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

Burger, A.M.

Part I

Extinction of Fear

Chapter 2

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

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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.

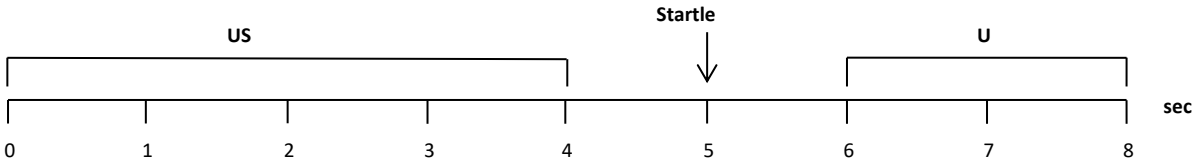

Figure 1. Schematic overview of the timing in a CS+ conditioning trial during the acquisition phase. CS+ trials during the extinction, retention and reinstatement phases were never followed by a US. CS- trials were never followed by a US. CS, conditioned stimulus; US, unconditioned stimulus.

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, $F(1, 713) = .82$, $p = .37$.

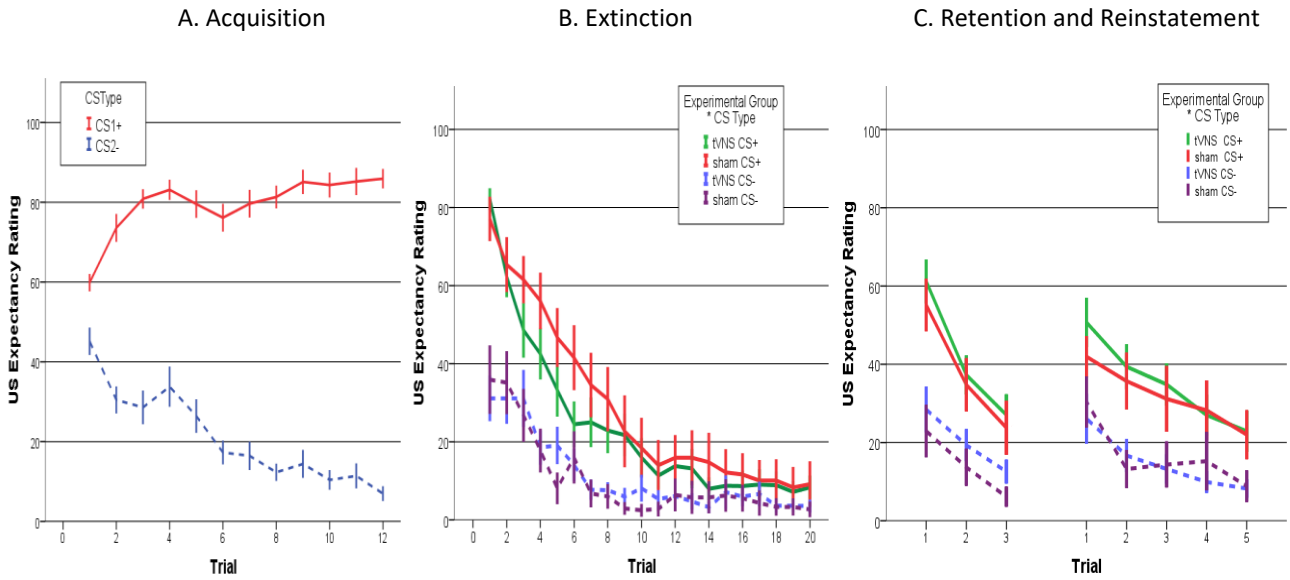

Figure 2. US expectancy ratings of the CS+ and CS- stimuli during acquisition for all participants (panel A). Panels B and C show US expectancy ratings of the CS+ and CS- stimuli during extinction (panel B), retention and reinstatement (panel C) for both experimental conditions. A significantly faster 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.