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

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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=0.79). 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.

Keywords: transcutaneous vagus nerve stimulation, norepinephrine, alpha amylase, cortisol, EEG, P3

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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 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 3h prior to the examination. Task order and treatment order were counterbalanced across participants. Figure 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 minutes. 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 μs 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: five minutes before taVNS began (baseline, t=-5), at t=45 and t=75 minutes 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 three-minute 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].

Figure 1. A) Depiction of the timing of each experiment. 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.

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 *X*s and *O*s 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.

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 kΩ. 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 35μV per sampling point were removed as were trials with slow drifts (>300 μV/200 ms) and low activity (<.5μV/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 μV; novelty oddball: 7.0 μV; **Fig. 3**), $F(1,22)=13.36$, $p=.001$, and modality (visual: 7.7 μV; auditory: 6.4 μV), $F(1,22)=28.65$, $p<.001$. 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 μV; sham: 7.0 μV), $F(1,22)=.020$, $p=.89$. Treatment did not interact with electrode, task, or modality.

Figure 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.

Figure 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.

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 μV), than in the visual modality (2.8 μV), $F(1,22)=82.36$, $p<.001$ (**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 μV; sham: 6.8 μV), $F(1,22)=0.65$, $p=.43$.

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.

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 (*X*s and *O*s) were the same, as were all the timing parameters.

The exact same stimulation protocol was used as in Experiment 1A. The first 55 minutes 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 Figure 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 seconds at 60 Hz using a Tobii T120 eye tracker (Tobii Technology, Stockholm, Sweden). Recordings were made roughly 10 minutes 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-second epoch at each time point. Treatment did not significantly affect pupil size (p=0.37), nor interact with time (p=0.79, 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**).

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

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.

Saliva sample processing (Experiments 1A and 2).

Fresh saliva samples were stored in ice for a maximum of 2.5 hours. Before freezing, samples were centrifuged at room temperature for 4 min at 4000 x *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 µg/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 (Fig. 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**).

Figure 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).

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}=.35, M_{t2}=.39, M_{t3}=.40), $F(2,68)=6.5$, p=.003, and between sessions (M_{s1}=.36, M_{s2}=.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 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 [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. [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.

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Figure1

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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

Figure2
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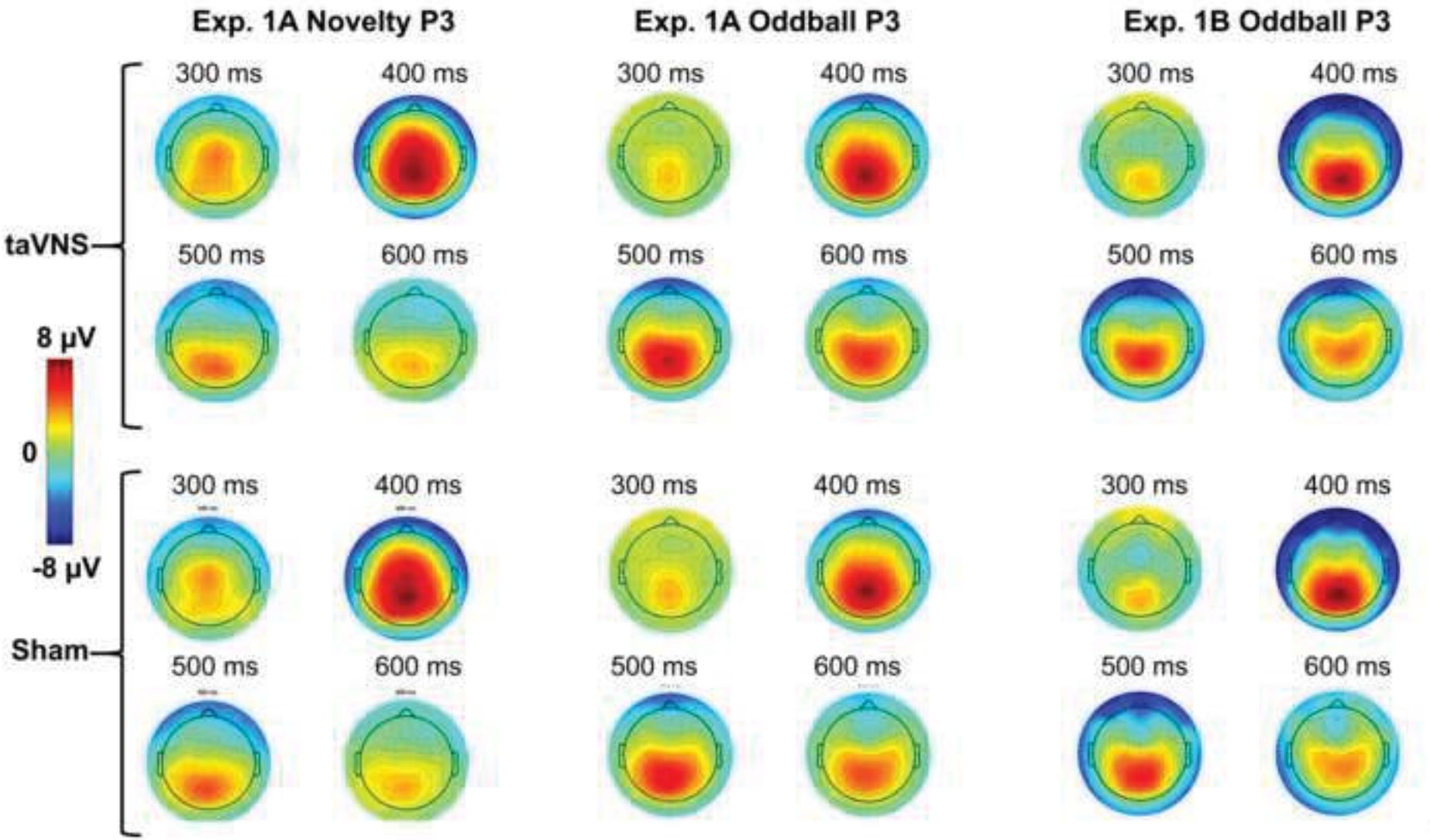

Figure3
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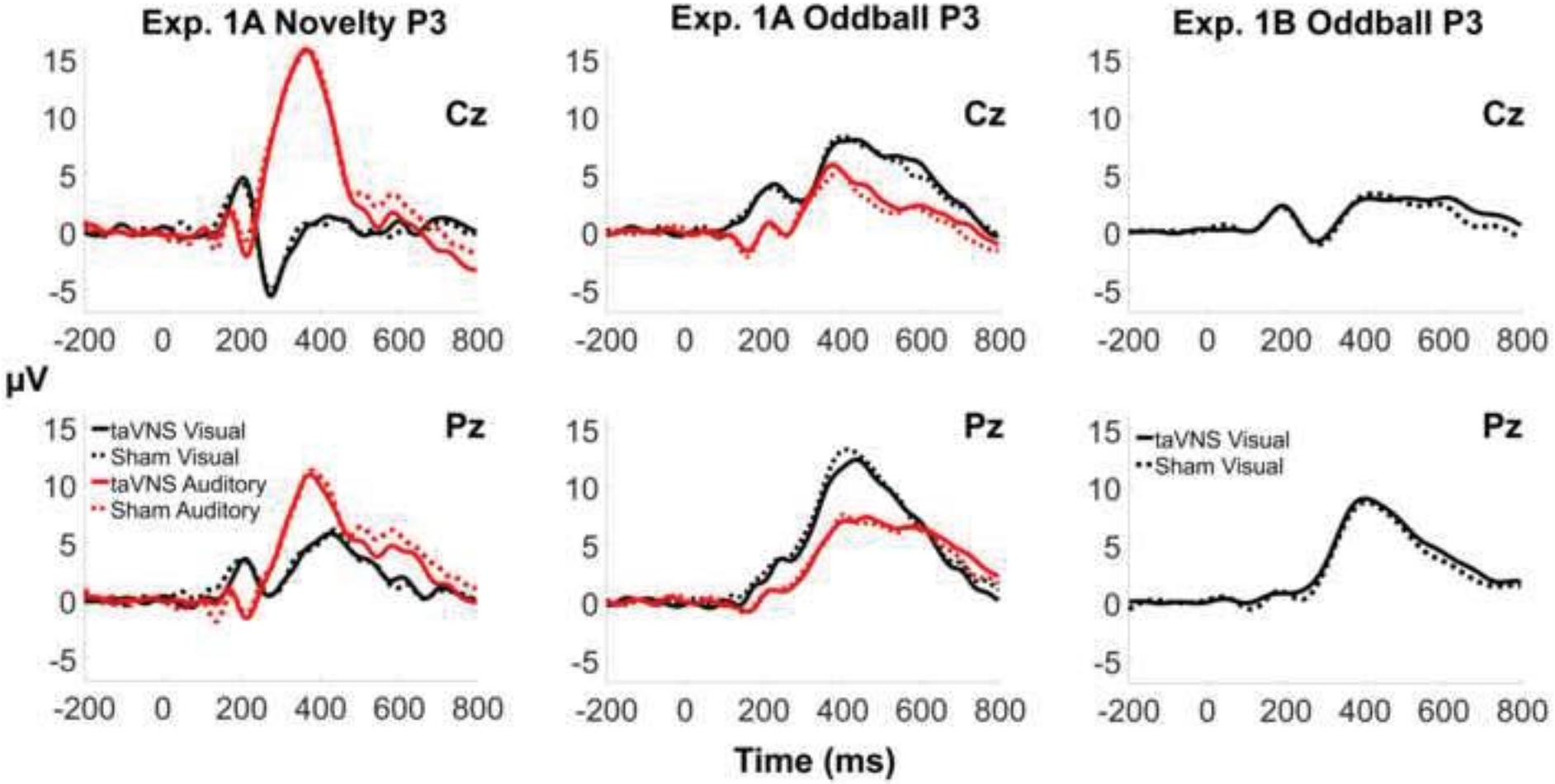

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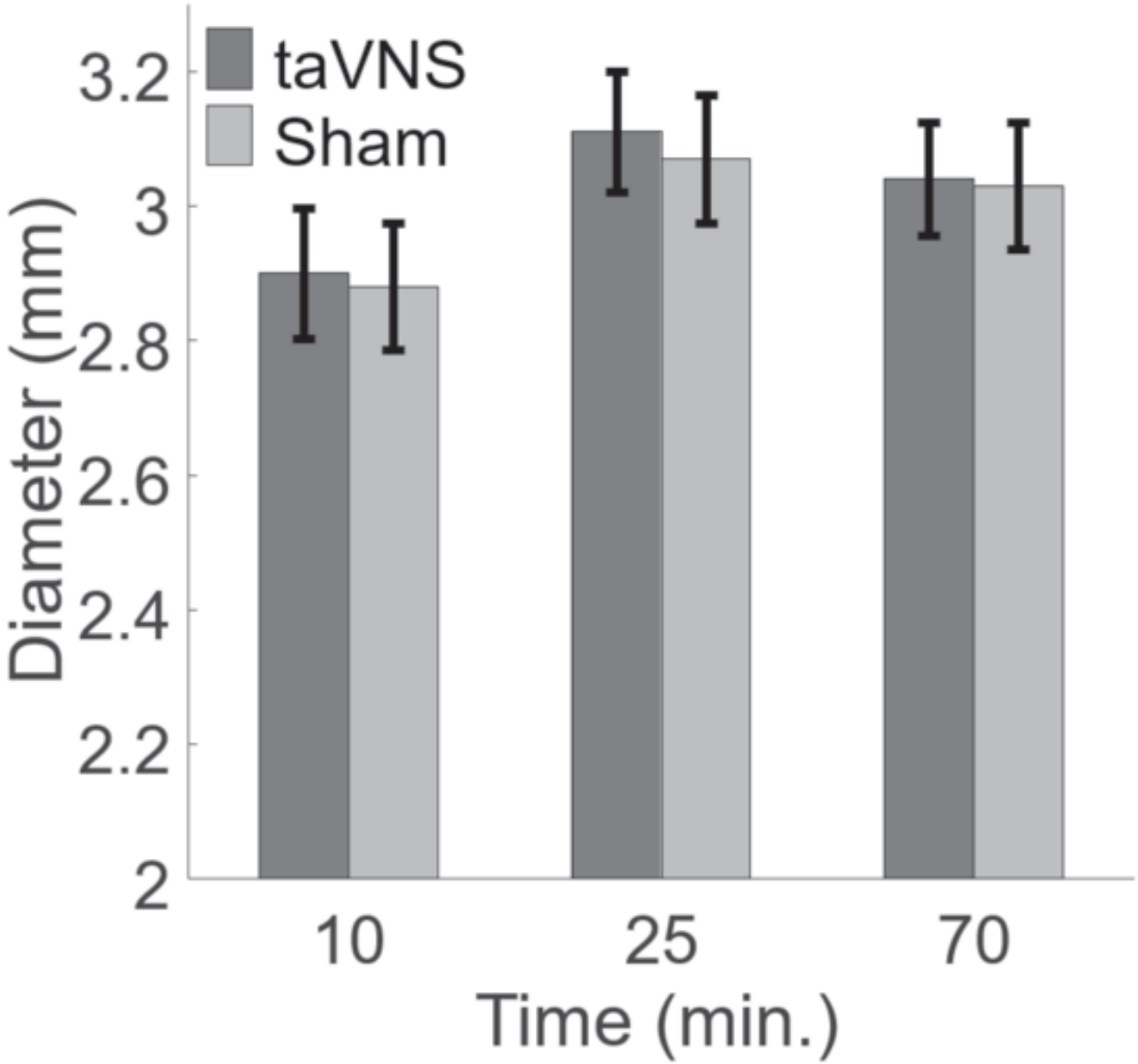

Figure5
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