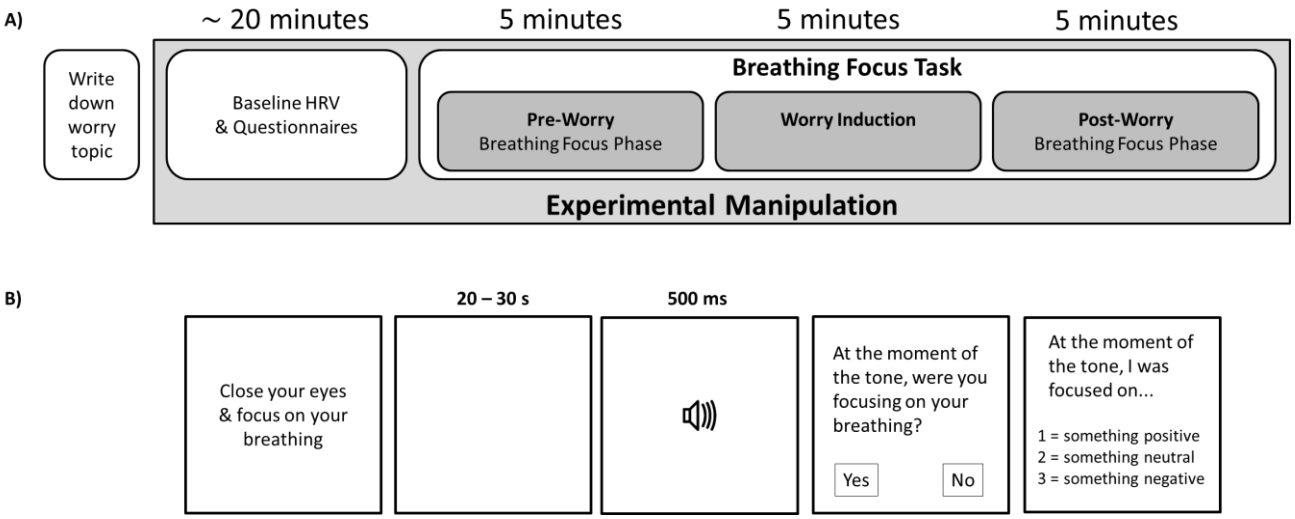


Figure 1. A) Overall experimental overview. After having signed an informed consent form, participants were asked to fill in their currently most pressing worry topic. Then, participants were randomly allocated to receive either tVNS or sham stimulation throughout the rest of the session. The Breathing Focus Task consisted of three separate phases; two Breathing Focus phases separated by a Worry Induction phase.

B) Graphical depiction of a trial during the Breathing Focus Phases. Participants were instructed to close their eyes and simply focus on their breathing. Then, after 20 or 30 seconds, they heard a soft tone prompting them to open their eyes and assess whether they were focused on their breathing at the time of the tone. If they were not, they were additionally asked to rate whether they were focused on something positive, neutral or negative. Both Breathing Focus phases consisted of 12 trials.

Statistical Analyses

To test for possible confounding baseline differences between participants in the tVNS and sham group, we conducted independent samples t-tests on test scores of all questionnaires. Similarly, we tested baseline differences in resting levels of RMSSD.

Since the dependent variable of our main analysis (*number of reported negative thought intrusions*) is a count variable, using conventional statistical procedures based on a Gaussian distribution of the data would produce unreliable results. Instead, we opted to use generalized linear mixed modelling using a Poisson distribution with a log link function. The variables *Condition* (0 = Sham, 1 = tVNS) and *Time* (0 = pre-worry, 1 = post-worry) were dummy coded and included as covariates in the generalized linear mixed model. Additionally, the model included a random intercept for every individual.

To test the effects of tVNS on HRV over the course of the BFT, we performed a linear mixed model analysis with log transformed RMSSD as a dependent variable, *Time* as a categorical independent variable (pre-worry, worry induction, post-worry; pre-worry being the reference category) and Experimental Condition as a dummy-coded covariate (0 = Sham, 1 = tVNS). To test the

effects of tVNS on HRV over the course of the BFT, we performed a linear mixed model analysis with log transformed RMSSD as a dependent variable, Time as a categorical independent variable (pre-worry as a reference category) and Experimental Condition as a dummy-coded covariate (sham group as a reference category). Prior to these analyses, we assessed the need to control for medication use, caffeine intake or smoking frequency on baseline RMSSD. Spearman's rho correlations between RMSSD and these potential confounders were all non-significant (all $\rho < .20$). As we found no clear effects of any of these factors on log transformed RMSSD in our sample, we decided to analyze the data for all participants without controlling for these variables, to increase the power of our analyses.

Between-groups differences on the side-effects ratings were not normally distributed. Therefore, we calculated between-group differences in side-effect severity by using Yates corrected Chi Square analyses.

Cohen's *d* effect sizes were calculated using the formula $d = \frac{\mathrm{b}}{\text{pooled SD}}$, where *b* denotes the regression coefficient of the corresponding effect and *SD* corresponds to the pooled within-group standard deviation [225]. Analyses were performed in SPSS version nr. 23.

Results

Descriptive Statistics

As shown in table 1, there were no significant differences between the groups in resting levels of RMSSD and the questionnaire scores. The average score on the PSWQ for both the tVNS and the control group corresponded to the 90th percentile of the general population and the 30th percentile of a GAD-patient population [142]. Likewise, the average score on the GAD-7 fell within the range of mild to moderate clinical anxiety, which is in the 90th percentile of the general population ($M_{\text{GAD-7}}$ =3.0,[279]).

Compared to the general population, participants in the current study also scored above average on state and trait anxiety (STAI; [143]). Similarly, compared to general student populations, participants scored above average on rumination (RRS; [286]), and below average on perceived attentional control (ACS; [287]).

On average, participants reported 1.25 (*SD* = 1.35) negative thought intrusions during the pre-worry period of the BFT. This number increased to an average of 1.80 (*SD* = 1.67) during the post-worry period.

Table 1. Descriptive statistics.

	Sham (*N* = 49)		tVNS (*N* = 48)		
	M	*SD*	*M*	*SD*	*p*
PSWQ	60.53	7.70	62.25	7.56	.27
GAD-7*	9.13	4.31	8.83	5.01	.76
STAI State	45.65	9.61	43.18	9.52	.21
STAI Trait	48.85	9.32	48.86	10.47	.99
RRS	50.69	12.25	48.94	13.24	.50
ACS	46.42	9.12	47.85	7.38	.40
Resting RMSSD	42.28	25.6	43.66	27.16	.81

Note. *N_{sham} = 40/ N_{tVNS} = 40 for the GAD-7. This questionnaire was added after data acquisition had already started as an additional check to ensure that the current sample consisted of high worriers.

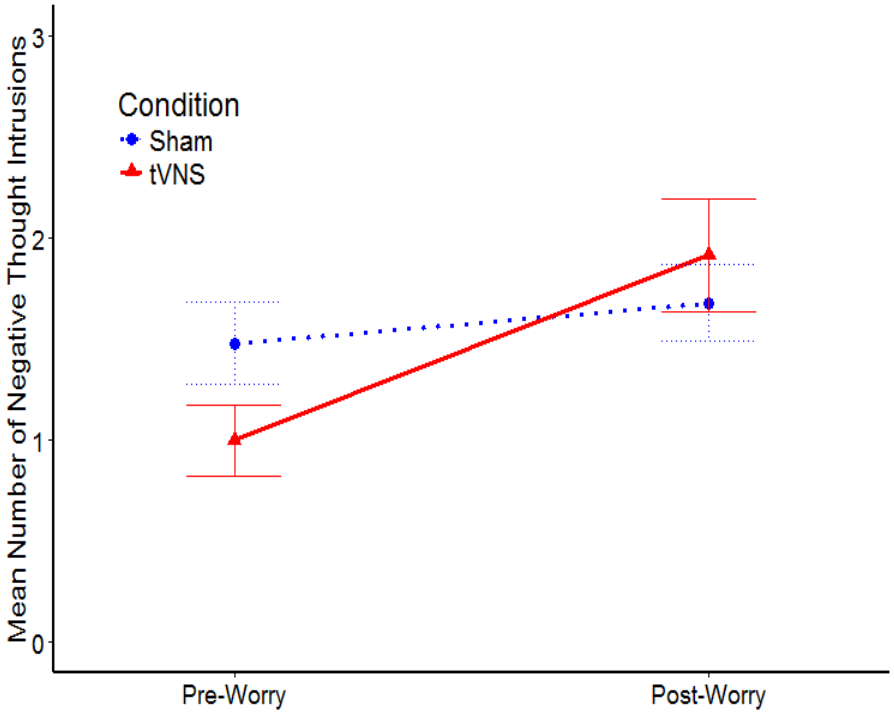


Figure 2. Line graph of average number of negative thought intrusions for every experimental condition before and after worry induction. Error bars denote ± 1 standard error.

Effects of tVNS on Negative Thought Intrusions

Figure 2 shows the mean number of negative thought intrusions per condition. During the pre-worry period, participants who received tVNS reported significantly fewer negative thought intrusions than participants in the sham condition (M_{tVNS} = 1.0 (*SD* = 1.2), M_{Sham} = 1.5 (*SD* = 1.4)), as reflected in the significant main effect of Condition, *b* = -.48 (.23), *t*(190) = -2.10, *p* = .037, *d* = -.36. After the worry induction, participants who received tVNS reported a stronger increase in negative thought intrusions than participants in the sham condition, as indicated by a significant Time*Condition interaction, *b* = .52 (.18), *t*(190) = 2.85, *p* = .005, *d* = .29. The number of negative thought intrusions reported by participants in the sham condition did not change significantly after the worry induction, as reflected by a non-significant main effect of Time, *b* = .13 (.12), *t*(190) = 1.10, *p* = .30, *d* = .08. Participants in the tVNS condition reported a slightly higher number of negative thought intrusions than participants in the sham condition after the worry induction (M_{tVNS} = 1.9 (*SD* = 1.9), M_{Sham} = 1.7 (*SD* = 1.4), see figure 2). To test whether the between-group difference during the post-worry period was significant, we performed a subsequent analysis where the post-worry period was used as the reference category. This analysis confirms that the between-group difference during the post-worry period is not significant, as reflected by the main effect of Condition, *b* = .05 (.21), *t*(190) = .22, *p* = .83, *d* = .03.

With regard to the retrospective worry assessments, we found no effects of tVNS on the time spent worrying during the breathing focus periods, either before (*p* = .92) or after the worry induction (*p* = .80). No effect of tVNS was observed on any of the other retrospective assessments (see Table 2).

Exploratory Analyses

To test whether the effect of tVNS on thought intrusions is specific to negative intrusions, or whether it reflects a reduction in mind wandering in general, we performed additional exploratory analyses to test whether tVNS also reduced the number of positive and neutral thought intrusions reported by participants. For both neutral and positive thought intrusion frequency, we found no effects of time, indicating that the number of positive or neutral intrusions were not affected by the worry induction. Additionally, neutral and positive thought intrusion frequency were not affected by tVNS in either breathing focus period, all *p* > .05.

Finally, we explored possible time-dependent effects of tVNS during the post-worry period. Visual inspection of the data suggested that during the post-worry period, participants in the tVNS condition were more likely to report negative thought intrusions at the start of the post-worry period, and became progressively less likely to report such thought intrusions as the period went on (see figure 3). To test this possible time-dependent effect of tVNS on negative thought intrusions, we conducted a logistic generalized linear mixed model analysis. To account for the differences in slopes between

the pre- and post-worry phase, we performed a piecewise regression analysis and included two independent *Time* variables in the model. The first Time variable, $Time_{pre\text{-}worry}$, is a continuous variable that counts the number of pre-worry probes that have been presented (ranging between 0 and 11 in the pre-worry phase, 11 throughout the post-worry phase). The second Time variable, $Time_{post\text{-}worry}$, is a continuous variable that counts the number of post-worry probes that have been presented (0 throughout the pre-worry phase, ranging between 1 and 12 during the post-worry phase). The model also includes Condition (0 = Sham, 1 = tVNS) and Phase (0 = pre-worry, 1 = post-worry) as dummy-coded covariates. The generalized linear mixed model included main effects of both Time variables, Condition, and Phase. Additionally, $Time_{pre\text{-}worry}$*Condition, $Time_{post\text{-}worry}$*Condition and Phase*Condition interactions were included in the model.

Over the course of the pre-worry phase, participants in both conditions showed a slight increase in the probability of reporting negative thought intrusions, as reflected by the main effect of $Time_{pre\text{-}worry}$, b = 0.07 (0.03), $t(2200) = 2.53$, $p = .011$, OR = 1.08 [1.02 – 1.15]. In line with the main analysis, the probability of reporting negative thought intrusions during the pre-worry phase was lower for participants in the tVNS condition, although this difference was no longer significant in this analysis, b = -0.73 (0.41), $t(2200) = -1.76$, $p = .078$, OR = 0.48 [0.21 – 1.09]. The linear increase in reported negative thought intrusions throughout the first breathing focus period did not differ between participants in the tVNS and sham conditions ($p = .97$).

As can be seen in figure 3, participants in the sham condition did not display a significant increase in the probability of reporting negative thought intrusions (main effect of Phase, $p = .82$). However, compared to participants in the sham condition, the proportion of participants in the tVNS condition that reported negative thought intrusions increased significantly from the end of the pre-worry phase to the start of the post-worry phase, as indicated by a significant Condition*Phase interaction, b = 1.71 (0.49), $t(2200) = 3.52$, $p < .001$, OR = 5.51 [2.13 – 14.25]. There was no significant decrease in the proportion of participants who reported negative thought intrusions in the sham condition, as reflected by the main effect of $Time_{post\text{-}worry}$ (p = .29). However, there was a significant Condition*$Time_{post\text{-}worry}$ interaction, b = -0.14 (.05), $t(2200) = -2.74$, $p = .006$, OR = .87 [.79 - .96], indicating that the proportion of participants reporting negative thought intrusions in the post-worry phase showed a significantly stronger reduction in the tVNS condition than in the sham condition.

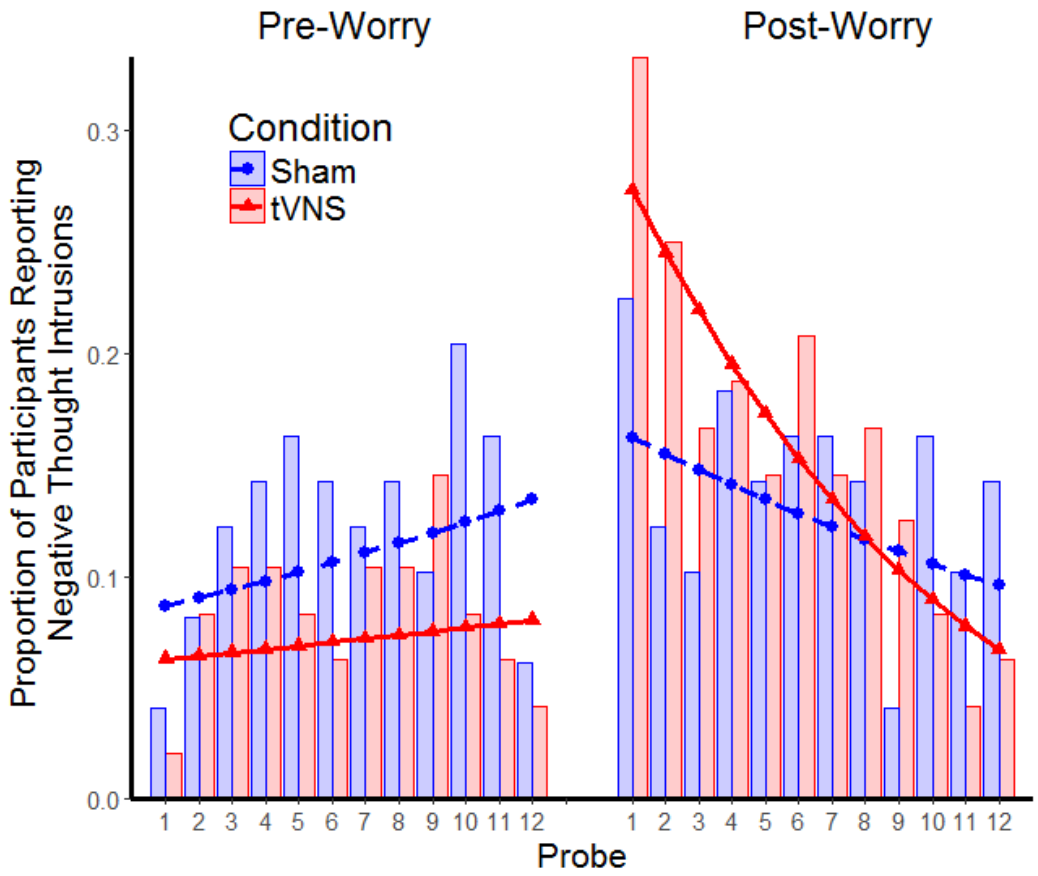


Figure 3. The bars denote the proportion of reported negative thought intrusions for every sound probe taken before and after the worry induction for participants in the sham (blue bars) and tVNS (red bars) condition. The lines depict the estimated marginal means of the logistic mixed model analysis for the sham (blue line) and tVNS (red line) condition.

Worry Induction

As displayed in table 2, the worry induction successfully led to a clear increase in time spent worrying compared to the first breathing focus period, b = 37.39 (2.88), $t(96)$ = 13.00, p < .001, d = 1.63. Additionally, participants spent more time worrying during the post-worry period compared to the pre-worry period, b = 6.95 (2.19), $t(96)$ = 3.17, p = .002, d = .29. There was no difference between conditions in the time that participants spent worrying, their perceived control over their worrying, or their worry intensity (all p > .05). Thus, the initial between-group difference in the number of negative thought intrusions during the post-worry period could not be attributed to a difference in the worry experienced during the worry induction between conditions.

Table 2. Mean self-report measures for tVNS and Sham stimulation conditions during pre- and post-worry breathing focus periods and the worry induction.

	Pre-worry			Worry Induction			Post-worry		
	Time spent worrying	Difficulty focusing on breathing	Time focused on breathing	Time spent worrying	Control over thoughts	Worry Intensity	Time spent worrying	Difficulty focusing on breathing	Time focused on breathing
tVNS	27.3%(25.0%)	46.4(28.5)	58.5%(23.7%)	66.0%(22.0%)	53.5(20.1)	52.7(21.5)	35.0%(24.5%)	58.5(25.7)	49.1%(25.3%)
Sham	28.5%(23.3%)	51.4(24.7)	50.5%(23.6%)	64.7%(22.0%)	49.4(20.8)	50.9(19.9)	34.8%(22.8%)	60.3(22.1)	48.1%(20.7%)

Note. Percentages denote percentage of time spent on an activity within the five minute period. Standard deviations are presented between brackets.

Heart Rate Variability

We explored the effects of tVNS on changes in log-transformed RMSSD over the course of the BFT. A significant decrease in RMSSD was observed during the worry induction and the post-worry period compared to the pre-worry period (main effect of Time, $F(2, 92.77) = 8.17$, $p < .001$). Regardless of experimental group, participants displayed a clear reduction in HRV during the worry induction, b = -.11 (.03), $t(90.00) = -4.04$, $p < .001$, $d = -.20$ (see figure 4). Participants showed a partial recovery of their HRV during the post-worry period, but HRV scores were still significantly lower than in the pre-worry period, $b = -.07$ (.03), $t(102.63) = -2.31$, $p = .023$, $d = -.13$. tVNS did not significantly affect the cardiac response to the worry induction ($p = .99$, $d < .01$) nor did it affect HRV during either of the breathing focus periods ($p_{\text{worry induction*Condition}} = .44$, $d = .05$, $p_{\text{post-worry*Condition}} = .83$, $d = .02$).

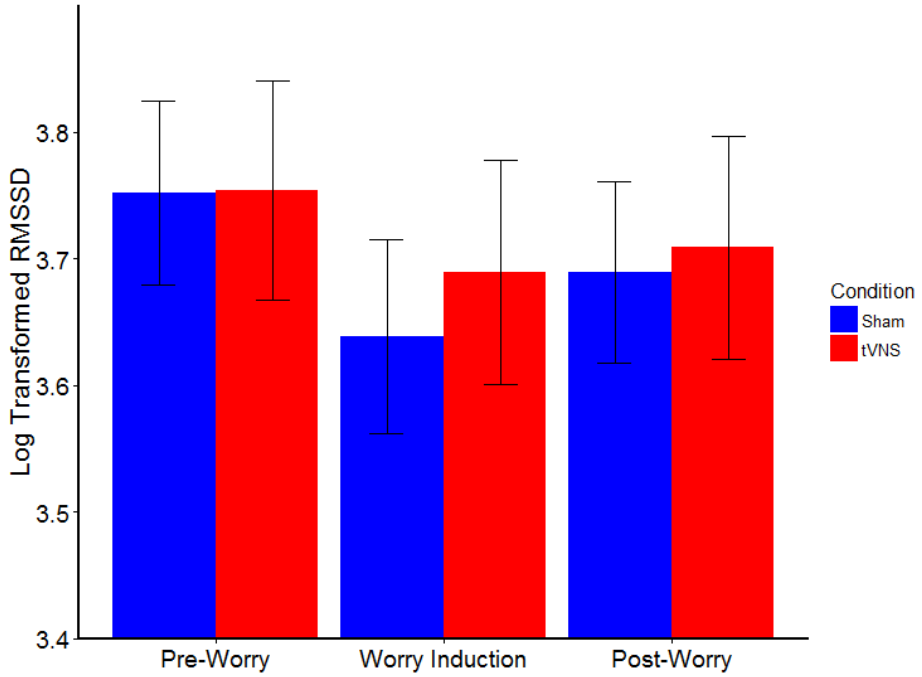


Figure 4. Log transformed RMSSD scores measured throughout the BFT (Error bars denote between-subjects standard errors). Participants in both conditions showed a decrease in RMSSD during the worry induction phase. During the post-worry period, RMSSD scores were still reduced. There were no significant effects of tVNS on RMSSD levels.

Side Effects

Participants in the tVNS condition reported more burning sensations ($Median_{tVNS}$ = 2.5 (Interquartile Range or IQR = 2 - 3), $Median_{Sham}$ = 1 (IQR = 1-2); $\chi^2(1) = 8.22$, $p = .004$) and more stinging sensations ($Median_{tVNS}$ = 4 (IQR = 3-4), $Median_{Sham}$ = 3 (IQR = 2-4); $\chi^2(1) = 7.97$, $p = .005$) as a result of stimulation compared to participants who received sham stimulation. There were no significant differences between groups on any of the other side-effects that were assessed (all $p > .08$).

Discussion

We tested whether short-term transcutaneous stimulation of the vagus nerve reduces worrying in a population of high worriers. Participants were randomized to receive either tVNS or sham stimulation during a Breathing Focus Task (BFT). The BFT measures the frequency of negative thought intrusions over two five minute periods as an index of worry propensity ('spontaneous' and 'induced' worry) [275]. Participants who received tVNS reported fewer negative thought intrusions during the pre-worry period. However, after a worry induction period, participants who received tVNS no longer significantly differed from participants who received sham stimulation in the amount of negative thought intrusions they reported during the post-worry period. We did observe an unexpected higher proportion of participants reporting negative thought intrusions in the tVNS group immediately after the start of post-worry period, which declined more rapidly over the course of the post-worry period than in the sham group.

Participants in the tVNS condition reported significantly fewer negative thought intrusions during the pre-worry breathing focus period. These results are in line with experimental studies indicating that tVNS has acute effects on cognitive processes, including inhibitory control [89,164], associative learning [165,196], and mood [123]. Additionally, these results correspond with treatment studies which have shown that tVNS may affect anxiety symptoms in patients suffering from major depressive disorders [91,92]. The results also seem to be in line with the neurovisceral integration model, which suggests that vagus nerve activity is associated with increased inhibitory control [64], which is believed to be impaired in chronic worriers and may contribute to the perceived uncontrollability of worrying [288].

We can only speculate on the neurobiological mechanisms underlying the effect of tVNS on worrying. Previous studies have found that stimulating the vagus nerve leads to increased functional connectivity between the PFC and the amygdala [50,80], which has been found to be attenuated in high worriers [82–84]. Alternatively, resting state fMRI studies have repeatedly, although not consistently, shown that tVNS is related to reduced activity in areas related to the Default Mode Network (DMN), notably the anterior cingulate gyrus, precuneus, and superior medial frontal gyrus (for an overview of tVNS effects on resting-state fMRI, see [270]). The DMN is thought to play an important role in self-referential thought and worry [73,83,289]. The reduction in negative thought intrusions during the pre-worry period could therefore be attributed to changes in activity in the DMN. However, stimulation of the earlobe (the sham stimulation condition in this experiment) has shown similar decreases in activity in these areas (possibly through the activation of the great auricular nerve) and no consistent significant differences in deactivation patterns were found in these areas between

participants who receive tVNS and participants who receive sham stimulation [92,270]. These results may indicate that earlobe stimulation may have also decreased the activation of the DMN and thereby the amount of negative thought intrusions.

After an explicit instruction to 'worry as you normally do' for 5 minutes, there was no longer an effect of tVNS on negative thought intrusions during the subsequent breathing focus period. An exploratory analysis of responses on a probe level within the post-worry period revealed that participants in the tVNS condition initially reported negative thought intrusions more frequently, although this difference between groups was not statistically significant. By contrast, the proportion of participants who reported negative thought intrusions decreased more rapidly in the tVNS condition than in the sham condition.

Despite being a group of high worriers, participants reported a relatively low amount of negative thought intrusions on average. Indeed, when compared to a study that tested participants without excessive worry complaints [290], participants in our current study reported a similar amount of negative thought intrusions during the post-worry period, and only reported slightly more negative thought intrusions in the pre-worry period. Previous studies in high worriers and GAD patients report higher average numbers of thought intrusions during either period of the BFT (high worriers: pre-worry = 2.3, post-worry = 3.4, [273]; GAD patients: pre-worry = 3.2, post-worry = 3.8, [274]). A methodological difference between the current study and previous studies that have employed the BFT is that these previous studies often asked participants to immediately give a short description of the content of their reported thought intrusions. Possibly this request to articulate thought intrusions may have increased the probability of new thought intrusions occurring at subsequent prompts. Alternatively, the relatively low number of negative thought intrusions reported in both groups in this study may have been affected by a reduction in DMN activity caused by both tVNS and stimulation of the earlobe, as mentioned above.

As participants were not required to articulate the contents of their thought intrusions, we cannot be certain that their negative thought intrusions always represented worry episodes. Indeed, the lack of significant effects of tVNS on retrospective worry assessments raises concerns about the validity of this assessment. On the other hand, there was a high correlation (.67) between the post-hoc worry assessment and negative intrusions reported during the breathing focus phases, indicating that 45% of the variance in negative intrusions can be explained by inter-individual differences in post-hoc worry assessment. This percentage is remarkably high considering the differences between what is being measured (repeated dichotomous point assessments compared to retrospective assessment of total worry duration) and confirms that the negative thought intrusions reported by participants reflect online assessments of worry frequency during the breathing focus phases.

The results of the current study may have been influenced by demand characteristics of the experimental procedure. During the worry assessments we asked our participants to report how long they had worried before and after a worry induction. Obviously, participants were aware of experimenter expectations. However, no other way exists to check the manipulation, and demand effects would affect both conditions. Our main outcome measure, negative thought intrusion frequency, may not have been impervious to demand characteristics, either. One could argue that in the present circumstances, with participants who were aware that they had been selected for being high worriers, the BFT may have acted as an implicit and unintentional thought suppression task. A high worrier may interpret the instruction 'focus on your breath only' as 'do not engage in worrying'. Although participants were not instructed to suppress worry, they were asked to report transgressions from the breathing focus instructions. If the BFT should indeed be interpreted as an unintentional thought suppression task, the increased proportion of participants in the tVNS condition who reported negative thought intrusions at the start of the post-worry period could reflect a stronger rebound effect, which in turn could be due to the stronger attentional control during the initial breathing focus ('thought suppression') period. Future studies should focus on using alternative paradigms to see whether this effect of tVNS on attentional control translates to worry behavior in general.

An additional limitation of the current study is that the sensations reported after tVNS and sham stimulation were not completely identical. Participants receiving tVNS reported more stinging and burning sensations than those reporting sham stimulation, which is in line with previous reports [240,291]. It remains unknown whether and in what way these differences in physical sensations may have affected performance on the BFT. On the one hand, these sensations may have distracted participants from potential negative thought intrusions. On the other hand, the sensations could have induced negative thought intrusions related to the physical sensations. To control for this second possibility, future research could consider reinstating the BFT protocol used in previous studies where the specific content of the thought intrusions are probed [273,274].

A final potential limitation of the current study was that the baseline HRV assessments, as well as the baseline questionnaires, were conducted during the ramp-up period of tVNS. Therefore, we cannot rule out that tVNS may have affected either of these measurements. Future studies should strongly consider administering these baseline measurements prior to the ramp-up period of tVNS to ensure that baseline effects cannot be affected by stimulation.

Corresponding to earlier studies that found a relation between vagal tone as indexed by HRV and worrying [73], participants showed a significant reduction in HRV during the worry induction period, which only partially recovered during the second breathing focus period. As could be expected from the stimulation parameters used in this study, there were no significant differences between the experimental conditions during any of the breathing focus phases, nor in the worry induction phase.

The finding that stimulating the vagus nerve does not subsequently affect HRV, the most widely used index of vagal tone, might strike some readers as a paradoxical finding. However, as mentioned in the introduction, this seemingly contradictory finding can easily be explained by the fact that in this study we stimulated the left branch of the vagus nerve. The left and right vagus nerve differentially innervate the heart, with the right side preferentially innervating the sinoatrial node and the left side innervating the atrioventricular node [15]. Stimulation of the sinoatrial node leads to stronger decreases in heart rate, which is reflected by stronger bradycardia after right-sided VNS [292]. In fact, due to this asymmetric cardiac innervation, invasive VNS has traditionally stimulated solely the left vagus nerve to avoid bradycardia. The lack of cardiac effects can also be explained by taking the different fiber types of the vagus nerve into account. Specifically, tVNS is likely to stimulate primarily thick myelinated *afferent* A-fibers. By contrast, cardiac effects of the vagus nerve are primarily determined by *efferent* B-fibers, which are thin myelinated efferent fibers that have a higher conduction threshold [11]. Specifically, studies in anesthetized dogs – which provide a good model for human stimulation thresholds due to the similarity in nerve diameter and total number of axons – showed that the stimulation intensity used in this study would be sufficient to stimulate *A*-fibers (threshold 0.4 mA), but not *B*- or *C*-fibers (3.8 and 17 mA, respectively; [276]).

One could argue that activation of afferent vagal fibers should still lead to significant cardiac effects indirectly by increasing prefrontal activity and increasing the functional connectivity between the prefrontal cortex and the amygdala. However, given the relatively small effect that experimental worry inductions have on HRV in subthreshold GAD participants [75,293], and the small effect size of tVNS on negative thought intrusions found in the current study, it seems likely that the current study did not have the statistical power to detect such an effect.

Thus, importantly, the lack of cardiac effects found in this study does not invalidate tVNS as a method of activating the vagus nerve. In fact, most manufacturers actively attempt to circumvent possible cardiac side-effects. This does pose an interesting new challenge however: there is a clear need for a sensitive biomarker of vagal activity, specifically a marker that activates when low intensity stimulation activates only the A-fibers of the vagus nerve. Possible candidates for such a measure of vagal activity may include the pupillary light reflex, pupil dilation or EEG measures such as the P300 as a measure of vagal effects on noradrenergic transmission [294,295].

To conclude, the current study showed that short-term tVNS may ameliorate spontaneously occurring worry in high worriers. However, the effects of tVNS after an explicitly induced worry period are mixed. In an exploratory analysis, we observed an unexpected higher proportion of participants in the tVNS group reporting negative thought intrusions immediately after the start of the post-worry period, which declined more rapidly over the course of the post-worry period than in the sham group. As such, the current study provides partial confirmation that activation of the vagus nerve may actively

reduce worrying. These results provide interesting indications for the validity of tVNS as an intervention for worry-related psychopathology.

Part III

Working Mechanisms

Chapter 7

Transcutaneous nerve stimulation via the tragus: are we really stimulating the vagus nerve?

Burger AM, Verkuil B (2018). *Brain Stimulation, 11,* 945-946.

Dear Editor,

Research on transcutaneous vagus nerve stimulation (tVNS) is accumulating. Several studies now assessed whether stimulating the auricular branch of the vagus nerve (ABVN) affects cognitive, emotional and neurological processes, similarly to the invasive stimulation of the vagus nerve at the cervix. Currently, the main areas that are targeted during auricular stimulation are the cymba concha and the tragus [270]. Recently, Badran et al. [296] published an important study on the brain activation patterns associated with tragus stimulation, showing that tragus stimulation was associated with stronger activation in afferent vagal cerebral areas compared to sham stimulation. Crucial to our interpretation of the findings on tragus stimulation as a method to stimulate the vagal nerve is anatomical evidence that the human tragus is indeed innervated by the auricular branch of the vagus nerve. Yet, we here describe inconsistencies in the reporting of this innervation pattern in the sole, but often cited cornerstone publication. These inconsistencies imply that interpreting tragus stimulation as a method to stimulate the vagus nerve is still too premature, and that it plausible that the tragus is innervated only by the great auricular nerve and the auriculotemporal nerve.

In 2002, Peuker and Filler published an article titled “The Nerve Supply of the Human Auricle” (Clinical Anatomy 15: 35-37), in which they described an anatomical study where the nerve supplies of the ears of seven cadavers was exposed. To this date, this article remains the only detailed description of the nerve distribution of different innervation areas of the lateral surface of the human auricle.

Although older studies provide some anatomical basis for a vagal innervation of the cymba concha [297,298], vagal innervation of the tragus is based solely on this article by Peuker and Filler [25].

Unfortunately, the article contains several inconsistencies, which limit the interpretability of their findings. Specifically, the main text in the results section does not correspond with their results presented in their Table 1. Below this text, we added a table summarizing the claims from both the first table as well as the main text from the original article. Importantly, there are contradictions between the text and the table regarding the innervation of the antihelix, the tragus and the cavity of the concha (bold and underlined in the table). According to the original Table 1, the tragus is innervated by the ABVN in 45% of the exposed auricles. However, in the main text, the authors mention that the tragus is innervated either by the great auricular nerve (45% of all exposed auricles) or by the auriculotemporal nerve (9%), or by both of these nerves (46%). They do not mention that the tragus is innervated by the ABVN, and this is inconsistent with their Table 1.

We have been in contact with professor Filler, who acknowledged the inconsistency and regretfully was unable to assess which of the assertions from the manuscript was correct. Given the current inconsistency in the original article, we would like to emphasize the clear need for a replication of this study to assess the nerve supply of the human auricle. As of yet, it is not possible to conclude that the tragus of the auricle is innervated by the ABVN and this impacts the interpretation of studies using this type of auricular stimulation.

Table 1. Inconsistencies in the innervation patterns between Table 1 and the main text by Peuker & Filler (2002).

	Table 1 by Peuker & Filler (2002)			Alternative Percentages in Main Text			
	ABVN	GAN	ATN	ABVN	GAN	ATN	Double Innervation
Crus of helix	20%		80%				
Spine of helix		9%	91%				
Tail of helix		100%					
Scapha		100%					
Crura of antihelix	9%	91%					
Antihelix	73%	9%	18%	73%	18%		9% (ABVN & GAN)
Antitragus		100%					
Tragus	45%	46%	9%		45%	9%	46% (GAN & ATN)
Cymba conchae	100%						
Cavity of concha	45%	55%		45%			55% (ABVN & GAN)
Lobule of auricle		100%					

Note: ABVN = auricular branch of the vagus nerve; GAN = great auricular nerve; ATN = auriculotemporal nerve. NB. Percentages reported here suggest that 11 auricles were reported on- although the paper mentions that 14 auricles were exposed. In the case of 14 auricles, one auricle would constitute 7% of the sample, yet, the smallest percentage reported here is 9%, suggesting that 11 auricles were examined.

Chapter 8

From ear to eye? No effect of transcutaneous vagus nerve stimulation on human pupil dilation: a report of three studies

Burger AM, Van der Does W, Brosschot JF, Verkuil B. *In preparation.*

Abstract

Transcutaneous stimulation of the auricular branch of the vagus nerve (tVNS) has been proposed as a treatment for a spectrum of physical and psychological disorders. One of the proposed working mechanisms of tVNS is a modulatory effect on the locus coeruleus – noradrenaline (LC-NA) network. We tested this hypothesis in humans in a series of three studies. In all three studies, we tested whether tVNS increases resting pupil diameter – as an index of LC-NA network activity. Additionally, we tested whether tVNS affects task performance and task-related pupil dilation during an Attentional Blink task. We found no evidence that tVNS increases pupil diameter or task-related pupil dilation in any of the tasks. No effects of tVNS on the attentional blink task were found in healthy populations. Overall, these studies indicate that tVNS does not affect these behavioral and physiological indices of noradrenergic activity.

Introduction

Since the development of devices that enable transcutaneous auricular vagus nerve stimulation (tVNS), and early studies showing that tVNS indeed leads to similar fMRI activation patterns as invasive VNS (iVNS) [122,270], researchers have quickly adopted this procedure and have tested its application in a wide variety of clinical and experimental paradigms. Echoing the widespread theorized applications of iVNS [299], tVNS has recently been proposed as a potential treatment for a wide spectrum of physical and psychological problems, including but not limited to epilepsy, depression, tinnitus, motor rehabilitation, autism, and pain (e.g. [300–303]). However, the working mechanisms of VNS are currently poorly understood, and are based primarily on preclinical iVNS research [94]. Thus, there is a clear need for more fundamental research on the working mechanisms underlying the effects of tVNS in humans.

The main working mechanism hypothesized to underlie the effects of tVNS on psychological and neurological disorders is the increased activity of the locus coeruleus – noradrenaline (LC-NA) system. Specifically, afferent fibers of the vagus nerve are known to terminate in the nucleus of the solitary tract, from which there are direct and indirect routes that both activate and inhibit neurons in the LC [12]. Indeed, animal studies that tested the effects of invasive VNS have repeatedly found that rats receiving VNS, compared to those that had undergone sham surgery, show increased firing rates in LC neurons both acutely [45,95–98] as well as over a longer timespan (after a period of 90 days: [95]; after 14 and 90 days: [99]). In line with these findings, several studies found increased concentrations of NE in brain areas to which the LC projects, including the hippocampus [304,305], basolateral amygdala [115] and medial PFC [144].

Although the effect of VNS on LC and noradrenergic activity is well established in animals, studies on the noradrenergic effects of (t)VNS in humans is lacking. Unfortunately, direct measurement of NE in humans requires an invasive procedure and suffers from poor reliability and sensitivity [100]. Several indirect physiological markers have been proposed as suitable measurements of NE in humans. One very recent pilot study has already assessed the effects of tVNS on two of these measures, the P300 and salivary alpha amylase (sAA) [271,291]. In that study, tVNS did not affect task performance on the oddball task [291], although tVNS did facilitate some indices of conflict processing during a Simon task [271] – a process that is believed to be mediated by the LC-NA network [306]. Physiologically, tVNS did not lead to a significantly stronger increase in sAA compared to sham stimulation. Additionally, tVNS did not significantly increase P300 during an oddball task [291], nor during the Simon task [271]. It should be noted, however, that this pilot study may have lacked statistical power, and effects of tVNS on sAA and the P300 did point in the hypothesized direction.

Here, we tested if tVNS affects the pupil diameter, as an index of noradrenergic activity. Pupil diameter has the distinct advantage that it can be used as an indicator of both tonic and phasic LC activity, by measuring pupil diameter during rest or task performance, respectively. Specifically, increased activity in the LC-NA system increases activity in the pupil's dilator muscle and inhibits activity in the sphincter muscle, thereby promoting pupil dilation [307]. Indeed, pupil diameter shows strong positive correlations with LC activity in monkeys [308,309]. In humans, these findings are corroborated by pharmacological studies showing that administration of α2-adrenoreceptor agonists leads to a constriction of the pupil, whereas α2-adrenoreceptor antagonists lead to a dilation of the pupil [310–312]. Finally, in line with the adaptive gain theory of LC-NE function [313], pupil diameter is larger during exploratory compared to exploitative task performance [294,314].

The effects of iVNS on pupillometry have only been described in three studies so far. In rats, iVNS has been shown to increase pupil diameter during rest, reflecting increased tonic LC-NA activity [315]. In humans, the effects of VNS on pupil diameter have been studied in patients suffering from refractory epilepsy. Although one study reported increased resting pupil diameters during periods when VNS was turned on compared to when it was turned off [102], a subsequent study failed to replicate this effect. Both studies on the effects of iVNS in humans suffered from relatively small sample sizes, and the lack of significant differences between stimulation turned off and on in the latter study may have been due to low statistical power. No studies have been published thus far that have assessed the effects of transcutaneous VNS on pupil dilation.

Next to testing the effects of tVNS on resting pupil diameter, we also tested the effects of tVNS on pupil dilation during an attentional blink (AB) task. Both pupil dilation and AB task performance have been suggested to reflect noradrenergic activity. During an AB task, participants are instructed to identify two distinct targets (e.g. digits) within a series of stimuli (e.g. letters) rapidly appearing on a computer screen. The difficulty of identifying the second target after having identified the first one is strongly related to the temporal proximity of the targets: when the second target appears approximately 200ms after the first one, it becomes a lot harder to identify the second target than when it appears considerably later (usually 700ms). This phenomenon is called the *attentional blink* (AB) and has is thought to be caused by with the temporary refractory period of LC neuron activity after the initial burst that occurred when the first target was correctly identified [316]. Indeed, attentional blink occurrence has been found to be positively related to other measures of noradrenergic activity such as pupil dilation [317] and P300 amplitude [295], and single cell recordings in monkeys have confirmed that the attentional blink timeframe coincides with the refractory period of LC neuron firing after seeing a first target [318]. Finally, a neuropharmacological study has shown that the β-adrenergic blockade with propranolol increases the magnitude of the attentional blink, whereas the selective NA reuptake inhibitor reboxetine decreases it (especially for emotionally salient

stimuli); [319]. Other neuropharmacological studies in which central NA levels were manipulated have failed to find these effects, however [320,321].

Considering the large number of tVNS papers in recent years, and the lack of effective and clinically meaningful biomarkers, we considered it timely to test the main monoaminergic working mechanism hypothesis of tVNS. In a series of three studies, we tested whether tVNS increased noradrenergic activity in humans. We measured noradrenergic activity indirectly both physiologically (i.e. dilation of the pupil) as well as through behavioral measures (i.e. accuracy at detecting the target stimuli during the attentional blink task). We hypothesized that tVNS would increase noradrenergic activity, as evidenced by a greater overall dilation as well as a greater task-related dilation of the pupil compared to sham stimulation. We also hypothesized that this increased noradrenergic activity associated with tVNS would be reflected in increased response accuracies during the AB task.

These hypotheses were tested in three separate studies. The first study was part of a larger project that aimed to test the effects of tVNS on negative thought intrusions in high-trait worriers [322]. In this first study, we assessed the effects of tVNS on resting pupil diameter and accuracy in a version of the AB task that included both neutral and negatively valenced trials. This was based on the finding that the NA reuptake inhibitor reboxetine selectively decreased the attentional blink for emotionally relevant stimuli, and not for neutral ones [319]. In the second study, we conducted a within-subject study to assess the effects of tVNS and sham stimulation on a non-emotional version of the AB task in a sample of healthy college students. The third study was a between-subject experiment in healthy college students, again using an emotional version of the AB task.. The second and third study also included task-related pupil dilation measurements in addition to resting pupil diameter and accuracy, to assess potential effects of tVNS on phasic LC-NA activity.

Overall methods

Instruments and Questionnaires

Transcutaneous vagus nerve stimulation

A tVNS device provided electrical stimulation using two titanium electrodes, positioned on top of a silicon earplug, which are connected by a wire to a portable neurostimulator (Nemos®, Cerbomed, Erlangen, Germany). The electrodes delivered 30-second waves of electrical stimulation (0.5mA, 25Hz, 250µs), alternated by 30-second breaks. In the tVNS condition, the electrodes were attached to the cymba conchae, an area of the outer ear that is innervated by the vagus nerve. In the sham condition, the electrodes were connected to the center of the earlobe, which is not innervated by the vagus nerve but is innervated by the great auricular nerve [25].

Questionnaires

We included several questionnaires to ensure that there were no large between-group differences on these potentially relevant indices. All studies included the Penn State Worry Questionnaire (PSWQ) [135,277], the State Trait Anxiety Inventory (STAI) [138,139] and the Attentional Control Scale (ACS) [280,281], and several study-specific questionnaires were added separately in each study.

Heart Rate Variability

In every study, participants were asked to wear a chest strap with a sensor worn at the base of the sternum to measure cardiovascular activity through two electrodes connected to the belt (Movisens, Gmhb, Karlsruhe, Germany). Raw ECG was measured at 1024Hz and was automatically cleaned for outliers and measurements artifacts by the Movisens Data-Analyzer software.

Every study included a 5-minute baseline recording of participants' heart rate variability (HRV) to test for possible differences in baseline vagal tone. Specifically, the root mean square of the successive differences (RMSSD) between heart rates was extracted from the raw ECG signal. Unfortunately, during study 3, we experienced technical difficulties with the heart rate monitors, and thus the ECG data for these participants was not included in this study.

Pupillometry

All three studies were performed in a lab room under moderate lighting conditions of approximately 100 lux to maximize cognitively-evoked pupil dilations [323]. Pupil diameter was measured using a Tobii T120 eye tracker, which is integrated into a 17" TFT monitor. The pupil dilation measurement was carried out using Eprime 2.0 software using the Tobii extension for E-Prime. Prior to the measurement, we conducted a baseline calibration using the calibration feature of the Tobii extension

to ensure that the eye tracker could correctly capture every participant's pupil. Pupil size data was gathered at 120Hz.

Raw pupil diameter data was filtered using a low-pass filter (4 Hz) to remove jittering. Linear interpolation was applied for missing data points when sections of missing data points did not exceed 250ms. Preprocessing of pupil size data was conducted using a customized open source MATLAB script [324].

All three studies included resting pupil size measurements before and after tVNS or sham stimulation. These resting pupil size measurements were collected over periods of 2 minutes, during which time participants were instructed to focus their gaze on a fixation cross in the middle of the screen.

To test the effects of tVNS on pupil dilation during cognitive processing (studies 2 and 3), we aggregated pupil diameters into 100ms bins to match the duration of stimulus presentations within the rapid serial visual presentation (RSVP). Trial-specific pupil dilation was calculated by subtracting the average pupil diameter during the a 200ms window just prior to RSVP onset from the average pupil diameters within the RSVP. Trial specific changes in pupil diameter were rescaled from millimeters to micrometers (µm) to improve the readability of the results.

Attentional Blink Task

During each trial of the AB task, a rapid serial visual presentation (RSVP) stream of stimuli is presented in the middle of the screen at a rate of 100ms per stimulus. The RSVP stream consists mostly of distractor stimuli, and includes 2 targets (the T1 and T2) embedded in the stream (see figure 1 for an overview of an AB trial). Some versions of the AB task also include trials containing 0 or 1 targets to decrease the predictability of the AB task and enable analyses of phasic pupil dilations to the presence versus the absence of a target. Participants are instructed to identify the target stimuli, and report them after presentation of the stream. The primary outcome measure of the AB is the proportion of trials where the second target (T2) is correctly identified given that the first target (T1) had also been correctly identified (in short: T2|T1). The position of the T2 relative to the T1 is experimentally manipulated to be either 200ms (i.e. Lag 2) or 700 ms (i.e. Lag 7) after the onset of the T1. Lag 2 trials are expected to be more difficult, as the presentation of the T2 coincides with the refractory period of neurons in the LC [316].

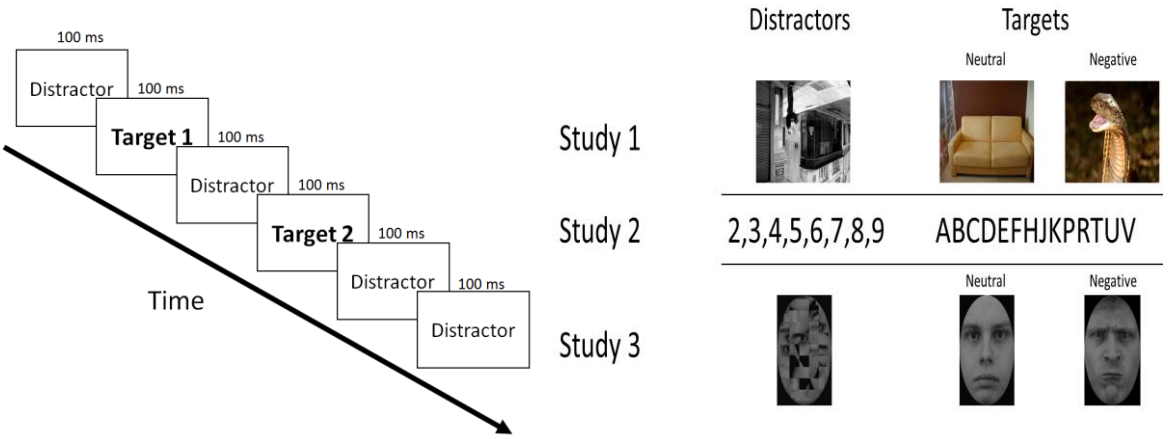


Figure 1. Overview of Attentional Blink paradigms. *Left*: Each trial consisted of a series of stimuli presented for 100 ms, immediately followed by a subsequent stimulus. Participants were instructed to identify the target pictures that were presented in the RSVP stream. In study 2 and 3, some trials consisted solely of distractor pictures, or included only one target picture. All other trials consisted of two target trials. The temporal lag between Target 1 (T1) and Target 2 (T2) was either 200ms (shown in the picture; 'Lag 2') or 700ms ('Lag 7'). 200ms stimulus onset asynchrony is believed to coincide with the refractory period of LC neuron firing, and will thus lead to larger attentional blinks. *Right*: Stimuli used as distractors and targets varied between studies. In study 1 and 3, we utilized an emotional AB task. In study 1, distractor pictures were neutral images selected from the IAPS, whereas target images were based on the ones used by de Oca [45]. In study 2, we used digits as distractors, and letters as targets. In study 3, we used cropped and framed greyscale pictures of the Karolinska Directed Emotional Faces Database.

Statistical Analyses

To test whether tVNS affects resting pupil diameter, we conducted linear mixed models in all three studies. Specifically, we tested how pupil diameter was affected by *Condition* (0 = sham stimulation, 1 = tVNS) and *Measurement* (categorical variable, reference category is the pre-stimulation baseline measurement).

The AB Task measures the proportion of trials where the second target (T2) is correctly identified given that the first target (T1) had also been correctly identified (in short: T2|T1). Since T2|T1 is a proportion and thus bound between 0 and 1 (or 0% accurate and 100% accurate), it does not fulfill the criterion for a continuous and normally distributed outcome variable. This is a point that has often been overlooked in prior studies on the AB task, but can hamper the validity and statistical power of analyses that rely on this assumption [325]. Therefore, we applied a logit transformation, $\log(\frac{p}{1-p})$, which makes the dependent variable unbounded and allows for regular linear mixed modelling [325]. As the logit transformation cannot be applied to proportions of 0 or 1, we added .001 to the scores of participants who were 0% accurate at detecting T2|T1. Similarly, we subtracted .001 from scores of participants who were 100% accurate at detecting T2|T1.

We conducted linear mixed models to test how T2|T1 is affected by *Condition* (sham vs tVNS), and *Lag* (temporal proximity between targets: a categorical variable with two levels: lag 2 and lag 7, reference category is lag 2). In studies 1 and 3, targets of the AB task varied in their emotional valence, and thus a variable *Valence* was included to differentiate $T1_{neutral}$-$T2_{neutral}$, $T1_{neutral}$-$T2_{negative}$, and $T1_{negative}$-$T2_{neutral}$ trials (categorical variable, reference category is $T1_{neutral}$ – $T2_{neutral}$).

Finally, in study 2 and 3 we additionally conducted linear mixed models to test the effects of tVNS on pupil dilation during trials of the AB task. Specifically, we tested the effects of *Condition* and *Time* (continuous variable, indicating the 100ms time bin corresponding with one stimulus presentation with an AB trial) on baseline-corrected pupil diameter.

All linear models included random intercepts. In study 2, we created a three-level nesting structure where random intercepts were included to the model for both testing sessions, to account for the fact that measurements were nested within sessions, which were nested within participants. Models that tested the effects of tVNS on pupil dilation during AB trials additionally included random slopes to account for inter-individual differences in pupil dilation over time.

All analyses were conducted in R using the *lme4* and *lmerTest* packages.

Study 1

Methods

Participants

We aimed to test 102 chronically worrying students between the ages 18-25. Participants could only participate in this study if they scored at least 45 on the Penn State Worry Questionnaire (PSWQ). Choosing a cut-off score of 45 ensured a selection that was highly sensitive for chronic worry in an advertised-for population [219]. Participants with internal or neurological comorbidities were excluded from the current study.

Ethical approval for this study was given by the ethical committee of the Institute of Psychology of Leiden University. Participants were rewarded with 10 euros or partial course credit for participating in this study.

Procedure

This study was part of a larger project focused on assessing the effects of tVNS on worry behavior as well as stress-related attentional biases. This larger study has been preregistered on the Open Science Framework, https://osf.io/za9mu.

After showing interest in this study, participants received a link via email asking them to fill in the PSWQ online. Participants who scored 45 or higher on the PSWQ were invited to the lab. In case participants scored lower than 45, researchers received a confirmation that the participant had not fulfilled the study criteria and the questionnaire was locked for that particular IP address, to ensure participants could not retake the questionnaire. Participants were subsequently informed that they did not fulfill the criteria for participating in the study.

All participants provided informed consent prior to the start of the experiment. Afterwards, participants were instructed to wear an ECG chest strap, which would measure their heart rate throughout the remained of the study. Subsequently, a 2-minute pupillometry measurement was conducted. During this baseline recording, participants were instructed to simply look at a fixation cross in the middle of a screen. Afterwards, the tVNS device was attached to the participant's left ear, and participants received either tVNS or sham stimulation throughout the rest of the experimental session.

Since not much is known about the temporal latency of the effects of tVNS [122], a short build-up period of the effects of tVNS was used during which participants were instructed to first complete a five-minute baseline recording of HRV and subsequently complete several questionnaires prior to the experimental tasks. The questionnaires included the ones mentioned in the General Methods

section, plus the Generalized Anxiety Disorder-7 (GAD-7) [278,279] and the Ruminative Response Scale (RRS) [282]. On average, filling in the questionnaires took approximately 15 minutes.

After filling in the questionnaires, participants were instructed to complete a Breathing Focus Task, which consisted of two breathing focus phases separated by a worry induction. Subsequent to the Breathing Focus Task, participants completed a second pupillometry measurement, followed by the Attentional Blink Task and an Inhibition of Return Task. Finally, participants were instructed to complete one final pupillometry measurement. The results of the Breathing Focus Task and the Inhibition of Return Task are beyond the scope of this article and are described elsewhere.

In total, the experimental procedure lasted approximately 90 minutes. Participants received tVNS or sham stimulation for roughly 80 minutes.

Instruments

Attentional Blink Task

The AB task consisted of 108 trials. During every trial, participants were presented with 16 pictures including 14 distractors and two targets (T1 and T2). Distractors were 118 pictures selected from the International Affective Picture System (IAPS), based on their low scores on arousal and valence [326]. Distractors were depicted in greyscale and were presented upside-down. In contrast, target pictures were presented as coloured, upright pictures. Target pictures were based on the ones chosen by De Oca and colleagues [327], and could be subdivided into three neutral categories (trees, sofas, lamps) and three negative categories (guns, blood/injuries, and snakes). Picture categories were matched on luminosity to reduce the risk of certain categories 'popping out' and thereby being easier to identify.

The AB task was subdivided into three order conditions, to test non-emotional attentional blinks ($T1_{neutral}$-$T2_{neutral}$), emotional disengagement ($T1_{negative}$-$T2_{neutral}$), and emotional engagement ($T1_{neutral}$-$T2_{negative}$) (cf. [319]). The first target always appeared at RSVP location 4, 5 or 6. The second target was presented either 200ms (*lag 2*) or 700ms (*lag* 7) after the onset of T1. Thus, participants completed 18 trials of every order-lag combination. For a graphical overview of the Attentional Blink task, see figure 1.

Recordings of pupil size at rest

As described above, participants were instructed complete a pupil size measurement three times over the course of the experiment: one time before starting tVNS or sham stimulation, once more after the first computer task, and one last time at the end of the experiment. During every recording of pupil size at rest, participants were instructed to sit still and look at a fixation cross in the middle of the screen for two minutes. Both the fixation cross and the background were presented in isoluminant colours [328].

Results

Participants

Out of 132 students who initially signed up for the study, 123 filled in the PSWQ that was sent prior to the experimental session. Of these 123 students, 114 scored 45 or higher and were invited to the lab. 98 students accepted the invitation and participated in the lab session. Unfortunately, due to mechanical problems with the Tobii eyetracker and the tVNS device, only 94 participants completed the experimental procedure and were included in the subsequent analyses.

As shown in table 1, there were no significant differences between participants in the tVNS and sham conditions on any of the questionnaires, nor on baseline resting levels of RMSSD. The average score on the PSWQ for both conditions falls in the 90th percentile of the general population and the 30th percentile of a GAD-patient population [142]. Likewise, the average score on the GAD-7 fell within the range of mild to moderate clinical anxiety, which is in the 90th percentile of the general population ($M_{GAD\text{-}7}$ =3.0, [279]). Thus, the scores on these questionnaires suggests that the sample included in this study is indeed a subclinical, high trait worrying sample.

Compared to the general population, participants in both conditions scored above average on state and trait anxiety (STAI; [143]). Similarly, compared to general student populations, participants scored above average on rumination (RRS; [286]), and below average on attentional control (ACS; [287]). This is in line with earlier studies showing that attentional control is reduced in chronic worriers [329,330].

Table 1. Baseline demographics for every study.

	Study 1		Study 2	Study 3	
	Sham (*N* = 49)	tVNS (*N* = 45)	(*N* = 30)	Sham (*N* = 40)	tVNS(*N* = 40)
PSWQ	60.41 (7.79)	62.16 (7.49)	45.90 (13.09)	49.95 (10.91)	47.25 (11.70)
STAI-S	45.65 (9.61)	43.67 (9.59)	-	-	-
STAI-T	48.85 (9.32)	49.09 (10.76)	35.97 (7.38)	35.25 (8.24)	35.73 (8.83)
ACS	46.42 (9.12)	47.80 (7.53)	52.37 (6.79)	52.78 (8.96)	50.63 (7.53)
RRS	50.69 (12.25)	49.31 (13.47)	-	-	-
GAD-7[1]	9.13 (4.31)	8.83 (5.01)	-	-	-
QIDS	-	-	4.31 (2.66)	4.28 (3.29)	4.90 (3.09)
Log Baseline RMSSD[2]	3.60 (0.53)	3.63 (0.64)	3.48 (0.59)	-	-

Note. Independent samples t-tests revealed no statistically significant differences between experimental conditions on any baseline questionnaire in study 1 and 3. Study 2 used a cross-over design, so questionnaire scores apply to both the tVNS group as well as the sham group.

[1]: N_{sham} = 40/ N_{tVNS} = 40 for the GAD-7. This questionnaire was added after data acquisition had already started as an additional check to ensure that the current sample consisted of high-trait worriers.

[2]: Due to connectivity issues with the ECG chest belt leading to excessive measurement artifacts, RMSSD data of 2 participants in study 1 was not recorded (n_{tVNS} = 1, n_{Sham} = 1). In study 2, RMSSD data of 8 baseline measurements had to be removed due to connectivity issues. In study 3, the chest belts malfunctioned altogether, and so the RMSSD data collected in this study is not reported.

Resting Pupil Diameter

Participants showed a significant decline in pupil diameter from the baseline measurement to 40 minutes after stimulation onset, b = -0.38 (0.03), $t(178)$ = -11.35, p < .001. 80 minutes after stimulation onset, pupil diameters were still reduced in both groups, b = -0.15 (0.03), $t(171)$ = -4.54, p < .001.

There were no significant between-group differences in pupil diameter between participants in the tVNS condition and those in the sham condition prior to stimulation onset, b = -0.08 (0.13), $t(94)$ = -0.73, p = .47. There were also no differences in pupil diameter between conditions after approximately 35 minutes of stimulation, b = -0.01 (0.05), $t(178)$ = 0.12, p = .89, or after approximately 80 minutes of stimulation, b =< -0.01 (0.05), $t(173)$ = < 0.01, p > .99.

Behavioral Effects

Participants in both conditions were significantly more accurate at detecting T2|T1 when the temporal lag between T1 and T2 was 700ms (i.e. lag 7) compared to when it was 200ms (i.e. lag 2), $t(470)$ = 9.75, p < .001, indicating an attentional blink at short temporal latencies. When the second target was

negative, T2|T1 accuracy was significantly increased, as indicated by the main effect of Valence-$_{T2=Negative}$, b = 2.13 (0.36), t(470) = 5.87, p < .001. This above-mentioned effects of T2 valence was smaller during lag 7 compared to lag 2, as reflected by the Lag*Valence$_{T2=Negative}$ interaction, b = -1.33 (0.51), t(470) = -2.59, p = .01. By contrast, when the first target was negative, T2|T1 accuracy significantly decreased, as reflected by the main effect of Valence$_{T1=Negative}$, b = -0.78 (0.36), t(470) = -2.15, p = .03.

There was no main effect of Condition on T2|T1 accuracy. However, there was a significant interaction between Valence$_{T2=Negative}$*Condition, b = -1.15 (0.53), t(465) = -2.19, p = .03. This effect indicates that participants in the tVNS condition showed less attention to threatening stimuli than participants in the sham condition, as suggested by the lower T2|T1 accuracies in trials that included a negative T2. All other main interaction effects of Condition, Lag, and Valence were not significant.

Discussion

In a group of high-trait worriers, no effect of tVNS on resting pupil diameter was observed. Our hypothesis that tVNS increases activity in the LC-NA system was not supported.

Participants who received tVNS displayed larger attentional blinks specifically during trials where the second target was threatening, indicating that participants receiving tVNS displayed reduced attentional engagement to threat compared to those who received sham stimulation. If tVNS would increase NE activity, as we hypothesized, this finding stands in contrast to a previous study, in which increasing NE activity through administration of reboxetine reduced the attentional blink for emotionally valenced stimuli, whereas the adrenergic receptor antagonist propranolol had an opposite effect [319]. These results would indicate that tVNS may have decreased instead of increased LC-NA activity. It should be noted, however, that this previous study tested a sample of healthy college students, whereas participants in the current study were specifically selected for being high-trait worriers. This sample may have already been experiencing more increased arousal during task performance than average participants would have, and a further increase in arousal through noradrenergic modulation may have actually worsened task performance in line with the inverted U-shape function of arousal [313].

Overall, the results from this study provide no clear indications that tVNS increases activity in the LC-NA system, although the effects of tVNS on the accuracy during emotional AB trials may suggest some involvement in emotional attentional control linked to LC activity. The current study had three clear limitations. Firstly, inter-individual differences in baseline pupil size may have limited our ability to assess the effects of tVNS on NA-mediated dilation in pupil size. Secondly, the stimuli used in the current AB task were not matched on luminance (i.e. the target trials were presented in colour, whereas the distractors were presented in greyscale), and thus we were unable to adequately assess

the effects of tVNS on task-related pupil dilation, a marker of phasic NA activity. Finally, it remains unclear whether the lack of effects that tVNS had on the resting pupil diameters in high-trait worriers is indicative of this population, or whether tVNS does not affect pupil dilation in general. We designed a second study to address these limitations and to test the effects of tVNS in the general population, using a within-subjects design.

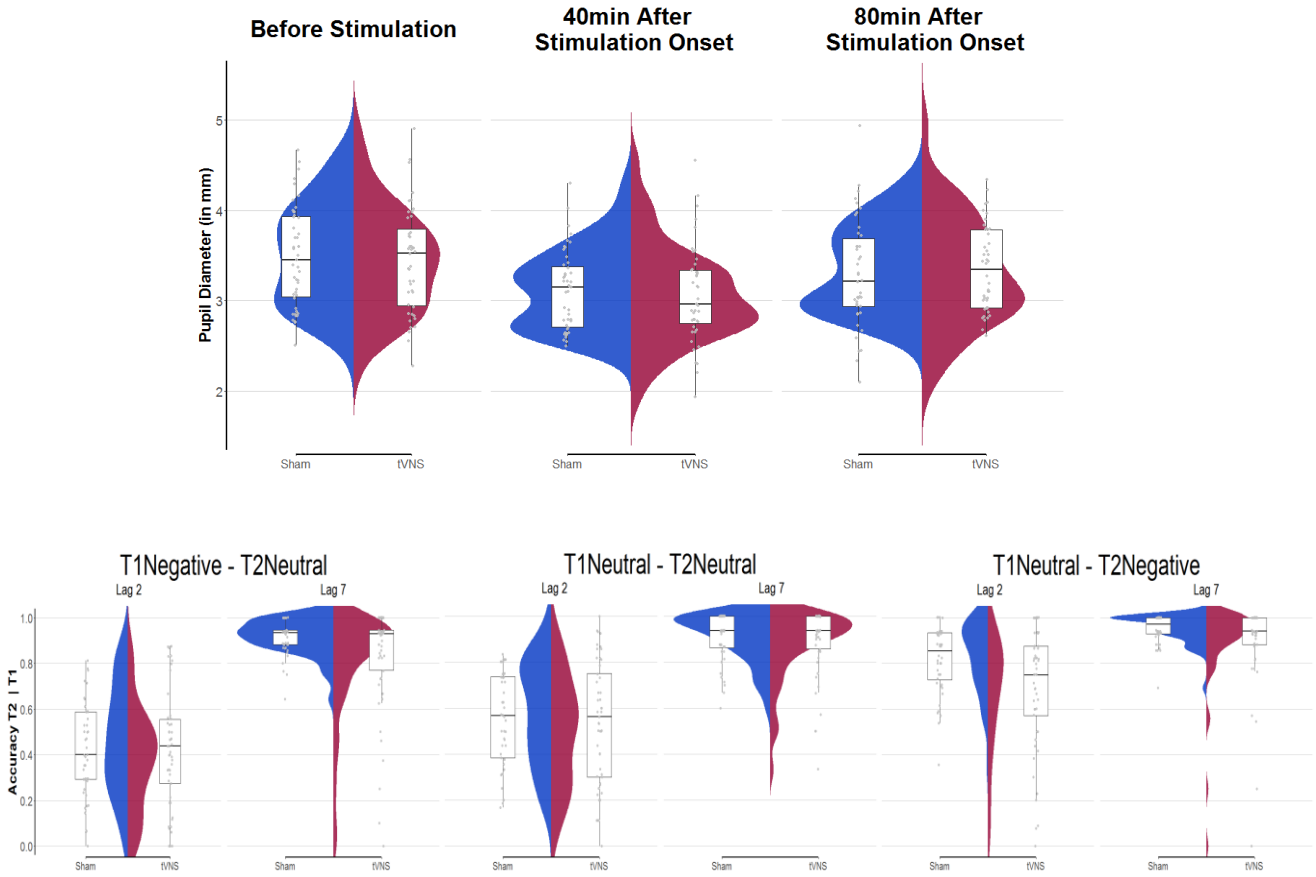


Figure 2. Accuracies and Resting Pupil Diameters for participants in the tVNS and sham condition in study 1. Top row: violin plots and boxplots of resting pupil diameters before stimulation, 40 minutes after stimulation onset, and 80 minutes after stimulation onset. Pupil diameter was recorded in 2-minute baseline recordings. Bottom row: Violinplots and boxplots of participants' accuracy at correctly identifying T2 after having correctly identified T1. Response accuracies are given separately for each T1-T2 valence condition and for different lags.

Study 2

Methods

Participants

We aimed to include 30 participants in this randomized crossover study. Participants with internal or neurological comorbidities were excluded from the current study.

Ethical approval for this study was given by the ethical committee of the Institute of Psychology of Leiden University. Participants were rewarded with 13 euros or partial course credit for participating in this study.

Design

The second study was a randomized crossover study where participants completed the AB task twice over 2 weeks, while receiving tVNS or sham stimulation during either phase. The order in which participants received tVNS was assigned randomly using the *RandomizR* function in R. The second test phase occurred one week after the first, at the same time of day as the first measurement so as to eliminate daily rhythmic changes in pupil dilation.

Procedure

Prior to the first session, participants received an email that contained a link to a set of questionnaires. Participants were asked to fill in these questionnaires, after which they were invited to the lab to complete the first experimental session. Participants provided informed consent prior to the start of the first experimental session. In case informed consent was not provided by the participant, any data from the questionnaire filled in by the individual was removed.

At the start of each test session, participants were fitted with the ECG chest strap. Afterwards, participants were instructed to complete a questionnaire asking them about sleep, caffeine intake and current mood and arousal. Afterwards, participants were instructed to complete a baseline measurement of pupil size as well as HRV.

After this initial baseline measurement, the tVNS device was placed on either the earlobe or the concha of the participant's left ear. Participants were allowed to read a magazine of their choosing for the next five minutes, to allow for a short build-up period of the effects of tVNS. After this five-minute break, another pupil size measurement was conducted.

After this second pupil size measurement, participants were instructed to complete an AB task. We measured pupil dilation throughout the task. After the AB task, participants were asked to complete

one final two-minute pupil size measurement. Finally, participants were prompted to answer several questions regarding the side-effects they had experienced during the task.

In total, the experimental procedure lasted approximately 40 minutes. Participants received tVNS or sham stimulation for roughly 32 minutes.

Attentional Blink Task

The AB task consisted of 180 trials, divided into three blocks of 60 trials. Participants were allowed to take a short break between every block. Every block contained 40 two-target trials, 10 one-target trials, and 10 zero target trials.

Each trial was preceded by a fixation cross which appeared in the middle of the screen for 2 seconds. Subsequently, participants watched an RSVP consisting of 19 stimuli. Stimuli consisted of the numbers 2-9 (distractors) and the capital letters ABCDEFHJKPRTUV (targets). These stimuli were selected because they present the least risk of distractor-target confusion (e.g. the letter L and the number 1 could easily be mistaken for each other) and are almost equal in size (e.g. W is larger than V, and thus may elicit a larger pupillary light reflex). Stimuli were presented on the screen for 100ms. The first target appeared at RSVP location 4, 5 or 6. After the first target, a second target could appear at lag 2 or lag 7 relative to the position of the first target. For a graphical overview of the Attentional Blink task, see figure 1.

At the end of each trial, the RSVP was followed by a dot or a semicolon. Participants had to report on what symbol was shown in order to ensure that the participants kept their attention on the trial until every target or distractor had been shown [331]. Participants were asked to type in which targets they had seen as well as whether the RSVP was followed by a dot or semicolon.

Results

Participants

Out of the 32 students who enrolled in this two-part cross-over study, 30 participants (5 male, 27 female) completed both experimental sessions of the experiment. Two participants dropped out after the first experimental session and were thus excluded from the statistical analyses.

Participants' scores on the baseline questionnaires and baseline resting RMSSD are presented in table 1. Scores on the PSWQ, ACS, STAI-T, QIDS, as well as baseline resting RMSSD corresponded with normative samples [142,143,287,332,333].

Resting Pupil

Participants showed a significant decrease in pupil diameter from pre-stimulation baseline to 5 minutes after stimulation onset, b = -0.11 (0.04), $t(118)$ = -3.00, p = .002. Thirty minutes after stimulation onset, participants showed a further decrease in pupil diameter compared to pre-stimulation baseline, b = 0.22 (0.03), $t(118)$ = -6.24, p < .001. There were no overall effects of Condition (p =.34) on pupil diameter. Additionally, there were no significant differences between conditions in the extent to which pupil dilated from pre-stimulation baseline to 5 minutes after stimulation onset (p = .71), nor from pre-stimulation baseline to 30 minutes after stimulation onset (p = .87).

Behavioral Effects

Participants displayed significantly higher T2|T1 accuracies in lag 7 trials compared to lag 2 trials, indicative of an attentional blink, b = 2.91 (0.33), $t(91.62)$ = 8.88, p <.001. Participants did not display higher accuracies at detecting T2|T1 in the session where they received tVNS compared to when they received sham stimulation, as reflected in the non-significant main effect of tVNS (p = .98) and the non-significant Condition*Lag interaction (p = .76).

Phasic Pupil Dilation

As can be seen in figure 3, participants displayed a significant pupillary dilation during trial presentation, as reflected in the main effect of Time, b = 8.54 (1.33), $t(35)$ = 6.44, p < .001. There was no significant effect of tVNS on the size of this dilatory response, as indicated by the non-significant main effect of Condition, p = .72, and the non-significant Time*Condition interaction, p = .83.

Discussion

In this within-subjects cross-over study, measurements of resting pupil diameter, AB task accuracy, and task-related pupil dilation showed no significant differences between sessions where participants received tVNS compared to when they received sham stimulation. Similarly to the first study – yet despite the methodological differences between these studies -, the results from this study are not in line with our hypotheses and provide no indications that tVNS increases activity in the LC-NA system.

Contrary to the first study, the second study included only a non-emotional variant of the AB task and found no differences between participants receiving tVNS and sham stimulation. We performed a final study to test the effects of tVNS on pupil diameter, task-related pupil dilation and task performance during an emotional AB task in a general student population.

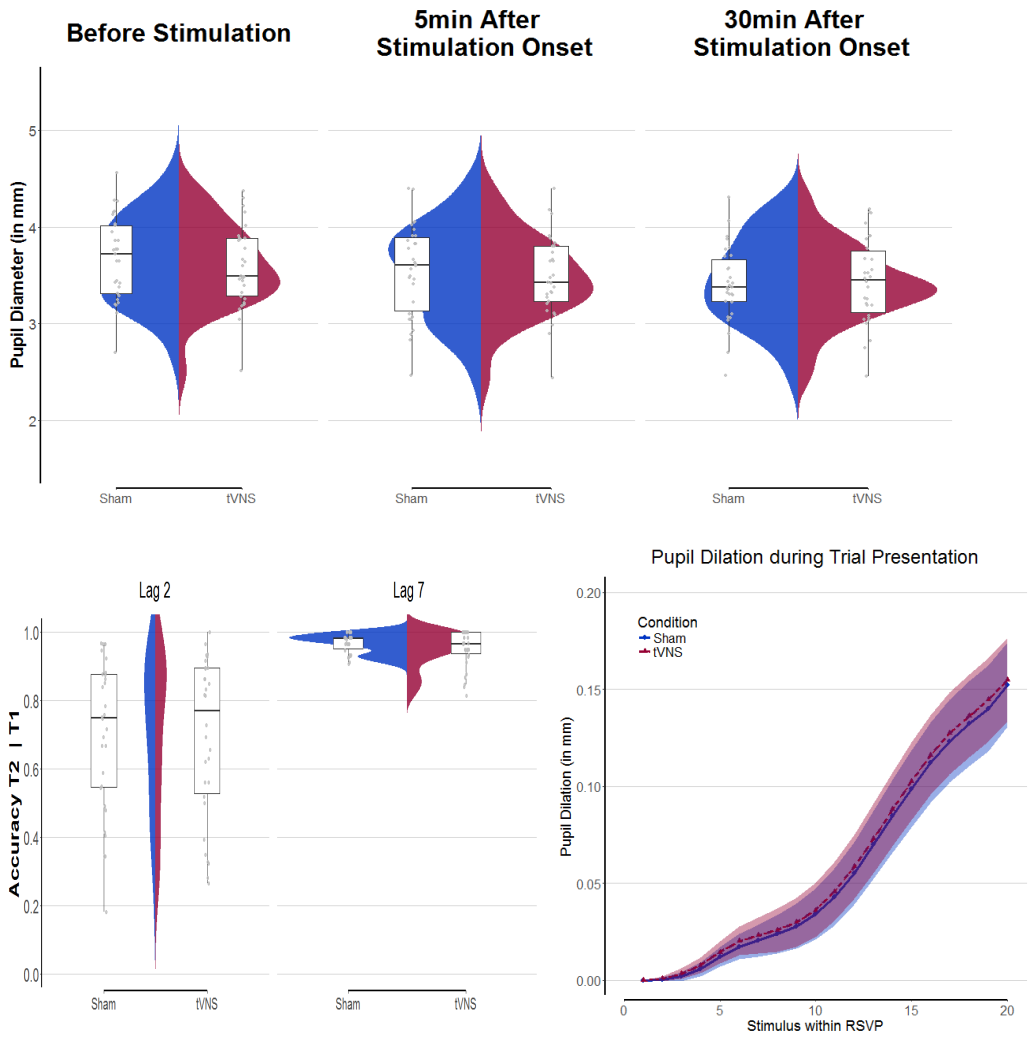


Figure 3. Accuracies, Pupil Dilation, and resting Pupil Diameters for participants in the tVNS and sham condition in study 2. *Top row*: Violinplots and boxplots of participants' accuracy at correctly identifying T2 after having correctly identified T1. Response accuracies are given separately for different lags. *Bottom Left*: violin plots and boxplots of resting pupil diameters before stimulation, directly after stimulation onset, and 30 minutes after stimulation onset. Pupil diameter was recorded in 2-minute baseline recordings. *Bottom Right*: Pupil dilation over the course of an AB trial for participants in the tVNS and sham conditions. Confidence interval reflects ± 1 standard error.

Study 3

Methods

Participants

We aimed to include 80 students from Leiden University between the ages 18-28 in this study. Participants with internal or neurological comorbidities were excluded from the current study. Ethical approval for this study was given by the ethical committee of the Institute of Psychology of Leiden University. Participants were rewarded with 7 euros or partial course credit for participating in this study.

Procedure

Participants applied to participate in this trial by signing up via a University-run website, or by sending an email to the first author. Participants then received a link via email, asking them to fill in several questionnaires. Once participants had done so, they were invited to the lab. All participants provided informed consent prior to the start of the experimental session. In case informed consent was not given by the participant, any questionnaire data was destroyed.

At the beginning of the lab session, after signing informed consent, participants were instructed to put on a heart rate monitor. Subsequently, they were asked to fill in several questions on the computer related to their coffee and alcohol consumption that day as well as their current mood and arousal, after which they had to complete the first baseline pupillometry measurement (same procedure as detailed in study 1 and 2). After the first pupillometry measurement, the tVNS device was attached to the participants' ear according to the experimental allocation (either concha or earlobe). Once the tVNS device had been attached, participants were instructed to complete the AB task. After the AB task, participants completed one last resting pupillometry measurement, and were subsequently debriefed about the goals of the task.

In total, the experimental procedure lasted approximately 40 minutes. Participants received tVNS or sham stimulation for roughly 32 minutes.

<u>Attentional Blink Task</u>

The AB task consisted of 10 practice trials and 136 test trials. Of these 136 test trials, 12 trials contained 0 targets, 16 trials had one target, and 108 had 2 targets. As target faces, we used cropped and framed pictures from the Karolinska Directed Emotional Faces Database. Specifically, we used 40 angry and 40 neutral images that had been most accurately been identified as such in a previous validation study [334]. Distractor stimuli were created by scrambling the neutral faces [335]. All target and distractor

stimuli were presented in greyscale and were matched on luminance. Every trial consisted of an RSVP of 30 stimuli, containing scrambled pictures of faces (distractors) and zero, one, or two unscrambled pictures of faces (targets). For a graphical overview of the Attentional Blink task, see figure 1.

Every stimulus appeared on the screen for 100ms. All distractor and target pictures were presented in greyscale and were matched on luminosity. The first target appeared at RSVP location 6, 7 or 8. The second target appeared at either lag 2 or lag 7 relative to the position of the first target. At the end of every trial, participants were asked to fill in whether they had seen zero, one or two targets, and were asked whether the targets they had seen had neutral or angry facial expressions. Out of 16 one-target-trials, 8 were $T1_{neutral}$, and 8 were $T1_{angry}$. The 108 two-target trials were evenly distributed into $T1_{neutral}T2_{neutral}$, $T1_{neutral}T2_{angry}$, and $T1_{angry}T2_{neutral}$ trials. In every two-target condition, the T2 was presented 18 times both at lag 2 and at lag 7.

Results

Participants

Out of 87 students who initially signed up for the study, 80 students (15 male, 65 female) participated in the experiment. All participants who came to the lab completed the study.

Participants' scores on the baseline questionnaires and baseline resting RMSSD are presented in table 1. Scores on the PSWQ, ACS, STAI-T, QIDS corresponded with normative samples [142,143,287,333].

Resting Pupil Diameter

Participants displayed a significant decrease in pupil diameter from the baseline measurement to after the experimental task, b = -0.40, (0.06), $t(78)$ = -6.61, p < .001. There were no between-group differences in pupil diameter prior to the experimental manipulation, p = .58, nor was there a differential increase in pupil diameter visible in the tVNS condition compared to the sham condition, p = .57.

Participants' scores on the baseline questionnaires and baseline resting RMSSD are presented in table 1. Scores on the PSWQ, ACS, STAI-T, QIDS, as well as baseline resting RMSSD corresponded with normative samples [142,143,287,332,333].

Behavioral Effects

Indicative of an attentional blink, participants displayed higher T2|T1 accuracies for lag 7 compared to lag 2 trials, as reflected by the main effect of Lag, b = 2.83 (0.48), $t(400)$ = 5.94, p < .001. When the first

target was negative, T2|T1 accuracies dropped significantly, as reflected by the main effect of $Valence_{T1=Negative}$, b = -1.97 (.48), t(400) = -4.15, p < .001. The effect of T1 valence mainly affects trials with a short temporal lag between T1 and T2 (i.e. lag 2 trials), as indicated by the $Valence_{T1=Negative}$*Lag interaction, b = 1.67 (0.67), t(400) = 2.48, p = .01.By contrast, the emotional valence of the T2 did not significantly affect T2|T1 accuracy, $Valence_{T2=Negative}$, b = 0.02 (0.48), t(400) = 0.04, p = .97.

There was no significant main effect of Condition, nor was there a significant interaction effect of Condition and Lag or Valence, all p > .05, as can also be seen in figure 4.

We performed an exploratory analysis in an attempt to replicate the results found in the first study. Specifically, in a group of high-trait worriers, we found that tVNS attenuated the attentional bias towards threat (i.e. participants receiving tVNS showed lower T2|T1 accuracy during trials with a negatively valenced T2). We therefore re-analyzed the subgroup of 58 out of the 80 participants who fit the PSWQ inclusion criterion of the first study (score of 45 or higher). Contrary to the first study, high worrying participants did not display an attentional bias in the engagement to threatening information, indicated by a non-significant effect of $Valence_{T2=Negative}$, p = .76. Additionally, participants who received tVNS did not differ from those who received sham stimulation, as reflected by the non-significant main effect of Condition and the non-significant interaction effects of Condition, Lag, and Valence.

Task-Related Pupil Dilation

Although we had minimized the differences in luminance between the different distractor and target pictures, the slight difference in luminance between the background and the stimuli in the RSVP still elicited a pupillary light reflex. As can be seen in figure 4, participants displayed a clear pupillary constriction in the first 600ms after RSVP onset, in line with pupillary light reflex latencies. As a result, participants' pupils undergo two opposite forces – an initial pupillary constriction due to the light reflex, and a subsequent pupil dilation due to cognitive effort in scanning for the targets during the RSVP.

We account for these two distinct processes by conducting a piecewise regression analysis. Specifically, by setting the knot value of the piecewise regression analysis at 600ms, two separate slopes focusing on regression lines before and after the knot value are fitted. This piecewise regression analysis was conducted in a mixed modelling framework, similar to prior analyses to account for the nested structure within our data. All models included random intercepts and two random slopes, one for each side of the knot.

As can be seen in figure 4, participants displayed a significant pupillary constriction during the first 600ms after trial onset, b = -32.58 (3.39), t(75) = -9.60, p < .001. Subsequent to this initial pupillary constriction, we observed a significant pupillary dilation, b = 44.58 (3.69), t(75) = 12.08, p < .001.

There was no significant main effect of Condition on pupil dilation, p = .46. Additionally, there were no significant differences between participants receiving tVNS and those receiving sham stimulation in the magnitude of the pupillary light reflex, as indexed by the Condition*Time interaction, p = .48. Finally, there were no significant differences between Conditions in subsequent pupillary dilation, as indexed by the interaction between Condition and the second sequential Time variable, p = .58.

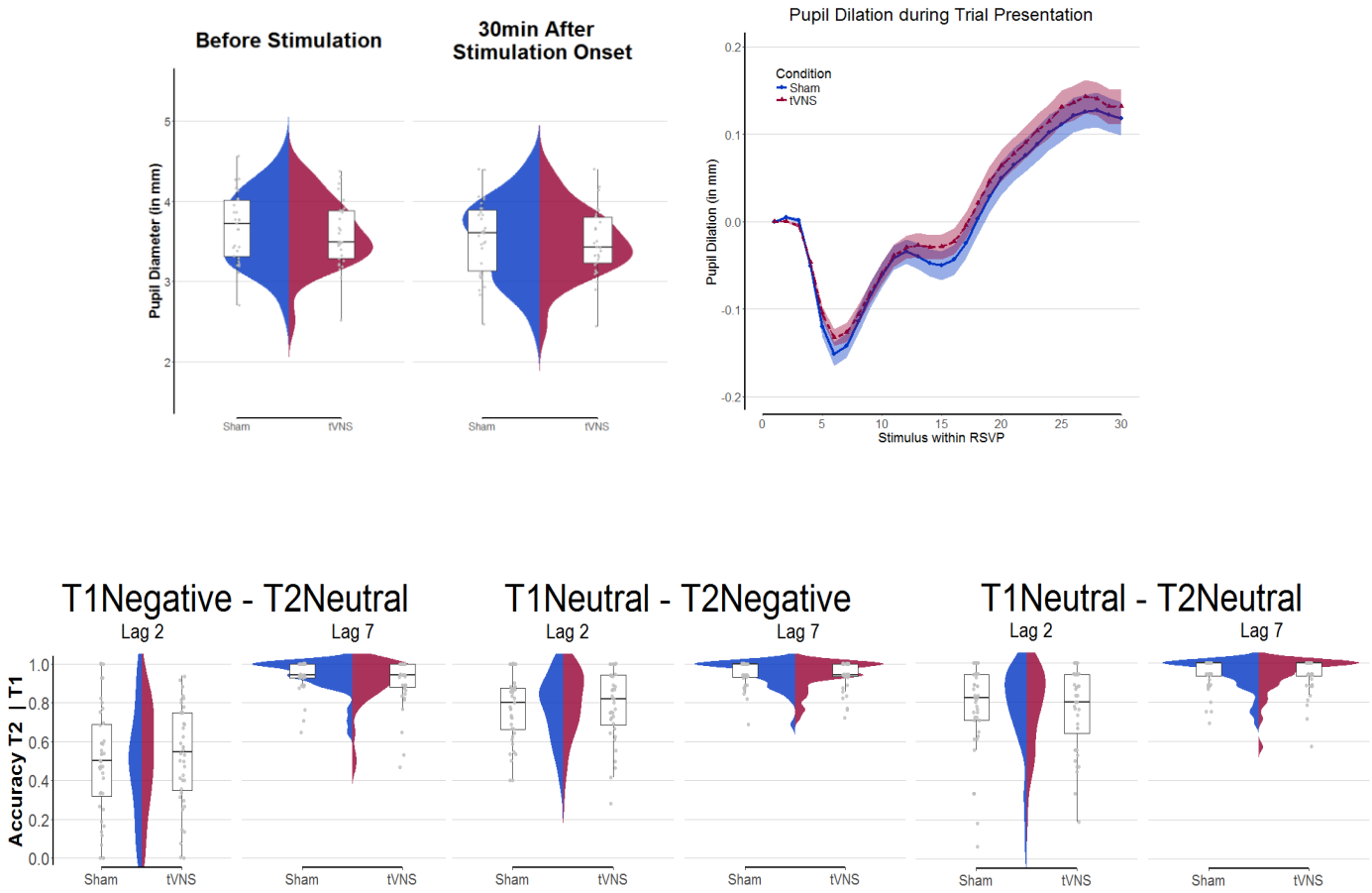


Figure 4. Accuracies, Pupil Dilation, and resting Pupil Diameters for participants in the tVNS and sham condition in study 2. *Top Left*: Violin plots and boxplots of resting pupil diameters before stimulation and 30 minutes after stimulation onset. Pupil diameter was recorded in 2-minute baseline recordings. *Top Right*: Pupil dilation over the course of an AB trial for participants in the tVNS and sham conditions. Confidence interval reflects ± 1 standard error. *Bottom*: Violinplots and boxplots of participants' accuracy at correctly identifying T2 after having correctly identified T1. Response accuracies are given separately for different lags.

Discussion

There was no effect of tVNS on resting pupil diameter, task-related pupil dilation, or accuracy during an emotional AB task. Thus, similarly to the previous two studies, there were no indications that tVNS affected the LC-NA network.

Contrary to the high-trait worriers in the first study, participants in the current study did not display an attentional engagement bias towards threat, which would be reflected in decreased attentional blink magnitudes when the second target had a negative valence. In an exploratory analysis, we re-analyzed the data on the high-worrying subset of our sample, and found no evidence for an attentional engagement bias towards threat. Participants who received tVNS or sham stimulation did not differ on attentional blink magnitude, irrespective of the emotional valence of either target, in both the main analysis and the exploratory analysis. It should be noted, however, that even though we used the same cut-off criteria to determine what constitutes 'high trait worrying', the samples may not be comparable. In the first study, we specifically advertised for and recruited participants who self-identified as 'chronic worriers', whereas study 3 recruited from a general student sample. As such, this subsample in study 3 may not be directly comparable to our high trait worry sample in study 1, which may explain the discrepancy between the findings.

General Discussion

In three separate studies, we tested the hypothesis that tVNS increases activity in the LC-NA network, as indexed by pupil diameter and performance on the AB task. Pupil diameter measurements provided no evidence to support this hypothesis: tVNS did not increase resting pupil diameter nor task-related pupil dilation compared to sham stimulation. Contrary to our hypotheses, high-trait worriers who received tVNS displayed less attentional engagement to threat than those who received sham stimulation (study 1). In general populations (study 2 and 3), there was no effect of tVNS on AB task performance, and when only high trait worriers were selected for an exploratory analysis in study 3, the behavioral effects of tVNS on attentional engagement from study 1 to threat could not be replicated. Overall, these studies provide no clear indications that tVNS affects either physiological or behavioral indices of noradrenergic activity.

The results found in this study are in stark contrast with preclinical studies, which consistently showed strong positive effects of iVNS on LC firing and central NA concentrations [45,95–99,115,144,304,305]. By contrast, studies on the effects of iVNS in humans have produced inconsistent results on indirect measures of LC-NA activity including pupil diameter and the P300 [101–104,336]. Previous studies on the effects of transcutaneous VNS in humans also found no significant effects of tVNS compared to sham stimulation on P300 and salivary alpha amylase [271,291]. It should be noted,

that these previous studies in humans included relatively small sample sizes, and thus their lack of significant effects may have been due to low statistical power. However, the current studies all included sufficient participants to detect at least medium effect sizes of tVNS. As such, these are the first adequately powered studies on the effects of vagus nerve stimulation on LC-NA activity in humans.

The reduced detection of emotional T2 stimuli found in high trait worriers who received tVNS during study 1 was contrary to our expectations. In a previous study, the administration of the noradrenergic agonists reboxetine enhanced emotional T2 detection in a group of healthy individuals [319], whereas noradrenergic antagonist propranolol decreased participants' accuracy during these trials. The reduced attentional bias found in study 1 would thus suggest that tVNS decreased rather than increased noradrenergic activity. However, as discussed by Aston-Jones [313], the effects of LC activity on task performance strongly resembles the inverted-U curve proposed to underlie the relation between arousal and task performance [337]. As such, given that participants were already performing very well on trials containing a negative T2, additional stimulation of the LC-NA network may have impaired performance on these trials. This may indicate some involvement of tVNS in the LC-NA network. However, in an exploratory analysis where only participants from study 3 who scored high on the PSWQ were included, we were unable to replicate this effect. Thus, we cannot exclude the possiblity that the effect found in study 1 was simply a type I error.

The current results pose a problem for the LC-NA-explanation that have repeatedly been suggested for the series of cognitive and emotional tVNS effects that have thus far been found (e.g. [164,165,196]), since one could argue that the null results found in this study demonstrate that these effects were not due to the modulation of the LC-NA network. Indeed, alternative working mechanisms have been identified in studies performed both in animals and in humans. Firstly, preclinical studies have shown that VNS increased neural plasticity through enhanced progenitor proliferation, cell survival, and cellular morphology (for a comprehensive review on this topic, see [94]). Moreover, a recent study in humans showed that tVNS increases the functional connectivity between the dorsal prefrontal cortex and the amygdala [80]. Thus, the modulation of the LC-NA network may not be a necessary requirement for the clinical efficacy of tVNS.

Alternatively, the lack of significant effects found in the current studies may have been a consequence of our choice of stimulation parameters, rather than a reflection of the effects of tVNS in general. The stimulation parameters that were used during active and sham stimulation were identical in all three studies. Participants received intermittent stimulation, alternating 30 seconds rest with 30 seconds active stimulation. Stimulation intensity was set at 0.5mA, administered at 25Hz and with a 250μs stimulation wavelength. These parameters were selected based on previous reports of parameter-dependent effects of iVNS following an inverted U-shape function [166,167]. However, it

remains unclear whether this stimulation intensity also produces the strongest cognitive effects for transcutaneous VNS.

An alternative tVNS stimulation paradigm that has been commonly used is to adjust the stimulation intensity to be above an individual's sensory threshold, yet below the individual pain threshold (cf. [52]). This calibration method is based on the assertion that any sensory information reported by participants at the level of the cymba concha can only be achieved by an activation of the vagus nerve. Indeed, a historical case report confirms that after sectioning the vagus roots at the level of the posterior fossa, a patient that had previously reported severe pain reported complete anesthesia at the level of the cymba concha [297]. However, even though this case report demonstrates that an intact vagus nerve is a necessary requirement for the processing of sensory information, it remains unknown whether sensory processing is sufficient for inducing noradrenergic effects. In study 3, participants were asked to rate whether they could feel when they were being stimulated, and thus whether tVNS was above the sensory threshold. Out of 40 participants who received tVNS stimulation, the stimulation intensity exceeded the individual sensory threshold for 33 participants. To assess whether tVNS increases NA activity in those participants where the stimulation intensity exceeded the sensory threshold, we performed additional exploratory analyses where the 7 participants that did not meet this criterion were excluded. These exploratory analyses revealed no differences between tVNS and sham stimulation in accuracy on the AB task, nor on resting pupil diameter or on pupil dilation during AB task performance (results not presented in this manuscript). As such, we would argue that although sensory processing may be necessary for any effects of tVNS to occur, it does not seem to be a sufficient requirement.

An important limitation to the current study is that pupil diameter is only an indirect measure LC-NA activity. A recent study in macaques showed that although pupil diameter is consistently associated with LC neuron firing during both passive viewing and cognitive processing, similar associations with pupil diameter were also found in other brain areas [308]. Thus, changes in pupil diameter cannot be attributed solely to activity in the LC and actually represent a complex interplay between different brain areas which indirectly affect the dilator and sphincter muscles of the pupil. It is unsurprising, therefore, that correlations between LC activity and pupil diameter are only small [308]. Consequently, even if tVNS did affect activity of the LC-NA system, the relatively small correlation between LC activity and pupil diameter may have resulted in effects of tVNS on pupil diameter that were too small to detect in our studies. Similarly, although the AB task has been associated with LC-NA activity [316,319], this association has not been found consistently [321]. Moreover, to our knowledge there is no 'golden standard' for AB task design, and it remains unclear whether one of the experimental designs used in this series of studies provided a better representation

of LC-NA activity than others. Clearly, these limitations highlight the need for more valid and reliable biomarkers for LC-NA activity in humans.

To summarize, we performed three studies to assess whether tVNS increases LC-NA activity in humans. Contrary to results from animal studies using iVNS, we found no evidence that transcutaneous VNS increases LC-NA activity, either on physiological or behavioral measures thought to be associated with LC-NA activity. These findings clearly highlight the need for more fundamental research to optimize stimulation parameters and study the working mechanisms underlying tVNS.

Chapter 9

General Discussion

The overarching goal of this dissertation was to assess whether transcutaneous vagus nerve stimulation (tVNS) has beneficial effects over sham stimulation in laboratory models of anxiety. Specifically, in *Part I*, the effects of tVNS were tested in a fear conditioning paradigm to test the utility of tVNS as an add-on treatment for exposure therapy. In *Part II*, the effects of tVNS as a stand-alone treatment for one of the core symptoms of anxiety – perseverative cognition – was tested within the experimental framework of a Breathing Focus task. *Part III* deals with working mechanisms: what is the optimal site of the ear to stimulate the auricular branch of the vagus nerve (ABVN) and what is the hypothesized working mechanism underlying the anxiolytic effects of tVNS? Specifically, the effects of tVNS on both physiological as well as behavioral indices of locus coeruleus – noradrenaline (LC-NA) network activity were tested.

In this last chapter, an overview of the results described in the previous chapters will be provided. Furthermore, theoretical and clinical implications of these results, the strengths and limitations of these empirical studies, and directions for future research will be discussed.

Summary

Part I: The extinction of fear

In a series of four classical cue conditioning studies, the effects of tVNS on the extinction, generalization, and retention of fear were tested. A general overview of the methods and results from these four studies is given in table 1. Below, a more elaborate summary of each individual chapter is provided.

Chapter 2 describes the first published experimental study that tested the effects of tVNS on the extinction and retention of fear in humans. This study utilized a differential cue conditioning paradigm including an immediate extinction phase (i.e. extinction occurring on the same day as acquisition) and a delayed extinction recall phase [222]. Participants were randomly allocated to receive either tVNS or sham stimulation throughout the extinction phase. Participants who received tVNS displayed faster extinction of differential declarative fear – as indexed by a steeper decline in US expectancy ratings for CS+ trials - than participants receiving sham stimulation. However, there were no between-group differences in US expectancy ratings during the extinction retention test twenty-four hours later, indicating that the consolidation of extinction memories had not been affected by tVNS. Participants did not display significant physiological responses indicative of differential fear learning during the acquisition phase, and consequently the effects of tVNS on the subsequent extinction of physiological fear could not be tested. This study provided the first indications that tVNS may accelerate the extinction of fear, although this could only be assessed on the declarative indices of fear, and not the physiological ones.

Chapter 3 describes a study that was conducted concurrently with the study described in **Chapter 2**. Although the general structure of the fear conditioning paradigm was the same, there were several important differences in experimental design: firstly, this study utilized a delayed extinction protocol, meaning that the extinction phase was conducted twenty-four hours after the initial acquisition phase. Secondly, all participants received sham stimulation throughout the fear acquisition and extinction retention phases. During fear extinction, half of the participants were randomly allocated to receive tVNS, whereas the other participants continued receiving sham stimulation. Consistent with the results in **Chapter 2**, tVNS accelerated the extinction of declarative fear, but did not promote a stronger retention of the extinction memory. Additionally, no effects of tVNS were found during fear generalization, reacquisition, or reinstatement tests. Finally, no clear effects of tVNS on physiological indices of extinction or retention were found. As such, this study confirmed the acceleratory effects of tVNS on declarative extinction and the lack of effects on memory retention that had been found in **Chapter 2**, while showing for the first time that tVNS did not affect the physiological indices of fear.

In **Chapter 4,** we attempted to replicate the finding that tVNS accelerated the extinction of fear in a larger sample than the previous two chapters. As we had not been able to find differential physiological fear learning in **Chapter 2**, several changes were made to the experimental paradigm to increase participants' arousal and the negative valence of the US, to facilitate physiological fear conditioning [217]. Firstly, the conditioned stimuli were changed from geometrical shapes to spiders, as the use of evolutionarily fear-relevant stimuli has been shown to lead to stronger acquisition of fear in previous studies [201]. Secondly, a 70dB background noise was added throughout the experimental paradigm, and the intensity of the startle probe was changed to be 2.5 times louder than before (i.e. a change from 100 to 104dB). Thirdly, the US was changed from a loud scream to an aversive electric shock that was individually calibrated to be very uncomfortable. Contrary to the previous studies, participants who received tVNS did not display an accelerated extinction of declarative or physiological fear compared to participants receiving sham stimulation. However, participants in the tVNS condition did report lower initial US expectancy ratings for CS− trials, which may indicate that tVNS facilitated the processing of safety cues. This finding is in line with the Generalized Unsafety Theory of Stress, which proposes that vagus nerve activity may increase the inhibition of the stress response in the presence of safety cues [339]. It should be noted, however, that this finding was not hypothesized beforehand, and had not been observed in the earlier chapters. The discrepancy between results from this study and the two earlier chapters may reflect the increased arousal that was induced in participants in **chapter 4**. Consequently, this study has resulted in a conceptual non-replication of the previous studies.

In **Chapter 5**, we conducted a fear conditioning study that focused on testing whether tVNS would decrease the generalization of fear – a process that has been proposed to be strongly associated with the onset and maintenance of anxiety disorders [108,109,229] – and accelerate the subsequent fear extinction. Based on preclinical evidence, tVNS was hypothesized to inhibit the generalization of fear by increasing activity in the dentate gyrus, which is believed to promote the distinction between novel memory traces with fear-relevant memory traces [187,241,242]. To test this hypothesis, we utilized the fear conditioning protocol designed by Lissek and colleagues [246]. In this protocol, participants initially underwent differential fear conditioning, using two circles of differing sizes as CSs. Participants were then randomly allocated to receive either tVNS or sham stimulation during the subsequent generalization and extinction phases. During fear generalization, participants were presented with circles of various sizes that were all intermediate to the two originally conditioned stimuli. Contrary to our hypotheses, participants who received tVNS did not display a different generalization of fear compared to participants in the sham condition, reflected in both declarative and physiological indices of fear. Similarly, tVNS did not affect physiological indices of fear during the extinction phase. However, compared to participants who received sham stimulation, those in the tVNS condition reported lower US expectancy ratings throughout the extinction phase, which is in line with our findings from **Chapters 2** and **3**.

Chapter	Total *N*	CS	US	Extinction	Retention	Reinstatement	Generalization	Reacquisition
2	31	*Geometrical Shapes*	*Scream*	**+**	**=**	**=**		
3	39	*Geometrical Shapes*	*Shock*	**+**	**=**		**=**	**=**
4	85	*Pictures of spiders*	*Shock*	**=**				
5	58	*Circles of different sizes*	*Shock*	**+**			**=**	

Table 1. Overview of results from the four fear conditioning studies presented in this thesis.
+: significant beneficial effect of tVNS compared to sham stimulation
= : no significant differences between tVNS and sham stimulation

<u>Part II: Negative Thought Intrusions</u>

Apart from the potential of tVNS as an add-on therapy for exposure therapy, we also tested the potential of tVNS as a stand-alone treatment for anxiety disorders. Specifically, in **Chapter 6**, we tested our pre-registered hypothesis that tVNS decreases negative thought intrusions in a Breathing Focus Task in a population of high trait worriers. Prior to the worry induction, participants who had received tVNS reported fewer negative thought intrusions than participants who had received sham

stimulation. No between-group differences were observed in self-reported worry intensity during the subsequent worry induction phase. After the worry induction there were no longer any differences between groups in the number of reported negative thought intrusions. Moreover, exploratory analyses revealed that, contrary to expectations, a higher proportion of participants in the tVNS condition reported negative thought intrusions immediately after the worry induction.

As an additional exploratory analysis, potential effects of tVNS on heart rate variability were tested throughout the Breathing Focus Task. Heart rate variability is often interpreted as an index of efferent vagal activity, which is predominantly affected by the changes in the inhibitory effect of the vagus nerve on the sinoatrial node [68,78]. Participants receiving tVNS did not differ in their heart rate variability from those receiving sham stimulation. This finding was unsurprising, given that the stimulation intensity utilized during tVNS was likely insufficient to activate cardiac *efferent* B-fibers of the vagus nerve [276]. Moreover, the vagus nerve's innervation on the sinoatrial node is predominantly innervated by the right vagus nerve, whereas the current study stimulated the left side, specifically to avoid cardiac side-effects [121,292]. Indeed, stimulation of the left ABVN was chosen specifically for ethical and safety purposes, to avoid the risk of adverse cardiac events.

Chapter	Total *N*	Pre-worry negative thought intrusions	Worry Induction	Post-Worry negative thought intrusions
6	97	**+**	**=**	**=/-**

Table 2. Overview of results of tVNS on the Breathing Focus Task.
+: significant beneficial effect of tVNS compared to sham stimulation
= : no significant differences between tVNS and sham stimulation
- : significant detrimental effect of tVNS compared to sham stimulation

Part III: Working Mechanisms

Whereas parts I and *II* of the thesis provided an experimental approach to the anxiolytic effects of tVNS, more fundamental questions surrounding tVNS had also remained unresolved and debated. In *Part III*, we focused on fundamental questions regarding the optimal stimulation site and working mechanisms of tVNS.

Firstly, in **Chapter 7**, we describe critical inconsistencies that had been overlooked in a cornerstone anatomical publication on the nerve supply in the human ear. Most importantly for the field of tVNS, the article that provided the anatomical basis for target areas of tVNS devices (Peuker and Filler, 2002) [25], contained a discrepancy. According to a table printed in this paper, the auricular branch of the vagus nerve (ABVN) innervated the cymba concha in 100% of all ears, and the tragus in 45% of all ears. Although the ABVN innervation of the cymba concha had already been demonstrated

in earlier accounts [24,298], the innervation of the tragus hinged solely on this one study. However, the article contained a discrepancy between the main text and the table; in the main text, the tragus was described as being innervated by the great auricular nerve, the auriculotemporal nerve, or a combination of the two. The ABVN was not mentioned to innervate the tragus of the ear. In a personal correspondence, the authors acknowledged the inconsistency but were unable to determine whether the main text or the table had been correct. The inconsistency that was brought to light in **chapter 7** leads us to conclude that researchers should be careful when interpreting results from studies that stimulated the tragus as reflecting vagus nerve stimulation, and emphasizes the need for further anatomical research on the innervation of the ABVN.

Secondly, in **chapter 8**, we attempted to test the working mechanisms underlying the anxiolytic effects of tVNS. Preclinical studies suggest clear involvement of the vagus nerve in LC-NA activity, and studies on the effects of invasive VNS in rats showed increased activity in the LC, resulting in higher noradrenaline levels in the LC and target brain areas [45,95–99,115,144,304,305]. However, studies on the effects of invasive VNS in humans have provided only mixed results [101–104,336]. This led to **Chapter 8**, where we tested the effects of tVNS on LC-NA activity in a series of three experimental studies. We assessed physiological and behavioral indices of LC-NA activity, namely increases in resting pupil diameter, phasic pupil dilation and performance on an Attentional Blink task [294,308,317,319]. These studies provided no clear indications for the modulation of tVNS on the LC-NA network: tVNS did not increase resting pupil diameter, nor did it increase task-related pupil dilation during an Attentional Blink task. Finally, there were no behavioral effects of tVNS on the Attentional Blink task itself. In conclusion, we found no evidence for the involvement of tVNS in LC-NA activity in these studies.

Study	Total *N*	Pupil Diameter	Attentional Blink Magnitude	Pupil Dilation
1	92	**=**	**-**	
2	30*	**=**	**=**	**=**
3	80	**=**	**=**	**=**

Table 3. Overview of results from the three studies presented in **chapter 8**.
+: significant increase in tVNS condition
= : no significant differences between tVNS and sham stimulation
- : significant decrease in tVNS condition
*: Study 2 utilized a within-subjects design, where every participant was tested using both tVNS and sham stimulation.

Synthesis and Implications

tVNS as an add-on treatment

In *Part I*, we assessed the effects of tVNS on the extinction of fear. These experimental studies were based on preclinical evidence for the involvement of afferent vagus nerve activity in the encoding and consolidation of memory [46–48], as well as consistent accounts showing that invasive VNS in rats strengthened the consolidation of extinction memories [50–53].

As summarized in table 1, the fear conditioning studies presented in this dissertation provide mixed evidence for a potential role of tVNS as an add-on for exposure therapy. Participants who received tVNS displayed an accelerated extinction of declarative fear (**Chapters 2**, **3**, and **5**), which may indicate that tVNS could facilitate inhibitory learning during exposure therapy [61]. Effect sizes of these effects varied, namely $\delta = 1.0$ (**chapter 2**), $\delta = 0.7$ (**chapter 3**), and $\delta = 0.5$ (**chapter 5**). However, in **Chapter 4**, this effect of tVNS on extinction learning was not replicated, and participants who received tVNS did not differ from those receiving sham stimulation in their rate of declarative fear extinction ($\delta < 0.01$, although tVNS did decrease expectancy ratings for CS- trials at the start of the extinction phase, $\delta = 0.4$). Although we cannot rule out that this inconsistency was simply due to either false positive findings in **Chapters 2**, **3**, and **5**, or a false negative finding in **Chapter 4**, these discrepant findings might also be caused by key differences in the design characteristics of these studies. Specifically, the discrepant results found in **Chapter 4** may have been a result of increased arousal experienced by participants due to changes in the experimental procedure (e.g. the use of spider pictures as a CS, continuous loud background tones), which may have caused increased afferent vagal activity even in the absence of active tVNS. Indeed, stressful or arousing situations lead to an increased secretion of adrenaline, which binds to beta-adrenergic receptors of the vagus nerve [340]. This receptor binding, in turn, triggers action potentials in the afferent vagus nerve, which subsequently increases memory encoding and consolidation through enhanced activity in the LC-NA network [45–47]. If tVNS would no longer accelerate the extinction of fear in arousing conditions – due to the vagus nerve already being activated through peripheral adrenergic pathways – this would greatly reduce the clinical applicability of tVNS as an add-on for exposure therapy, because this behavioral intervention is inherently arousing and stressful [341].

In contrast to US expectancy ratings, physiological indices of fear – skin conductance, fear potentiated startle, and phasic heart rate responses - were not affected by tVNS in any of the conditioning experiments. One possible explanation for the null results of tVNS on physiological indices of fear would be that physiological indices are relatively more variable and contain a lower signal-to-

noise ratio compared to US expectancy ratings[4]. This decreased signal-to-noise ratio in the dependent variables directly translates to reduced model fit of our data and reduced statistical power to detect differences between tVNS and sham stimulation. Alternatively, this discrepancy can be explained by the two-factor account of emotional memory proposed by Phelps [184]. In short, this theory proposes that distinct aspects of fear are controlled by at least two independent memory systems: the first memory system, linked to the amygdala, is mainly involved in the processing of the emotional load of an event, and would therefore affect the physiological responses of fear. By contrast, the second memory system specializes in forming declarative memories of an event. This second memory system is mainly linked to the hippocampus, and affects the declarative indices of fear including US expectancy ratings. Although these two memory systems often interact with each other, studies in patients with damage to either brain area have revealed that either memory system also operates independently (e.g. [185,186]). Since tVNS in our studies mainly affected the declarative extinction of fear, tVNS may lead to more prominent changes in activity of the hippocampal complex than in the amygdala. Increased hippocampal activity after tVNS would be consistent with animal studies that have shown increased NE activity and increased cellular proliferation in the hippocampus after VNS [95,187,242]. Finally, it should be noted that although US expectancy ratings have been argued to provide a valid representation of conditioned fear [146], we did not explicitly measure declarative *fear* (an emotion), but *expectation* (a cognition) of an unconditioned stimulus (scream or shock). We cannot rule out that relatively low expectancy ratings (e.g., 30%) were accompanied by rather high fear ratings in some participants (or vice versa, low fear despite high expectancy).

Although tVNS affected the extinction of declarative fear at least under certain conditions, the potential clinical efficacy of tVNS is dampened by the lack of effects on the retention of extinction memories during a test phase 24 hours after extinction learning (**Chapters 2** and **3**). These results are in contrast to preclinical studies in rats, which show that 24h extinction memory retention improved significantly after invasive VNS [50–53]. As discussed in **Chapter 3**, this discrepancy between human and animal studies may simply have been a consequence of the high number of extinction trials included in **Chapters 2 and 3**. Indeed, the effects of tVNS on the consolidation of extinction memories may have been confounded by the high number of extinction trials which allowed participants to

[4] To illustrate this point, I calculated the root mean squared errors (RMSE) of linear mixed models for every dependent variable (i.e. US expectancy ratings, Skin Conductance Responses, Fear Potentiated Startle responses) during the Acquisition phase of Chapter 3 [365]. To facilitate comparison of these metrics, each dependent variable was standardized into a T-score prior to the analysis. The independent variables for every model were *Time* and CS type. US expectancy ratings showed a lower RMSE ($RMSE_{USexp}$ = 6.33) compared to the physiological indices ($RMSE_{startle}$ = 8.81, $RMSE_{SCR}$ = 8.47), indicating a larger standard deviation of residuals and thus a larger error variance for physiological compared to declarative measures.

consolidate their extinction memories, irrespective of whether they had received tVNS or sham stimulation.

Taken together, *Part I* of this dissertation has yielded mixed preliminary evidence for an effect of tVNS on extinction learning. These results are in contrast to the robust effects of invasive VNS found in animal studies [50–53]. It remains unknown whether this inconsistency reflects translational differences in mechanisms underlying extinction learning, differences between invasive and transcuteanous VNS, or the use of suboptimal tVNS parameters. Given the low costs, ease of use, and mild side-effect profile of tVNS, as well as the initial beneficial results on extinction memory encoding found in **Chapters 2**, **3**, and **5**, I would argue that it is worthwhile that the clinical potential of tVNS as an add-on for exposure therapy is investigated further.

tVNS as a stand-alone treatment

Next to the potential effects of tVNS as an add-on for exposure therapy, there are studies that point towards a general anxiolytic effect of tVNS, suggesting that tVNS may be used as a stand-alone treatment for anxiety disorders. Firstly, a recent study indicated that four weeks of tVNS significantly decreased symptoms of depression and anxiety in patients suffering from a major depressive disorder, compared to sham stimulation [300]. Moreover, after four weeks of tVNS, these patients displayed significantly higher resting state functional connectivity between the amygdala and the dorsolateral prefrontal cortex [80]. Reduced connectivity between these brain areas has been suggested to reflect diminished prefrontal inhibitory control, which is believed to underlie perseverative cognition [84]. In line with this finding, several studies have indicated that tVNS affects cognitive functions that rely on prefrontal activity, including action control [87] and task-irrelevant information processing [88,271,342]. Critically, however, none of these studies had tested perseverative cognition directly, and **chapter 6** provides the first indications that tVNS may decrease perseverative cognition in a population of high trait worriers, a population that is especially characterized by reduced amygdala - prefrontal cortex connectivity [275]. However, after a brief worry induction, there was no longer an effect of tVNS on the number of negative thought intrusions reported by participants. In fact, exploratory analyses revealed that a higher proportion of participants in the tVNS condition reported negative thought intrusions directly after the worry induction, which might reflect a failure to disengage attention from threatening information. **Chapter 6** provides mixed indications of the clinical applicability of tVNS for perseverative cognition in anxious individuals, and more ecologically valid studies are warranted to further examine the possible effects of tVNS on perseverative cognitions.

tVNS in general

The mixed effects of tVNS found in **chapters 2-6** may raise fundamental questions about whether electrical stimulation at the level of the cymba concha truly increases afferent vagus nerve activity, and if so, via what mechanisms it affects cognitive and emotional processes. Based on animal literature, the effects of tVNS on cognitive and affective processes were hypothesized to be mediated primarily through a modulation of the LC-NA network [45,95–98]. However, in **Chapter 8**, we were unable to find any consistent indications for the involvement of tVNS in the LC-NA system, as indexed by pupillometry and performance on an attentional blink task. This is in line with a recent pilot study that also found no significant effects of tVNS on two other indirect markers of LC-NA activity, namely the P300 magnitude and salivary alpha amylase (sAA) [271,291].

The theoretical and clinical implications of these null findings are unclear at this point. On the one hand, these results could indicate that tVNS does not affect the LC-NA network in humans at all. If so, the same could hold true for invasive VNS in humans, since there are only inconsistent results for an involvement of VNS on P300 or pupil dilation as well [101–104,336]. However, I would argue that this interpretation is premature. Indeed, neuroimaging studies have repeatedly shown that tVNS targeted at the location of the cymba concha increases BOLD activity in the LC compared to earlobe stimulation [79,122,343]. Additionally, effects of tVNS on cognitive processes including post-error slowing [164], action cascading [272], and associative learning [56, this thesis] suggest the activation of the LC-NA network as a result of tVNS. Nonetheless, the null findings in **Chapter 8** are puzzling and do not support the hypothesis that tVNS leads to increased activity in the LC-NA network.

Strengths and limitations

Considering the recent replication crisis in Psychology [344], we have attempted to establish research lines through the repeated attempts to replicate previous findings and investigate their robustness. For example, in **Chapter 2**, participants who received tVNS showed accelerated extinction of fear compared to sham stimulation, and **Chapters 3-5** constituted attempts to replicate this effect using slightly different experimental paradigms. Similarly, **Chapter 8** describes three studies that tested the effects of tVNS on pupil diameter and the attentional blink task with slight deviations in the experimental design. Recent controversies in psychological science have once again highlighted the importance of replication research [344], which should be viewed as a fundamental pillar of science, especially given the large researcher degrees of freedom awarded to scientists when designing a study and analyzing the results, as well as the inherent uncertainty surrounding statistical inference of significance based on one study. Unfortunately, whereas conceptual replication can offer additional knowledge in terms of generalizability of findings in the case of convergent results, divergent results

of conceptual replications are more difficult to interpret compared to direct replication studies. For example, the discrepancy between the results of **Chapters 2** and **3** on the one hand, and **Chapter 4** on the other hand, could be due to differences in experimental design or may reflect type I or type II errors in one or all of these chapters. The differences between the experimental designs mean that we cannot disentangle these options yet, and therefore call for additional research. Unfortunately, these conceptual replications were unavoidable for this thesis, as progressive understanding of tVNS and the fear conditioning paradigm inspired us to make changes to the experimental paradigms.

Another strength of this dissertation is the use of (generalized) linear mixed model analyses, which permits a more tailored data analysis approach compared to conventional RM ANOVA. Mixed model analyses have clear advantages over RM ANOVA when analyzing repeated measures data: Firstly, RM ANOVA is incapable of dealing with missing data, and will remove all data of participants in case of missing data for a single trial. To circumvent this problem, researchers often aggregate their data into blocks, which strongly reduces their statistical power. Additionally, the 'sphericity' assumption of RM ANOVA (i.e. the variance of errors is identical for each repeated measurements, and errors are completely independent of each other) is not realistic for most repeated measures data, especially fear conditioning data [345]. As a consequence, researchers are forced to perform corrections to their RM ANOVA which further decrease their statistical power [346,347]. Finally, this approach allows us to flexibly adapt our analyses to accommodate dependent variables that are clearly not normally distributed. For example, in the case of the Breathing Focus task, the amount of negative thought intrusions reported by participants was heavily zero-inflated, and consequently does not approximate a normal distribution. The use of generalized linear mixed model analyses, where negative thought intrusions were modelled within a negative binomial distribution, provides a much better fit to the actual data. The choice for (generalized) linear mixed models increased the statistical power and the validity of our analyses.

Another strength of this thesis is that the effects of tVNS on inhibitory learning were not limited to an assessment of fear extinction, but also included tests of fear generalization, retention, reacquisition, and reinstatement. These experimental tests all relate to specific aspects of the etiology, treatment, and return of fear, and enabled a broader understanding of the clinical applicability and utility of tVNS. An additional strength of the fear conditioning studies included in this thesis is the use of a multimodal approach towards fear. Specifically, all studies included both physiological indices (i.e. fear potentiated startle responses, skin conductance responses, heart rate) as well as declarative indices (i.e. US expectancy ratings) of fear. In contrast to the declarative indices of fear extinction, psychophysiological indices of fear extinction were not affected by tVNS.

Parts I and II of this dissertation were devoted to testing tVNS as an add-on or standalone treatment in clinical experimental models of anxiety, respectively. These models offer a valid

experimental representation of specific concepts related to anxiety (e.g. fear development, treatment, and relapse, as well as specific components related to anxiety including fear generalization and worrying). One should keep in mind, however, that the translation of these findings in relatively healthy individuals to clinical practice with patients will require more elaborate testing using more ecologically valid designs.

A clear limitation of the current thesis, and indeed of the research domain of tVNS as a whole, is that there is no research that has studied the optimization of tVNS stimulation parameters. All studies presented in this thesis utilized identical stimulation parameters (0.5mA stimulation intensity, 250µs wavelength, 25Hz frequency). The stimulation intensity was largely based on research in animals and humans on the optimal stimulation intensities to achieve cognitive effects using invasive VNS [116,167]. However, it seems unlikely that these stimulation parameters can be directly translated from invasive to transcutaneous VNS: during invasive VNS, the stimulator is wrapped directly around the nerve, and the electrical current only needs to penetrate the epineurium to reach the nerve fibers. By contrast, tVNS is applied on the skin of the ear, and will need to penetrate the skin prior to reaching the epineurium, which increases the impedance of the electrical charge. It seems likely that tVNS needs to apply a higher current to achieve the same effects on afferent fibers of the vagus nerve as invasive VNS. On the other hand, similar stimulation intensities as the ones used in **chapters 2-8** significantly increased activation of the nucleus tractus soliatarii (the primary central relay of afferent vagus nerve fibers) as well as the LC, compared to sham stimulation [122]. Thus, although the tVNS parameters utilized in **chapters 2-8** appear to at least successfully stimulate the ABVN, there is a clear need for additional research to find the optimal stimulation parameters of tVNS.

Contrary to preclinical studies, LC-NA activity in humans can only be assessed indirectly (for example via measurements of pupil diameter, P300, or sAA). Unfortunately, these indirect measures of LC-NA activity suffer from relatively low reliability. For example, the Spearman's correlation between LC neuron spike rate and mean pupil diameter in macaques is around $\rho = .15$, indicating a low signal-to-noise ratio and a limited criterion validity of pupil diameter as an index of LC-NA activity [308]. This low reliability of pupil diameter as a measure of LC-NA activity negatively impacts the power of our statistical analyses to detect meaningful effects. As such, although the findings in **Chapter 8** may truly be a testament to the relatively modest effect size of tVNS on LC-NA activity, it seems likely that the low reliability of pupillometry as an index of LC-NA activity has also negatively affected our ability to detect noradrenergic effects of tVNS.

Future Directions

Although tVNS is a relatively new research area, positive initial reports and the non-invasiveness of the technique itself have greatly increased researchers' interest in this field. In recent years, tVNS has been studied for the treatment of anxiety (this thesis), depression [91], chronic cluster headache [348–350], epilepsy [303,351], diabetes [352,353], pain [354,355], and tinnitus [356,357]. However, as discussed previously, the working mechanisms underlying these effects are still poorly understood, which makes it difficult to explain how tVNS could achieve such a myriad of positive effects on mental health. There is a clear need for more fundamental research on the working mechanisms of tVNS.

The optimal stimulation site for performing tVNS remains a hotly debated topic. It seems clear that to answer this question, the anatomical distribution of the ABVN in the human auricle has to be studied further. Given the inconsistencies in the anatomical study by Peuker and Filler [25] that were brought to light in **Chapter 7**, there is currently no reliable empirical evidence that the ABVN innervates the tragus of the ear. Nonetheless, the tragus is still being used as a target site for tVNS (e.g. [296]). Similarly, the validity of the cymba concha as a target site for tVNS hinges on one anatomical study in humans that we now know – based on **chapter 7** - contains crucial inconsistencies [25], one anatomical study in macaques from 1897 [298], and one surgical case study from 1927 [24]. The neck has been suggested and studied as an alternative stimulation site for tVNS (e.g. [348]), and recent anatomical evidence suggests that the vagus nerve traverses the neck lateral to the common carotid artery [358]. Nonetheless, the neck does not seem to be a practical stimulation target area; given the location of the vagus nerve in relation to the carotid artery, we know that the vagus nerve lies roughly 23mm medial to the skin surface [359], which means that the electrical resistance would strongly increase compared to auricular stimulation.

The most basic requirement for tVNS to work would be that the electrical stimulation of the tVNS device should induce an action potential in afferent fibers of the ABVN that propagate through the vagus nerve towards the NST. This process has been verified in humans and in animals for invasive VNS [41,276]. To measure the compound action potentials elicited by VNS, researchers attached a recording electrode around the nerve near the location of the stimulator [41,276]. These studies were able to obtain high-resolution recordings that differentiated the conduction velocities of the different vagal fiber types. Unfortunately, these direct measurements of compound action potentials have not yet been obtained from tVNS. One explanation for why this basic effect has not been tested yet is that the recording of compound action potentials requires the vagus nerve to be exposed through a surgical procedure, which goes against the noninvasive nature of tVNS. Nonetheless, a recording of compound action potentials may be obtainable from a patient undergoing a surgical procedure for VNS implantation. Specifically, during VNS implantation, a recording electrode can be placed around the

exposed vagus nerve at the level of the neck. Although this area does not receive afferent vagal projections from the ABVN, high-intensity tVNS aimed at stimulating vagal efferent B-fibers should theoretically be able to elicit downstream action potentials that could be measured at the level of the neck. This direct test could strengthen the basic premise underlying tVNS that transcutaneous electrical stimulation of the outer ear is capable of stimulating the vagus nerve.

There is also a clear need for more elaborate research on the central working mechanisms of tVNS. Although preclinical studies seem to have produced robust effects of invasive VNS on LC-NA activity in rats, these results have not been reliably replicated in humans. It is unclear whether this failure to replicate these fundamental effects reflect neuroanatomical differences between species, differences between VNS and tVNS, high signal-to-noise ratios of indirect measurements of LC-NA activity in humans, or simply the use of suboptimal stimulation parameters for tVNS. Future research would greatly benefit from parametric studies on the effects of tVNS on varying indices of LC-NA activity, not limited to just pupil diameter, but also including P300, and salivary alpha amylase. Alternatively, one could argue that future research should focus on testing alternative working mechanisms of tVNS, including increased functional connectivity between the amygdala and the prefrontal cortex [80], increased serotonergic activity [94], increased synaptic plasticity in the hippocampus, or GABAergic modulation [197]. From a measurement standpoint, testing the effects of tVNS on GABA seems particularly promising; contrary to NA, GABA levels can be measured directly in the brain through the use of magnetic spectroscopy [360]. It should be noted, however, that although two recent studies found significant effects of tVNS compared to sham stimulation on indirect markers of GABA (i.e. cortical excitability [361] and EEG readiness potentials [362]), the only direct assessments of GABAergic modulation of invasive VNS did not produce significant results (although the results were interpreted as such by the authors) [197,363]. It should be noted, however, that all studies on the GABAergic effects of invasive or transcutaneous VNS have employed very small sample sizes. These small sample sizes strongly decrease the statistical power to detect true effects, but also decrease the likelihood that statistically significant effects that are found reflect a true effect [364]. Regardless of whether future research focuses on testing the effects of tVNS on LC-NA activity, GABA, or any alternative working mechanisms, statistical power should be taken into account when designing these studies.

From a clinical perspective, the studies presented in this thesis provide some initial support for the hypothesis that tVNS may accelerate the extinction of fear. However, the non-significant effect found in **Chapter 4** could suggest that arousing situations already cause increased afferent vagal activation through α2 vagal receptor binding of peripheral adrenaline. If this were indeed the case, this would greatly reduce the clinical applicability of tVNS for exposure therapy, given that exposure therapy is inherently arousing and stressful [341]. Future research should assess whether potential

effects of tVNS on extinction learning are indeed dependent on the arousal experienced by participants, by manipulating either the experimental context (e.g. manipulating the predictability or aversiveness of a US or experimental context) or through the administration of a pharmacological agent (e.g. direct administration of an α2-adrenergic receptor agonist such as clonidine, or a β-adrenergic receptor antagonist such as propranolol).

More generally, research on the use of tVNS as an add-on or stand-alone treatment deserves further exploration. Focusing on the applicability of tVNS for the treatment of anxiety disorders, future studies could focus on expanding our knowledge in a number of ways. Firstly, research may move to (sub)clinically anxious individuals. With the possible exception of **chapter 6** which included high trait worriers, all chapters that tested the clinical applicability of tVNS included healthy (i.e., unselected) student samples. Secondly, all studies included in this dissertation focused on studying the effects of acute tVNS, administered within a single session. Although acute tVNS has been shown to increase LC activity within minutes after stimulation onset [122,270], preclinical studies have shown that activity in the LC may increase further during prolonged VNS, and serotonergic effects of VNS have been demonstrated only after prolonged stimulation of the vagus nerve (i.e. after 2 weeks of VNS) [95]. Indeed, prolonged tVNS has been shown to decrease symptoms of depression and anxiety in a depressed patient population [300], although this study did not include a properly randomized sham control group. Thus, moving tVNS research towards studying prolonged stimulation protocols, and doing this in (sub)clinical patients will greatly improve our ability to gauge the clinical applicability of tVNS as a standalone or add-on treatment for anxiety disorders.

Conclusion

The studies presented in this thesis provide mixed support for the hypothesis that tVNS is a useful add-on or standalone intervention for the treatment of anxiety disorders. Most notably, we found that tVNS may accelerate the extinction of fear, although this effect may be diminished in arousing contexts, which would limit the clinical applicability of tVNS as an add-on for exposure treatment. Unexpectedly, we found no support for a modulatory effect of tVNS on LC-NA activity – the main hypothesized working mechanism underlying tVNS – as indirectly indexed by pupil dilation and attentional blink magnitude. The field of tVNS is currently left with a number of small-scale clinical and experimental studies boasting significant effects of tVNS, but no reliable working mechanism to explain them. All in all, this thesis provides preliminary support for the notion that tVNS may be a useful tool in the treatment of anxiety, but emphasizes the need for more elaborate fundamental studies to assess the working mechanisms, the optimal stimulation parameters, and boundary conditions of tVNS in clinical and nonclinical populations.

Dutch Summary
Nederlandse Samenvatting

Het overkoepelende doel van dit proefschrift was om vast te stellen of symptomen van angst verminderd kunnen worden door gebruik te maken van transcutane nervus vagus stimulatie (tVNS), een innovatieve en non-invasieve zenuwstimulatie techniek. De nervus vagus is een craniale zenuw die signalen verstuurd tussen de hersenen en de perifere organen, en waarvan gedacht wordt dat deze een belangrijke rol speelt in associatief leren en geheugen [114]. Tijdens tVNS wordt de nervus vagus geactiveerd door elektrische impulsen te sturen naar een specifiek deel van het oor dat door deze zenuw wordt geïnnerveerd. Voorgaand dieronderzoek in ratten heeft aangetoond dat invasieve stimulatie van de nervus vagus gebruikt kan worden om angst sneller te doen verminderen. Binnen dit proefschrift werden enkele experimentele studies uitgevoerd om de effecten van tVNS op symptomen van angst te testen bij mensen. In deel I werden de effecten van tVNS getest in angstconditioneringsparadigma's, om de bruikbaarheid van tVNS als toevoeging voor exposure therapie te testen. In deel II werd onderzocht of tVNS als opzichzelfstaande therapie effect heeft op een van de symptomen die ten grondslag liggen aan angst: perseveratieve cognities. In deel III werd gekeken naar werkingsmechanismen: wat is de optimale plek in het oor om de auriculaire vertakking van de nervus vagus (ABVN) te stimuleren, en wat is het werkingsmechanisme dat de mogelijke angst remmende werking van tVNS veroorzaakt? Specifiek is gekeken of tVNS fysiologische en gedragsmatige indexen van activiteit in het locus coeruleus – noradrenaline (LC-NA) netwerk beïnvloedt.

In dit hoofdstuk wordt een overzicht gegeven van de resultaten die in de voorgaande hoofdstukken is beschreven.

Deel I: Het uitdoven van angst

In een reeks van vier klassieke conditioneringsstudies hebben we de effecten van tVNS op de extinctie, generalisatie, en retentie van angst getest. Hieronder zal een samenvatting van ieder individueel hoofdstuk gegeven worden.

Hoofdstuk 2 beschrijft de eerste gepubliceerde experimentele studie die de effecten van tVNS op de extinctie en retentie van angst heeft onderzocht in mensen. In deze studie werd gebruik gemaakt van een differentieel conditioneringsparadigma, waarbij de extinctie fase op dezelfde dag plaatsvond als de acquisitie fase en de retentie fase 24 uur later [222]. Deelnemers werden willekeurig toegewezen om tVNS of placebo stimulatie te ontvangen. Deelnemers die tVNS kregen lieten een snellere extinctie van differentiële declaratieve angst zien - zoals bleek uit de steilere afname in de verwachting om een harde, aversieve gil te horen – vergeleken met deelnemers die placebo stimulatie kregen. Daarentegen waren er geen verschillen tussen de groepen in verwachtingen om de gil te horen tijdens de retentietest vierentwintig uur later, wat suggereert dat de verdere verwerking van extinctie herinneringen niet beïnvloed werd door tVNS. Deelnemers lieten echter geen fysiologische responsen

zien die zouden suggereren dat er differentiële angst is aangeleerd gedurende de acquisitie fase. Hierdoor konden de effecten van tVNS op de extinctie van fysiologische angst niet getest worden. In deze studie werden de eerste indicaties gepresenteerd die suggereren dat tVNS de extinctie van angst zou kunnen versnellen, hoewel dit enkel kon worden getest in declaratieve maten van angst en niet in fysiologische maten.

Hoofdstuk 3 beschrijft een onderzoek dat gelijktijdig werd uitgevoerd met de studie die in hoofdstuk 2 is beschreven. Hoewel de algemene structuur van het angstconditioneringsparadigma hetzelfde was, waren er meerdere belangrijke verschillen tussen de experimentele designs: ten eerste werd in deze studie de extinctiefase pas 24 uur na de acquisitiefase uitgevoerd. Ten tweede ontvingen alle deelnemers placebostimulatie gedurende de acquisitie- en retentiefasen van het onderzoek. Tijdens de extinctiefase werd de helft van de deelnemers willekeurig toegewezen aan een conditie waarin ze tVNS ontvingen, terwijl de andere helft van de deelnemers placebostimulatie kregen. In overeenstemming met de resultaten uit hoofdstuk 2, zagen we opnieuw dat tVNS de extinctie van declaratieve angst versnelde, maar geen sterkere retentie van extinctiegeheugen teweegbracht. Daarnaast waren er geen effecten van tVNS op de generalisatie, re-acquisitie, en het herstel van angst. Tot slot werden er geen eenduidige effecten van tVNS op fysiologische metingen van angst gevonden. Deze studie bevestigde het effect van tVNS op de versnelde extinctie van angst die gevonden werden in **hoofdstuk 2**, terwijl er voor het eerst werd aangetoond dat tVNS geen effect heeft op fysiologische maten van angst.

In **hoofdstuk 4** hebben we geprobeerd de bevinding uit de twee eerdere hoofdstukken dat tVNS de extinctie van angst versnelt te repliceren in een grotere steekproef. Aangezien we niet in staat waren om differentiële angstconditionering aan te tonen in hoofdstuk 2, werden meerdere aanpassingen gemaakt aan het experimentele paradigma om de opwinding van deelnemers en de negatieve valentie van de US te verhogen [217], om de fysiologische angstconditionering te versterken. Ten eerste werden de geconditioneerde stimuli veranderd van geometrische figuren naar plaatjes van spinnen, omdat eerdere studies hebben gesuggereerd dat het gebruik van dergelijke stimuli tot sterkere acquisitie van angst leidt [201]. Ten tweede werd er gedurende het gehele experiment een achtergrondgeluid van 70dB gepresenteerd, en werd de intensiteit van de *startle probe* aangepast om 2.5 keer luider te zijn dan daarvoor (van 100 naar 104dB). Tot slot werd er gebruik gemaakt van een aversieve elektrische schok die individueel gekalibreerd werd om erg oncomfortabel te zijn. In tegenstelling tot de eerdere studies, lieten deelnemers die tVNS ontvingen geen versnelde extinctie van declaratieve of fysiologische angst zien ten opzichte van deelnemers die placebostimulatie ontvingen. Deelnemers die tVNS ontvingen rapporteerden wel lagere verwachtingen om een schok te ontvangen tijdens presentaties van de CS- aan het begin van de extinctie fase, wat een mogelijke indicatie zou kunnen zijn dat tVNS het verwerken van veiligheidssignalen vergemakkelijkt. Deze

bevinding zou in lijn zijn met de *Generalized Unsafety Theory of Stress*, welke onder meer poneert dat activiteit van de nervus vagus de inhibitie van stressresponsen versterkt in aanwezigheid van veiligheidssignalen [339]. Wij hadden deze bevinding vooraf niet voorspeld, en deze ook niet gevonden in de twee eerdere studies. De discrepantie tussen de resultaten van deze studie en de twee eerdere hoofdstukken zou een gevolg kunnen zijn van de verhoogde opwinding die is geïnduceerd in deelnemers in hoofdstuk 4. Mogelijk als gevolg hiervan heeft deze studie geresulteerd in een conceptuele non-replicatie van de twee eerdere studies.

In **hoofdstuk 5** hebben we een onderzoek uitgevoerd om te testen of tVNS een vermindering teweegbrengt in de generalisatie van angst – een proces dat gesuggereerd wordt ten grondslag te liggen aan het ontstaan en voortbestaan van angststoornissen [108,109,229] – en vervolgens de extinctie van angst kan versnellen zoals ook bleek in hoofdstuk 2 en 3. Op basis van preklinisch bewijs in ratten, verwachtten wij dat tVNS de generalisatie van angst zou afzwakken door activiteit in de gyrus dentatus juist te verhogen. Van dit deel in de hersenen wordt gedacht dat deze het onderscheid tussen nieuwe geheugensporen en oude angst-gerelateerde geheugensporen versterkt [187,241,242]. Om deze hypothese te testen, werd gebruik gemaakt van een angstconditioneringsprotocol dat ontworpen is door Lissek en collega's [246]. In dit protocol worden deelnemers aanvankelijk blootgesteld aan differentiële angstconditionering, waarbij twee cirkels van verschillende grootte gebruikt worden als geconditioneerde stimuli. Vervolgens werden deelnemers willekeurig ingedeeld in een tVNS en een placebostimulatiegroep gedurende de daaropvolgende generalisatie- en extinctiefases. Tijdens de generalisatiefase werden cirkels gepresenteerd met verschillende grootten tussen de oorspronkelijke geconditioneerde stimuli in. In tegenstelling tot onze hypotheses, was er geen effect van tVNS op de generalisatie van angst, noch op fysiologische of declaratieve maten van angst. Tijdens de daaropvolgende extinctiefase was er wederom geen effect van tVNS op fysiologische maten van angst. Daarentegen – en in overeenstemming met de positieve vondsten uit hoofdstukken 2 en 3 - rapporteerden deelnemers in de tVNS conditie gedurende de extinctiefase lagere verwachtingen om een schok te ontvangen dan deelnemers in de placebo conditie.

Deel II: Negatieve gedachtenintrusies

Naast de mogelijke toepassing van tVNS als een *add on* voor exposure-therapie, hebben we ook onderzocht of tVNS mogelijk als behandeling voor angststoornissen zou kunnen worden ingezet. In **hoofdstuk 6** hebben we onze (gepreregistreerde) hypothese onderzocht dat tVNS het aantal negatieve gedachtenintrusies zou verminderen dat gerapporteerd wordt door chronische piekeraars tijdens een 'Breathing Focus'-taak – een taak waarin deelnemers gevraagd werd om hun aandacht op hun ademhaling te richten. Deze studie werd gedaan in een sample van studenten die hoog scoorden op een vragenlijst die de neiging tot pathologisch piekeren meet, die weer *at random* werden toegewezen

aan een tVNS of placeboconditiie. Tijdens de eerste ademhalingstaak rapporteerden deelnemers die tVNS ontvingen minder negatieve gedachtenintrusies dan deelnemers in de placebogroep. Tijdens een piekerinductie werden er echter geen verschillen gerapporteerd tussen de groepen in de intensiteit van het gepieker. Na afloop van deze piekerinductie waren er ook geen verschillen meer tussen de groepen in de hoeveelheid negatieve gedachtenintrusies die werden gerapporteerd in een volgende fase van de Breathing Focus taak. Sterker nog, in tegenstelling tot onze hypotheses bleek uit een exploratieve analyse dat een hoger percentage deelnemers in de tVNS conditie negatieve gedachten-intrusies rapporteerde direct na de piekerinductie.

Deel III: Werkingsmechanismen

Waar delen I en II van dit proefschrift zich richten op een experimentele benadering om de mogelijke angstverminderende effecten van tVNS te onderzoeken, waren er ook fundamentelere vraagstukken rondom tVNS onbeantwoord gebleven. In Deel III richtten we ons op fundamentele vraagstukken met betrekking tot de optimale locatie om de vagus te stimuleren en de werkingsmechanismen van tVNS.

In **hoofdstuk 7** beschrijven wij fundamentele inconsistenties die over het hoofd zijn gezien in een wijd geciteerde publicatie over de zenuwbanen in het menselijk oor. Dit *paper* van Peuker en Filler uit 2002, dat de anatomische basis vormt voor de bewering dat elektrische stimulatie van het oor door tVNS apparaten daadwerkelijk de nervus vagus stimuleerden, bevat een kritieke inconsistentie. Volgens een tabel uit het oorspronkelijke artikel innerveert de auriculaire vertakking van de nervus vagus de cymba concha van het oor in 100% van alle onderzochte kadavers, terwijl de tragus werd geïnnerveerd in 45% van alle oren. Hoewel de innervatie van de vagale zenuw in de cymba concha al in eerdere studies werd aangetoond [24,298], en ook als basis diende voor de studies in de dissertatie, wordt de innervatie van de vagale zenuw in de tragus enkel beschreven in deze studie. In tegenstelling tot de tabel wordt er in de hoofdtekst van het *paper* geschreven dat de tragus wordt geïnnerveerd door de nervus auricularis magnus, de nervus auriculotemporalis, of een combinatie van de twee. De nervus vagus wordt niet genoemd als een zenuw die de tragus innerveert. In een persoonlijke correspondentie erkennen de oorspronkelijke auteurs de inconsistentie maar geven zij aan niet meer in staat te zijn te zien of de informatie uit de tabel of die uit de tekst correct is. Deze inconsistentie, die beschreven staat in hoofdstuk 7, suggereert dat onderzoekers heel voorzichtig moeten zijn in het interpreteren van resultaten van studies waarbij stimulatie van de tragus werd geïnterpreteerd als nervus vagus stimulatie. Daarnaast bevestigt dit de noodzaak van meer onderzoek naar de innervatie-patronen van de auriculaire vertakking van de nervus vagus.

In **hoofdstuk 8** werden de werkingsmechanismen die mogelijk ten grondslag liggen aan het angstremmende effect van tVNS onderzocht. Preklinische studies suggereren duidelijk betrokkenheid

van de nervus vagus in activiteit van het locus coeruleus – noradrenaline (LC-NA) netwerk: invasieve VNS in ratten leidt tot een verhoogde activiteit in de LC, wat op diens beurt hogere noradrenerge activiteit in de LC en andere hersengebieden tot gevolg had [45,95–99,115,144,304,305]. Studies naar de effecten van invasieve VNS bij mensen hebben echter wisselende resultaten opgeleverd [101–104,336]. In hoofdstuk 8 hebben wij dus getest wat het effect is van tVNS op LC-NA activiteit in een serie van drie experimentele studies. We deden onderzoek naar verschillende fysiologische en gedragsmatige indexen van LC-NA activiteit: veranderingen in pupildiameter tijdens rust, taak-gerelateerde veranderingen in pupil diameter, en prestaties tijdens een 'Attentional Blink'-taak [294,308,317,319]. In drie studies die we naar dit onderwerp gedaan hebben, zijn geen duidelijke indicaties naar voren gekomen dat tVNS de activiteit in het LC-NA netwerk moduleert: tVNS verhoogde niet de pupil diameter tijdens rust, vergrootte niet de taak-gerelateerde pupildilatatie, en had geen effecten op prestaties tijdens de uitvoering van de Attentional Blink taak. Wij hebben in deze studies dus geen indicaties gevonden dat tVNS een effect heeft op het LC-NA netwerk.

De studies in dit proefschrift bieden wisselende en gemende ondersteuning voor de hypothese dat tVNS een bruikbare (ondersteunende) interventie zou kunnen zijn voor de behandeling van angstklachten. In deel I van het proefschrift vonden we indicaties dat tVNS de extinctie van angst kan versnellen, hoewel dit effect mogelijk verminderd is in opwindende situaties, wat de klinische toepasbaarheid van tVNS als *add on* voor exposure therapie zou verminderen. In deel II vonden we dat deelnemers die tVNS ontvingen minder negatieve gedachtenintrusies rapporteerden, maar dat dit effect verdween na een korte piekerinductie. In deel III vonden we geen ondersteuning voor onze hypothese dat tVNS een effect heeft op LC-NA activiteit, het verwachtte werkingsmechanisme dat ten grondslag zou liggen aan de effecten van tVNS. Het onderzoeksveld bestaat momenteel uit vele kleinschalige klinische en experimentele studies die verscheidene significante effecten van tVNS laten zien, maar er zijn geen betrouwbare bevindingen over de werkingsmechanismen die deze effecten zouden kunnen verklaren. Er is een grote belang bij meer uitgebreide fundamentele studies naar de werkingsmechanismen, optimale stimulatie parameters van tVNS, in zowel klinische en non-klinische populaties.

Curriculum Vitae

Andreas Burger was born in Reutlingen, Germany on the 23rd of December 1989. His family moved to Hoofddorp, The Netherlands, in 1990. Andreas finished high school at the Katholieke Scholengemeenschap Hoofddorp in 2008, and started his Bachelor's degree in Psychology at Leiden University later that year. In the third year of his Bachelor, he moved to Scotland, to spend one semester studying Psychology and Philosophy at the University of Glasgow. Afterwards, he finished his Bachelor's in Leiden and started a Research Master Clinical, Health and Neuropsychology at Leiden University.

During his Master's, Andreas completed a research internship at the Chronobiology department of PsyQ in The Hague, under the supervision of Dr. Judith Haffmans and Tess Naus, studying the clinical applicability of bright light therapy for patients suffering from Bipolar Disorder. Afterwards, he completed a clinical internship at the Anxiety and Chronobiology departments of PsyQ The Hague, under the supervision of Margreet Blaauw and Dr. Judith Haffmans. After graduation in 2014, Andreas continued working as a junior researcher at the Chronobiology department of PsyQ The Hague for half a year, before starting his PhD project.

In November 2014, Andreas started his PhD project at the department of Clinical Psychology at Leiden University, under the supervision of Prof. Dr. Willem van der Does, Prof. Dr. Jos Brosschot, and Dr. Bart Verkuil. During his PhD, Andreas spent three months at the KU Leuven, to further strengthen the collaboration with Prof. Dr. Ilse Van Diest and her lab. He finished his PhD project in November 2018 and started a postdoctoral position at the KU Leuven in Belgium, under the supervision of Prof. Dr. Ilse Van Diest.

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