Effects of transcutaneous auricular vagus nerve stimulation on P300 magnitudes and salivary alpha-amylase during an auditory oddball task

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Abstract

Transcutaneous auricular vagus nerve stimulation (taVNS) is a non-invasive neurostimulation technique that is thought to modulate noradrenergic activity. Previous studies have demonstrated inconsistent effects of taVNS on noradrenergic activity, which is possibly due to insufficient statistical power, suboptimal stimulation parameter settings, and data collection procedures. In this preregistered within-subject experiment, 44 healthy participants received taVNS and sham (earlobe) stimulation during two separate experimental sessions. Stimulation intensity was individually calibrated to the maximum level below pain. During each session, participants received the stimulation continuously ten minutes before an auditory novelty oddball task till the end of the experimental session. The P3b component of the event-related potential served as a marker of phasic noradrenergic activity, whereas P3a magnitude was explored as an index of dopaminergic activity. Salivary alpha-amylase (sAA) was measured as an index of tonic noradrenergic activity before and at the end of the stimulation. The taVNS and sham conditions did not differ in P3a or P3b magnitudes, nor sAA secretion. These findings call into question whether taVNS, administered continuously at high, nonpainful stimulation intensities, reliably augments noradrenergic activity via the vagus nerve.

Keywords

Transcutaneous auricular vagus nerve stimulation; EEG; auditory oddball; salivary alpha-amylase; P300

Introduction

Transcutaneous auricular stimulation of the vagus nerve (taVNS) seeks to upregulate afferent vagal activity via electrical stimulation of the auricular branch of the vagus nerve. This neurostimulation technique has been proposed as a promising non-invasive alternative to surgical cervical VNS (Burger, D'Agostini, et al., 2020a; Ventureyra, 2000). Despite growing interest in potential therapeutic applications of taVNS, fundamental questions regarding the working mechanisms and optimal stimulation parameters of this technique remain unanswered (Farmer et al., 2021).

The auricular branch of the vagus nerve consists of afferent fibers that project to the nucleus of the solitary tract and the spinal nucleus of the trigeminal nerve (Frangos et al., 2014; Yuan & Silberstein, 2016). taVNS is believed to increase afferent vagal activity and, in turn, raise the activity in the locus coeruleus (LC)-noradrenaline (NA) network via the nucleus of the solitary tract. This indirect neuromodulatory effect has been demonstrated repeatedly using invasive cervical VNS (iVNS) in mice (e.g., Dorr & Debonnel, 2006; Hulsey et al., 2017; Manta et al., 2009; Mridha et al., 2021). Evidence for a noradrenergic mechanism of taVNS primarily relies on fMRI studies showing that taVNS increases LC activity in humans (Frangos et al., 2014; Sclocco et al., 2019; 2020; Yakunina et al., 2016, 2018; Zhang et al., 2019). Although promising, these results should be treated cautiously given all studies except two (Sclocco et al., 2019; 2020) employed a 3 Tesla MRI, which has a low signal-to-noise ratio in the brainstem and is thus unlikely to precisely characterize taVNS-evoked changes in LC activity (Sclocco et al., 2018). More research is thus required to support the hypothesis of a noradrenergic mechanism of taVNS in humans.

The LC-NA network plays a crucial role in many cognitive functions including alertness, memory, and attention (Chamberlain & Robbins, 2013; Sara, 2009). While tonic LC activity corresponds to the background LC activity which supports alertness, phasic LC activity refers to a transient increase in activity in response to salient stimuli that capture attention (Aston-Jones & Cohen, 2005). An inverted U shape relation between phasic and tonic LC-NA activity has been described (Aston-Jones & Cohen, 2005), meaning that phasic activity is maximal at intermediate levels of tonic activity. Direct

measurements of LC-NA activity are not feasible in humans due to the invasiveness of the required procedures (Grassi & Esler, 1999). Instead, researchers have relied on indirect, physiological markers of LC-NA activity to investigate the noradrenergic mechanism of taVNS. Examples of these indirect indices include salivary alpha-amylase (sAA) and the P300 scalp-recorded event-related potential (ERP) in the electroencephalogram (Burger, D'Agostini, Verkuil, & Van Diest, 2020). Salivary alpha-amylase is a protein released by the salivary glands in response to local sympathetic nervous system activity. sAA has been proposed as a marker of tonic noradrenergic activity given pharmacological noradrenergic manipulations have been shown to affect sAA secretion (Ehlert et al., 2006; Warren et al., 2017). The P300 is an ERP component typically observed approximately 300 ms after the onset of task-relevant or rare stimuli. Two subcomponents of the P300 are distinguished in the literature: P3b and P3a. The P3b is observed in the temporo-parietal junction and has been shown to be sensitive to noradrenergic pharmacological manipulations (Brown et al., 2016; De Rover et al., 2015; Nieuwenhuis et al., 2005). The P3a is located more frontally and is thought to reflect dopaminergic activity (Polich, 2007). While P3b is maximally evoked by infrequent task-relevant stimuli, P3a is maximal when novel, deviant stimuli are infrequently presented. A reliable paradigm to evoke the P300 is the oddball task. In a classical oddball task, one has to respond to infrequent target stimuli while ignoring standard frequent stimuli. To be able to distinguish the P3b from P3a, researchers employ the novelty oddball task which includes a third novelty stimulus that is infrequently presented and for which no response is required (Polich, 2007). The P3b amplitude is the largest for target stimuli while the P3a is the greatest for novelty stimuli (Polich, 2007).

Previous studies in humans have provided rather inconsistent evidence for an effect of taVNS on P3b and sAA (Burger, D'Agostini, Verkuil, & Van Diest, 2020). While three studies found taVNS to increase P3b for at least a subset of stimuli (Rufener et al., 2018; Ventura-Bort et al., 2018; Warren et al., 2020), three other studies did not find such an effect (Fischer et al., 2018; Gadeyne et al., 2022; Warren et al., 2019). Importantly, one of the studies with positive findings revealed an effect of taVNS on the P3b only in a post-hoc exploratory analysis on a subset of trials, but no overall difference

between taVNS and sham stimulation (Ventura-Bort et al., 2018). Also, the effects of taVNS on sAA have been studied in several studies. Three studies (Ventura-Bort et al., 2018, 2021; Warren et al., 2019) out of nine found preliminary evidence that taVNS increases sAA in post-hoc analyses (D'Agostini, Burger, Franssen, et al., 2023; D'Agostini, Burger, Villca Ponce, et al., 2022; D'Agostini et al., 2021; Giraudier et al., 2020; Höper et al., 2022; Koenig et al., 2019). Furthermore, in a mega-analysis pooling data of 371 participants, Giraudier and colleagues (2022) found taVNS to increase sAA using a mixed model approach but not when relying on a meta-analytic approach. While these mixed findings may reflect that taVNS does not affect the same LC-NA pathways as iVNS, the inconsistent findings may also result from methodological shortcomings of previous studies as discussed in detail in the next paragraphs.

One potential point of concern in previous taVNS studies is the adopted stimulation pattern, which may have been suboptimal to increase noradrenergic activity and see any effect on P3b and sAA. Specifically, the vast majority of studies administered the stimulation intermittently, typically 30 sec on followed by 30 sec off (D'Agostini et al., 2021; D'Agostini, Burger, Franssen, et al., 2023; Gadeyne et al., 2022; Giraudier et al., 2020; Höper et al., 2022; Koenig et al., 2019; Rufener et al., 2018; Warren et al., 2019; Warren et al., 2020). Studies in mice have shown that noradrenergic activity drops soon after iVNS is switched off (Hulsey et al., 2017; Mridha et al., 2021). One possibility is that administering taVNS intermittently dampens the effect of taVNS on noradrenergic markers due to the off periods during which noradrenergic activity may transiently decrease (D'Agostini, Burger, Villca Ponce, et al., 2022). As a solution, taVNS could be administered continuously to maximize its noradrenergic-enhancing effects. Furthermore, half of taVNS studies on sAA and P3b administered a stimulation intensity equal to 0.5mA (D'Agostini et al., 2021; Höper et al., 2022; Koenig et al., 2019; Rufener et al., 2018; Warren et al., 2019; Warren et al., 2020). The other half adopted a stimulation intensity individually tailored to the maximum level below pain (D'Agostini, Burger, Villca Ponce, et al., 2022; Gadeyne et al., 2022; Ventura-Bort et al., 2018; 2021; Fischer et al., 2018; Giraudier et al., 2020). Parametric studies on iVNS in mice and humans have shown that greater stimulation intensities evoke

greater noradrenergic activity (mice: Hulsey et al., 2017; Mridha et al., 2021; humans: Vespa et al., 2022). One possibility is that high stimulation intensities of taVNS are required to see an effect of taVNS on noradrenergic markers (D'Agostini, Burger, Villca Ponce, et al., 2022). In line with this idea, one parametric study on taVNS has reported an effect of taVNS on evoked pupil dilation (i.e., phasic noradrenergic marker) when considering the trials with the maximum intensity below pain threshold but not 0.5mA (D'Agostini, Burger, Franssen, et al., 2023).

Another limitation of previous taVNS studies on sAA is the employment of suboptimal saliva collection procedures (Burger, D'Agostini, Verkuil, & Van Diest, 2020). Six taVNS studies out of nine measured sAA using cotton sponges, which require chewing without assessing salivary flow rate (D'Agostini et al., 2021; Giraudier et al., 2020; Höper et al., 2022; Koenig et al., 2019; Ventura-Bort et al., 2018, 2021). Chewing and salivary flow rate (i.e., index of parasympathetic activity) are well-known to influence sAA secretion independently of central noradrenergic involvement (Bosch et al., 2011) and are thus potentially important confounding factors (Burger, D'Agostini, Verkuil, & Van Diest, 2020). It follows that the lack of adherence to state-of-the-art measurement methods (i.e., methods that do not entail chewing) might partly explain the inconsistent results (Burger, D'Agostini, Verkuil, & Van Diest, 2020).

A final shortcoming of the majority of P3b studies is the limited statistical power. Four studies out of six utilized rather small sample sizes (range of N's = 20-25) (Fischer et al., 2018b; Rufener et al., 2018; Ventura-Bort et al., 2018; Warren et al., 2019), which reduces the statistical power and increases the risk of false negatives (Button et al., 2013). Low statistical power may therefore additionally underlie the inconsistent results on P3b.

The current within-subjects cross-over study aimed to study the effects of taVNS on indirect indexes of phasic and tonic noradrenergic activity using continuous stimulation at the maximum level below pain in a larger study sample. Specifically, we focused on the effects of taVNS on P3b, a marker of phasic noradrenergic activity, and sAA, a marker of tonic noradrenergic activity, which have shown some promise in earlier taVNS research. The selected stimulation set-up is expected to maximize the

effect of taVNS on noradrenergic activity and, therefore, on sAA and P3b. To our knowledge, this is the first study on taVNS and P3b testing the selected stimulation pattern in a well-powered experiment. To distinguish P3b from P3a, we adopted an auditory novelty oddball task. We also addressed the shortcomings of previous taVNS studies on sAA by collecting saliva with a method that does not entail chewing and by assessing the salivary flow rate to rule out a potential parasympathetic influence on sAA. We hypothesized that participants would display larger P3b magnitudes for target stimuli of an auditory novelty oddball task during taVNS compared to sham stimulation. Additionally, we hypothesized that participants would show larger increases in sAA secretion during taVNS compared to sham stimulation. Finally, we explored the effects of taVNS on P3a magnitudes for novelty stimuli during the auditory novelty oddball task. Only two studies have previously investigated a potential taVNS modulation of P3a and reported no significant differences compared to sham (Ventura-Bort et al., 2018; Warren et al., 2019). All confirmatory hypotheses, as well as the procedures and planned statistical analyses, were preregistered on the Open Science Framework, https://osf.io/jf247.

Methods

Participants

We used the software program G*Power (v 3.1) to conduct a power analysis for a paired-samples t-test, given that our main hypothesis concerns a within-subjects comparison of the effects of taVNS versus sham stimulation. Our goal was to obtain a power of at least 0.80 to detect a medium effect size of $\delta = 0.5$ at the standard 0.05 alpha error probability. The power analysis was based on a simple paired-samples t-test analysis, as this is a simplified alternative to the multilevel model that was preregistered and performed in this analysis. Based on this power analysis, the current study needed to include a minimum of 34 participants to reach the desired statistical power.

To ensure that the analyses would still have sufficient power in case some participants would have to be excluded due to electrical interference between the taVNS device and the EEG measurement, 44 healthy participants between 18 and 30 years old were included in this experiment

(22 male/22 female, M_{age} = 23 years). Participants were recruited using fliers, designated university webpages, and social media. Participants received partial course credit or 40 euros for participation in this experiment. Participants were allowed to participate unless they had a history of or current neurological disorder, or suffered from a current cardiac or psychiatric disorder. Additional exclusion criteria included pregnancy, recovering from serious trauma or surgery, having untreated hearing problems, having participated in a study using the taVNS device before, wearing any implants, the use of illicit drugs in the past three months, and chronic or ongoing use of medication (oral contraceptives excluded). Finally, participants were asked to complete the Patient Health Questionnaire (PHQ-9; Kroenke et al., 2001) and the Generalized Anxiety Disorder scale (GAD-7; Löwe et al., 2008) prior to the experimental session, and were only allowed to participate if their score on either scale was lower than 10. These scores correspond to recommended cut-off scores for further evaluation of depressive and anxiety symptoms, as they correspond to at least moderately severe depressive or anxiety symptoms (Manea et al., 2012; Spitzer et al., 2006).

Instruments

Vagus nerve stimulation

Electrical stimulation of the ear was provided using a bipolar constant current stimulator (DS5 stimulator, Welwyn Garden City, UK) connected to two titan electrodes. We used electrodes designed for transcutaneous stimulation of the cymba concha of the ear (NEMOS®, Cerbomed, Erlangen, Germany) to ensure proper placement. In the taVNS condition, the electrodes were attached to the cymba concha of the left ear, an area of the outer ear that is innervated by the vagus nerve. In the sham stimulation 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). In both conditions, continuous biphasic stimulation was provided at a frequency of 25 Hz and a pulse width of 250 μs. The stimulation intensity was individually calibrated to be above the detection threshold and below the pain threshold for both the taVNS and the sham condition, with a maximum stimulation intensity of 4 mA.

The stimulation intensity was calibrated by determining the average intensity that was perceived as 'highly intense but not painful' in two subsequent ramp-up and ramp-down series. During the calibration phase, participants received increasing and decreasing series of 5-s stimulation trials. After each trial, they rated the subjective sensation on a VAS ranging from no sensation (0) to highly intense and painful (100). The increasing series of trials started from an intensity of 0.3 mA and increased in 0.1 mA increments until participants reported a ''highly intense and slightly painful" sensation of 90 on the VAS. The same intensity was repeated and then reduced in 0.1 mA decrements until participants rated the sensation as ''intense" (70 on the VAS). This ramp-up and ramp-down procedure was then repeated a second time. The stimulation intensity used for the rest of the experimental session was calculated based on the average of the four intensities rated as "highly intense, but not painful" (80 on VASs; 2 from increasing and 2 from decreasing series). A similar procedure has been used in other studies with healthy participants (D'Agostini, Burger, Franssen, et al., 2023; D'Agostini, Burger, Villca Ponce, et al., 2022; Ventura-Bort et al., 2018; Yakunina et al., 2016). Participants received a mean stimulation intensity of 2.42 mA (sd = 1.02 mA) during taVNS and 2.86 mA (sd = 0.96 mA) during sham stimulation.

Task

During the auditory novelty oddball paradigm (Chourchese et al., 1975; Polich et al., 2007; Warren et al., 2019), participants had to respond with a key press when presented with an infrequently occurring target tone (10% of trials), while ignoring frequent standard tones (80%) and novel, surprising tones (10%). Low (350 Hz) and high (500 Hz) sine wave tones were used as standard and target tones, counterbalanced across participants. The novel stimuli were short environmental sounds extracted from a set by Fabiani and Friedman (Fabiani & Friedman, 1995). All tones were presented binaurally for 300 ms and separated by jittered inter-stimulus intervals (range: 2.1 to 2.9 sec). Trials were presented in a pseudorandomized order, to ensure that at least 3 standard tones were presented between each target and/or novel distractor tone. The task consisted of 540 trials and lasted approximately 40 minutes.

Salivary alpha-amylase (sAA) and salivary flow rate

Participants were instructed to passively pool saliva under their tongue for a minute and then spit into a test tube. This procedure was repeated three times over the course of three minutes. All samples were kept in a freezer at -20 degrees Celsius. The saliva samples were sent to a laboratory (Dresden LabService GmbH, Germany), where the concentration of sAA and the saliva volume were measured for each sample. To account for potential confounding effects of parasympathetic activity on sAA concentrations (Bosch et al., 2011), sAA secretion (U/min) was calculated by multiplying sAA concentration (i.e., net sAA per milliliter of fluid - U/ml) with the salivary flow rate (i.e., salivary fluid output per minute - ml/min). Salivary flow rate was measured exploratively as an index of parasympathetic activity (Bosch et al., 2011).

EEG

A high-density 129-channel EEG sensor net (Philips EGI, Eugene, USA) was used to measure EEG throughout the auditory oddball task with a sampling rate of 250 Hz and using Cz as the online reference. ERP amplitudes were extracted from a representative fronto-central cluster to examine the P3a, and from a representative centro-parietal cluster to examine the P3b as an index of phasic LC-NA activity (Ventura-Bort et al., 2018). The raw EEG data were filtered using BESA 6.0 analysis software (BESA GmbH, Gräfelfing, Germany). The following filters were applied: a 0.1 Hz high-pass filter, 20 Hz low-pass filter to filter out the 25 Hz taVNS signals, and an additional notch filter of 50 Hz (band with: 2 Hz) to reduce line noise.

The filtered EEG data were visually inspected for irregular artifacts. EEG channels with noisy data were removed from the analyses or interpolated based on surrounding artifact-free channels using a spherical spline procedure. A median of 3 channels was interpolated and a median of 6 channels was removed per participant, with a set maximum of 12 channels per participant in total. After marking artifacts and removing or interpolating channels, a blink detection algorithm was run and variance due to eyeblinks was removed from the EEG signal (Ille et al., 2002). Finally, filtered and cleaned data was re-referenced to the average reference.

After pre-processing, time windows surrounding stimulus presentations (-200 ms prior to stimulus onset to 800 ms post stimulus onset) were extracted and averaged. A researcher blinded to the condition (sham vs. taVNS) visually inspected the individual averages as well as the grand average to determine the most representative latency windows of P300 amplitudes. The selected P300 time window corresponded to ±15 samples (120ms) around the most frequently occurring P300 peak latency. The response window for P3a was thus determined as 220-340 ms following stimulus onset, whereas the response window for P3b was 300-420 ms following stimulus onset (similar time windows for auditory oddball stimuli were used in Warren et al., 2019). Visual inspection of grand average ERPs for each stimulus type also allowed us to determine suitable clusters of electrodes to capture both the P3a and P3b. Both the P3a and P3b amplitudes were quantified as the average of a cluster of 4 electrodes around Cz for P3a, and a cluster of 4 electrodes around Pz for P3b. These electrodes have been commonly used to quantify these components (e.g., Warren et al., 2017; Polich, 2007). P3a and P3b amplitudes were calculated as the mean baseline-corrected (200ms before onset) amplitude in the selected time window across the selected electrodes. The amplitudes of either component were averaged across trials of a stimulus type per participant's session.

Questionnaires

Positive and Negative Affect Schedule (PANAS): The PANAS was administered at the start of both lab visits, to measure emotional state levels at the beginning of both experimental sessions. The PANAS-state version is a 20-item self-report measure comprising two sub-scales consisting of 10 items each, respectively measuring positive affect (PA, range: 10 - 50) and negative affect (NA, range: 10 - 50) (Watson et al., 1988).

Side effects and Distraction by stimulation items: participants were asked to rate the following potential stimulation-related side effects at the end of the experimental session: 1. Headache, 2. Neck pain, 3. Numbness of a limb, 4. Nausea, 5. Drowsiness, 6. General feelings of discomfort, as well as 7. Painful-, 8. Redness-, 9. Tingling-, 10. Itching-, or 11. Burning sensations at the location of the electrode. All side effects were rated on a 7-point Likert scale ranging from 0 (not at all) to 6 (very strong).

Participants also indicated to what extent they were distracted from the oddball task by the stimulation on a VAS (0 = not at all - 100 = very much; *Distraction by stimulation*).

Stimulation perceived intensity and unpleasantness items: the perceived intensity of the stimulation was rated on a VAS ranging from no sensation (0), light (10), mild (30), moderate (60), intense (70), highly intense, but not painful (80), highly intense and slightly painful (90), highly intense and painful (100). (Un)pleasantness was rated on a VAS labeled at both extremes (-50 = very unpleasant and 50 = very pleasant) as well as at the midpoint (0 = neither unpleasant nor pleasant).

Other administered questionnaires/subjective ratings include the short version of the Depression Anxiety Stress Scale (DASS-21; Lovibond & Lovibond, 1995), Childhood Trauma Questionnaire (CTQ-SF; Bernstein et al., 2003), subjective ratings on distress and uncomfortableness due to the equipment, and appraisal of the influence of the stimulation on performance during the task (for an overview see https://osf.io/jf247). These questionnaires are not reported because out of the scope of this manuscript.

DS5 output

The Matlab script used to administer the stimulation stored the DS5 output, which corresponds to the current and voltage applied in a session. The DS5 output was measured to verify the complete stimulation pattern.

Procedure

The experimental protocol was approved by the Ethics Committee of KU Leuven (EC code S62386). The experiment utilized a two-part cross-over single-blind design, where participants completed an auditory novelty oddball task while receiving taVNS in one session and sham stimulation in the other session, in a counterbalanced order with approximately one week in between, at the same time of day. Participants were only tested in the afternoon and at the same time of day for both sessions, to avoid potential confounding effects of the circadian rhythm. Participants were asked to abstain from brushing their teeth and chewing gum, eating and drinking (1 hour before the experiment), smoking

(2 hours before), drinking caffeinated beverages (6 hours before), and taking medication, drinking alcohol, or doing intense physical exercise (24 hours before).

At the start of the first experimental session, participants were asked to fill in the PHQ-9 and the GAD-7. After this screening process, eligible participants were provided with oral and written information about the study and the in- and exclusion criteria before signing an informed consent form.

Electrodes were attached to the participants' skin to enable the continuous measurement of heart rate and skin conductance as exploratory measures of peripheral arousal. Participants were instructed to provide the first saliva sample and, then, asked to fill in the Positive and Negative Affect Schedule (PANAS; Watson et al., 1988). The EEG net was applied to the participants, as well as a nasal air sampling cannula connected to a capnograph (Nonin Medical Inc., Plymouth, UK) to continuously monitor respiratory rate[1]. Afterward, a five-minute baseline measurement of heart rate variability was conducted.

The taVNS electrode was attached to the participants' left ear, stimulating either the cymba concha (taVNS) or the earlobe (sham). After a 20-trial practice run for the auditory oddball task, the stimulation intensity was individually calibrated. The stimulation was applied 10 minutes before and during the auditory oddball task till the end of the experiment. No instruction on blinking was provided given attentional control is required to suppress the blinks. After completing the task, the second saliva sample was collected during active auricular stimulation, after which the auricular stimulator was removed. The stimulation lasted approximately 40 minutes. Participants were then asked to rate to what extent they experienced a list of side effects, and they rated how intense, unpleasant, and distracting was the stimulation.

[1] Due to a mechanical error, the respiratory rate was not measured reliably and will therefore not be presented in this manuscript. Analysis of the skin conductance data falls outside of the scope of this manuscript.

Statistical Analyses

The statistical analyses were carried out as described in our preregistration, https://osf.io/jf247. Unblinded analyses were conducted meaning that the experimenter was not blind to the stimulation condition while analyzing the data. We performed linear mixed model analyses (maximum likelihood modeling) in *R* using the *nlme* package unless stated otherwise. In the next sections, we first describe the undertaken steps to model the fixed and random structures that are common to all linear mixed models. Next, we describe the pre-registered and selected models specific to each outcome.

While the fixed part of each model was outlined a priori, the random structure was defined using a bottom-up procedure starting from the simplest model (i.e., fixed part plus random intercept) towards a more complex model (Hox et al., 2010). Intercepts were allowed to vary randomly across participants. Random slopes of theoretical interest were specified in the pre-registration. To take into account intra-individual variance due to multiple days of testing, we deviated from the pre-registration and tested whether adding the random effect session number nested within participants improves the fit of the model. We performed a likelihood ratio test to compare the relative fit of two competing models that differ for the random structure only and chose the model based on the log-likelihood statistics. We found that the tested models met the normality and homoscedasticity assumption based on the visualization of models' residuals (see Finch, Bolin, & Kelley, 2019). Along with the regression coefficient of the fixed structure, we report the corresponding t-test statistics, degrees of freedom (df), and the p-values. The *nlme* package in R estimates the df using the method described by Pinhero and Bates (2000; P 91). Pre-registered analyses on P3b and sAA utilized one-tailed testing, following our pre-registration. We conducted two-tailed testing in our exploratory analyses. The alpha level was set equal to 0.5.

Pre-registered analyses

P3a/P3b: data from 7 participants (of which 4 for one session) were excluded based on the following pre-registered exclusion criteria: 1) bad performance on the oddball task (i.e., <30% accurate responses given to targets and/or >10% incorrect responses given to non-targets); 2) excessive

measurement artifacts in EEG data (i.e., less than one-third of the trials left to be analyzed). Furthermore, the data of one participant in one session was not properly stored. We conducted two separate linear mixed model analyses to test the main and interaction effects of *Condition* (Sham - reference category - vs. taVNS) and *Stimulus Type* (Standard – reference category, vs. Novelty/Target; dummy coded) on P3a and P3b amplitude. The random slope for Stimulus Type and the random effect Session nested were excluded from both models (no improvement of model fit).

sAA secretion: we conducted a linear mixed model analysis to test the main and interaction effects of Condition and *Time* (pre-stimulation – reference – vs. end stimulation) on sAA levels. sAA secretion was natural log transformed to improve adherence to the normality assumption of linear mixed models. The random slope Time and nesting Session within participants did not improve model fit and were thus excluded.

Pre-registered sensitivity analyses

In the pre-registration, we stated we would perform the pre-registered analyses excluding those participants that received the stimulation for less than 90% of the intended duration. Nevertheless, 27% of the DS5 output data was not stored, preventing us to identify those participants with a short administration of the stimulation. We thus decided not to perform the pre-registered sensitivity analyses.

Un-preregistered Bayesian analyses

*W*e conducted Bayesian analyses to provide evidence for the lack of an effect of taVNS on the pre-registered outcomes, P3a, P3b, and sAA. The Bayes factors (BF_{01}) estimate the probability of the data to occur under the null hypothesis (H_0; model excluding the main and interaction effects of Condition) compared to the alternative hypothesis (H1). The Bayesian Information Criteria of the models' output was used to calculate the BF_{01} (see Wagenmakers, 2007). A larger BF_{01} indicates greater evidence for H_0. The random structure and covariance-variance matrix of both models (H_0 and H_1) were the same.

Un-preregistered exploratory analyses

We tested the main and interaction effect of Condition and Time on flow rate. To meet the normality assumption, we transformed flow rate using a natural log transformation. The random slope Time and random effect nesting session within participants were left out (no improvement of model fit). We also conducted exploratory analyses on the effect of taVNS on HRV, self-reported side effects, distraction by stimulation, and stimulation-perceived intensity/unpleasantness items. The description and results of such analyses can be found in the appendix.

We explored whether the administration order of sham and taVNS influence P3a and P3b. For this purpose, we ran two mixed model analyses adding the main effect of administration order (Order: taVNS first - reference vs. Sham first) to the fixed structure of the pre-registered analyses. The random structure of the exploratory and pre-registered analyses on P3a and P3b was the same. The results indicate that the administration order did not influence either P3a or P3b (see Table S2 in the Appendix).

Sex-based analyses

In line with the definition provided by the Biological Psychology Journal, we define sex as "a set of biological attributes that are associated with physical and physiological features". We did not conduct any sex-based analyses as we did not have the statistical power to investigate whether sex moderates the interaction effect between stimulation and time (sAA)/stimulation type (P3a/P3b). The lack of such analyses prevents us from knowing if the effect of taVNS varies as a function of sex. Nevertheless, we collected the data from half female and half male, which approximately reflects the ratio in the general population.

Results

PANAS scores

On average, participants in the taVNS condition had a negative (NA) and positive affect (PA) score equal to 12.65 (SD_{NA} = 3.75) and 28.27 (SD_{PA} = 6.91), respectively. In the sham condition, on average, they had a NA score of 12.42 (SD = 3.22) and a PA score of 28.86 (SD = 6.56).

P3b

Participants displayed a significantly larger P3b amplitude for novelty stimuli compared to standard stimuli (t(182) = 3.95, $p < .001$), and an even larger increase in amplitude for target stimuli ($t(182) = 9.26$, $p < .001$). There were no differences in P3b amplitude between the taVNS and sham stimulation conditions overall ($t(182) = -0.47$, $p = 0.18$) and for the target ($t(182) = 0.43$, $p = 0.34$) and standard stimuli ($t(182) = 0.7$, $p = 0.24$; see Figures 1, 2 and Table 1).

P3a

Participants displayed a significantly larger P3a amplitude for target stimuli compared to standard stimuli ($t(182) = 2.44$, $p = 0.015$) and an even larger increase in amplitude for novel stimuli ($t(182) = 11.51$, $p < .001$). There were no differences in P3a amplitude between the taVNS and Sham stimulation conditions overall ($t(182) = -1.07$, $p = 0.29$) and for target ($t(182) = 0.64$, $p = 0.52$) and standard stimuli ($t(182) = 0.67$, $p = 0.5$; see Figures 1, 3 and Table 1).

Table 1
Unstandardized regression weights (b), standard errors (SE), and p-values for mixed model analyses predicting P3a and P3b.

	P3a			P3b		
	b	SE	p	b	SE	p
Intercept	0.08	0.44	0.86	0.59	0.35	0.091
Condition $_{taVNS\ vs.\ Sham}$	-0.53	0.5	0.29	-0.2	0.42	0.18#
Stimulus type $_{Novelty\ vs.\ Standard}$	5.7	0.5	<0.001	1.67	0.42	<0.001#
Stimulus type $_{Target\ vs.\ Standard}$	1.21	0.5	0.015	3.91	0.42	<0.001#
Condition $_{taVNS\ vs.\ Sham}$ * Stimulus type $_{Novelty\ vs.\ Standard}$	0.47	0.7	0.50	0.42	0.6	0.24#
Condition $_{taVNS\ vs.\ Sham}$ * Stimulus type $_{Target\ vs.\ Standard}$	0.45	0.7	0.52	0.25	0.6	0.34#

Notes. Regression weights represent the difference in mean P3a/P3b between the condition under study and the reference condition; Condition (Sham - reference category - vs. taVNS) and Stimulus Type (Standard – reference category, vs. Novelty/Target; dummy coded). The symbol # indicates the predictors for which a one-sided hypothesis test was conducted.

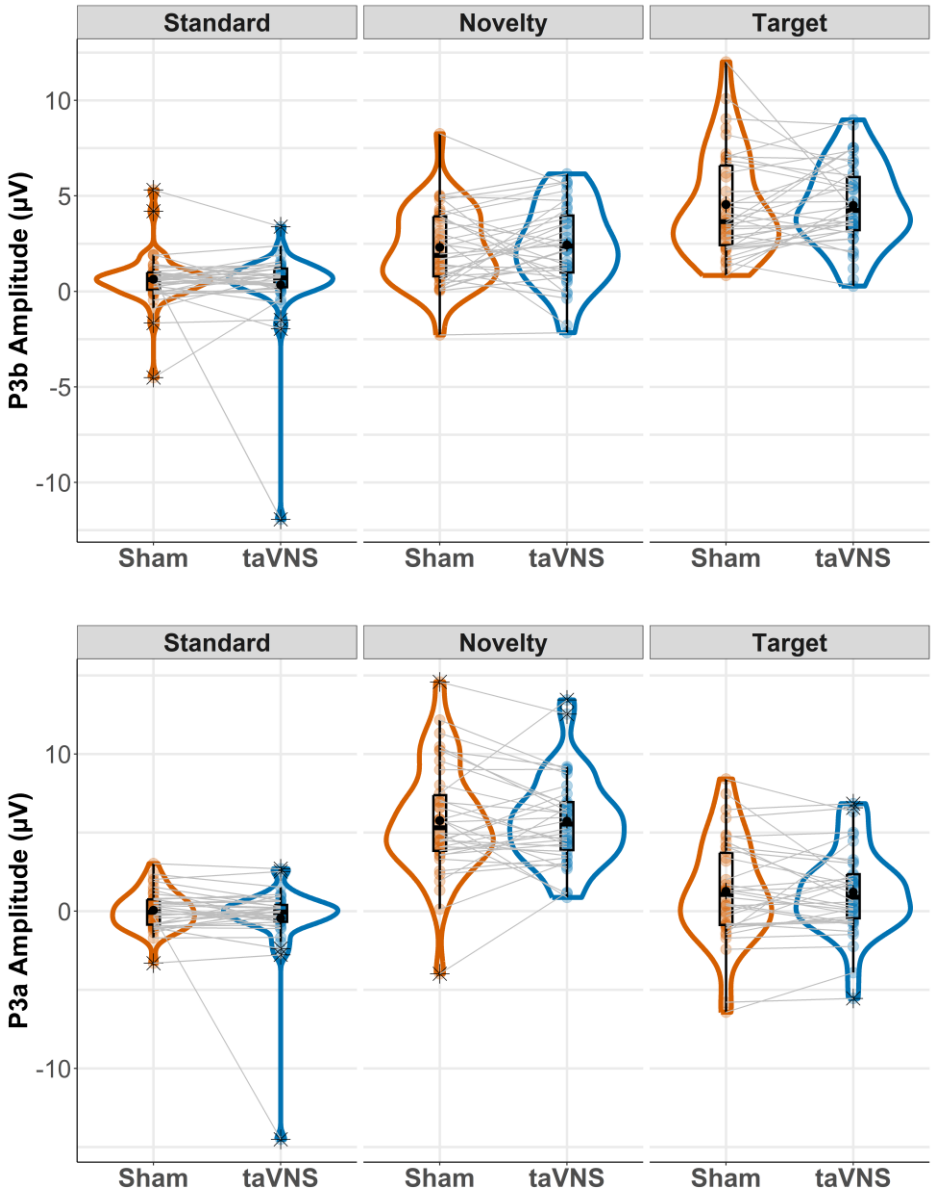


Figure 1. Violin plots, boxplots, and mean (black dot) of P3b/P3a as a function of Condition and Stimulus Type. The violin plot displays the full distribution of the data. The boxplot shows the median, minimum, maximum, and interquartile ranges. Individual data is plotted (dots connected with lines).

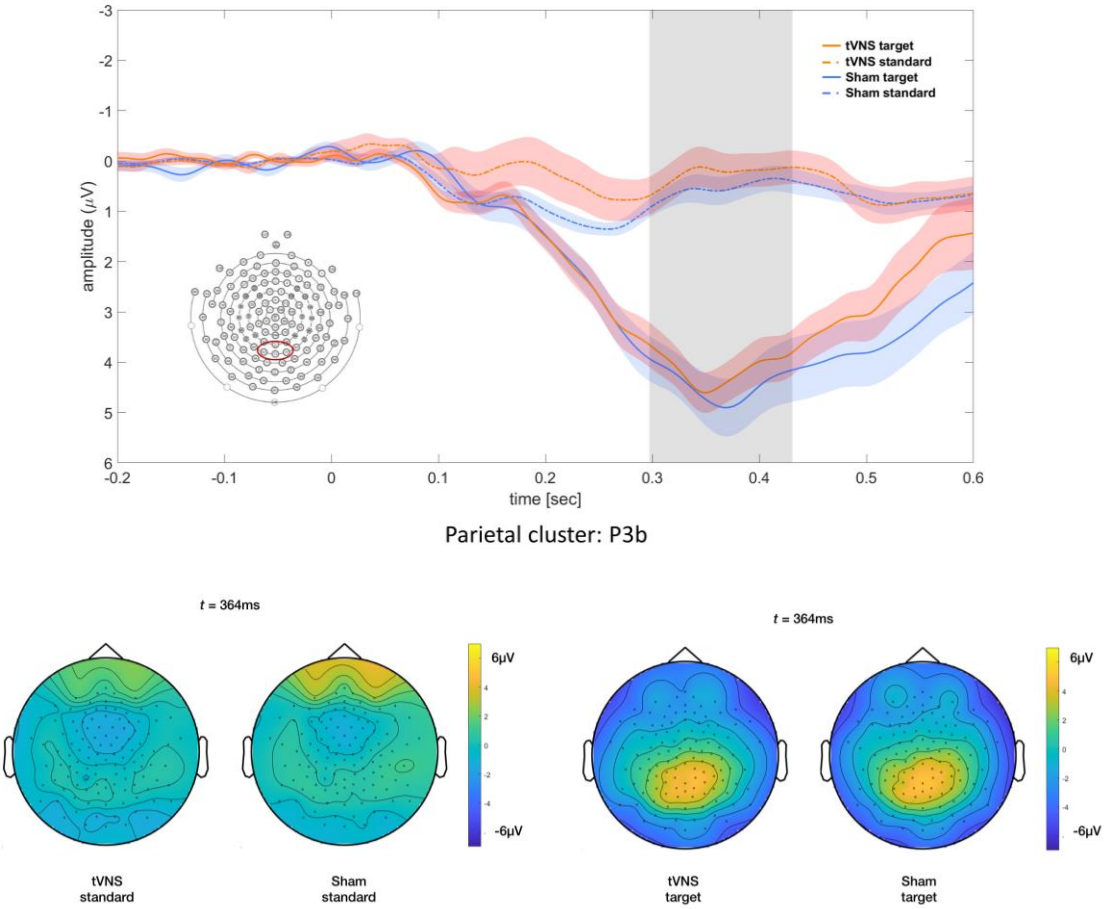
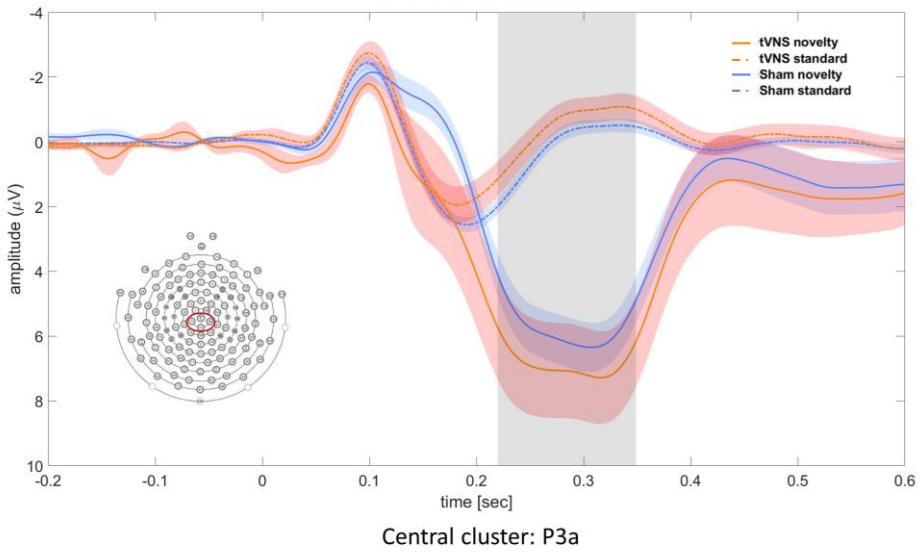
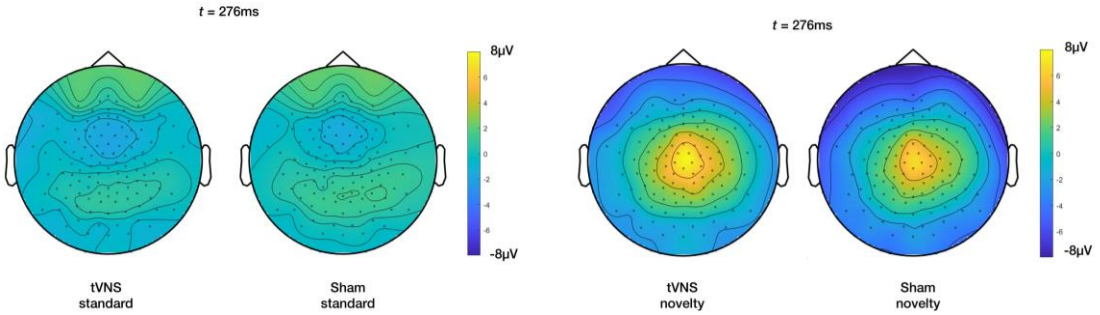


Figure 2. (*Top*) Grand average P3b ERPs for Target (black lines) and Standard (grey lines) stimuli, presented for both the taVNS (solid line) and Sham (dotted line) condition. The band bounds represent the standard errors. The response window is represented by the grey box. (*Bottom)* The scalp topography for standard and target stimuli during the peak P3b response for target stimuli is presented for each stimulus type and stimulation condition.



1



2

Figure 3. (*Top*) Grand average P3a ERPs for novelty (black lines) and standard (grey lines) stimuli, presented for both the taVNS (solid line) and Sham (dotted line) condition. The band bounds represent the standard errors. The response window is represented by the grey box. (*Bottom)* The scalp topography for standard and novelty stimuli during the peak P3a response for target stimuli is presented for each stimulus type and stimulation condition.

Salivary Alpha-Amylase

The average (untransformed) sAA secretion during pre-stimulation was 20.37 U/min (median of 13.35 U/min and standard deviation equal to 18.13 U/min). sAA did not significantly differ between conditions ($t(127) = -0.3$, $p = 0.12$) and did not significantly increase over time ($t(127) = 1.2$, $p = 0.12$). Furthermore, there was no significant difference between conditions in the change of sAA secretion over time ($t(127) = -0.39$, $p = 0.15$; see Figure 4 & Table 2).

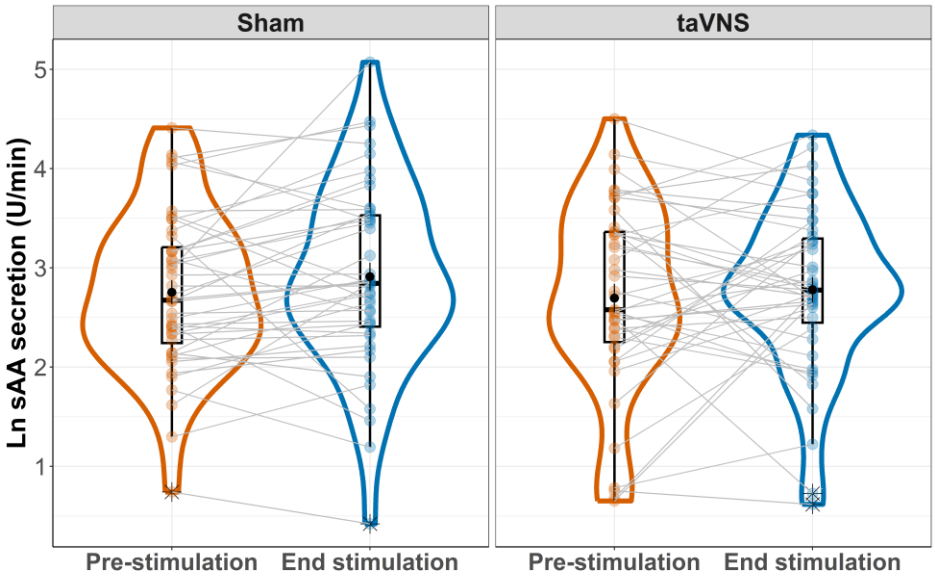


Figure 4. Violin plots, boxplots and mean (black dot) of ln sAA secretion at pre-stimulation and the end of stimulation for sham and taVNS. Individual data is plotted (dots connected with lines).

Flow rate

The average (untransformed) flow rate during pre-stimulation was 0.36 ml/min with a median of 0.33 ml/min and a standard deviation equal to 0.23 ml/min. Flow rate did not differ between sham and taVNS overall ($t(42) = 0.10$, $p = 0.92$) and over time ($t(85) = -1.08$, $p = 0.29$). Furthermore, it did not change as a function of time ($t(85) = 1.2$, $p = 0.24$; see Table 2).

Table 2

Unstandardized regression weights (b), standard errors (SE), and p-values for mixed model analyses predicting Ln sAA and Ln flow rate.

	Ln sAA			Ln salivary flow rate		
	b	SE	p	b	SE	p
Intercept	2.75	0.13	< 0.001	0.3	0.02	< 0.001
Condition *taVNS vs. Sham*	-0.04	0.13	0.12#	0.002	0.02	0.92
Time *End vs. Pre-stim.*	0.16	0.13	0.12#	0.02	0.02	0.24
Condition *taVNS vs. Sham* * Time *End vs. Pre-stim.*	-0.07	0.19	0.15#	-0.02	0.02	0.29

Notes. Regression weights represent the difference in mean Ln sAA/Ln flow rate between the condition under study and the reference condition; Ln sAA = salivary alpha-amylase secretion (U/min) ln transformed; Ln flow rate = flow rate (ml/min) ln transformed; Time = Pre-stimulation – reference category – vs. End Stimulation; Condition = Sham – reference category – vs. taVNS. The symbol # indicates the predictors for which one-sided hypothesis tests were conducted.

Bayesian analyses

All Bayes factors (BF_{01}) were larger than 20, providing strong to very strong evidence for a lack of an effect of taVNS on sAA, P3b, and P3a (see Table 3).

Table 3.

Bayes Factors (BF_{01}) in favor of the null hypothesis (H_0) estimated for P3b, P3a, and Ln sAA.

Fixed structure H_0 model	Fixed structure H_1 model	Outcome	BF_{10}
Stimulus type *Target vs. Standard* Stimulus type *Novelty vs. Standard*	H_0 model + Condition *taVNS vs. Sham* + Condition *Stimulus type *Target vs. Standard* + Condition * Stimulus type *Novelty vs. Standard*	P3b	2648.45
		P3a	1892.33
Time *End vs. Pre-stim.*	H_0 model + Condition *taVNS vs. Sham* + Condition * Time *End vs. Pre-stim.*	Ln sAA	114.57

Notes. Reference values to interpret the Bayes Factor as evidence in favor of H0 (cf. Raftery, 1995): weak = 1-3; positive = 3-20; strong = 20–150; very strong =/>150.

Discussion

Previous studies provided rather inconclusive evidence for a noradrenergic mechanism of taVNS using physiological markers known to reflect central LC-NA activity including the P3b component of the event-related potential (phasic noradrenergic marker) and salivary alpha-amylase (tonic noradrenergic marker). We argued that these contrasting results may be due to three main methodological shortcomings: 1) suboptimal stimulation parameters, 2) low statistical power, and 3) suboptimal saliva collection methods. In a well-powered study adhering to state-of-the-art sAA assessment methods, we addressed these limitations by testing whether continuous taVNS at the maximum intensity below the

pain threshold increases P3b magnitude and sAA. Despite the implementation of these methodological improvements, we found no differences between taVNS and sham stimulation on either noradrenergic marker.

The observed lack of an effect of taVNS on P3b converges with the inconsistent evidence for a modulation of phasic noradrenergic markers by taVNS. Specifically, three studies (Fischer et al., 2018; Gadeyne et al., 2022; Warren et al., 2019) out of six showed no effect of taVNS on P3b (Rufener et al., 2018; Ventura-Bort et al., 2018; Warren et al., 2020). Interestingly, all studies except for Fischer and colleagues (2018) measured P3b in the context of a similar oddball task. Also, there is inconsistent evidence for an effect of taVNS on evoked pupil dilation, an index of phasic noradrenergic activity. While three studies found no effect of taVNS on evoked pupil dilation in a task (Borges et al., 2021; Burger, Van der Does, et al., 2020; D'Agostini, Burger, Villca Ponce, et al., 2022; Keute et al., 2019), three other studies showed short bursts of taVNS to increase evoked pupil dilation in a resting state (D'Agostini, Burger, Franssen, et al., 2022; Sharon et al., 2020; Urbin et al., 2021).

The null results on taVNS and sAA secretion in this study are in line with the findings of the majority of previous human studies. On one hand, four studies showed taVNS to increase sAA in post hoc analyses (Ventura-Bort et al., 2018; 2021; Warren et al., 2019) or a sub-set of analyses (Giraudier, 2022). On the other hand, six studies found no evidence for an effect of taVNS on sAA (D'Agostini, Burger, Franssen, et al., 2023; D'Agostini, Burger, Villca Ponce, et al., 2022; D'Agostini et al., 2021; Giraudier et al., 2020; Höper et al., 2022; Koenig et al., 2019). Importantly, our zero-finding mirrors what was observed in another well-powered study using the same stimulation set-up and saliva collection method and very similar stimulation duration and experimental design (D'Agostini, Burger, Villca Ponce, et al., 2022). Evidence for an effect of taVNS on other markers of tonic noradrenergic activity is also rather inconclusive. No study reported an effect of taVNS on tonic pupil size measures (Borges et al., 2021; Burger, Van der Does, et al., 2020; Keute et al., 2019) and two studies out of three found no difference in cortisol between sham and taVNS conditions (D'Agostini, Burger, Villca Ponce, et al., 2022; D'Agostini et al., 2021; Warren et al., 2019).

The exploratory aim of this study was also to test whether taVNS modulates P3a magnitude, an indirect biomarker of dopaminergic activity, and flow rate, a marker of parasympathetic activity. The observed zero-finding on P3a converges with the results of two other studies showing no effect of taVNS on P3a (Ventura-Bort et al., 2018; Warren et al., 2019). Furthermore, in line with our finding on flow rate, two studies reported no significant difference in flow rate between sham and taVNS (D'Agostini, Burger, Villca Ponce, et al., 2022; Warren et al., 2019). On the contrary, one study showed taVNS to increase flow rate from pre-stimulation (D'Agostini, Burger, Franssen, et al., 2023). Evidence for an effect of taVNS on flow rate in D'Agostini et al. (2023) should however be treated cautiously given flow rate was significantly different between sham and taVNS at pre-stimulation.

Current and previous studies have reported inconsistent evidence for an effect of taVNS on physiological biomarkers of tonic and phasic noradrenergic activity. On the contrary, fMRI studies (N = 6) have consistently reported taVNS to increase LC activation (Frangos et al., 2014; Sclocco et al., 2019, 2020; Yakunina et al., 2016, 2018; Zhang et al., 2019). It is important to note that these fMRI studies largely differ for adopted stimulation parameters, tested population (healthy vs. patients), control condition (earlobe vs. no stimulation), and tested taVNS form (taVNS vs. respiratory gated taVNS). Furthermore, half of these studies have small sample sizes (within-subject design, N range: 12 – 26) (Frangos et al., 2014; Sclocco et al., 2019; Zhang et al., 2019), which limits the statistical power and, in turn, inflates type I error (Button et al., 2013). Therefore, caution is required when interpreting positive findings from fMRI studies on taVNS. Moreover, our results are in sharp contrast with the consistent finding that iVNS increase LC activity and pupil size in mice (e.g., Hulsey et al., 2017; Mridha et al., 2021). In summary, the overall evidence for a noradrenergic mechanism of taVNS in humans remains rather weak and calls into question to what extent animal research using iVNS can be translated to taVNS in humans.

An important point of discussion remains the selected stimulation parameters. Our results indicate that continuous taVNS at the maximum intensity below pain-threshold and a pulse width of 250 µs does not modulate noradrenergic markers. Previous studies reporting inconsistent results on

taVNS and noradrenergic biomarkers used the same pulse width, administered a long stimulation pattern (continuous or intermittent), and differed for the selected stimulation intensity (see Burger et al., 2020). Altogether, these results indicate that long taVNS with a pulse width of 250 µs does not increase noradrenergic biomarkers independently of the selected stimulation intensity (see Burger et al., 2020). Recent findings on iVNS (mice) and taVNS (humans) indicate that the interaction between the pulse width and intensity (i.e., charge per pulse) determines the change in noradrenergic activity during VNS. Specifically, two parametric studies in mice have shown that charge per pulse during iVNS increases LC activity (Hulsey et al., 2017) and evoked pupil dilation (Mridha et al., 2021) linearly up until a plateau is reached. Intriguingly, one parametric study on taVNS replicated such a finding in humans by showing that short bursts of taVNS increase evoked pupil dilation as a function of the charge per pulse (D'Agostini, Burger, Franssen, et al., 2023). These results are promising and invite further investigation of the taVNS parameters. Specifically, D'Agostini and colleagues (2023) administered a low pulse width similar to the one selected in this study (200 µs) and a higher one (400 µs). One possibility is that the current study employed a rather low pulse width to optimally stimulate the vagus nerve and see any effect on P3b and sAA. Future studies investigating the noradrenergic mechanism of taVNS should adopt higher pulse widths and systematically test the effect of charge per pulse on P3b and sAA.

Another related point of discussion is the administration of a continuous stimulation pattern. We selected a continuous stimulation pattern based on the expectation that intermittent taVNS leads to a transient decrease in noradrenergic activity due to the off periods, which could explain previous studies' mixed results. Nevertheless, we found that continuous taVNS does not reliably modulate P3b and SAA, which is in line with findings from some previous studies (e.g., D'Agostini, Burger, Villca Ponce, et al., 2022; Fischer et al., 2018). Inconclusive evidence from studies with a long stimulation pattern (continuous or intermittent) suggests that the effectiveness of taVNS does not depend on the On-Off time, meaning the ratio of time in which the stimulation is ON and is OFF. On the contrary, growing evidence indicates that short bursts of taVNS increase phasic noradrenergic activity as indexed

by evoked pupil dilation (D'Agostini, Burger, Franssen, et al., 2023; Sharon et al., 2020; Urbin et al., 2021). Whether short bursts of taVNS also modulate P3b is currently unknown. It is unclear why short rather than long taVNS would reliably modulate phasic noradrenergic activity. Authors have already proposed that long taVNS shifts tonic LC activity further away from the intermediate level to see any difference in phasic LC activity between sham and taVNS (Aston-Jones & Cohen, 2005; D'Agostini, Burger, Franssen, et al., 2022; Sharon et al., 2020). This interpretation, however, contrasts with consistent evidence that long taVNS does not reliably modulate tonic noradrenergic markers (Burger, D'Agostini, Verkuil, & Van Diest, 2020). Methodological differences between studies may also underlie the inconsistent results. While studies using long taVNS measured phasic noradrenergic markers (P3b and evoked pupil dilation) in the context of a task, those with positive findings on evoked pupil dilation administered short bursts of taVNS during a resting state. Cortical control of the LC is theorized to regulate tonic and phasic LC activity and, in turn, fine-tune behavioral performance during a task (Aston-Jones & Cohen, 2005). A possibility for the observed null results with longer taVNS is that the task boosts cortical control of the LC, increasing phasic responses in both sham and taVNS. As a result, the potential effects of taVNS on phasic noradrenergic activity may be overruled. This would imply that taVNS does not reliably modulate phasic noradrenergic activity during ongoing behavior/tasks. A systematic investigation of the potential interaction between the stimulation pattern and the presence of a task seems therefore warranted. While evoked pupil dilation can be measured in both a resting state and task, P3b can be measured only in the context of a task preventing any comparison between the effects of taVNS under the task and no task conditions. Future studies should systematically test the effect of short bursts of taVNS on evoked pupil dilation in a task and in a resting state.

An obvious limitation of the current study is that the experimenter was not blinded to the stimulation condition during data collection and that the first author was not blinded when performing the statistical analyses. In addition to this limitation, our study may have not been successful in blinding the participants, as participants reported having experienced different sensations during cymba concha stimulation (taVNS) compared to earlobe stimulation (sham). The blinding challenges of the

current study potentially extend to previous studies observing positive findings of taVNS on noradrenergic biomarkers. Therefore, future studies contrasting earlobe and auricular vagal nerve stimulation would greatly benefit from establishing and consistently applying double-blind procedures to overcome this potential problem. The fact that we conducted unblinded statistical analyses may also be a potential source of noise. Future studies on taVNS should thus adopt blinding procedures to prevent potential bias in the statistical analyses.

To conclude, this well-powered study showed that continuous taVNS at the maximum level below the pain threshold does not increase P3b and sAA in the context of an oddball task. Key questions regarding stimulation parameters and patterns (long vs. short taVNS) remain unanswered. To further develop the field, taVNS researchers may want to systematically manipulate the stimulation parameters and pattern to understand whether and how the stimulation setup modulates the effect of taVNS on noradrenergic activity.

Disclosure Statement

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