



ELSEVIER

Contents lists available at ScienceDirect

Brain Stimulation

journal homepage: <http://www.journals.elsevier.com/brain-stimulation>

The neuromodulatory and hormonal effects of transcutaneous vagus nerve stimulation as evidenced by salivary alpha amylase, salivary cortisol, pupil diameter, and the P3 event-related potential

C.M. Warren^{a, b, *, 1}, K.D. Tona^{a, b}, L. Ouwerkerk^a, J. van Paridon^{a, c}, F. Poletiek^{a, c},
Henk van Steenbergen^{a, b}, J.A. Bosch^{d, e}, S. Nieuwenhuis^{a, b}

^a Institute of Psychology, Leiden University, Leiden, 2333, AK, Netherlands

^b Leiden Institute for Brain and Cognition, Leiden University, Leiden, 2300, RC, Netherlands

^c Max Planck Institute of Psycholinguistics, Nijmegen, 6525, XD, Netherlands

^d Department of Clinical Psychology, University of Amsterdam, Amsterdam, 1018, XA, Netherlands

^e Mannheim Institute of Public Health, Heidelberg University, Mannheim, 68167, Germany

ARTICLE INFO

Article history:

Received 17 September 2018

Received in revised form

7 December 2018

Accepted 12 December 2018

Available online xxx

Keywords:

Transcutaneous vagus nerve stimulation

Norepinephrine

Alpha amylase

Cortisol

EEG

P3

ABSTRACT

Background: Transcutaneous vagus nerve stimulation (tVNS) is a new, non-invasive technique being investigated as an intervention for a variety of clinical disorders, including epilepsy and depression. It is thought to exert its therapeutic effect by increasing central norepinephrine (NE) activity, but the evidence supporting this notion is limited.

Objective: In order to test for an impact of tVNS on psychophysiological and hormonal indices of noradrenergic function, we applied tVNS in concert with assessment of salivary alpha amylase (SAA) and cortisol, pupil size, and electroencephalograph (EEG) recordings.

Methods: Across three experiments, we applied real and sham tVNS to 61 healthy participants while they performed a set of simple stimulus-discrimination tasks. Before and after the task, as well as during one break, participants provided saliva samples and had their pupil size recorded. EEG was recorded throughout the task. The target for tVNS was the cymba conchae, which is heavily innervated by the auricular branch of the vagus nerve. Sham stimulation was applied to the ear lobe.

Results: P3 amplitude was not affected by tVNS (Experiment 1A: N = 24; Experiment 1B: N = 20; Bayes factor supporting null model = 4.53), nor was pupil size (Experiment 2: N = 16; interaction of treatment and time: $p = .79$). However, tVNS increased SAA (Experiments 1A and 2: N = 25) and attenuated the decline of salivary cortisol compared to sham (Experiment 2: N = 17), as indicated by significant interactions involving treatment and time ($p = .023$ and $p = .040$, respectively).

Conclusion: These findings suggest that tVNS modulates hormonal indices but not psychophysiological indices of noradrenergic function.

© 2018 Elsevier Inc. All rights reserved.

Introduction

Invasive vagus nerve stimulation (VNS) is a somewhat promising treatment for depression [1–3] and epilepsy [4,5] that likely exerts part of its therapeutic effect by increasing norepinephrine (NE) release from the locus coeruleus (LC). The vagus nerve projects to

the nucleus tractus solitarius, which projects both directly and indirectly to the LC [1–3]. Transcutaneous VNS can be achieved by delivering electrical impulses to the cervical or the auricular branches of the vagus nerve, which are situated close to the surface of the skin of the neck and outer ear respectively [4]. Functional magnetic resonance imaging (fMRI) studies in healthy humans demonstrate that the more commonly applied transcutaneous auricular VNS (taVNS) elicits widespread changes in cortical and brainstem activity [5–8]. In light of the clinical potential of taVNS, it would be valuable to establish if taVNS, like invasive VNS, affects NE, using relatively inexpensive and easy-to-use biomarkers of NE. Here we evaluated the effect of taVNS on NE levels using three accepted

* Corresponding author. Institute of Psychology, Leiden University Leiden, 2333 AK, the Netherlands.

E-mail address: chris.warren@usu.edu (C.M. Warren).

¹ Present Address: Utah State University, Department of Psychology, Logan, UT 84322.

biomarkers and one putative biomarker of central NE activity: salivary alpha amylase (SAA), salivary cortisol, pupil size, and the P3 component of the event-related brain potential (ERP), respectively.

SAA is a digestive enzyme that is released by the saliva glands in response to local sympathetic nervous system activity [9]. SAA secretion is increased during stress and correlates with blood plasma NE during exercise [10,11]. SAA is a proxy marker of sympathetic-adreno-medullary activation [9,12], which is driven by central NE, leading to the assumption that SAA marks central NE activity [13–16]. One preliminary study [17] has reported suggestive evidence that taVNS increases SAA relative to sham stimulation—reason to be optimistic that a larger study with a more targeted methodology might reveal a robust effect of taVNS on SAA.

Salivary cortisol is a glucocorticoid stress hormone that correlates with hypothalamo-pituitary-adrenal axis activation [12,18]. Salivary cortisol may likewise be a reliable index of central NE activity, mediated in part by noradrenergic inputs to the hypothalamus [12,18,19]. Salivary cortisol is sensitive to pharmacologically induced changes in central NE activity [16,20].

Pupil size is correlated with activity of NE-releasing neurons in the LC [21–24]. This relationship may be mediated by activity in the rostral ventrolateral medulla, which projects to the LC and also innervates the peripheral sympathetic ganglia regulating the pupil [25]. Studies of primates and rodents show that LC activity correlates with baseline pupil diameter [21,24] and the magnitude of task-evoked pupil dilations [21,23]. In human participants, BOLD activity in the LC covaries with pupil size at rest and during simple decision-making tasks [22,26]. In rats, direct stimulation of the central stump of the vagus nerve provokes pupil dilation [27], but results in humans have been mixed [28,29].

Phasic changes in cortical NE levels are associated with the scalp-recorded P3 component [30–37]. Events that lead to increased phasic firing of the LC also lead to increased P3 amplitude [30]. Noradrenergic drugs influence P3 amplitude in both animals [38] and humans [39–41], and lesion of the LC eliminates the P3 in monkeys [42]. Of interest here, the amplitude of the P3 is increased by invasive VNS [29,31,35].

Although LC-NE activity is associated with changes in SAA, salivary cortisol, pupil size and the P3, these psychophysiological measures are not exclusively diagnostic of changes in LC-NE activity. For example, fluctuations in pupil size have been shown to track activity in a number of neuromodulatory brainstem centers, including the LC, the dopaminergic ventral tegmental area, and the cholinergic basal forebrain [24,26]. Also, P3 amplitude can be modulated by dopaminergic and cholinergic pharmacological manipulations, suggesting a role for those systems in P3 generation [30]. Thus, although our study is well-equipped to pick up converging evidence for taVNS effects on the noradrenergic system, it does not allow us to fully discriminate between noradrenergic and other neuromodulatory and hormonal effects of taVNS.

To explore the claim that taVNS increases central NE, we assayed SAA, salivary cortisol, pupil size and P3 amplitude across three experiments. In Experiments 1A and 2, we collected saliva samples and analyzed these samples all together. In Experiment 2, we also recorded pupil size. Experiment 1B was a partial replication of 1A, wherein we recorded EEG data during a classic oddball task, the seminal task for eliciting a P3 [43].

Experiment 1A

Method

Participants

Twenty-four students at Leiden University (6 male, mean age 22.6) participated in return for €30. We used the following

exclusion criteria: history of psychiatric or neurological disorders, head trauma, migraine, current use of psychoactive drugs, pregnancy, active implants (cochlear implant, pacemaker) and a permanent ear-piercing.

Procedure

The study was a participant-blind crossover design consisting of two sessions, separated by at least three days. In one session participants received taVNS, and in the other they received sham stimulation. Time of day, recent exercise, mental effort, and time since last meal are all factors that can affect SAA and salivary cortisol levels. Accordingly, participants performed the same tasks in each session at the same time of day, and participants were asked to refrain from excessive exercise, alcohol, caffeine and meals within 3 h prior to the examination. Task order and treatment order were counterbalanced across participants. Fig. 1A shows the timeline of all three experiments reported here.

The taVNS device was attached during EEG set-up, but not turned on until set-up was almost complete. Set-up was paused to obtain a baseline saliva sample before turning on the device. Participants did not begin the task until the device had been active for 20 min. A second saliva sample was taken halfway through the task, and a final sample taken after task completion. Participants did not report any notable adverse after-effects of taVNS.

Stimulation

We applied taVNS (NEMOS®, Cerbomed, Germany) with an intensity of 0.5 mA and a pulse width of 200–300 μ s at 25 Hz, alternating between on and off periods every 30 s [44–46]. In the taVNS condition, the electrodes were applied to the cymba conchae region, which is heavily innervated by the auricular branch of the vagus nerve [47,48] (Fig. 1B). In the sham condition, the electrodes were placed on the left ear lobe (Fig. 1C), which should not induce any significant brainstem or cortical activation [5,44–46].

Saliva sample collection

Saliva samples were collected at three points in time: 5 min before taVNS began (baseline, $t = -5$), at $t = 45$ and $t = 75$ min after taVNS began. Whole saliva was collected by instructing participants to let saliva collect passively in their mouth and spit the accumulated saliva into a polypropylene tube once per minute, over a 3-min period [49–51]. We calculated SAA and salivary cortisol secretion as the flow rate multiplied by the concentration values, as in our previous work [16] and as is considered the “gold standard” in the field [9,51].

Task

Participants performed “classic oddball” and “novelty oddball” tasks in both the visual and auditory modality, in which they had to respond with a key press on rare target oddball trials (12% of trials) interspersed among frequent standards/distracters (as well as rare novels in the novelty oddball task). In the classic oddball, there were 36 oddball trials and 264 standard trials. In the novelty oddball there were 36 oddball trials, 36 novel trials, and 228 standard trials. In both the visual and auditory modality, the stimulus was presented for 400 ms with an interstimulus interval of 1000 ms.

In the visual modality, targets and standards consisted of black Xs and Os on a white background. The novel stimuli were symbols derived from the Glagolitic and Cyrillic scripts (Fig. 1D). Participants were seated at a viewing distance of 70 cm, such that target and standard stimuli subtended 1.2° of visual angle, and novels subtended 1.7° of visual angle. A black-on-white fixation cross was presented during the interstimulus interval.

A																									
Time (min)	-30	-25	-20	-15	-10	-5	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90
Exp 1a	IL, IC, Qs			EEG Prep			SA1 Start Stim	Continue EEG Prep				Modality 1: Classic Oddball, Novelty Oddball					SA2	Modality 2: Classic Oddball, Novelty Oddball					SA3, Stop Stim	Qs, DB	
Exp 1b	IL, IC			EEG Prep			Start Stim	Continue EEG Prep				Visual Classic Oddball		Reading EEG Task										Stop Stim	DB
Exp 2	IL, IC, Qs			HR	Qs, PU1		SA1 Start Stim	Practice Task				Qs, PU2	Block 1: Task Switch		SA2	Block 2: Task Switch		PU3		SA3, Stop Stim	Qs, DB				

IL = Information Letter
 IC = Informed Consent
 HR = Heart Rate Measure
 DB = Debriefing
 SA (1, 2, 3) = Saliva Sample
 PU (1, 2, 3) = Pupil Size Measure
 Qs = Questionnaire data not reported [52-54]

B



C



D



Fig. 1. A) Depiction of the timing of each experiment. For questionnaires see [52–54]. B) Position of the taVNS device that gives targeted stimulation to auricular branch of the vagus nerve. C) Position of the taVNS device for “sham” stimulation that putatively does not impact activity in the vagus nerve. D) Examples of the visual novelty stimuli used in the novelty oddball P3 task in Experiment 1A.

In the auditory modality, the fixation cross remained on-screen constantly. The auditory stimuli were tones of high (500 Hz) or low pitch (350 Hz), presented at 70 dB through speakers. Target frequency was counterbalanced across participants. The novel stimuli consisted of unusual noises pulled from the set of Fabiani and Friedman [55].

EEG collection and processing

EEG was recorded from 64 channels (ActiveTwo system, Biosemi B.V., Netherlands) in the standard 10–20 configuration. Data were pre-amplified at the electrode site and recorded with a sampling rate of 512 Hz with reference to a common mode sense. Impedances were kept below 32 k Ω . EEG recordings were processed using Brain Vision Analyzer 2 (Brain Products GmbH, Germany). Data were re-referenced offline to the right mastoid, and band-pass filtered (0.1 Hz–20.0 Hz). Ocular artifacts were removed [56]. Epochs were extracted from the EEG from –200 ms to 800 ms relative to stimulus onset, using the first 200 ms for baseline correction. Trials in which the change in voltage at any channel exceeded 35 μ V per sampling point were removed as were trials with slow drifts (>300 μ V/200 ms) and low activity (<5 μ V/100 ms).

We created difference waves to simplify figures and analyses, to isolate the topography of the P3, and to distinguish the P3 to oddballs (“oddball P3”) and the P3 to novel stimuli (“novelty P3”). To isolate the oddball P3 we subtracted the standard ERP from the oddball ERP, and to isolate the novelty P3 we subtracted the standard ERP from the novelty ERP. In each case P3 amplitude was quantified as the most positive mean amplitude from a 200-ms sliding window across the entire difference wave.

The oddball P3 was analyzed using an ANOVA including the factors treatment (taVNS vs sham), modality (visual vs auditory), task (classic oddball vs novelty oddball) and electrode (Fz, Cz, Pz). The novelty P3 obtained in the novelty oddball task was analyzed

using an ANOVA including the factors treatment (taVNS vs sham), modality (visual vs auditory) and electrode (Fz, Cz, Pz). In addition, treatment order was added as a between-subjects variable-of-no-interest, to account for additional error variance.

Results

Oddball P3

The amplitude of the oddball P3 showed significant main effects of electrode, indicating a typical parietal distribution (Fig. 2), $F(2,44) = 60.82$, $p < .001$, task (classic oddball: 7.7 μ V; novelty oddball: 7.0 μ V; Fig. 3), $F(1,22) = 13.36$, $p = .001$, and modality (visual: 7.7 μ V; auditory: 6.4 μ V), $F(1,22) = 28.65$, $p < .001$. In addition, electrode interacted with task, $F(2,44) = 5.15$, $p = .010$, and exhibited a three-way interaction with task and modality, $F(2,44) = 3.56$, $p = .037$. Treatment did not significantly affect oddball P3 amplitude (taVNS: 7.1 μ V; sham: 7.0 μ V), $F(1,22) = 0.020$, $p = .89$. Treatment did not interact with electrode, task, or modality.

Novelty P3

As expected, the novelty P3 had a more frontal distribution than the oddball P3, with largest amplitude at electrode Cz, $F(2,44) = 14.05$, $p < .001$ (Fig. 2). The novelty P3 was larger in the auditory modality (10.5 μ V), than in the visual modality (2.8 μ V), $F(1,22) = 82.36$, $p < .001$ (Fig. 3). In addition, the effect of modality on novelty P3 amplitude was larger at Fz and Cz than at Pz, $F(2,44) = 34.17$, $p < .001$. Treatment did not significantly affect novelty P3 amplitude (taVNS: 6.4 μ V; sham: 6.8 μ V), $F(1,22) = 0.65$, $p = .43$.

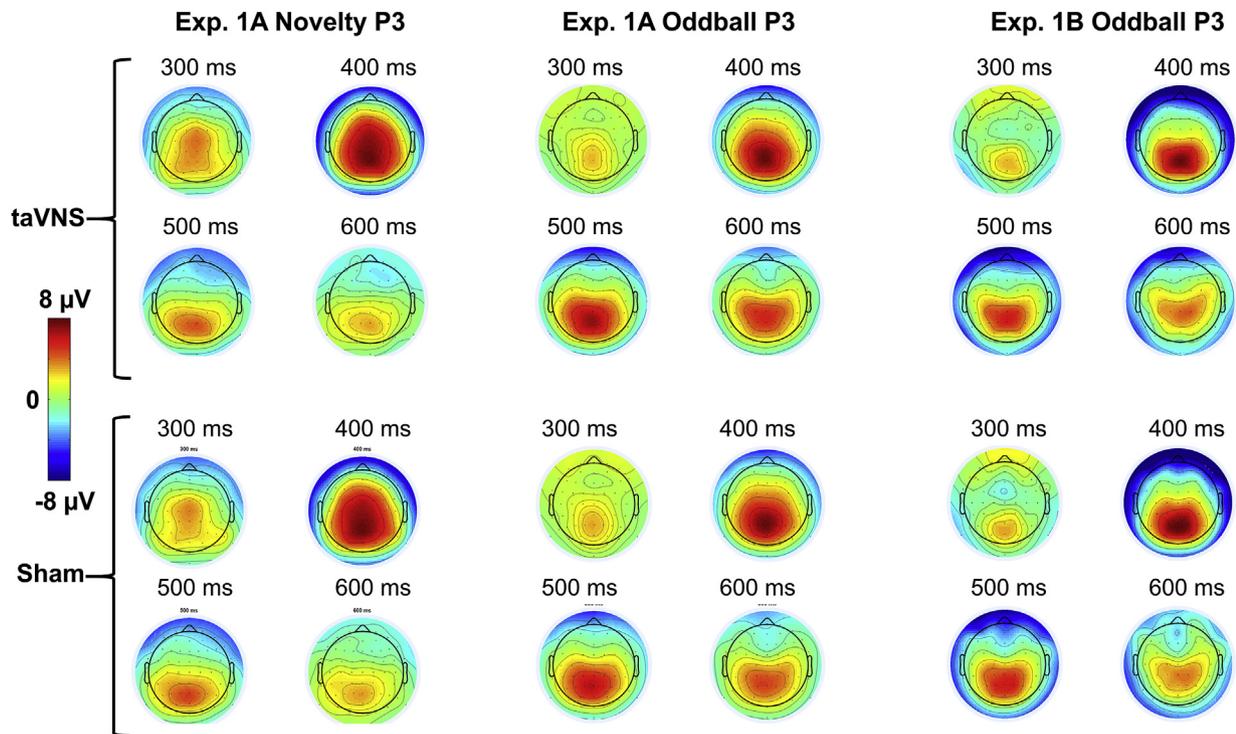


Fig. 2. Scalp distribution of the oddball P3 and novelty P3 for taVNS (top row) and sham stimulation (bottom row), for the novelty and classic oddball tasks in Exp. 1A, and the classic oddball task in Exp. 1B. For Exp. 1A, scalp distributions are collapsed across the visual and auditory modalities, and for the oddball P3 they are collapsed across tasks as well.

Experiment 1B

Our task in Experiment 1A yielded a typical oddball P3 and novelty P3, exhibiting characteristic effects of electrode, task and modality. There were no effects of treatment on the oddball P3 or novelty P3. Given that P3 amplitude was shown to be increased by

invasive VNS, we considered the possibility that our null effect of treatment on P3 amplitude was a type-2 error, and followed up with a simplified experiment on new participants, composed of a single classic oddball task with a greater number of trials, and with the exact same stimulation protocol.

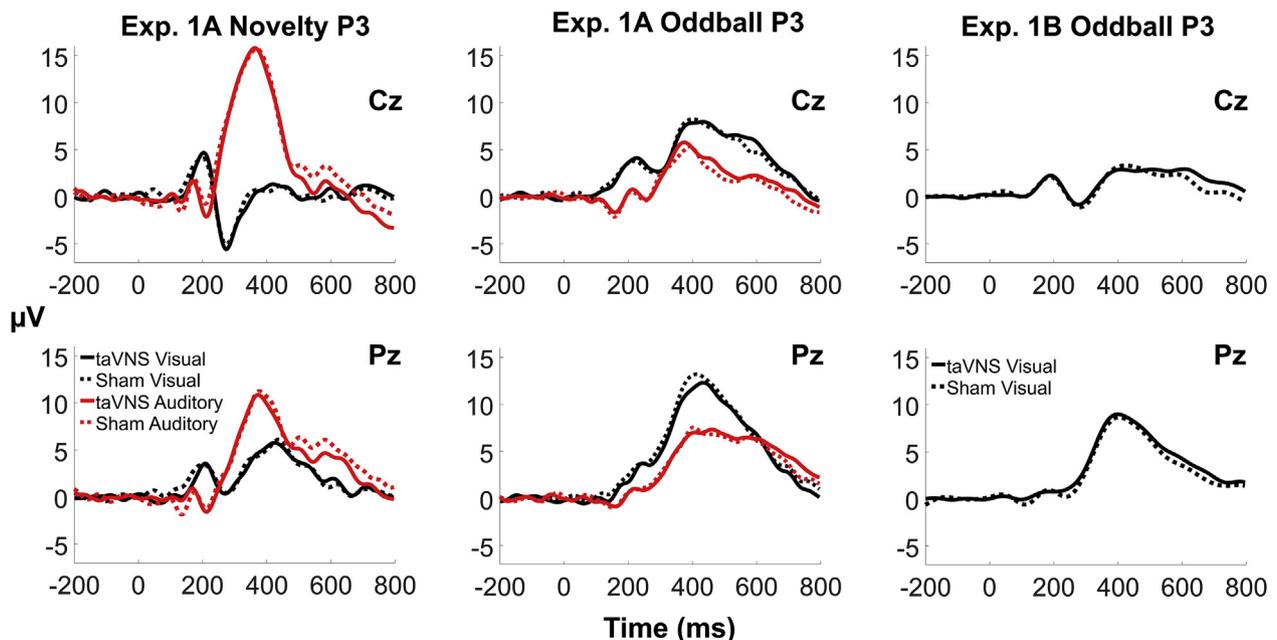


Fig. 3. ERP waveforms from Experiments 1A and 1B for electrodes Cz (top) and Pz (bottom) were not affected by taVNS. Exp. 1A included both a novelty oddball task and a classic oddball task, in both the visual and auditory modality. Experiment 1B included only a classic oddball task in the visual modality.

Method

Participants

Twenty Leiden University students (11 males, mean age 23.6) participated in return for €25. Exclusion criteria were the same as Experiment 1A.

Task and procedure

The task was an abbreviated version of the task used in Experiment 1A. Experiment 1B included only the visual version of the classic oddball paradigm. We increased the number of trials from 300 to 400 (352 standards, 48 targets). The materials (Xs and Os) were the same, as were all the timing parameters.

The exact same stimulation protocol was used as in Experiment 1A. The first 55 min of each session in Experiment 1B were identical to Experiment 1A, except that participants did not fill out any questionnaires, and participants did not provide saliva samples (Fig. 1A).

EEG collection and processing

EEG collection and processing methods as well as statistical analysis were identical as described for Experiment 1A, except as follows. Recordings for five participants had noisy signals at one electrode. For these participants the bad electrode was removed before ocular correction, and its signal interpolated from the remaining electrodes using a spline-based method.

Results

The oddball P3 in Experiment 1B exhibited a parietal distribution (Fig. 2), with largest amplitude at Pz, $F(2,34) = 32.54$, $p < .001$. The oddball P3 was larger in the taVNS condition (7.6 μV) than in the sham condition (6.5 μV). This difference was not significant, $F(1,17) = 2.32$, $p = .15$ (Fig. 3).

Bayesian analyses

In Experiment 1A taVNS did not affect the size of the oddball P3 or novelty P3. We ran Experiment 1B to perform a second examination of this potential effect, and found no effect ($p = .15$). The combined results suggest that taVNS does not affect P3 amplitude, but such a conclusion cannot be firm under the rules of null hypothesis statistical testing. In order to quantify evidence for the null hypothesis, we ran a Bayesian-evidence synthesis analysis [57] on the visual oddball P3 data from Experiments 1A and 1B.

Method

Data were analyzed using JASP (JASP Team, 2016), which yields Bayes factors that give the relative probability of competing models of the data. We ran a Bayesian repeated-measures ANOVA with treatment as the repeated factor, and two between-subjects factors: treatment order and experiment.

Results

Bayes factor comparisons favored the null model over a model including an effect of treatment ($BF_{01} = 4.53$). This quantity means no effect of treatment is 4.53 times more probable, given the data, than an effect of treatment, which constitutes “substantial evidence” for the null hypothesis [58]. The null model was also superior to a model including an interaction of treatment with experiment ($BF_{01} = 13.48$). This Bayes factor constitutes “strong evidence” in favor of the null model [58]. Taken together with the

frequentist methods reported above, the appropriate conclusion is that taVNS does not affect the (visual) oddball P3.

Experiment 2

Method

Participants

Seventeen young adults (all males, mean age 22.1) participated in Experiment 2. One participant gave saliva samples but did not have his pupil data recorded. Exclusion criteria were the same as for Experiment 1A.

Procedure

The timeline of Experiment 2 is displayed in Fig. 1A. Pupil data were recorded at three time points during each session. Saliva samples were collected with the exact same methodology and timing relative to taVNS as described in Experiment 1A. taVNS was applied with the exact same timing, positioning and parameters as in Experiments 1A and 1B. Participants performed a cued task-switching task [62] and other tests from which data will be reported elsewhere.

Pupil size recording and analysis

Participants sat in a dimly lit room with head held steady in a chin rest, and fixated on a luminance-controlled, salmon-colored fixation cross on a slate blue computer screen. Pupil diameter was recorded for 96 s at 60 Hz using a Tobii T120 eye tracker (Tobii Technology, Stockholm, Sweden). Recordings were made roughly 10 min before stimulation began ($t = -10$), between practice and critical task-switching trials ($t = 25$) and upon completion of the task ($t = 70$). Pupil data were analyzed using custom-made macros in BrainVision Analyzer 2.0 (Brain Products GmbH). Linear interpolation was applied to artifacts such as blinks and missing data.

Results

We analyzed mean pupil size across the 96-s epoch at each time point. Treatment did not significantly affect pupil size ($p = .37$), nor interact with time ($p = .79$, Greenhouse-Geisser corrected). There was an effect of time on pupil size whereby pupil size increased from baseline during task performance, $F(1,15) = 9.78$, $p = .007$ (Greenhouse-Geisser corrected) (Fig. 4).

Analysis of saliva data

Method

Participants

Experiments 1a and 2 were run simultaneously with identical taVNS protocol and identical saliva collection, processing and analysis protocol. Saliva samples were collected from twenty participants in Experiment 1a, and seventeen participants in Experiment 2. Twenty-five participants provided SAA samples with concentrations within the sensitivity range of our assay in all six cells of the design (3 samples x 2 treatment conditions). In the follow-up analyses of the effect of time in each treatment condition, thirty-three participants provided utilizable samples in all 3 cells of the taVNS condition, and twenty-seven participants provided utilizable samples in all 3 cells of the sham condition. Salivary cortisol was determined only for the saliva samples from Experiment 2. All seventeen participants provided sufficient samples to include cortisol secretion data in every cell of the design.

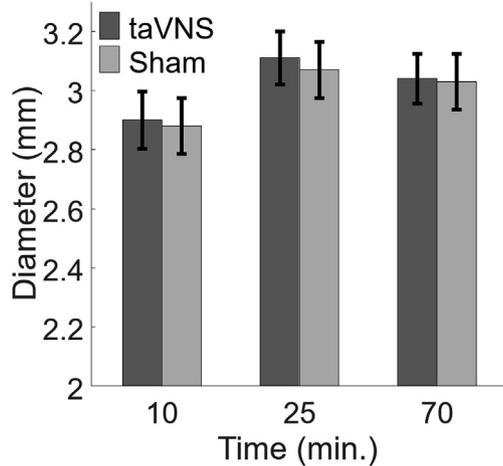


Fig. 4. Pupil size increased over time but was not affected by taVNS. Error bars reflect SEM.

Saliva sample processing (experiments 1A and 2)

Fresh saliva samples were stored in ice for a maximum of 2.5 h. Before freezing, samples were centrifuged at room temperature for 4 min at $4000\times g$ to force debris and bacteria to the bottom of the tube. The clear supernatant was pipetted into a smaller polypropylene tube and frozen at -60°C until the assay procedure.

Hormonal analyses

SAA was assayed using a quantitative kinetic determination kit (IBL, Hamburg, Germany) [50]. The assay has a sensitivity of 12.5 U/ml. The CV% was 2.5. Each participant's samples were assayed for SAA at the same time. 25/222 samples gave values outside the sensitivity range of the SAA assay on two successive attempts. These samples were considered outliers and not assayed a third time.

Cortisol was assayed using a competitive enzyme-linked immunosorbent assay, according to the manufacturer's instructions (IBL, Hamburg, Germany), with a sensitivity of 0.045 $\mu\text{g}/\text{mL}$, and intra-assay variability (CV%) was 2.4. All samples of the same participant were assayed for cortisol simultaneously. Treatment order and experiment (1a vs. 2) were included as between-subjects variables. Here we report statistical terms involving treatment order because it allows us to deconstruct the omnibus ANOVA in a way that demonstrates a potentially important effect of treatment on this measure. Note that the direction of the main

effect of time (Figs. 4, 5a and 5b) may reflect the influence of treatment, activities the participants carried out during each session (see Fig. 1A), mental fatigue and other factors.

Results

Saliva alpha amylase secretion

An analysis of the pooled data from Experiments 1A and 2 indicated that taVNS affected SAA, exhibited as an interaction of treatment with treatment order and time, $F(2,42) = 4.1, p = .023$. Other main effects or interactions, including the interaction of treatment with time, $F(2,42) = 0.3, p = .73$, were not significant in the omnibus ANOVA. We decomposed the significant three-way interaction by running mixed ANOVAs (including treatment order and time) for the taVNS and sham conditions separately. This analysis indicated that SAA increased from baseline to later samples in the taVNS condition, $F(2,58) = 7.1, p = .002$, but not in the sham condition, $F(2,46) = 1.2, p = .31$ (Fig. 5A).

Salivary cortisol secretion

Treatment affected salivary cortisol secretion, exhibited as an interaction of treatment with time, $F(2,30) = 3.6, p = .040$. There were no other significant effects in the omnibus ANOVA. Separate ANOVAs for each treatment revealed that salivary cortisol did not change from baseline in the taVNS condition, $F(2,30) = 0.5, p = .63$, but significantly decreased in the sham condition, $F(2,30) = 4.5, p = .020$, (Fig. 5B). This suggests that taVNS worked against a general tendency for salivary cortisol secretion to decrease over the course of a session.

Salivary flow rate

As with the SAA analysis, we pooled flow rate data from Experiments 1a and 2 for a single analysis. Participants tended to provide more saliva as they became practiced and comfortable with the collection method, both within sessions ($M_{T1} = 0.35, M_{T2} = 0.39, M_{T3} = 0.40$), $F(2,68) = 6.5, p = .003$, and between sessions ($M_{S1} = 0.36, M_{S2} = 0.40$), $F(1,34) = 4.5, p = .042$. There was no significant effect of treatment on flow rate ($p = .46$).

General discussion

We examined the neuromodulatory and hormonal effects of taVNS, analyzing several putative markers of central NE activity. Relative to baseline, taVNS increased SAA and attenuated a decrease in salivary cortisol that was observed with sham stimulation. We also found that baseline pupil size was not affected by

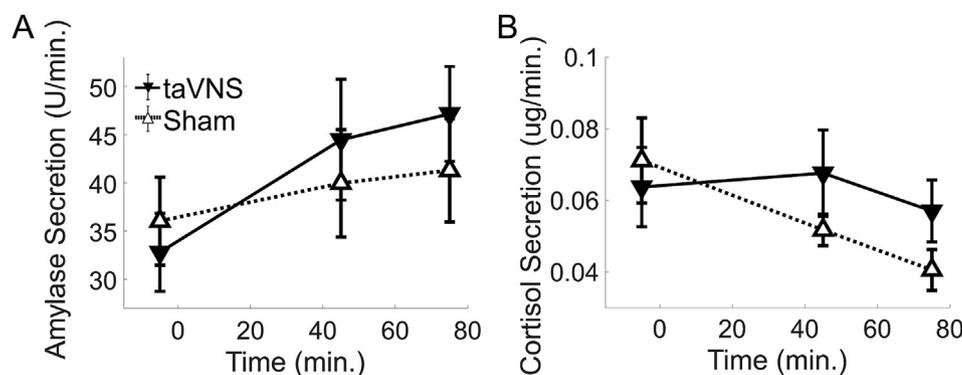


Fig. 5. Effects of treatment on SAA and cortisol. SAA increased from baseline in the taVNS condition ($p = .002$) but not the sham condition ($p = .31$). Cortisol dropped from baseline in the sham condition ($p = .020$) but not in the taVNS condition ($p = .63$). Error bars reflect SEM. Means and SEM for the SAA data points come from variable sample sizes due to missing data points (taVNS: $n = 34, 36, 34$; sham: $n = 34, 30, 29$).

taVNS, and that taVNS did not affect P3 amplitude. Thus, two of our four physiological markers responded sensitively to taVNS, consistent with increases in central NE.

Our results compliment work by Ventura-Bort and colleagues [17], who reported preliminary evidence that taVNS increases SAA. We demonstrate a more robust effect, using a larger sample size (25 vs. 18 participants), more post-stimulation saliva samples (2 vs. 1), and a superior method of saliva collection (whole saliva method vs. absorbent cotton sponges) [9,51]. In addition, we report the first evidence that taVNS influences salivary cortisol. Together, these hormonal analyses add to an accumulating pharmacological literature suggesting that SAA and salivary cortisol might be effective markers of central NE activity [13–16,20].

The relationship between pupil size and NE activity has been supported by direct recordings from the LC [21,23], direct stimulation of the LC [24], and by fMRI data from human participants [22,26]. We found no effect of taVNS on pupil size, suggesting that taVNS might not increase NE. An alternative possibility is that our pupil experiment was underpowered in terms of sample size or recording duration, or otherwise not sensitive enough to the taVNS manipulation. Our null result resonates with Schevernels and colleagues [29], who found no effect on pupil size of invasive VNS. Although two other invasive VNS studies found significant pupil effects, we know of no other taVNS study that has measured pupil data. Thus, our work serves as a first exploration that should be revisited with methods adjusted accordingly.

The LC-P3 hypothesis proposes that the P3 reflects the change in neural gain produced by a phasic burst of NE release [30]. Only one research group has reported an effect of taVNS on P3 amplitude [17,59]. In one study [59] these researchers analyzed data from a Simon task and report that taVNS increased both conflict adaptation and N2 amplitude on incompatible trials, but not P3 amplitude. In a separate study they found taVNS increased P3 amplitude [17]. This study was exploratory, reporting significant simple effects in specific cells of their design, without justification from interactions in the omnibus ANOVA. In light of their other null effect, and our Bayesian evidence in favor of no effect, we must acknowledge that the evidence that the P3 is directly affected by invasive or transcutaneous VNS is mixed at best.

We note some limitations to this work. Saliva data was pooled across two experiments. In Experiment 1a participants were being set up with EEG between the baseline sample and subsequent samples, whereas in Experiment 2 participants were practicing the task-switching experiment. The stimulation protocols and collection methods were identical, and no statistical tests were interpreted between experiments, but the difference likely introduced some variability to the data. This could have contributed to the relatively weak statistical support for the effect of taVNS on SAA. That is, the two-way interaction of treatment with time was not significant so we relied on the three-way interaction of treatment with time and treatment order to justify decomposing the omnibus ANOVA. The salivary cortisol results gave more straightforward evidence of an effect of taVNS, but the sample size was smallish ($n = 17$), though still within an appropriate range of sample sizes for investigating phasic changes in salivary cortisol (for a review see Ref. [51]). An additional limitation of this work concerns the general parameters and targets used for taVNS. We used a stimulation intensity of 0.5 mA for all participants. In contrast, some studies titrate stimulus intensity to the participant's perceptual threshold (e.g. Ref. [60] titrated to an intensity of 3.14 mA). In addition, if stimulation in the sham condition was painful, it could have increased central NE, creating a ceiling effect that minimized our ability to detect a further increase due to taVNS. Our stimulation intensity was low, but pre-treating with taVNS or sham first and then running the oddball task with no simultaneous stimulation

could address this concern. Finally, there is an unresolved question as to whether the cymba conchae is the best target for taVNS, though both sites yield significant changes in cortical activity [47,61,62]. It is possible that some of our null effects were due to our use of a weak current, a ceiling effect, or a sub-optimal target. Nevertheless, our entire protocol was based on previous work [44–46] and use of this protocol lead to significant effects on salivary markers of NE activity. Keeping the discussed null effects and limitations in mind, our results provide support for the clinical and experimental use of taVNS.

Declarations of interest

None.

Funding

This work was supported by a Starting Grant of the European Research Council (#283314, 2011).

References

- [1] Vonck K, Raedt R, Naulaerts J, De Vogelaere F, Thiery E, Van Roost D, et al. Vagus nerve stimulation. 25 years later! what do we know about the effects on cognition? *Neurosci Biobehav Rev* 2014;45:63–71. <https://doi.org/10.1016/j.neubiorev.2014.05.005>.
- [2] Nemeroff CB, Mayberg HS, Krahl SE, McNamara J, Frazer A, Henry TR, et al. VNS therapy in treatment-resistant depression: clinical evidence and putative neurobiological mechanisms. *Neuropsychopharmacology* 2006;31:1345–55. <https://doi.org/10.1038/sj.npp.1301082>.
- [3] George MS, Aston-Jones G. Noninvasive techniques for probing neurocircuitry and treating illness: vagus nerve stimulation (VNS), transcranial magnetic stimulation (TMS) and transcranial direct current stimulation (tDCS). *Neuropsychopharmacology* 2010;35:301–16. <https://doi.org/10.1038/npp.2009.87>.
- [4] Ellrich J. Transcutaneous vagus nerve stimulation. *Eur Neurol Rev* 2011;6:254–6. <https://doi.org/10.17925/ENR.2011.06.04.254>.
- [5] Kraus T, Kiess O, Hösl K, Terekhin P, Kornhuber J, Forster C. CNS BOLD fMRI effects of sham-controlled transcutaneous electrical nerve stimulation in the left outer auditory canal - a pilot study. *Brain Stimul* 2013;6:798–804. <https://doi.org/10.1016/j.brs.2013.01.011>.
- [6] Kraus T, Hösl K, Kiess O, Schanze A, Kornhuber J, Forster C. BOLD fMRI deactivation of limbic and temporal brain structures and mood enhancing effect by transcutaneous vagus nerve stimulation. *J Neural Transm* 2007;114:1485–93. <https://doi.org/10.1007/s00702-007-0755-z>.
- [7] Frangos E, Ellrich J, Komisaruk BR. Non-invasive access to the vagus nerve central projections via electrical stimulation of the external ear: fMRI evidence in humans. *Brain Stimul* 2015;8:624–36. <https://doi.org/10.1016/j.brs.2014.11.018>.
- [8] Yakunina N, Kim SS, Nam E-C. Optimization of transcutaneous vagus nerve stimulation using functional MRI. *Neuromodulation Technol Neural Interface* 2017;20:290–300. <https://doi.org/10.1111/ner.12541>.
- [9] Bosch JA, Veerman ECI, de Geus EJ, Proctor GB. A-amylase as a reliable and convenient measure of sympathetic activity: don't start salivating just yet! *Psychoneuroendocrinology* 2011;36:449–53. <https://doi.org/10.1016/j.psyneuen.2010.12.019>.
- [10] Chatterton RT, Vogelsong KM, Lu Y, Ellman AB, Hudgens GA. Salivary α -amylase as a measure of endogenous adrenergic activity. *Clin Physiol* 1996;16:433–48. <https://doi.org/10.1111/j.1475-097X.1996.tb00731.x>.
- [11] Bosch JA, Brand HS, Ligtenberg TJM, Bermond B, Hoogstraten J, Amerongen AVN. Psychological stress as a determinant of protein levels and salivary-induced aggregation of *Streptococcus gordonii* in human whole saliva. *Psychosom Med* 1996;58.
- [12] Bosch JA, de Geus EJC, Carroll D, Goedhart AD, Anane LA, van Zanten JJV, et al. A general enhancement of autonomic and cortisol responses during social evaluative threat. *Psychosom Med* 2009;71:877–85. <https://doi.org/10.1097/PSY.0b013e3181baef05>.
- [13] Speirs RL, Herring J, Cooper WD, Hardy CC, Hind CRK. The influence of sympathetic activity and isoprenaline on the secretion of amylase from the human parotid gland. *Arch Oral Biol* 1974;19:747–52. [https://doi.org/10.1016/0003-9969\(74\)90161-7](https://doi.org/10.1016/0003-9969(74)90161-7).
- [14] Ehlert U, Erni K, Hebisch G, Nater U. Salivary α -amylase levels after yohimbine challenge in healthy men. *J Clin Endocrinol Metab* 2006;91:5130–3. <https://doi.org/10.1210/jc.2006-0461>.
- [15] Van Stegeren A, Rohleder N, Everaerd W, Wolf OT. Salivary alpha amylase as marker for adrenergic activity during stress: effect of betablockade. *Psychoneuroendocrinology* 2006;31:137–41. <https://doi.org/10.1016/j.psyneuen.2005.05.012>.

- [16] Warren CM, van den Brink RL, Nieuwenhuis S, Bosch JA. Norepinephrine transporter blocker atomoxetine increases salivary alpha amylase. *Psychoneuroendocrinology* 2017;78. <https://doi.org/10.1016/j.psyneuen.2017.01.029>.
- [17] Ventura-Bort C, Wirkner J, Genheimer H, Wendt J, Hamm AO, Weymar M. Effects of transcutaneous vagus nerve stimulation (tVNS) on the P300 and alpha-amylase level: a pilot study. *Front Hum Neurosci* 2018;12:1–12. <https://doi.org/10.3389/fnhum.2018.00202>.
- [18] Hill SA, Taylor MJ, Harmer CJ, Cowen PJ. Acute reboxetine administration increases plasma and salivary cortisol. *J Psychopharmacol* 2003;17:273–5. <https://doi.org/10.1177/02698811030173008>.
- [19] Dunn AJ, Swiergiel AH, Palamarchouk V. Brain circuits involved in corticotropin-releasing factor-norepinephrine interactions during stress. *Ann N Y Acad Sci* 2004;1018:25–34. <https://doi.org/10.1196/annals.1296.003>.
- [20] Chamberlain SR, Müller U, Cleary S, Robbins TW, Sahakian BJ. Atomoxetine increases salivary cortisol in healthy volunteers. *J Psychopharmacol* 2007;21:545–9. <https://doi.org/10.1177/0269881106075274>.
- [21] Joshi S, Li Y, Kalwani RM, Gold JJ. Relationships between pupil diameter and neuronal activity in the locus coeruleus, colliculi, and cingulate cortex. *Neuron* 2016;89:221–34. <https://doi.org/10.1016/j.neuron.2015.11.028>.
- [22] Murphy PR, O'Connell RG, O'Sullivan M, Robertson IH, Balsters JH. Pupil diameter covaries with BOLD activity in human locus coeruleus. *Hum Brain Mapp* 2014;35:4140–54. <https://doi.org/10.1002/hbm.22466>.
- [23] Varazzani C, San-Galli A, Gilardeau S, Bouret S. Noradrenaline and dopamine neurons in the reward/effort trade-off: a direct electrophysiological comparison in behaving monkeys. *J Neurosci* 2015;35:7866–77. <https://doi.org/10.1523/JNEUROSCI.0454-15.2015>.
- [24] Reimer J, McGinley MJ, Liu Y, Rodenkirch C, Wang Q, McCormick DA, et al. Pupil fluctuations track rapid changes in adrenergic and cholinergic activity in cortex. *Nat Commun* 2016;7:1–7. <https://doi.org/10.1038/ncomms13289>.
- [25] Nieuwenhuis S, De Geus EJ, Aston-Jones G. The anatomical and functional relationship between the P3 and autonomic components of the orienting response. *Psychophysiology* 2011;48:162–75. <https://doi.org/10.1111/j.1469-8986.2010.01057.x>.
- [26] de Gee JW, Colizoli O, Kloosterman NA, Knäpen T, Nieuwenhuis S, Donner TH. Dynamic modulation of decision biases by brainstem arousal systems. *Life* 2017;6:1–36. <https://doi.org/10.7554/eLife.23232>.
- [27] Bianca R, Komisaruk BR. Pupil dilatation in response to vagal afferent electrical stimulation is mediated by inhibition of parasympathetic outflow in the rat. *Brain Res* 2007;1177:29–36. <https://doi.org/10.1016/j.brainres.2007.06.104>.
- [28] Desbeaumes Jodoin V, Lespérance P, Nguyen DK, Fournier-Gosselin MP, Richer F. Effects of vagus nerve stimulation on pupillary function. *Int J Psychophysiol* 2015;98:455–9. <https://doi.org/10.1016/j.ijpsycho.2015.10.001>.
- [29] Schevernels H, van Bochove ME, De Taeye L, Bombeke K, Vonck K, Van Roost D, et al. The effect of vagus nerve stimulation on response inhibition. *Epilepsy Behav* 2016;64:171–9. <https://doi.org/10.1016/j.yebeh.2016.09.014>.
- [30] Nieuwenhuis S, Aston-Jones G, Cohen JD. Decision making, the P3, and the locus coeruleus–norepinephrine system. *Psychol Bull* 2005;131:510.
- [31] Neuhaus AH, Luborzewski A, Rentzsch J, Brakemeier EL, Oppen-Rhein C, Gallinat J, et al. P300 is enhanced in responders to vagus nerve stimulation for treatment of major depressive disorder. *J Affect Disord* 2007;100:123–8. <https://doi.org/10.1016/j.jad.2006.10.005>.
- [32] Murphy PR, Robertson IH, Balsters JH, O'Connell RG. Pupilometry and P3 index the locus coeruleus–noradrenergic arousal function in humans. *Psychophysiology* 2011;48:1532–43.
- [33] Warren CM, Tanaka JW, Holroyd CB. What can topology changes in the oddball N2 reveal about underlying processes? *Neuroreport* 2011;22. <https://doi.org/10.1097/WNR.0b013e32834bbe1f>.
- [34] Warren CM, Holroyd CB. The impact of deliberative strategy dissociates ERP components related to conflict processing vs. Reinforcement learning. *Front Neurosci* 2012. <https://doi.org/10.3389/fnins.2012.00043>.
- [35] De Taeye L, Vonck K, van Bochove M, Boon P, Van Roost D, Mollet L, et al. The P3 event-related potential is a biomarker for the efficacy of vagus nerve stimulation in patients with epilepsy. *Neurotherapeutics* 2014;11:612–22. <https://doi.org/10.1007/s13311-014-0272-3>.
- [36] Wolff N, Mückschel M, Ziemssen T, Beste C. The role of phasic norepinephrine modulations during task switching: evidence for specific effects in parietal areas. *Brain Struct Funct* 2018;223:925–40.
- [37] Chmielewski WX, Mückschel M, Ziemssen T, Beste C. The norepinephrine system affects specific neurophysiological subprocesses in the modulation of inhibitory control by working memory demands. *Hum Brain Mapp* 2017;38:68–81.
- [38] Swick D, Pineda JA, Foote SL. Effects of systemic clonidine on auditory event-related potentials in squirrel monkeys. *Brain Res Bull* 1994;33:79–86.
- [39] Brown SBRE, Slatger HA, van Noorden MS, Giltay EJ, van der Wee NJA, Nieuwenhuis S. Effects of clonidine and scopolamine on multiple target detection in rapid serial visual presentation. *Psychopharmacology (Berlin)* 2016;233:341–50.
- [40] Brown SBRE, Van der Wee NJA, Van Noorden MS, Giltay EJ, Nieuwenhuis S. Noradrenergic and cholinergic modulation of late ERP responses to deviant stimuli. *Psychophysiology* 2015;52:1620–31.
- [41] De Rover M, Brown SBRE, Band GP, Giltay EJ, Van Noorden MS, Van Der Wee NJA, et al. Beta receptor-mediated modulation of the oddball P3 but not error-related ERP components in humans. *Psychopharmacology (Berlin)* 2015;232:3161–72. <https://doi.org/10.1007/s00213-015-3966-2>.
- [42] Pineda JA, Foote SL, Neville HJ. The effects of locus coeruleus lesions on a squirrel monkey late positive component: a preliminary study. *Electroencephalogr Clin Neurophysiol Suppl* 1987;40:481–486.
- [43] Sutton S, Braren M, Zubin J, John ER. Evoked-potential correlates of stimulus uncertainty. *Science* 1965;150:1187–8. 80-.
- [44] Beste C, Steenbergen L, Sellaro R, Grigoriadou S, Zhang R, Chmielewski W, et al. Effects of concomitant stimulation of the GABAergic and norepinephrine system on inhibitory control – a study using transcutaneous vagus nerve stimulation. *Brain Stimul* 2016;9:811–8. <https://doi.org/10.1016/j.brs.2016.07.004>.
- [45] Steenbergen L, Sellaro R, Stock AK, Verkuil B, Beste C, Colzato LS. Transcutaneous vagus nerve stimulation (tVNS) enhances response selection during action cascading processes. *Eur Neuropsychopharmacol* 2015;25:773–8. <https://doi.org/10.1016/j.euroneuro.2015.03.015>.
- [46] Sellaro R, van Leusden JWR, Tona K-D, Verkuil B, Nieuwenhuis S, Colzato LS. Transcutaneous vagus nerve stimulation enhances post-error slowing. *J Cognit Neurosci* 2015;27:2126–32. https://doi.org/10.1162/jocn_a.00851.
- [47] Badran BW, Brown JC, Dowdle LT, Mithoefer OJ, LaBate NT, Coatsworth J, et al. Tragus or cymba conchae? Investigating the anatomical foundation of transcutaneous auricular vagus nerve stimulation (taVNS). *Brain Stimul* 2018;11:947–8. <https://doi.org/10.1016/j.brs.2018.06.003>.
- [48] Peuker ET, Filler TJ. The nerve supply of the human auricle. *Clin Anat* 2002;15:35–7. <https://doi.org/10.1002/ca.1089>.
- [49] Beltzer EK, Fortunato CK, Guaderrama MM, Peckins MK, Garramone BM, Granger DA. Salivary flow and alpha-amylase: collection technique, duration, and oral fluid type. *Physiol Behav* 2010;101:289–96. <https://doi.org/10.1016/j.physbeh.2010.05.016>.
- [50] Nagy T, van Lien R, Willemsen G, Proctor G, Efting M, Fülöp M, et al. A fluid response: alpha-amylase reactions to acute laboratory stress are related to sample timing and saliva flow rate. *Biol Psychol* 2015;109:111–9. <https://doi.org/10.1016/j.biopsycho.2015.04.012>.
- [51] Strahler J, Skoluda N, Kappert MB, Nater UM. Simultaneous measurement of salivary cortisol and alpha-amylase: application and recommendations. *Neurosci Biobehav Rev* 2017;83:657–77. <https://doi.org/10.1016/j.neubiorev.2017.08.015>.
- [52] Spielberger CD, Gorsuch RL, Lushene RE. *State-trait anxiety inventory*. Palo Alto, 1970.
- [53] Russell JA, Weiss A, Mendelsohn GA. Affect grid: a single-item scale of pleasure and arousal. *J Pers Soc Psychol* 1989;57:493–502. <https://doi.org/10.1037/0022-3514.57.3.493>.
- [54] Benedict RHB, Schretlen D, Groninger L, Brandt J. Hopkins verbal learning Test? Revised: normative data and analysis of inter-form and test-retest reliability. *Clin Neuropsychol* 1998;12:43–55. <https://doi.org/10.1076/clin.12.1.43.1726>.
- [55] Fabiana M, Friedman D. Changes in brain activity patterns in aging: the novelty oddball. *Psychophysiology* 1995;32:579–94. <https://doi.org/10.1111/j.1469-8986.1995.tb01234.x>.
- [56] Gratton C, Coles MGH, Donchin E. A new method for off-line removal of ocular artifact. *Electroencephalogr Clin Neurophysiol* 1983;55:468–84.
- [57] Scheibehenne B, Jamil T, Wagenmakers EJ. Bayesian evidence synthesis can reconcile seemingly inconsistent results: the case of hotel tower rescue. *Psychol Sci* 2016;27:1043–6. <https://doi.org/10.1177/0956797616644081>.
- [58] Jeffreys H. *Theory of Probability* 1961. <https://doi.org/10.1038/163464a0>.
- [59] Fischer R, Ventura-Bort C, Hamm A, Weymar M. Transcutaneous vagus nerve stimulation (tVNS) enhances conflict-triggered adjustment of cognitive control. *Cognit Affect Behav Neurosci* 2018;1–14. <https://doi.org/10.3758/s13415-018-0596-2>.
- [60] Badran BW, Dowdle LT, Mithoefer OJ, LaBate NT, Coatsworth J, Brown JC, et al. Neurophysiologic effects of transcutaneous auricular vagus nerve stimulation (taVNS) via electrical stimulation of the tragus: a concurrent taVNS/fMRI study and review. *Brain Stimul* 2018;11:492–500. <https://doi.org/10.1016/j.brs.2017.12.009>.
- [61] Fallgatter AJ, Neuhauser B, Herrmann MJ, Ehlis A-C, Wägenar A, Scheuerpflug P, et al. Far field potentials from the brain stem after transcutaneous vagus nerve stimulation. *J Neural Transm* 2003;110:1437–43. <https://doi.org/10.1007/s00702-003-0087-6>.
- [62] Burger AM, Verkuil B. Transcutaneous vagus nerve stimulation via the tragus: are we really stimulating the vagus nerve? *Brain Stimul* 2018;11:945–6. <https://doi.org/10.1016/j.brs.2018.03.018>.