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

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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.