



## Transcutaneous vagus nerve stimulation via tragus or cymba conchae: Are its psychophysiological effects dependent on the stimulation area?

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### ABSTRACT

Efforts in optimizing transcutaneous vagus nerve stimulation (tVNS) are crucial to further develop its potential in improving cognitive and autonomic regulation. The present study focused on this topic. The aim was to compare for the first time the main stimulation areas of the ear currently used in studies with tVNS, taking cognitive as well as neurophysiological effects into account. The main areas to be compared with one another were tragus, cymba conchae, and earlobe (sham) stimulation. Post-error slowing, which has already been shown to be influenced by tVNS, was used to investigate the cognitive effects of tVNS when applied on the different auricular areas. On the neurophysiological level, we measured pupillary responses as an index of norepinephrine activity during post-error slowing, and cardiac vagal activity to investigate the activation of neural pathways involved in post-error slowing. Stimulation of different auricular areas led to no differences in post-error slowing and in pupillary responses. However, the neurological processes involved in post-error slowing could be observed, since norepinephrine activity increased after committing an error. Further, there was an increase in cardiac vagal activity over the test period that was independent of the stimulation areas. The results suggest that tVNS targeting the ear might have a non-specific effect on the processing of error commission, on pupillary responses, and on cardiac vagal activity. We conclude that it is necessary to consider alternatives for sham conditions other than electrical earlobe stimulation.

### 1. Introduction

Transcutaneous vagus nerve stimulation (tVNS) is a noninvasive technology used to electrically modulate brain activity via afferent vagal pathways (Colzato and Vonck, 2017). In 2019, 59 studies using the term “transcutaneous vagus nerve stimulation” appeared in Web of Science.<sup>1</sup> Compared to only two publications in 2009, this represents a growth of 2850% within 10 years. Many of these studies have investigated how tVNS enhances cognitive (e.g., Beste et al., 2016) and neurophysiological (e.g., Antonino et al., 2017) processes in healthy humans. Nevertheless, because of the novelty of this technology and the absence of standards regarding stimulation protocols, the tVNS-related stimulation

parameters have not been used consistently in research (Badran, Mithoefer, et al., 2018), which impedes the comparability of such studies. Currently, a hot topic in this regard is the debate about the stimulation of different parts of the ear. The present work addresses this issue and investigates for the first time the influence of applying tVNS on different parts of the ear regarding behavioral (cognitive) and neurophysiological processes. On a behavioral level, we considered post-error slowing (PES), and on a neurophysiological level we took norepinephrine-related pupillary responses and cardiac vagal activity (CVA) into account.

The working mechanism of tVNS in the brain has been profusely investigated by means of functional magnetic resonance imaging (fMRI).

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In comparison to sham stimulation or baseline measurement, active stimulation has shown to increase nucleus tractus solitarius activity, providing evidence that an electrical signal transcutaneously applied at the ear is projected to the medulla oblongata in the brainstem (Frangos et al., 2015; Frangos and Komisaruk, 2017; Sclocco et al., 2019; Yakunina and Kim, 2017). Moreover, the locus coeruleus—a brain area that is highly connected with the nucleus tractus solitarius and is considered to be the primary source of norepinephrine in the brain (Foote et al., 1983)—was found to have an increased activity during tVNS (Dietrich et al., 2008; Kraus et al., 2013). Furthermore, activations in the spinal trigeminal nucleus and insula have been reported (Dietrich et al., 2008; Frangos et al., 2015; Kraus et al., 2013). The activity of brain areas such as the hypothalamus and the amygdala have shown heterogeneous results, i.e., in some studies they increased and in others decreased (Dietrich et al., 2008; Frangos et al., 2015; Kraus et al., 2007, 2013; Yakunina and Kim, 2017). Importantly, cortical areas such as cingulate and prefrontal cortices, which are crucial brain areas for executive control, response selection, error monitoring, and conflict adaptation (Aston-Jones and Cohen, 2005; Logue and Gould, 2014; Ullsperger et al., 2014), have also been reported to show increased activity (Badran, Mithoefer, et al., 2018; Dietrich et al., 2008; Frangos and Komisaruk, 2017). To summarize, these studies showed that tVNS can activate “classical” vagal pathways (Frangos and Komisaruk, 2017).

The areas affected by tVNS in the fMRI studies are part of the central autonomic network, an internal regulation system through which the brain controls autonomic processes (Benarroch, 1993). According to the neurovisceral integration model (Thayer et al., 2009), the brain areas that form the central autonomic network are an integral part of neuro-anatomical pathways of the vagus nerve. Accordingly, the optimal activation of the neural pathways within this network is crucial for performing tasks that require executive functioning (Thayer et al., 2009).

Despite providing substantial evidence towards tVNS producing a significant activation of central vagal projections, the reviewed fMRI studies do not show consistent results regarding brain areas affected by tVNS. The heterogeneity of results might be partly explained by the use of different stimulation parameters across these fMRI studies (Borges et al., 2019; Butt et al., 2019). Given the substantial heterogeneity in tVNS literature regarding the choice of stimulation parameters, the lack of knowledge about optimal stimulation parameters can be seen as a general limitation in this research field (Badran, Mithoefer, et al., 2018; Butt et al., 2019; Clancy et al., 2014). Varying electrode placement may play a crucial role in the divergence of these results (Butt et al., 2019).

Recently, tVNS electrode placement on the ear has become an important topic of debate in research. This is likely due to the fact that mainly two auricular areas have been established as target areas for tVNS, namely cymba conchae and tragus, with both of them showing increased brain activation patterns compared to sham stimulation (Badran, Dowdle, et al., 2018; Yakunina and Kim, 2017). Yakunina and Kim (2017) compared both auricular areas, among others, with sham in an fMRI study and found activation of vagal pathways in the brain during both cymba conchae and tragus stimulation. However, cymba conchae stimulation led to stronger activations compared to tragus stimulation. However, because they only used fMRI, no insights into either cognitive or autonomic regulation were possible.

The justification used for choosing cymba conchae or tragus to deliver tVNS mainly relies on one single anatomical study in which the nerve supply of the ears of seven cadavers were exposed (Peuker and Filler, 2002). According to this study, the tragus is 45% innervated by the auricular branch of the vagus nerve (ABVN), whereas the cymba conchae has 100% of its fibers from the ABVN. Importantly, this study remains to date the only cadaver ear dissection study with a detailed description of the vagal innervation in the tragus (Burger and Verkuil, 2018). On the one hand, results from studies using tragus stimulation have been questioned due to inconsistencies in the reporting of innervation patterns in Peuker and Filler's (2002) study, meaning that it is

still too premature to interpret tragus stimulation as a reliable way to stimulate the ABVN (Burger and Verkuil, 2018). On the other hand, and giving support to findings by Peuker and Filler (2002), it has been thought that both locations, tragus and cymba conchae, likely engage vagal fibers (Badran, Brown, et al., 2018; Butt et al., 2019). The current literature lacks a clear consensus on the auricular area that is most densely innervated by the ABVN, thus rendering it necessary for further studies to address this gap (Badran, Brown, et al., 2018; Burger and Verkuil, 2018; Butt et al., 2019). Concretely, it is essential to investigate the effect of stimulation area on biomarkers and behavioral (cognitive) effects in order to optimize the effects of tVNS (Badran, Brown, et al., 2018).

Regarding effects on cognition, there is promising evidence that tVNS can affect the processing of error commissions. Error monitoring is assumed to be regulated by prefrontal and cingulate areas (Hoffmann and Beste, 2015), which are targeted by tVNS. As stated by the inhibitory account (Ridderinkhof, 2002), error commission is typically followed by increased inhibitory control. This leads to a slowdown of the task performance after committing an error, a phenomenon known as PES. A previous study found increased PES during tVNS compared to sham stimulation (Sellaro et al., 2014). It has long been proposed that slowing after unforeseen errors is linked to increased norepinephrine release (Ullsperger et al., 2010). Yet, the work of Sellaro et al. (2014) is one of the few studies investigating the causal role of norepinephrine—allegedly upregulated by tVNS—in increasing PES. Nonetheless, they did not address measurements that reflect mechanisms involving PES at the physiological level. Sellaro et al. (2014) analyzed heart rate at different time points. However, heart rate is the result of mixed inputs from the sympathetic and parasympathetic (vagus) nerves, so that results on heart rate may not necessarily correlate with the outcomes of interest (Goldberger et al., 2019). Thus, the interpretation of findings provided by Sellaro et al. (2014) currently rather lies on mere speculations about the mechanisms underlying tVNS which involve norepinephrine activity and PES.

Pupil dilation is considered the most reliable noninvasive marker of norepinephrine activity in the brain given constant illuminance (Joshi et al., 2016). Pupil dilation is linked to effort in actions involving cognitive control (van der Wel and van Steenbergen, 2018). The iris dilator muscle is controlled by the sympathetic system via locus coeruleus activity (Mathôt, 2018), which controls norepinephrine release in the brain and has shown to be increased by tVNS (Dietrich et al., 2008; Kraus et al., 2013). Despite this promising relationship, studies investigating tVNS and pupillary responses are still scarce. No modulation evoked by tVNS has been found in this small amount of studies (Burger et al., 2020; Keute et al., 2019; Warren et al., 2019), however none of them investigated PES.

Conversely, despite expecting a sympathetic reaction such as pupil dilation to be evoked by tVNS, there is an array of studies that investigate the enhancing effect of tVNS on the parasympathetic processes related to the vagus nerve (Butt et al., 2019). Because of the neural pathways that constitute the brain-heart axis, CVA—the activity of the vagus nerve regulating cardiac functioning—has been thought to be affected by tVNS (Murray et al., 2016). This is in line with the neurovisceral integration model, which states that the central autonomic network links the prefrontal cortex to the heart (Thayer et al., 2009). Using vagally-related heart rate variability (vmHRV) parameters as an index of CVA (Malik et al., 1996), some studies have shown that tVNS can increase CVA (Bretherton et al., 2019; De Couck et al., 2017; Ylikoski et al., 2017) and simultaneously suppress sympathetic activity (Clancy et al., 2014). However, this positive effect of tVNS on CVA could not be shown in other studies (Burger et al., 2017; Burger et al., 2019; Burger et al., 2016). Furthermore, two studies have shown that CVA can increase during both active and sham stimulation (Borges et al., 2019, 2020). These contradictory results might, similarly to the fMRI studies, be explained by the use of different stimulation parameters, including the use of different auricular areas.

In summary, previous studies showed that tVNS can affect cognitive processes such as PES, whereas results for pupil sizes and CVA are still inconsistent. Importantly, these studies stimulated different areas of the ear, with this possibly leading to heterogeneous results. Inspired by the debate on the best ear target for tVNS, the present study goes beyond existing research on tVNS and addresses the main stimulation areas of the ear currently used in the state of the art. For the first time, tragus, cyma conchae, and earlobe (as a sham stimulation) are compared to one another by taking cognitive as well as neurophysiological effects into account. To investigate the cognitive effects of tVNS, we chose PES, which has already been shown to be influenced by tVNS with medium to large effect sizes (Sellaro et al., 2014). On the neurophysiological level, we measured pupil dilation as an index of norepinephrine activity involved in PES. Furthermore, we used vmHRV to measure CVA, which allows for addressing the current inconsistency in HRV measurements related to tVNS. These results might contribute to the efforts in optimizing the tVNS signal in order to further improve its effects on cognitive and autonomic regulation.

The objective of the present work is to investigate whether stimulating different auricular areas, namely cyma conchae and tragus, affects PES on the behavioral level, and pupillary responses as well as CVA on the neurophysiological level compared to sham condition (earlobe stimulation). Given that the cyma conchae might be more strongly innervated by the ABVN than the tragus (Peuker and Filler, 2002) and based on findings of a previous fMRI study (Yakunina and Kim, 2017), we expected that cyma conchae stimulation, when compared to tragus and sham stimulation, provokes higher PES ( $H_{1a}$ ), higher pupil dilation after committing an error ( $H_{2a}$ ), and higher cardiac vagal activity ( $H_{3a}$ ). Furthermore, we hypothesized that tragus stimulation, when compared to sham stimulation, provokes higher PES ( $H_{1b}$ ), higher pupil dilation after committing an error ( $H_{2b}$ ), and higher CVA ( $H_{3b}$ ).

## 2. Method

### 2.1. Participants

As it is not possible to run power analyses for multi-factorial repeated-measures designs with G\*Power 3.1 (Faul et al., 2007), we followed the same procedure found in previous studies with similar design (Liepelt et al., 2019). Accordingly, we matched the average number of participants in interventional studies using tVNS and invasive VNS that investigated a) PES (Sellaro et al., 2014), b) pupillary responses (Desbeaumes Jodoin et al., 2015; Keute et al., 2019; Warren et al., 2019), and c) vmHRV parameters (Borges et al., 2019; Bretherton et al., 2019; Burger et al., 2019, 2017, 2016; De Couck et al., 2017). Forty-two participants were calculated to find effects on these dependent variables. We recruited 49 participants, but due to technical problems with electrocardiogram (ECG) signals of five participants and two dropouts, 42 participants (24 females,  $M_{age} = 23.2$  years,  $SD = 3.1$ ) were included in the analysis.

The sample consisted of healthy sport science students at the local university. Participants were eligible if they were free of cardiovascular, neurological diseases or major mental conditions, not using a pacemaker or piercings, did not need glasses, and were not pregnant at the time of the experiment. They were asked not to smoke, exercise, or consume food, alcohol, or caffeine for at least 2 h before participation. These potentially confounding variables as well as tVNS safety-related questions were assessed by means of an adapted version of the demographics questionnaire for HRV psychophysiological experiments (Laborde et al., 2017). All participants gave written informed consent prior to the experiment. The study was approved by the local ethical committee (ethics approval number 041/2019).

### 2.2. Transcutaneous vagus nerve stimulation

For anatomical reasons, two tVNS devices with different electrodes

but with identical stimulation parameters were used to compare the three different auricular parts (Fig. 1). To stimulate the cyma conchae, we employed the NEMOS tVNS device (Cerbomed, Erlangen, Germany) with modified duty cycle in order for it to perform continuous stimulation. Two electrodes located in a structure similar to an earphone were placed along the skin surface of the cyma conchae. For stimulation at the tragus, the ParaSym tVNS device (ParaSym, London, UK), was used. An ear clip with two electrodes was attached to the tragus, enabling the electrical current to pass through this area. In order to have a control condition, a sham stimulation was used, which had the same characteristics as normal tVNS, but instead of the electrodes being attached to the ABVN, they were attached to the left earlobe. The earlobe is thought to be free of vagal innervation (Peuker and Filler, 2002). The ear clip electrode was chosen for the sham condition as it is easier to attach to the earlobe compared to the NEMOS device. As shown in a pilot testing, the ear clip enabled a stable attachment at the earlobe, whereas the earlobe stimulation with NEMOS as proposed by van Leusden et al. (2015) fell off easily and repeatedly. Both constant current devices delivered an electrical current with a pulse width of 200–300  $\mu$ s at 25 Hz. The stimulation intensity was determined by the participants themselves based on the method used by De Couck et al. (2017). According to this protocol, the stimulation intensity is determined by taking the mean of the individually detectable stimulation and the personal uncomfortable stimulation intensity. The intensity was determined for each session. The average chosen stimulation intensity in the tragus condition was  $M = 2.18$  mA ( $SD = 0.69$ ),  $M = 0.94$  mA ( $SD = 0.57$ ) in the cyma conchae condition and  $M = 2.19$  mA ( $SD = 0.71$ ) in the sham condition. These stimulation intensities differed significantly from each other,  $F(2, 82) = 82.743$ ,  $p < .001$ ,  $\eta_p^2 = 0.669$ . Post-hoc  $t$ -tests (Bonferroni-corrected  $p = .017$ ) revealed that the intensity chosen during the cyma conchae stimulation was significantly lower than the one chosen during tragus stimulation,  $t(41) = 10.389$ ,  $p < .001$ ,  $d = 1.603$ , and during sham stimulation,  $t(41) = 10.494$ ,  $p < .001$ ,  $d = 1.619$ .

Aligned with several studies using tVNS (e.g. Kreuzer et al., 2012; Sellaro et al., 2014; Yakunina and Kim, 2017), we performed electrode placement on the left side of the ear in order to control for cardiac side effects. This is because fibers originating from the left vagus nerve supply the atrioventricular node, causing decremental conduction, and those from the right vagus nerve innervate the sinoatrial node, which is able to reduce depolarization rates and produce bradycardia (Krahl, 2012).

### 2.3. Post-error slowing

In order to conceptually replicate Sellaro et al.'s (2014) findings regarding PES, participants performed a modified version of the Flanker task (Eriksen and Eriksen, 1974), adapted from Brink et al. (2014). In each trial, participants were presented with a target stimulus (“H”, “K”, “C”, or “S”) flanked on each side by four additional letters which differed from the target stimuli but belonged to the same set of letters (e.g., HHHHCHHHH). Participants were asked to concentrate only on the middle letter (target stimulus) and ignore the other letters. Each target stimulus required a different response on the keyboard keys (“1” and “2” on left hand and “7” and “8” on right hand). To ensure a sufficient high error rate, the task had a total of 1040 trials and target stimuli were always incongruent with the flanker letters. Further, target stimuli also differed from the flanker letters concerning the hand required to respond. Participants were asked to respond as fast as possible.

Stimuli were shown in white on a grey background to reduce incidence of light, for 200 ms. During the intertrial interval, a white fixation cross was presented. The intertrial intervals randomly varied between 1000 and 1300 ms in steps of 50 ms in order to ensure relatively short response stimulus intervals. After stimulus onset, participants had 1000 ms to respond (Fig. 2). Participants first completed 120 practice trials after which they always received a feedback with the message “correct”

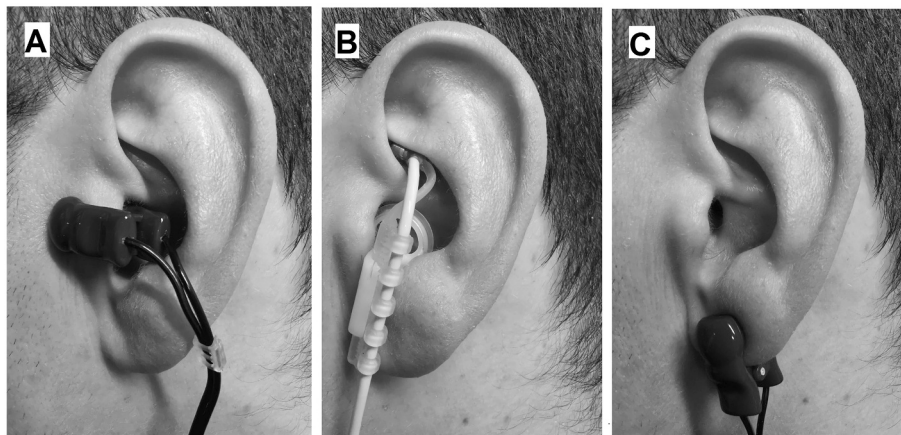


Fig. 1. Placement of the electrodes on the ear. A. Tragus stimulation; B. cymba conchae stimulation; C. earlobe stimulation.

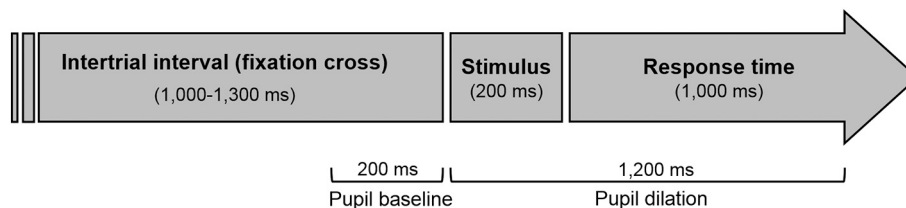


Fig. 2. Trial structure in the cognitive task und pupil measurements.

or “wrong” in green and red, respectively. The experimental task included 10 blocks of 104 trials each. Each block lasted 4 min. After each block, participants could take a break of approx. 30 s, were given reaction time (RT) and accuracy feedback and were pressed for speed. The experimental task took approx. 40 min. We used a 24-in. flat-screen monitor (1920 × 1080 pixels at 60 Hz) to present the task and ran it with PsychoPy3 (Peirce et al., 2019).

Similar to Sellaro et al. (2014), PES was analyzed according to a method described in Dutilh et al. (2012). This method considers only errors that are preceded and followed by at least one correct trial. In order to calculate PES for each triplet (correct-wrong-correct), a pairwise comparison of the two correct trials was computed ( $RT_{\text{post-error}} - RT_{\text{pre-error}}$ ). Mean PES for each participant was computed by averaging all single PES values. This method controls for global fluctuations over the task (Dutilh et al., 2012). In addition to mean PES, mean correct RT, error rates, and post-error change in accuracy (percentage of correct answers in post-error trials – percentage of correct answers in post-correct trials) were included in our analysis (Sellaro et al., 2014).

#### 2.4. Pupillary responses

Pupil diameter was measured with participants comfortably sitting in an adjustable chair in a well-lit room with lowered window shades, with their head lying on a desk-mounted chinrest at a distance of 60 cm to the screen throughout the experiment. Pupil responses of the right eye were measured with the SMI Eye Tracking Glasses® (SensoMotoric Instruments GmbH, Germany). This device has a sampling rate of 60 Hz, a 1280 × 960-pixel resolution scene camera, and operates with an infrared light and a video camera. The eye tracker was calibrated using the three-point method. SMI’s proprietary software, BeGaze 3.2, was used to export pupil diameter in millimeters. Following recommendations of Mathôt et al. (2018), blinks and missing data were dealt with smoothing and cubic-spline interpolation, and subtractive baseline correction was preferred in order to minimize distortion of pupil-size data. After preprocessing the pupillary data, five participants had to be excluded from the pupil analysis due to the high amount of missing

data (>30% of the total dataset). Pupil sizes were then averaged according to the response given trial-by-trial (error or correct response).

We analyzed pupil baseline and pupil dilation separately. Pupil baseline consists in the averaged pupil diameter during the last 200 ms of the pre-trial period and was calculated to check whether the pupil sizes showed differences between the groups shortly before the stimulus onset. For the period after stimulus onset (pupil dilation period), the baseline-corrected pupillary change was calculated by considering the time window of 1200 ms between stimulus onset and the next fixation cross on a trial-by-trial basis (Fig. 2). This approach is recommended by pupillometry literature because baseline correction takes into account random fluctuations in pupil size over time, thus improving statistical power (Mathôt et al., 2018). All preprocessing steps were performed using RStudio 1.2.1335 with the package *dr-JT/pupillometry*.<sup>2</sup> To control for possible daylight fluctuations despite controlled illumination of the room, we measured with a luxmeter (Votcraft LX-10, Conrad GmbH, Germany) how much incident light illuminates the area at which the participant’s eyes were directed to during the experiment. This measurement took place four times: first within one day, by comparing during sunny weather with direct light incidence on the room and later after sunset, and second within a pilot session, by comparing the response phase (only a grey background) with the stimulus phase (stimulus in white with a grey background). In all situations, the values were identical with 255 lx or 32 footcandles, meaning that the illumination could be kept constant over the data collection.

#### 2.5. Cardiac vagal activity

To assess CVA, we measured vmHRV parameters using the ECG device Faros 180° (Mega Electronics, Kuopio, Finland) with a set sampling rate of 500 Hz. This device enables users to measure the ECG signal as recommended by current guidelines on HRV measurement (Laborde et al., 2017). We placed two disposable ECG pre-gelled electrodes

<sup>2</sup> URL: <https://dr-jt.github.io/pupillometry/>.

(Ambu L-00-S/25, Ambu GmbH, Bad Nauheim, Germany) on the body, the positive electrode on the right infraclavicular fossa and the negative one on the left anterior axillary line below the 12th rib.

Root mean square of successive differences (RMSSD) as well as high frequency (HF) (0.15 Hz to 0.40 Hz band) transformed with autoregressive modeling were chosen as vmHRV parameters that are known to index CVA (Malik et al., 1996). From ECG recordings, we extracted HRV with Kubios software (University of Eastern Finland, Kuopio, Finland), visually inspected the full ECG recording, and manually corrected artifacts (Laborde et al., 2017). Since HF is only influenced by breathing when breathing cycles are between 9 cycles per minute (0.15 Hz) and up to 24 cycles per minute (0.40 Hz) (Malik et al., 1996), four participants with a respiratory rate out of this range were excluded from analyses with HF. The respiratory frequency (the number of respiratory cycles per minute) was obtained multiplying the ECG-derived respiration value obtained via the Kubios algorithm by 60 (Tarvainen et al., 2013) and was also separately analyzed. Because the measurement time windows need to be kept constant across the time measurements in order for them to be comparable with each other (Malik et al., 1996), the time windows were defined according to the duration of the blocks of the cognitive task, i.e. 4 min. This is in accordance with the range suggested by recent recommendations for experiment planning with HRV in psychophysiological research (Laborde et al., 2017). The CVA values of the blocks were then averaged, resulting in a single task value.

## 2.6. Procedure

We conducted a single-blind experiment with a balanced crossover within-subject design, as recommended by Quintana and Heathers (2014) to address the high interindividual variation and the complex interactions influencing CVA and pupil responses. All participants underwent all three stimulation conditions in a counterbalanced order to cancel out order and learning effects, and were randomly assigned to the different possible order sequences. To reduce carryover effects for tVNS and the Flanker task, the three sessions were on different days, and took place at approximately the same time of the day, given that time of the day may influence physiological processes and cognitive performance (Folkard and Rosen, 1990). There was a break of 1 min between the test phases to reduce possible effects after the stimulation period. Upon arrival to the laboratory, participants were asked to fill out an informed consent form and the demographic questionnaire to assess any exclusion criteria. After attaching all devices and calibrating the eye tracker, a 4-min resting phase took place. Subsequently, a 4-min tVNS phase (one of the three conditions per session) took place. In this phase, participants determined their individual stimulation intensity and were habituated to the stimulation. Following this, participants performed the cognitive task on the computer while receiving stimulation. Directly after the task and before the recovery phase, the stimulation stopped. The recovery phase followed the task phase with a final 4-min measurement. During all time periods around the task, the participants were instructed to keep their gaze on a white fixation cross presented centrally against a grey background on the screen and not to move their head from the chinrest. Keeping the same color characteristics on the screen compared to during the cognitive task, the light emission from the screen could be kept constant. Pupil sizes and CVA were recorded throughout the testing

session, whose protocol is depicted in Fig. 3.

## 2.7. Data analysis

Outliers (less than 1% of the data) were winsorized, meaning that values higher/lower than two standard deviations from the mean were transformed into a value of two standard deviations from the mean. Since the HRV as well as the Flanker task data were still not normally distributed afterwards, they were log-transformed to obtain a normal distribution. To check whether PES took place within each stimulation condition, one-sample *t*-test per condition has been performed. To analyze the effect of tVNS on cognitive data, four separate three-way repeated-measure analyses of variance (rmANOVAs) with stimulation conditions (tragus, cymba conchae, and sham stimulation) were performed. The relevant cognitive measurements were PES, RT of the correct trials, error rates, and post-error change in accuracy. Both measurements of CVA, RMSSD and HF, and additionally respiratory frequency, were analyzed with three separated 3 (stimulation: tragus, cymba conchae, and sham stimulation) x 4 (time: resting, tVNS, task and recovery phases) rmANOVAs. Regarding pupil measurements, the pupil baselines of the stimulation conditions were compared to each other in a 3 (stimulation: tragus, cymba conchae and sham stimulation) x 2 (response: error and correct response) rmANOVA, and the same type of rmANOVA was performed for baseline-corrected pupil dilation. Greenhouse–Geisser correction was used when sphericity was violated. In the case of a significant main or interaction effect, post-hoc *t*-tests with aggregated means were conducted using Bonferroni correction. To quantify evidence for the hypotheses found and counteract bias in the rmANOVAs given possible lack of power in specific measurements, we ran Bayesian statistics using Bayesian information criteria (Wagenmakers, 2007) for all analyses. Terms used to discuss the reported Bayes factors are based on Wetzels et al.'s (2011) recommendations. Accordingly, values higher than 1 provide evidence for alternative hypotheses, whereas values lower than 1 provide evidence for null hypotheses. The Bayes factor can have the following meanings: anecdotal or worth no more than a bare mention ( $0.333 < B_{10} < 3$ ), substantial ( $0.100 < B_{10} \leq 0.333$  or  $3 \leq B_{10} < 10$ ), strong ( $0.033 < B_{10} \leq 0.100$  or  $10 < B_{10} < 30$ ), very strong ( $0.010 < B_{10} \leq 0.033$  or  $30 \leq B_{10} < 100$ ), and decisive ( $B_{10} \leq 0.010$  or  $B_{10} \geq 100$ ) evidence. To control for learning effects on the cognitive task parameters, which potentially arose due to repeating the same task across the three testing days, we tested the order effect. We sorted the measures according to the testing day (i.e., first, second, and third day) and ran four separated one-way rmANOVAs, one for each task parameter, with stimulation as a factor. In case learning effects on task performance were found, we performed an additional analysis to check whether the absence of learning effects in a subsample would lead to differences in performance regarding the stimulation conditions, thus having a more comparable statistical analysis to what has been reported by Sellaro et al. (2014). For these cases, we ran separated one-way ANOVAs with the stimulation conditions that have been applied only on Day 1 as a factor. We used RStudio 1.2.1335 to prepare the data and JASP 0.11.1 to analyze it. Significance level was  $\alpha = 0.05$ .

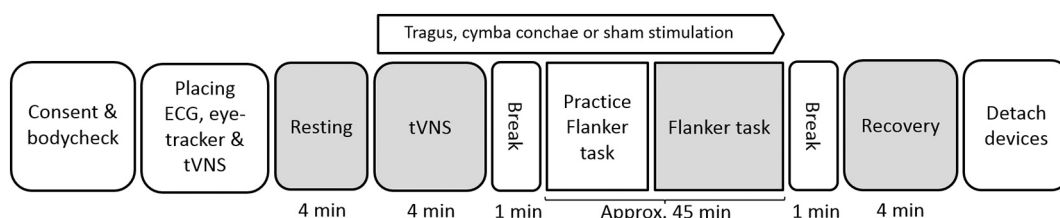


Fig. 3. Experimental overview. ECG = electrocardiogram; tVNS = transcutaneous vagus nerve stimulation.

### 3. Results

#### 3.1. Effects of tVNS on cognitive measurements

Descriptive statistics are presented in Table 1. Separated one-sample t-tests revealed that PES could be found in cymba conchae condition,  $t(41) = 3.970, p < .001, d = 0.613$ , tragus condition,  $t(41) = 5.048, p < .001, d = 0.779$ , and in sham condition,  $t(41) = 3.088, p = .004, d = 0.476$ . There was no difference between the stimulation conditions regarding RT,  $F(2, 82) = 0.031, p = .969$ , and error rates,  $F(1.724, 70.695) = 1.179, p = .308$ . These results were supported by Bayesian estimations ( $B_{10} = 0.077$  for RT and  $B_{10} = 0.196$  for error rates). Regarding PES, there was no effect of stimulation,  $F(2, 82) = 1.064, p = .350$ , with this result being supported by Bayes factor ( $B_{10} = 0.190$ ). Post-error change in accuracy showed no differences between stimulation conditions neither,  $F(2, 82) = 1.565, p = .215$ , with Bayes factor supporting this result ( $B_{10} = 0.333$ ).

#### 3.2. Effects of tVNS on pupillary responses

Descriptive statistics for effects of ear areas on pupil sizes are presented in Table 1 and depicted in Fig. 4. Pupil baselines did not differ significantly between stimulation conditions,  $F(2, 58) = 0.722, p = .467$ , with Bayesian statistics supporting this evidence ( $B_{10} = 0.275$ ). There was no difference regarding the trial-to-trial responses,  $F(1, 29) = 4.036, p = .054$ , with Bayesian estimation supporting this result ( $B_{10} = 0.210$ ). There was no interaction effect between stimulation and response,  $F(2, 66) = 0.185, p = .831$ , which was confirmed by Bayesian statistics ( $B_{10}$

**Table 1**  
Means (standard deviations) for all task and physiological measurements.

		Tragus	Cymba conchae	Sham	
Flanker task	RT	641.36 (72.10)	640.14 (67.78)	639.66 (84.66)	
	Error rates	5.21 (3.39)	5.09 (2.53)	4.62 (2.58)	
	Post-error slowing	15.41 (32.34)	23.83 (38.89)	23.58 (30.27)	
	Post-error change	-1.85 (6.72)	-3.85 (7.21)	-1.59 (4.85)	
Pupil sizes	Baseline				
	Correct response	3.78 (0.48)	3.85 (0.50)	3.81 (0.47)	
	Error	3.75 (0.45)	3.83 (0.50)	3.79 (0.49)	
Dilation	Correct response	0.15 (0.08)	0.15 (0.10)	0.14 (0.09)	
	Error	0.23 (0.14)	0.22 (0.14)	0.23 (0.14)	
Cardiac vagal activity	RMSSD	Resting	43.81 (23.67)	45.59 (22.98)	44.23 (24.96)
		tVNS	47.77 (23.76)	50.49 (25.08)	47.29 (25.49)
	Flanker	Resting	46.71 (20.62)	48.35 (20.58)	47.28 (20.74)
		Recovery	52.61 (24.28)	55.63 (23.20)	56.35 (26.88)
	HF	Resting	861.83 (931.19)	922.22 (1042.2)	895.34 (1092.58)
		tVNS	997.36 (1107.70)	1114.87 (1300.28)	887.65 (846.46)
	Flanker	Resting	816.78 (743.70)	837.63 (691.67)	775.51 (616.86)
		Recovery	1167.18 (1077.03)	1208.18 (1015.39)	1431.78 (1383.19)
	Respiratory frequency	Resting	14.52 (2.41)	14.67 (2.53)	14.23 (2.86)
		tVNS	14.23 (2.13)	14.09 (2.12)	14.30 (2.33)
Flanker		14.39 (2.59)	14.76 (2.58)	15.00 (2.58)	
Recovery		13.35 (2.74)	13.41 (2.47)	13.15 (2.27)	

Note. RT = reaction time; RMSSD = root mean square of successive differences; tVNS = transcutaneous vagus nerve stimulation; HF = high frequency.

= 0.090).

Regarding pupil dilation, there was no main effect of stimulation,  $F(2, 58) = 0.004, p = .996$ , which was supported by Bayesian statistics ( $B_{10} = 0.056$ ). There was a main effect of response,  $F(1, 29) = 35.214, p < .001, \eta_p^2 = 0.548$ , with post-hoc analyses (no Bonferroni correction needed) showing that pupil dilation during error ( $M = 0.22$  mm,  $SD = 0.13$ ) was significantly higher than the pupil dilation during correct responses ( $M = 0.15$  mm,  $SD = 0.08$ ),  $t(37) = 5.877, p < .001, d = 0.953$ . Bayesian estimation supported this main effect ( $B_{10} = 1.557e+8$ ). No interaction effect could be found,  $F(2, 58) = 0.078, p = .925$ , with Bayesian factor supporting this lack of effect ( $B_{10} = 1.070e-4$ ).

#### 3.3. Effects of tVNS on cardiac vagal activity

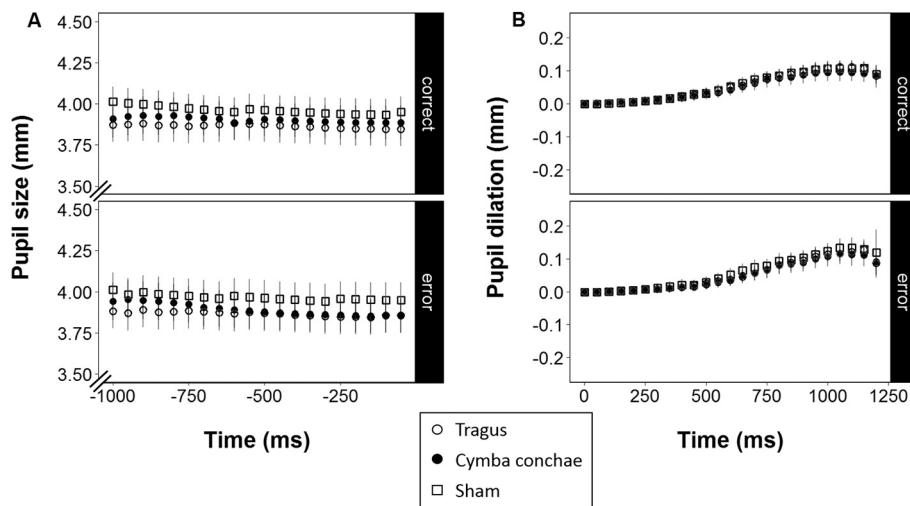
Descriptive statistics for effects of auricular areas on CVA are presented in Table 1. Regarding RMSSD, there was no main effect of stimulation,  $F(2, 82) = 0.953, p = .390$ . There was an effect of time,  $F(1.974, 80.945) = 17.628, p < .001, \eta_p^2 = 0.301$ . Post-hoc analyses (Bonferroni-corrected  $p = .008$ ) pointed out a significant increase from resting RMSSD ( $M = 44.55$  ms,  $SD = 21.86$ ) to tVNS RMSSD ( $M = 48.52$  ms,  $SD = 22.28$ ),  $t(41) = 4.632, p < .001, d = 0.715$ , and from task RMSSD ( $M = 47.45$  ms,  $SD = 19.05$ ) to recovery RMSSD ( $M = 54.86$  ms,  $SD = 22.34$ ),  $t(41) = 4.823, p < .001, d = 0.744$ . Moreover, recovery RMSSD was significantly higher than resting RMSSD,  $t(41) = 5.766, p < .001, d = 0.890$ , and tVNS RMSSD,  $t(41) = 4.206, p < .001, d = 0.649$ . There was no interaction effect of stimulation with time,  $F(4.250, 174.261) = 0.795, p = .537$  (Fig. 5A). Bayesian statistics gave support for the main effects in the rmANOVA ( $B_{10} = 0.268$  for main effect of stimulation,  $B_{10} = 5.006e+7$  for effect of time), but not for the lack of interaction ( $B_{10} = 6.378$ ).

HF controlled for respiration showed the same pattern: There was no main effect of stimulation,  $F(2, 74) = 0.803, p = .452$ , but of time,  $F(2.150, 79.536) = 16.636, p < .001, \eta_p^2 = 0.310$ . Post-hoc analyses (Bonferroni-corrected  $p = .008$ ) showed a significant increase from resting HF ( $M = 893.13$  ms<sup>2</sup>,  $SD = 946.63$ ) to tVNS HF ( $M = 999.96$  ms<sup>2</sup>,  $SD = 971.98$ ),  $t(37) = 4.060, p < .001, d = 0.659$ . There was a significant increase from task HF ( $M = 809.98$  ms<sup>2</sup>,  $SD = 627.64$ ) to recovery HF ( $M = 1269.05$  ms<sup>2</sup>,  $SD = 1078.99$ ),  $t(37) = 6.068, p < .001, d = 0.984$ . Moreover, recovery HF was significantly higher than resting HF,  $t(37) = 5.727, p < .001, d = 0.929$ , and tVNS HF,  $t(37) = 3.805, p < .001, d = 0.617$ . There was no interaction effect of stimulation with time,  $F(4.241, 156.907) = 1.262, p = .286$  (Fig. 5B). Bayesian estimations supported these results ( $B_{10} = 0.153$  for stimulation,  $B_{10} = 2.032e+8$  for time, and  $B_{10} = 0.011$  for interaction).

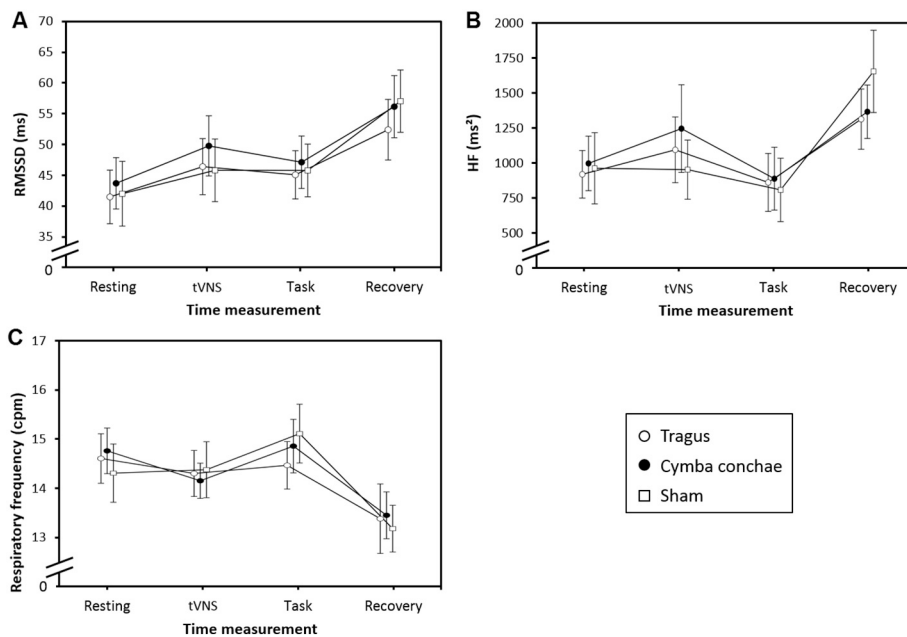
Regarding respiratory frequency, there was also no effect of stimulation,  $F(1.526, 62.575) = 0.117, p = .836$ , but of time,  $F(2.228, 91.355) = 13.036, p < .001, \eta_p^2 = 0.241$ . Post-hoc analyses (Bonferroni-corrected  $p = .008$ ) showed a decrease of respiratory frequency from task ( $M = 14.72$  times per minute,  $SD = 2.31$ ) to recovery phase ( $M = 13.31$  times per minute,  $SD = 2.09$ ),  $t(41) = 6.396, p < .001, d = 0.987$ . Furthermore, respiratory frequency was reduced in the recovery phase compared to the resting ( $M = 14.47$  times per minute,  $SD = 2.34$ ),  $t(41) = 4.504, p < .001, d = 0.695$ , and the tVNS phase ( $M = 14.21$  times per minute,  $SD = 1.88$ ),  $t(41) = 4.132, p < .001, d = 0.638$ . There was no interaction effect of stimulation with time,  $F(6, 246) = 1.678, p = .127$  (Fig. 5C). Bayesian factor supported these results ( $B_{10} = 0.027$  for stimulation,  $B_{10} = 2.182e+8$  for time, and  $B_{10} = 0.027$  for interaction).

#### 3.4. Learning effects analyses

To investigate whether there was a learning effect for the cognitive task, four separated rmANOVAs were performed. We checked whether the testing days, when arranged chronologically, differed from one another regarding RT, error rates, PES and post-error accuracy, respectively. There was a difference between the days regarding RT,  $F(2, 82) = 38.905, p < .001, \eta_p^2 = 0.487$  (Fig. 6A). Post-hoc analyses



**Fig. 4.** Pupil measurements, averaged according to response accuracy and stimulation condition. A. Pupil baseline 1000 ms before stimulus onset until stimulus onset; B. baseline-corrected pupil dilation after stimulus onset at time zero.



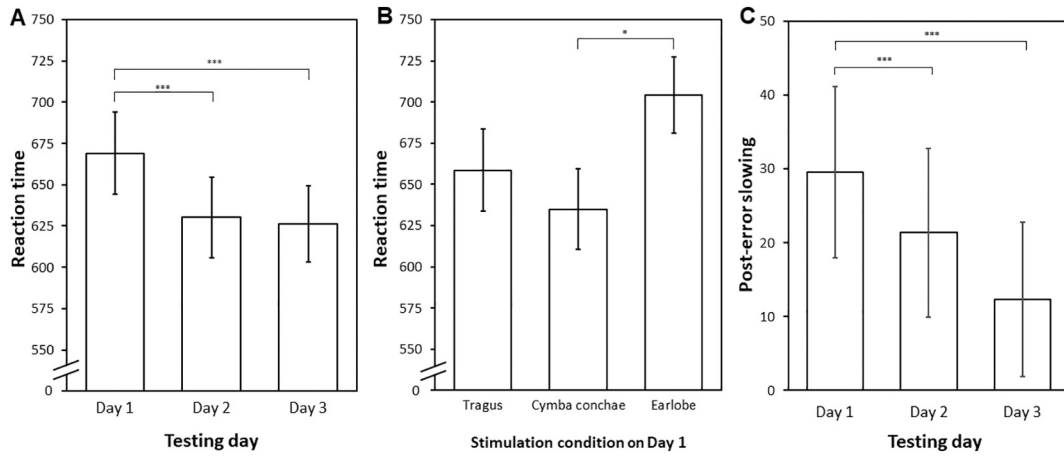
**Fig. 5.** Mean scores of heart rate variability parameters and respiration over time with confidence interval as error bars. A. Root mean square of successive differences (RMSSD); B. high frequency (HF); C. respiratory frequency.

(Bonferroni-corrected  $p = .017$ ) revealed that RT on Day 1 ( $M = 666.45$  ms,  $SD = 74.18$ ) was significantly higher than on Day 2 ( $M = 628.92$  ms,  $SD = 75.34$ ),  $t(41) = 7.354$ ,  $p < .001$ ,  $d = 1.135$ , and Day 3 ( $M = 626.12$  ms,  $SD = 72.60$ ),  $t(41) = 7.320$ ,  $p < .001$ ,  $d = 1.129$ . There were no differences between the three testing days regarding error rates,  $F(2, 82) = 2.523$ ,  $p = .086$ . Regarding PES, there was a significant difference between the days,  $F(2, 82) = 4.052$ ,  $p = .021$ ,  $\eta_p^2 = 0.090$  (Fig. 6C). Post-hoc analyses (Bonferroni-corrected  $p = .017$ ) showed that PES on Day 1 ( $M = 29.76$  ms,  $SD = 35.34$ ) was significantly higher than on Day 3 ( $M = 11.83$  ms,  $SD = 31.16$ ),  $t(41) = 2.493$ ,  $p = .016$ ,  $d = 0.338$ .

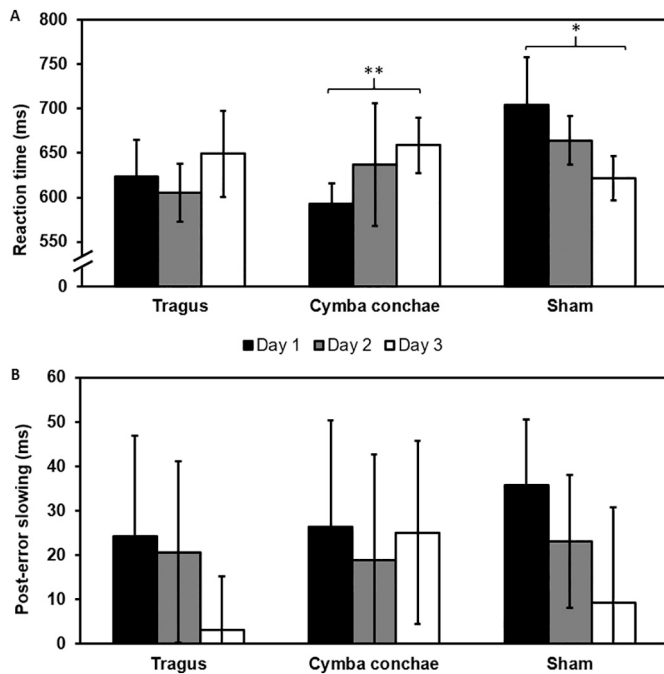
Because learning effects were found for RT and PES, we ran two separated one-way ANOVAs with the stimulation conditions that have been applied only on Day 1 as a factor and RT and PES and dependent variables. Only RT showed a significant difference regarding stimulation condition on Session Day 1,  $F(2, 39) = 3.829$ ,  $p = .030$ ,  $\eta_p^2 = 0.164$  (Fig. 6B). Post-hoc analyses (Bonferroni-corrected  $p = .017$ ) were performed using Welch's  $t$ -tests, as the equal variation assumption was

violated (Levene's test was significant with  $p < .05$ ). The tests revealed that participants who received cymba conchae stimulation on Day 1 showed lower RT ( $M = 634.96$ ,  $SD = 39.44$ ) than participants who received earlobe stimulation on Day 1 ( $M = 704.23$ ,  $SD = 96.04$ ),  $t(18.591) = 2.584$ ,  $p = .015$ ,  $d = 0.944$ . Regarding PES, there was no difference between the different stimulation areas when they took place on Day 1,  $F(2, 39) = 0.455$ ,  $p = .638$ ,  $\eta_p^2 = 0.023$ .

To further investigate the learning effects found for RT and PES, we ran one-way ANOVAs for each stimulation condition over the three testing days arranged chronologically (Fig. 7). Regarding RT, no effect of day was found in the tragus condition,  $F(2, 39) = 1.428$ ,  $p = .252$ , but in the cymba conchae condition,  $F(2, 39) = 3.348$ ,  $p = .046$ ,  $\eta_p^2 = 0.147$ . Post-hoc  $t$ -tests (Bonferroni-corrected  $p = .017$ ) revealed that RT during cymba conchae stimulation was significantly lower when this condition took place on Day 1 ( $M = 592.48$ ,  $SD = 42.35$ ) compared to Day 3 ( $M = 658.78$ ,  $SD = 56.38$ ),  $t(28) = 3.641$ ,  $p = .001$ ,  $d = 1.330$ . Furthermore, there was an effect of testing days on sham condition,  $F(2, 39) = 4.882$ ,



**Fig. 6.** Learning effects on task performance with confidence interval as error bars. A. Reaction time over the three testing days; B. reaction time of the three stimulation conditions when they took place on Day 1; C. post-error slowing over the three testing days. \* $p < .05$ ; \*\*\* $p < .001$ .



**Fig. 7.** Learning effects on task performance with confidence interval as error bars. A. Reaction time over the three testing days per stimulation condition; B. post-error slowing over the three testing days per stimulation condition. \* $p < .05$ ; \*\* $p < .01$ .

$p = .013$ ,  $\eta_p^2 = 0.200$ . Post-hoc t-tests (Bonferroni-corrected  $p = .017$ ) revealed that RT during cymba conchae stimulation was significantly higher when this stimulation condition took place on Day 1 ( $M = 704.23$ ,  $SD = 96.04$ ) compared to Day 3 ( $M = 622.02$ ,  $SD = 39.35$ ),  $t(25) = 2.776$ ,  $p = .010$ ,  $d = 1.075$ .

#### 4. Discussion

The aim of this study was to compare the effects of tVNS on cognitive and neurophysiological regulation when applied at different areas of the ear, namely tragus, cymba conchae and earlobe (sham). We expected cymba conchae stimulation to evoke the highest PES ( $H_{1a}$ ), followed by tragus stimulation ( $H_{1b}$ ). None of the stimulation areas showed significant differences regarding PES, thus neither of the  $H_1$ -hypotheses could be confirmed. We also hypothesized that cymba conchae stimulation

would lead to increased pupil dilation as a consequence of error commitment ( $H_{2a}$ ), followed by tragus stimulation ( $H_{2b}$ ), which would indicate an increased norepinephrine release. Pupil dilation was indeed higher during errors than during correct responses, but this increase was not different between the stimulation conditions. Thus, neither of the  $H_2$ -hypotheses could be confirmed. Finally, vmHRV parameters as indices of CVA were expected to increase during cymba conchae stimulation ( $H_{3a}$ ), followed by tragus stimulation ( $H_{3b}$ ). As stated by the neurovisceral integration model (Thayer et al., 2009), this would indicate that the neural pathways involved in PES (Ridderinkhof, 2002) have been optimized. Both RMSSD and HF increased during tVNS compared to resting, with them being at highest after finalizing the task (recovery phase). However, similar to pupillary responses during error commitment, there was no difference between the stimulation areas. Consequently, neither of the  $H_3$ -hypotheses could be confirmed.

Taken together, the core neurological basis for PES could be observed, since there was an increased norepinephrine release after committing an error, but differences regarding PES per se due to tVNS could not be found. Similar results were found in a recent study investigating the effect of tVNS on pupillary responses and on attentional blink: Pupil increased after stimulus onset, but there was no effect of cymba conchae stimulation compared to earlobe stimulation (Burger et al., 2020). In the present study, at the same time that this index of sympathetic activity (Mathôt, 2018) increased, the same pattern was found in CVA, an index of parasympathetic activity (Malik et al., 1996). It has been shown that pupillary light reflex and CVA do not generally correlate with each other (Daluwatte et al., 2012). That means, one autonomic process does not necessarily exclude the other, rather both represent different aspects of autonomic activity. In the opposite direction, it has already been shown that CVA can predict decreased pupil size while viewing positive emotional stimuli (Macatee et al., 2017). Therefore, both pupillary responses and CVA seem to present context-dependent adjustments. This is in line with the extended neurovisceral integration model (Smith et al., 2017), which states that attention provides a direct means of adjusting the strength of the functional interactions between structurally connected regions in a context-specific manner. In the case of the present study, the need to reduce errors in the task, which involves attention, might have led to the predicted need for visceral-motor adjustments to support expected behavioral demands (Smith et al., 2017). Such context-specific adjustment might have led both pupil and CVA to concomitantly activate.

Regarding CVA, previous studies from our research group (Borges et al., 2019, 2020) have also found an increase of CVA from resting to tVNS phase for both active and sham stimulation conditions. However, in contrast to the present study with only one resting phase, one tVNS phase, one task phase, and one recovery phase measurement per session,

these previous studies grouped different measurement blocks within one single session. Consequently, CVA was measured in these studies at least in two resting and single tVNS phases within one session. Yet, despite a slight increase from one resting measurement to the other, there was no linear increase of CVA across the measurement blocks (Borges et al., 2019, 2020). Instead, in one study RMSSD increased from resting to tVNS phase for both active and sham stimulation (Borges et al., 2019), and the same pattern was observed in the other study for HF within blocks with cognitive flexibility tasks (Borges et al., 2020). Thus, taking together the evidence found in previous studies with the findings reported here, tVNS might increase CVA regardless of stimulation area. At the same time, it is possible that other confounders, instead of tVNS, have influenced—or were even responsible for—this increase during the tVNS phase. The present study does not provide a clear evidence that tVNS, regardless of stimulation area, positively influenced CVA. It cannot be ruled out that CVA increased because of relaxation that occurred while performing a monotonous task for 40 min. Moreover, the overall respiratory frequency decreased during tVNS and after the task phase. Since respiration can have a high impact on CVA (Brown et al., 1993; Houtveen et al., 2002), it is possible that CVA increased not due to tVNS, but to a change in respiration that either was caused by the task or was a result of the possible relaxation that occurred during the task. Thus, it is recommended that future studies measurement the level of the relaxation during or after the task, and use further strategies to control for respiration, for instance taking into account the moderating role of respiration in the statistical analyses.

Among all measurements presented here, only the task-related measurements were the ones for which no effects could be found. Interestingly, this is also the only variable for which no time component was considered in the analyses. Thus, it is possible that tVNS had effects on the neurophysiological measurements that were independent of the stimulation area, and that this effect could only be found because of the comparison between before and after a relevant event, which was not possible for the cognitive measurements. The relevant event for pupillary responses might have been the stimulus response, whereas for CVA might have been the beginning of the stimulation. In the present study, both of these events were expected to engage the brain areas whose activity is modulated by tVNS. If this possibility is true, then this would implicate that the effects of tVNS on PES may have been overlooked, and that the sham condition showed the same effects as active stimulation. This idea is supported by another study that also found an increase of CVA across three experiments independent of the stimulation condition used, including sham (Borges et al., 2019). This would also explain why some studies had opposite results to what was hypothesized (Colzato et al., 2017; Keute et al., 2018), since these studies also did not consider a time component, which would enable a time-related comparison. Such findings reinforce the questions about the suitability of the earlobe as a sham condition.

According to Rangon (2018), the fact that the earlobe is not supplied by the vagus nerve does not mean that earlobe stimulation has no effect on the variables investigated. She argues that it is possible to activate cortical and limbic areas by using acupuncture on the anti-tragus, an area located just above the earlobe (Rangon, 2018). Supporting the argument against earlobe as a sham stimulation, it has been argued that a precise cutaneous map of the external ear is not practical for three reasons: a) there is a high interindividual variation regarding nerve distribution, b) some nerves cross-communicate with other nerve fibers along their intracranial course, and c) the boundaries between particular dermatomes often overlap (Butt et al., 2019). Although there are sparse attempts to create a sham condition independent on the earlobe, there is still no sham stimulation during which a) the participants cannot differentiate it from active stimulation, and b) no nerve is stimulated. Studies addressing this issue are essential to further improve tVNS.

The present study aimed to conceptually replicate the findings from Sellaro et al. (2014) by using a Flanker task. Aligned with that study, the present study did also not find improvement in task performance,

represented by higher RT and less errors, via tVNS. However, contrary to Sellaro et al. (2014), we did not find a stronger PES during tVNS compared to sham stimulation. Importantly, the present study showed different values when compared to the original study (Sellaro et al., 2014): Overall, the present study reports higher RT, lower error rates, and lower post-error slowing than the original one. Furthermore, the standard deviation found in the present study is much higher than in the previous study. Our study made use of varying measurement and analysis approaches, which is aligned with the idea of a conceptual replication (Walker et al., 2017). In the following paragraphs, we briefly discuss these variations.

First, we used a within-subject design whereas Sellaro et al. (2014) used a between-subjects design. Besides the advantage of having more power by using a within-subject design compared to a between-subjects design (Thompson and Campbell, 2004), this approach can lead to learning effects. Since there was a strong decrease from Day 1 to Day 2 in RT, and PES decreased over the three days, learning effects could indeed be observed in the present study. Although we counterbalanced the stimulation conditions, learning effect might have played a role in this considerable difference regarding results between both studies. The learning effects analysis showed reaction time in the cymba conchae condition to be lower on Day 1 in comparison to reaction time in the earlobe condition on Day 1. However, this analysis has been performed on very small groups, ranging from 12 to 15 participants per group. Thus, an array of biases can have influenced these results (Button et al., 2013). To counteract these possible biases, future studies with between-subjects design and an appropriate power should further investigate this effect.

Second, we defined stimulation intensity based on individual threshold levels, whereas Sellaro et al. (2014) set the stimulation intensity as 0.5 mA for all participants. In the present study, we adopted this method because of the lack of comparability between stimulation during cymba conchae and tragus stimulation regarding sensitivity. Tragus stimulation is usually done with a much higher amplitude when compared to cymba conchae stimulation (e.g., Antonino et al., 2017; Bretherton et al., 2019; Clancy et al., 2014), so that it renders difficult to use the same set intensity for all participants. Despite the significant differences between the auricular areas regarding chosen stimulation intensity, the intensities chosen by the participants in the three conditions are in line with previous research. This discrepancy might have anatomical origins, for instance because of possible different skin thicknesses between both auricular areas, or by the inherent difference between electrodes that are placed along the skin surface (for cymba conchae stimulation) vs. ear clip electrodes (for tragus stimulation). Varying the intensity of tVNS has been shown not to impact on CVA in healthy adults, and this may be valid for other outcomes of tVNS (Borges et al., 2019). However, because the effect of different stimulation intensities on psychophysiological measurements has so far only been tested in the context of cymba conchae stimulation, and using only one type of electrode (Borges et al., 2019), these significant differences regarding stimulation intensity might still act as a confounder. Moreover, the method to choose the stimulation intensity, which is based on individual threshold levels, may have led to different sensations on the cymba conchae and on the earlobe that are potentially relevant for the assessed effects of tVNS. Instead of considering the mean between the individually detectable stimulation and the uncomfortable stimulation intensity as described by De Couck et al. (2017), the free stimulation method as described by Borges et al. (2019) possibly provides more similar sensations of the stimulation, thus potentially eliciting different effects as reported in the current study. More research addressing these questions is necessary.

Third, we used a different electrode placement on the earlobe for sham condition. Whereas Sellaro et al. (2014) placed two surface electrodes side by side, we used ear clips that allow the signal to pass through the earlobe. Possibly stimulation with ear clips allows a real stimulation of the nerves in the earlobe, whereas placing electrodes side

by side does not. Alternatively, the higher possibility of signal disturbance because of the placement being side by side reduces the potential effect of the stimulation on the earlobe, which would explain the lower PES during earlobe stimulation in Sellaro et al. (2014). Finally, it is possible that different types of electrodes with different sizes produce different electrical field maps produce different effects. The potential effect caused by different types of electrodes should be investigated in future studies.

Forth, we tested sport science students, who are possibly a population with relevant differences from the sample recruited by Sellaro et al. (2014). Concretely, possible differences in autonomic responses between sport students and less athletic students (Martinelli, 2005) cannot be ruled out. These possible differences might explain in part the differences in the results reported in the present study and by Sellaro et al. (2014). A comparison between samples might be relevant since we found in the present study a higher tendency to slower responses, higher accuracy, and more varied PES compared to Sellaro et al. (2014). In the same sense, it is important to highlight that different results may be observed in different populations, for instance comparing patients with healthy participants, or young with older participants. Furthermore, given that sex differences can influence cardiac vagal activity (Koenig and Thayer, 2016), it is possible that this difference in the sample influenced pupillary reaction, PES, and responsiveness to tVNS. Our study was better balanced regarding gender distribution, with 18 male participants out of 42 participants, compared to the sample reported by Sellaro et al. (2014) with only five male participants out of 40. Hence, Possibly differences in the gender distribution between our study and the study reported by Sellaro et al. (2014) have played a role in the different findings. Taken together, it is recommendable for future studies to carry out an exact replication instead of a conceptual one (Walker et al., 2017), and in a next step to investigate whether testing different populations leads to different results. Future studies in this direction might contribute to a better understanding of the heterogeneity of the results reported in both studies.

#### 4.1. Limitations

There are limitations to our study that should be addressed. First, learning effects were observed, which may serve as a confounder in the results. Second, respiratory frequency was obtained via a dedicated algorithm from Kubios (Tarvainen et al., 2013). However, a more precise assessment of respiratory frequency such as a respiration belt or a pneumotachograph is recommendable (Quintana et al., 2016). Third, earlobe stimulation with the Cerbomed's tVNS device was not tested. Although earlobe stimulation by means of ear clip electrodes is very common in research with tVNS (e.g., Antonino et al., 2017; Bretherton et al., 2019; Clancy et al., 2014), comparing both earlobe stimulations with each other would have been useful to control for possible effects arose due to the use of different placements. Fourth, the present study lacks a condition in which no stimulation is administered. Since it cannot be ruled out that the sham stimulation evoked a similar effect as the tragus and the cymba conchae stimulations, putting electrodes on the ear with the complete absence of electrical signal might be a further step to investigate the mechanisms of action of tVNS. PES seems to be an adequate cognitive phenomenon to investigate the suitability of this kind of sham stimulation since it might be less conscientiously influenced when compared to task performance parameters.

#### 4.2. Conclusion

The present study represents the first attempt to compare two major auricular areas that are targeted by tVNS regarding both cognitive and autonomic regulation. On the one hand, PES did not differ regarding stimulation of different auricular areas. On the other hand, error commission led to an increase in the sympathetic control of pupils via norepinephrine, and there was an undifferentiated increase in CVA

which might not necessarily have been triggered by tVNS. The results put question marks on the effectiveness of tVNS in influencing the mechanisms underlying PES and on the suitability of sham as a control condition. Future studies with tVNS should consider using neurophysiological measurements in order to explain more concretely the mechanisms underlying tVNS. Finally, this study showed again how timely it is to develop new possibilities for sham condition as an alternative for earlobe stimulation.

#### Acknowledgments

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#### Open practices statement

The raw data are available at <https://doi.org/10.7910/DVN/L2ID75>.

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