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

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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: one focusing on high trait worriers, and two in healthy populations. 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 consistent effects of tVNS on performance on the attentional blink task were found. Overall, the results of these studies indicate that tVNS does not affect these behavioral and physiological indices of noradrenergic activity.

1. 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) (Frangos, Ellrich, & Komisaruk, 2014; Yakunina, Kim, & Nam, 2016), 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 (Johnson & Wilson, 2018), 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. ; Aihua et al., 2014; Jin & Kong, 2017; Redgrave et al., 2018; Rong et al., 2016). However, the working mechanisms of VNS are currently poorly understood, and are based primarily on preclinical iVNS research (Grimonprez, Raedt, Baeken, Boon, & Vonck, 2015). 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. LC neurons are known to exhibit tonic and phasic activity modes. Tonic LC activity is related to arousal; this baseline LC firing rate is low during sleep or relaxation, moderate during engaged task performance, and high in moments of distractedness (Aston-Jones & Cohen, 2005; Gilzenrat, Nieuwenhuis, Jepma, & Cohen, 2010). In other words, this tonic LC activity displays an inverted U-shape relationship with task performance such that task performance is highest when tonic LC activity is at an intermediate level. By contrast, phasic LC activity can be observed in response to salient and task-relevant stimuli. While the exact function and mechanism of action of phasic LC activity is still under debate (Corbetta, Patel, & Shulman, 2008; Mather, Clewett, Sakaki, & Harley, 2016; Sara & Bouret, 2012), phasic LC activity seems to facilitate of attention to salient stimuli. These phasic and transient peaks in LC activity are more pronounced during intermediate compared to either high or low levels of tonic LC activity. 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 tonic firing rates in LC neurons both acutely (Chen & Williams, 2012; Dorr & Debonnel, 2006; Groves, Bowman, & Brown, 2005; Hulsey et al., 2017; Manta, El Mansari, Debonnel, & Blier, 2013) as well as over a longer timespan (after a period of 90 days: Dorr & Debonnel, 2006; after 14 and 90 days: Manta, Dong, Debonnel, & Blier, 2009). In line with these findings, several studies found increased concentrations of NE in brain areas to which the LC projects, including

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the hippocampus (Raedt et al., 2011; Roosevelt, Smith, Clough, Jensen, & Browning, 2006), basolateral amygdala (Hassert, Miyashita, & Williams, 2004) and medial PFC (Follesa et al., 2007).

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 are scarce. Unfortunately, direct measurement of NE in humans requires an invasive procedure and suffers from poor reliability and sensitivity (Grassi & Esler, 1999). Several indirect physiological markers have been proposed as suitable measurements of NE in humans. Three recent pilot studies have already assessed the effects of tVNS on these markers. In a series of three subsequent pilot studies, Warren and colleagues assessed the effects of tVNS on P300 amplitude (study 1 and 3), pupil diameter (study 2), salivary alpha amylase (sAA; study 1 and 2), and salivary cortisol (study 2). tVNS did not affect P300 amplitude or pupil diameter, but did increase sAA and decreased the decline in salivary cortisol in comparison to sham stimulation (Warren et al., 2018). Another recent pilot study assessed the effects of tVNS on two of these measures, the P300 and salivary alpha amylase (sAA) (Fischer, Ventura-Bort, Hamm, & Weymar, 2018; Ventura-Bort et al., 2018). In that study, tVNS did not affect task performance during an oddball task (Ventura-Bort et al., 2018), although tVNS did facilitate some indices of conflict processing during a Simon task (Fischer et al., 2018) – a process that is believed to be mediated by the LC-NA network (Verguts & Notebaert, 2009). 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 (Ventura-Bort et al., 2018), nor during the Simon task (Fischer et al., 2018). 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 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 baseline pupil diameter during rest or by measuring pupil dilations during 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 (Samuels & Szabadi, 2008). Indeed, pupil diameter shows strong positive correlations with LC activity and NA levels in animal studies (Joshi, Li, Kalwani, & Gold, 2016; Rajkowski, Kubiak, & Aston-Jones, 1993; Reimer et al., 2016; Varazzani, San-galli, Gilardeau, & Bouret, 2015). 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 (Hou, Freeman, Langley, Szabadi, & Bradshaw, 2005; Hou, Langley, Szabadi, & Bradshaw, 2007; Phillips, Szabadi, & Bradshaw, 2000). Moreover, pupil diameter has been shown to correlate with BOLD activity in the locus coeruleus in humans (Murphy, O'Connell, O'Sullivan, Robertson, & Balsters, 2014). Finally, in line with the adaptive gain theory of LC-NE function (Aston-Jones & Cohen, 2005), pupil diameter is larger during exploratory compared to exploitative task performance (Gilzenrat et al., 2010; Jepma & Nieuwenhuis, 2011).

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 (Bianca & Komisaruk, 2007). 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 (Desbeaumes Jodoin et al., 2015), 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.

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 200 ms after the first one, it becomes a lot harder to identify the second target than when it appears considerably later (usually 700 ms). This phenomenon is called the *attentional blink* (AB) and is thought to be caused by the temporary refractory period of LC neuron activity after the initial burst that occurred when the first target was correctly identified (Nieuwenhuis, Gilzenrat, Holmes, & Cohen, 2005). Indeed, AB occurrence is dependent on the temporal lag between the first and second target, and not on the amount of distractors that are presented in between the two targets, which reinforces the theory that the refractory period of LC neuron activity is the driving force behind the AB phenomenon (Warren et al., 2009). Indeed, attentional blink occurrence has been found to be positively related to other measures of noradrenergic activity such as pupil dilation (Zylberberg, Oliva, & Sigman, 2012) and P300 amplitude (Nieuwenhuis, Aston-Jones, & Cohen, 2005), 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 (Usher, Cohen, Servan-Schreiber, Rajkowski, & Aston-Jones, 1999). 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) (De Martino, Strange, & Dolan, 2008). Other neuropharmacological studies in which central NA levels were manipulated have failed to find these effects, however (Brown et al., 2016; Nieuwenhuis, Van Nieuwpoort, Veltman, & Drent, 2007).

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 (Burger, Van der Does, Thayer, Brosschot, & Verkuil, 2019). 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 (De Martino et al., 2008). 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.

2. Overall experimental procedures

2.1. Ethics

Ethical approval for this all three studies was provided by the ethical committee of the Institute of Psychology of Leiden University. Participants were compensated for participating in one of the experiment with partial course credit or 8 euro's per hour that the experiment lasted. Monetary compensation was 10 euros for study 1, 13 euros for study 2, and 7 euros for study 3.

2.2. Instruments and questionnaires

2.2.1. 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-s waves of electrical stimulation (0.5 mA, 25 Hz, 250μs), alternated by 30-s 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 (Peuker & Filler, 2002). 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 (Peuker & Filler, 2002).

2.2.2. 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) (Meyer, Miller, Metzger, & Borkovec, 1990; Verkuil & Brosschot, 2012), the State Trait Anxiety Inventory (STAI) (Barnes, Harp, & Jung, 2002; Spielberger, Gorsuch, & Lushene, 1970) and the Attentional Control Scale (ACS) (Derryberry & Reed, 2002; Judah, Grant, Mills, & Lechner, 2014), and several study-specific questionnaires were added separately in each study.

2.2.3. 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 1024 Hz and was automatically cleaned for outliers and measurements artifacts by the Movisens Data-Analyzer software.

Every study included a 5-min 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.

2.2.4. Pupillometry

All three studies were performed in a lab room under moderate lighting conditions of approximately 100 lx to maximize cognitively-evoked pupil dilations (Steinhauer, Siegle, Condray, & Pless, 2004). Luminance was measured using a Lutron LX1108 lx m. Pupil diameter was measured using a Tobii T120 eye tracker, which is integrated into a 17″ TFT monitor. Participants were instructed to place their head in a chinrest during pupil dilation measurements, to avoid measurement artifacts due to head movements. 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 120 Hz.

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 250 ms. Preprocessing of pupil size data was conducted using a customized open source MATLAB script (Kret & Sjak-Shie, 2020).

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 min, 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 100 ms 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 200 ms 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.

2.3. 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 100 ms per stimulus. The RSVP stream consists mostly of distractor stimuli, and includes 2 targets (T1 and T2) embedded in the stream (see Fig. 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 200 ms (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 (Nieuwenhuis, Gilzenrat et al., 2005).

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 (Warton & Francis, 2011). Therefore, we applied a logit transformation, $\log(\frac{p}{1-p})$, which makes the dependent variable unbounded and allows for regular linear mixed modelling (Warton & Francis, 2011). As the logit transformation cannot be applied to proportions of 0 or 1, we added 0.001 to the scores of participants who were 0 % accurate at detecting T2|T1. Similarly, we subtracted 0.001 from scores of participants who were 100 % accurate at detecting T2|T1.

2.4. Statistical analyses

We conducted linear mixed model analyses to test the effects of tVNS on pupil diameter at rest (all three studies), AB task performance (all three studies), and task-related pupil dilation (studies 2 and 3). We allowed intercepts to vary randomly across participants. In the models for pupil diameter and task-related pupil dilation, we added random slopes for *Measurement* or *Time* when this increased the BIC model fit compared to models that included only a random intercept (see Table 1). All analyses were conducted using full information maximum likelihood modelling.

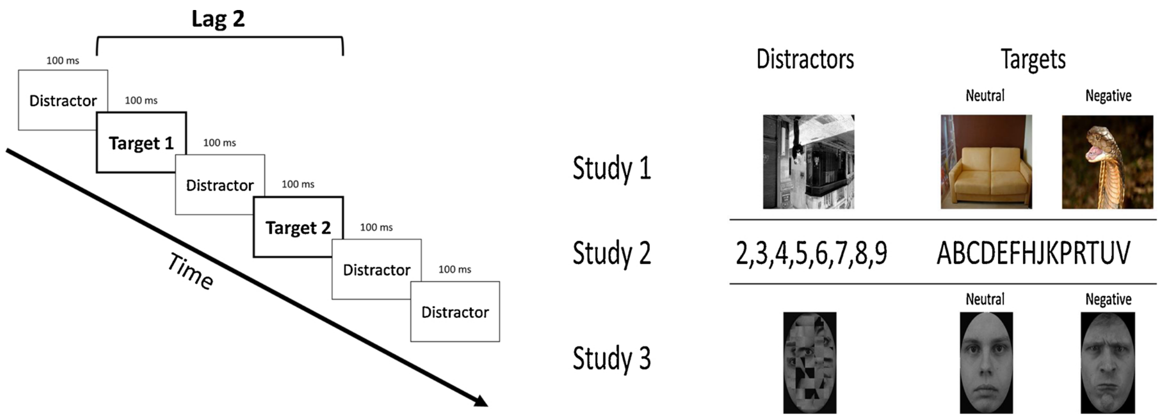

Fig. 1. Overview of Attentional Blink paradigms. *Left*: Each trial consisted of a series of stimuli presented for 100 ms, immediately followed by a subsequent stimulus. Participants were instructed to identify the target pictures that were presented in the RSVP stream. In study 2 and 3, some trials 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 200 ms (shown in the picture; 'Lag 2') or 700 ms (Lag 7). 200 ms 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 (Usher et al., 1999). 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.

To assess whether tVNS affects resting pupil diameter, 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).

To assess whether tVNS affects AB task performance, we tested 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 the three valence task manipulations present in the task: 1) both T1 and T2 were neutral (henceforth this will be described as $T1_{neut}$-$T2_{neut}$), 2) T1 was negative but T2 was neutral ($T1_{neg}$-$T2_{neut}$); 3) T1 was neutral but T2 was negative ($T1_{neut}$-$T2_{neg}$). *Valence* was added as a categorical variable, with the reference category being $T1_{neut}$–$T2_{neut}$).

To assess the effects of tVNS on task-related pupil dilation, we tested the effects of *Condition* and *Time* (continuous variable, indicating the 100 ms time bin corresponding with one stimulus presentation with an AB trial) on baseline-corrected pupil diameter.

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 Fig. 4, participants displayed a clear pupillary constriction in the first 600 ms 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 600 ms, 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. The model included random intercepts and two random slopes, one for each side of the knot.

All analyses were conducted in R version 3.5.2 using the *lme4* and *lmerTest* packages.

3. Study 1

3.1. Methods study 1

3.1.1. Participants

We aimed to test 102 chronically worrying students between the ages 18–25. The sample size calculation for this study was based on testing effects of tVNS on worry behavior as described in (Burger et al., 2019). Given that 68 participants should be included to detect at least a medium effect size δ = 0.5 for a Condition*Measurement interaction in a repeated measures analysis with 3 repeated measurements, given α = 0.05 and a power of .80, the current study was highly powered to detect small-to-medium effects sizes.

Table 1
Mixed model analyses.

AB task performance	
Study 1 and 3	T2|T1 ~ Valence*Lag*Condition + (1 | Subject)
Study 2	T2|T1 ~ Lag*Condition + (1 | Subject)
Pupil Diameter	
Study 1 and 3	Pupil Diameter ~ Time*Measurement + (1 | Subject)
Study 2	Pupil Diameter ~ Time* Measurement + (1 + Session | Subject)[a]
Pupil Dilation	
Study 2	$Diameter_{Baseline\ Corrected}$ ~ Condition*Time + (1 + Time | Subject)
Study 3	$Diameter_{Baseline\ Corrected}$ ~ Condition*$Time_{0\text{-}600ms}$ + Condition*$Time_{600\text{-}3000ms}$ + (1 + $Time_{0\text{-}600ms}$ + $Time_{600\text{-}3000ms}$ | Subject)

Note. Description of the final models described in the results sections of studies 1-3. All models that included interaction between variables also included the lower-order interactions and main effects of the variables included in the interactions (e.g. a model containing Valence*Lag*Condition interaction also included all main effects and two-way interactions of these variables.
Models are built up as: Score on Dependent Variable ~ Fixed Effects + (Random Intercept + Random Slope Terms | Grouping Factor).
[a] A random slope for Session was added in this model, as participants' baseline pupil diameter varied between the first and second session, irrespective of whether they received tVNS or sham stimulation.

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 (Behar, Alcaine, Zuellig, & Borkovec, 2003). Participants suffering from current or past neurological, psychological, or cardiac disorders were excluded from the current study.

3.1.2. Procedure

This study was part of a larger project focused on assessing the effects of tVNS on worry behavior and stress-related attentional biases (Burger et al., 2019; Verkuil & Burger, 2019). 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 remainder of the study. Subsequently, a 2-min 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.

With the tVNS device activated, participants were instructed to complete a five-minute baseline recording of HRV. Subsequently, participants completed several questionnaires. The questionnaires included the ones mentioned in the Overall Experimental Procedures section, plus the Generalized Anxiety Disorder-7 (GAD-7) (Löwe et al., 2008; Spitzer, Kroenke, Williams, & Löwe, 2006) and the Ruminative Response Scale (RRS) (Just & Alloy, 1997). On average, filling in the questionnaires took approximately 15 min.

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 (this task and the effects of tVNS are described in (Burger et al., 2019)). Subsequent to the Breathing Focus Task, participants completed a second pupillometry measurement, followed by the Attentional Blink Task and an Inhibition of Return Task (described elsewhere Verkuil & Burger, 2019). 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 min. Participants received tVNS or sham stimulation for roughly 80 min.

3.1.3. Instruments

3.1.3.1. 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 (Lang, Bradley, & Cuthbert, 2008). 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 (de Oca, Villa, Cervantes, & Welbourne, 2012), 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 ($T1_{neut}$-$T2_{neut}$), emotional disengagement ($T1_{neg}$-$T2_{neut}$), and emotional engagement ($T1_{neut}$-$T2_{neg}$) (cf. De Martino et al., 2008). The first target always appeared at RSVP location 4, 5 or 6. The second target was presented either 200 ms (*lag 2*) or 700 ms (*lag* 7) after the onset of T1. Thus, participants completed 18 trials of every order-lag combination.

3.1.3.2. Recordings of pupil size at rest. As described above, participants were instructed to 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 (Teufel & Wehrhahn, 2000).

3.2. Results Study 1

3.2.1. Demographics

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 2, 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 (Van Der Heiden, Muris, Bos, Van Der Molen, & Oostra, 2009). 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-7} = 3.0, Löwe et al., 2008). 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; Crawford, Cayley, Lovibond, Wilson, & Hartley, 2011). Similarly, compared to general student populations, participants scored above average on rumination (RRS; Schoofs, Hermans, & Raes, 2010), and below average on attentional control (ACS; Fajkowska & Derryberry, 2010). This is in line with earlier studies showing that attentional control is reduced in chronic worriers (Fox, Russo, & Dutton, 2002; Stefanopoulou, Hirsch, Hayes, Adlam, & Coker, 2014).

3.2.2. Resting pupil diameter

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

As shown in Fig. 2, 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 min of stimulation, $b = -0.01$ (0.05), $t(178) = 0.12$, $p = .89$, or after approximately 80 min of stimulation, $b = < -0.01$ (0.05), $t(173) = < 0.01$, $p > .99$.

Table 2
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[a]	9.13 (4.31)	8.83 (5.01)	–	–	–
QIDS	–	–	4.31 (2.66)	4.28 (3.29)	4.90 (3.09)
Log Baseline RMSSD[b]	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.

[a] 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.

[b] 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.

3.2.3. Behavioral effects

Participants in both conditions were significantly more accurate at detecting T2|T1 when the temporal lag between T1 and T2 was 700 ms (i.e. lag 7) compared to when it was 200 ms (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 $\text{Valence}_{T2=\text{Neg}}$, $b = 2.13$ (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*$\text{Valence}_{T2=\text{Neg}}$ 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 $\text{Valence}_{T1=\text{Neg}}$, $b = -0.78$ (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 $\text{Valence}_{T2=\text{Neg}}$*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.

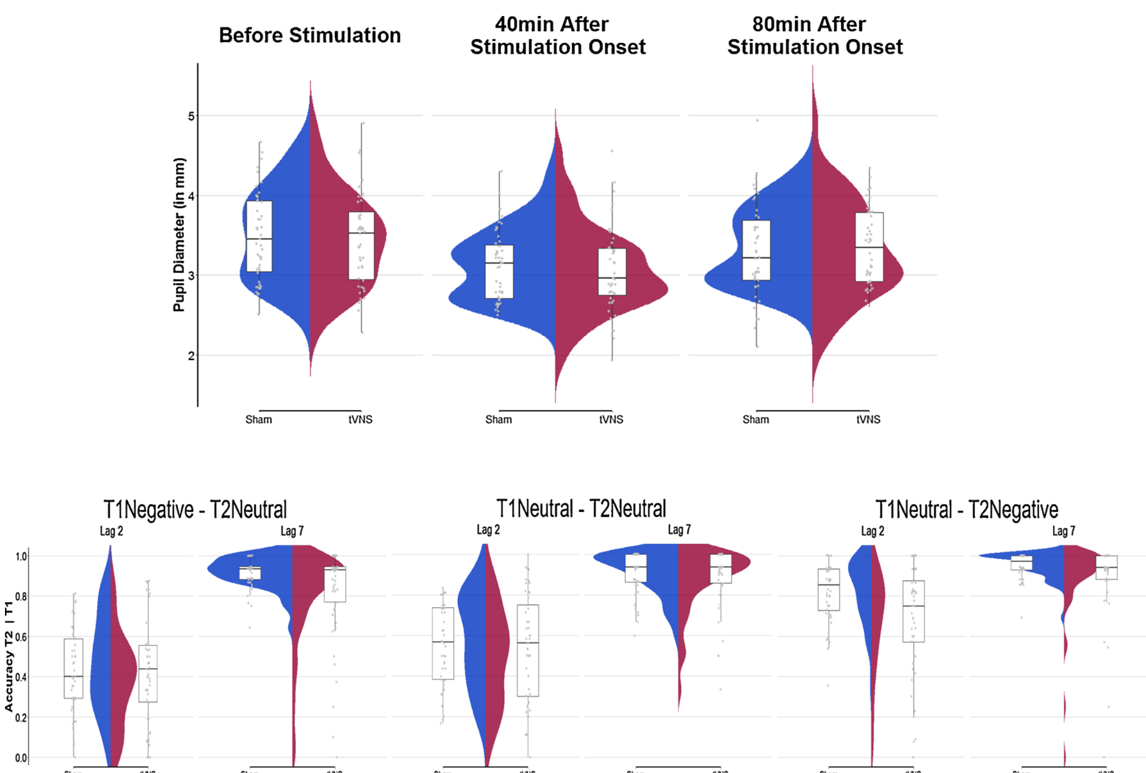

Fig. 2. Accuracies and Resting Pupil Diameters for participants in the tVNS and sham condition in study 1. Top row: violin plots and boxplots of resting pupil diameters before stimulation, directly after the first computer task (after stimulation onset), and at the end of the experimental procedure (after stimulation onset). Pupil diameter was recorded in 2-min 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.

3.3. Discussion study 1

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 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. 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 (Aston-Jones & Cohen, 2005).

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.

4. Study 2

4.1. Methods Study 2

4.1.1. Participants

We aimed to include 30 healthy participants in this randomized crossover study. This sample size was based on a power analysis that was performed beforehand, that showed that 28 participants should be included to detect at least a medium effect size $\delta = 0.5$ of the main effect of Condition in a within-subjects repeated measures analysis, given $\alpha = 0.05$ and a power of .80. Participants suffering from current or past neurological, psychological, or cardiac disorders 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.

4.1.2. 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 *complete_ra* function of the *RandomizR* package (v.0.20.0) 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.

4.1.3. 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 min. Participants received tVNS or sham stimulation for roughly 32 min.

4.1.4. 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 s. 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 100 ms. 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 Fig. 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 (Wierda, van Rijn, Taatgen, & Martens, 2012). Participants were asked to type in which targets they had seen as well as whether the RSVP was followed by a dot or semicolon.

4.2. Results study 2

4.2.1. Demographics

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 2. Scores on the PSWQ, ACS, STAI-T, QIDS, as well as baseline resting RMSSD corresponded with normative samples (Crawford et al., 2011; Fajkowska & Derryberry, 2010; Nunan, Sandercock, & Brodie, 2010; Rush et al., 2003; Van Der Heiden et al., 2009).

4.2.2. Resting pupil

Participants showed a significant decrease in pupil diameter from pre-stimulation baseline to 5 min 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 min after stimulation onset ($p = .71$), nor from pre-stimulation baseline to 30 min after stimulation onset ($p = .87$).

4.2.3. 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 ($p = .76$).

4.2.4. Phasic pupil dilation

As can be seen in Fig. 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, $p = .83$.

4.3. Discussion study 2

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.

5. Study 3

5.1. Methods study 3

5.1.1. Participants

We aimed to include 80 students from Leiden University between the ages 18–28 in this study. This sample size was based on a power analysis that was performed beforehand, that showed that 68 participants should be included to detect at least a medium effect size $\delta = 0.5$ for a Condition*Measurement interaction in a repeated measures analysis, given $\alpha = 0.05$ and a power of .80. Eighty participants were recruited to account for the risk of having to drop participants because of measurement artifacts. Participants suffering from current or past neurological, psychological, or cardiac disorders 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.

5.1.2. Procedure

Participants applied to participate in this experiment 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 min. Participants received tVNS or sham stimulation for roughly 32 min.

5.1.3. Attentional blink task

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 (Calvo & Lundqvist, 2008). Distractor stimuli were created by scrambling the neutral faces (Müsch, Engel, & Schneider, 2012). 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 Fig. 1.

Every stimulus appeared on the screen for 100 ms. 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 $T1_{neut}$, and 8 were $T1_{angry}$. The 108 two-target trials were evenly distributed into $T1_{neut}$-$T2_{neut}$, $T1_{neut}$-$T2_{angry}$, and $T1_{angry}$-$T2_{neut}$ trials. In every two-target condition, the T2 was presented 18 times both at lag 2 and at lag 7.

5.2. Results study 3

5.2.1. Demographics

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 2. Scores on the PSWQ, ACS, STAI-T, QIDS corresponded with normative samples (Crawford et al., 2011; Fajkowska & Derryberry, 2010; Rush et al., 2003; Van Der Heiden et al., 2009).

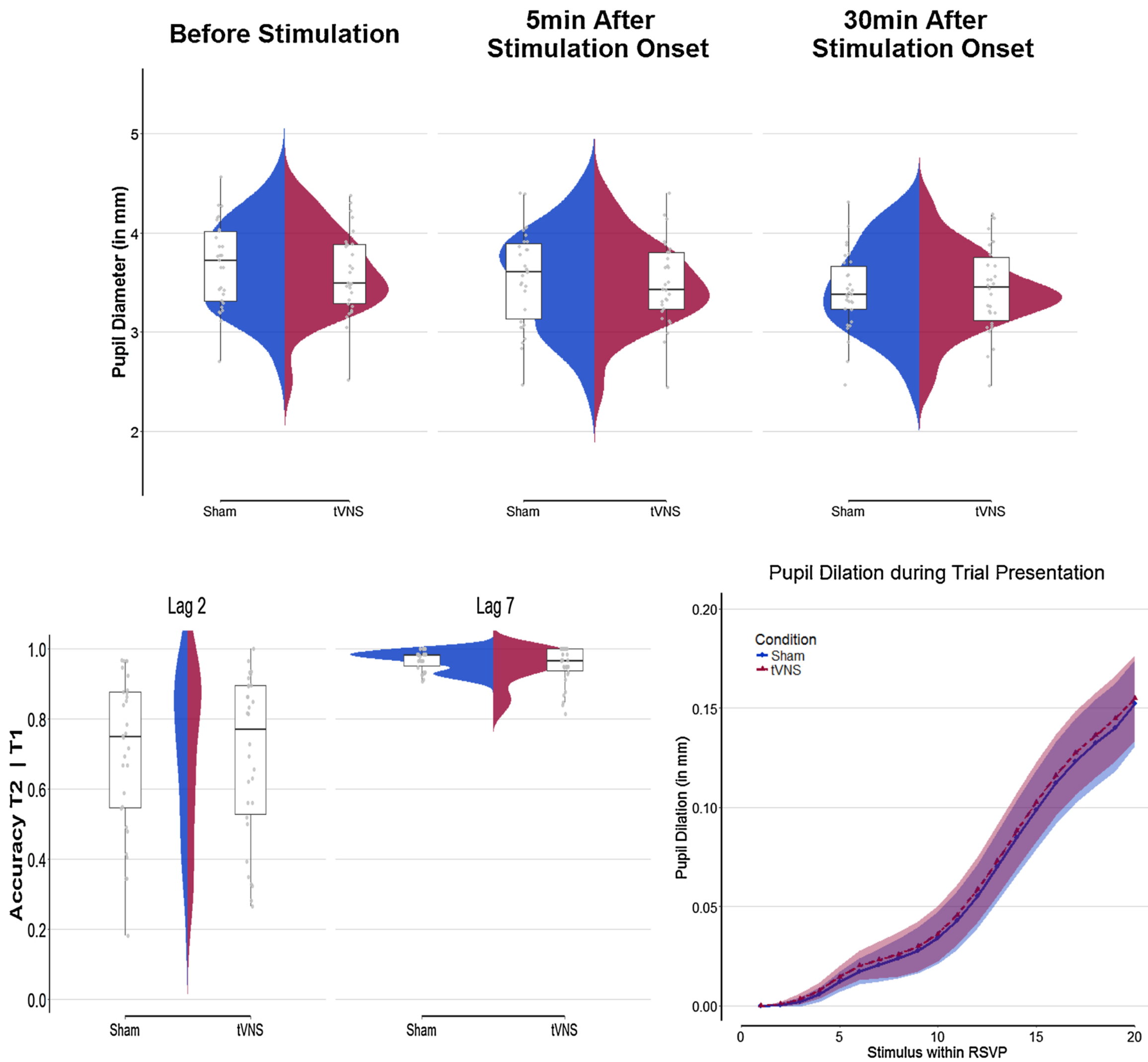

Fig. 3. Accuracies, Pupil Dilation, and resting Pupil Diameters for participants in the tVNS and sham condition in study 2. *Top row*: violin plots and boxplots of resting pupil diameters before stimulation, after stimulation onset, and directly after the AB task (after stimulation onset). Pupil diameter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

5.2.2. *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 2. Scores on the PSWQ, ACS, STAI-T, QIDS, as well as baseline resting RMSSD corresponded with normative samples (Crawford et al., 2011; Fajkowska & Derryberry, 2010; Nunan et al., 2010; Rush et al., 2003; Van Der Heiden et al., 2009).

5.2.3. *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 $\text{Valence}_{T1=\text{Neg}}$, $b = -1.97$ (.48), $t(400) = -4.15$, $p < .001$. The effect of T1 valence was specific for lag 2 trials, as indicated by the $\text{Valence}_{T1=\text{Neg}}$*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, $\text{Valence}_{T2=\text{Neg}}$, 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 Fig. 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 $\text{Valence}_{T2=\text{Neg}}$, 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

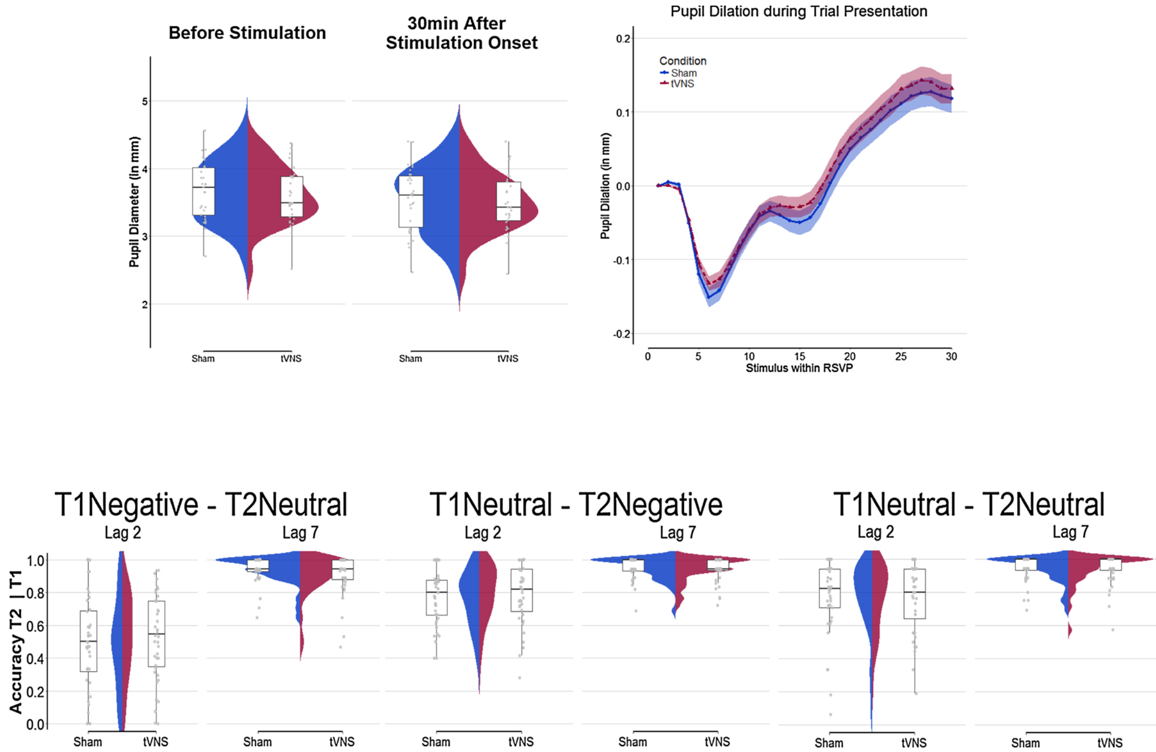

Fig. 4. Accuracies, Pupil Dilation, and resting Pupil Diameters for participants in the tVNS and sham condition in study 3. *Top Left*: Violin plots and boxplots of resting pupil diameters before stimulation and directly after the AB task (after stimulation onset). Pupil diameter was recorded in 2-min baseline recordings. *Top Right*: Pupil dilation over the course of an AB trial for participants in the tVNS and sham conditions. The shaded ribbon areas reflect confidence interval of ± 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.

and the non-significant interaction effects of Condition, Lag, and Valence.

5.2.4. Task-related pupil dilation

As can be seen in Fig. 4, participants displayed a significant pupillary constriction during the first 600 ms 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, $p = .58$.

5.3. 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.

6. 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 (Chen & Williams, 2012; Dorr & Debonnel, 2006; Follesa et al., 2007; Groves et al., 2005; Hassert et al., 2004; Hulsey et al., 2017; Manta et al., 2009, 2013; Raedt et al., 2011;

Roosevelt et al., 2006), as well as fMRI studies, which showed increased activity in the NTS and the LC after tVNS compared to sham stimulation (Frangos et al., 2014; Yakunina et al., 2016). 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 (Brázdil et al., 2001; De Taeye et al., 2014; Desbeaumes Jodoin et al., 2015; Hammond, Uthman, Reid, & Wilder, 1992; Schevernels et al., 2016). A recent study on the effects of transcutaneous VNS in humans also found no significant effects of tVNS compared to sham stimulation on pupil dilation (Warren et al., 2018). Finally, effects of tVNS on alternative indices of LC-NA activity, including P300 and salivary alpha amylase, also did not produce significant effects (Fischer et al., 2018; Ventura-Bort et al., 2018; Warren et al., 2018). 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 indirect markers of 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 (De Martino et al., 2008), 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 (Aston-Jones & Cohen, 2005), the effects of LC activity on task performance strongly resembles the inverted-U curve proposed to underlie the relation between arousal and task performance (Yerkes & Dodson, 1908). 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 challenge for the LC-NA explanation that has repeatedly been suggested for the cognitive and emotional tVNS effects that have thus far been found (e.g. (Burger et al., 2016, 2017; Sellaro, Van, & Colzato, 2015)), 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 Grimonprez et al., 2015). Moreover, a recent study in humans showed that tVNS increases the functional connectivity between the dorsal prefrontal cortex and the amygdala (Liu et al., 2016). 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 s rest with 30 s active stimulation. Stimulation consisted of square wave pulses with a 250μs pulse width, delivered at 25 Hz with an intensity set at 0.5 mA. These parameters were selected based on previous reports of parameter-dependent effects of iVNS following an inverted U-shape function (Clark et al., 1998; Clark, Naritoku, Smith, Browning, & Jensen, 1999). 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. Verkuil & Brosschot, 2012). 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 (Fay, 1927). 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.

A second consideration in selecting stimulation parameters for tVNS is the duty cycle. In all three studies, we utilized the preprogrammed 30 s ON/30 s OFF duty cycle of the Nemos device. However, recent research in rats indicated that while VNS increases activity in the LC almost instantaneously after stimulation onset, the effects also dissipate rapidly after stimulation offset (Hulsey et al., 2017). This would suggest that tVNS may have affected LC activity in only half of the trials during all three experiments. Future researchers should consider utilizing either a continuous stimulation duty cycle, or continuously measuring the electrical tVNS output to differentiate between periods where stimulation is on or off.

One could argue that the null results in this study are partly due to the experimental paradigm that was used. Indeed, although the AB task has been associated with LC-NA activity (De Martino et al., 2008; Nieuwenhuis, Gilzenrat et al., 2005), this association has not been found consistently (Brown et al., 2016). 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. The pupillary light reflex that was only present in study 3 and not in study 2 – a result of differences in luminosity between intertrial intervals and trial presentations in study 3 but not in study 2 - inadvertently produced a manipulation check for our paradigm, showing that our experimental paradigm allowed us to accurately track changes in pupil diameter. This strengthens our confidence in the null findings described in this study to truly reflect that tVNS did not affect pupil dilation. Nonetheless, we urge researchers interested in studying the effects of tVNS on LC-NA activity to consider alternative experimental paradigms. The oddball paradigm has been used in earlier studies on the effects of tVNS on LC-NA activity (Ventura-Bort et al., 2018; Warren et al., 2018), and has repeatedly been shown to be sensitive to noradrenergic manipulations (Polich & Criado, 2006). 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.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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