Transcutaneous auricular vagus nerve stimulation increases long-latency event-related potentials, but does not affect neural gating or alpha oscillations

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# Abstract

Transcutaneous auricular vagus nerve stimulation (taVNS) is a non-invasive technique stimulating vagal afferent fibers, showing promise in treating neurological and mental disorders. taVNS is believed to activate the locus coeruleus (LC), promoting noradrenergic activation (NA), which enhances arousal and attention. However, evidence for the LC-NA hypothesis is mixed, and investigations in different sensory modalities are lacking. This study investigated whether taVNS enhances standard NA markers along with neural processing in three sensory modalities (auditory, respiratory, and somatosensory). In a two-day Sham-controlled crossover protocol, 45 healthy adults received taVNS at the *cymba concha* and Sham stimulation at the earlobe. During stimulation, participants experienced paired auditory clicks, inspiratory occlusions and electrocutaneous stimuli, while EEG was acquired. Salivary alpha-amylase (sAA) and subjective experienced arousal were measured at pre-/end-stimulation. Resting-state EEG was measured pre-/post-stimulation to assess alpha-band (8-13Hz) oscillation power, and participants rated the intensity and unpleasantness of all stimuli. Auditory-, respiratory-related-, and somatosensory evoked potentials were measured, specifically P50, N1, and P2 components, as well as the P50/N1 amplitude difference of the second and the first stimulus in the pair (neural gating; S2-S1). Although no effects in P50 or N1 amplitudes were observed, P2 amplitudes in auditory and somatosensory blocks increased during taVNS. Self-reported arousal increased in the taVNS condition, with no effects on neural gating, sAA concentration, or resting-state alpha power. taVNS had no effect on self-reported intensity/unpleasantness of stimuli. These results highlight certain limitations posed by combining taVNS and EEG, and underline the need for further mechanistic and clinical taVNS research.

*Keywords:* transcutaneous vagus nerve stimulation, interoception, attention, EEG, event-related potentials

# 1. Introduction

The vagus nerve, named for the Latin verb *vagari,* to wander, is the longest cranial nerve, with afferent and efferent branches enveloping the abdominal viscera, as well as lungs, heart, esophagus and trachea (Butt et al., 2020). The vagus nerve enters the brain at the brainstem, and its projections travel through the midbrain nuclei, including the *nucleus tractus solitarii* (NTS) and *locus coeruleus* (LC) (Nonis et al., 2017). The primary role of the vagus nerve within the autonomic nervous system (ANS) is supporting homeostasis, however it also has a marked influence on brain function (Narayanan et al., 2002; Takahashi et al., 2020). Invasive vagus nerve stimulation (iVNS), achieved via the implantation of electrodes at the cervical vagus nerve, has been used for decades as treatment for drug-resistant epilepsy and depression (Beekwilder & Beems, 2010; Nemeroff et al., 2006; Rush et al., 2000)

Transcutaneous auricular vagus nerve stimulation (taVNS) is a non-invasive approach to VNS, targeting the vagus nerve through its afferent, auricular branch, via electrodes placed over the skin of the outer ear (Butt et al., 2020; Farmer et al., 2021). taVNS has been used therapeutically for the treatment of drug-resistant depression (Kong et al., 2018; Liu et al., 2016), tinnitus (Wu et al., 2024), and chronic pain (Nesbitt et al., 2015; Vieira et al., 2018), however its potential therapeutic mechanisms are still not well-understood. The most prominent hypothesis, based on rodent models and neuroanatomical studies, posits that stimulating the afferent fibers of the auricular vagal branch exerts a top-down influence on the sympathetic ANS (Butt et al., 2020). The afferent vagal fibers, via the projections to NTS and LC, promote noradrenergic activation (NA), increasing arousal and attentional processing (Burger et al., 2020). In an effort to test the LC-NA hypothesis, research into the (neuro)physiological markers of taVNS proliferated in the last ten years (Burger et al., 2020; Farmer et al., 2021). Most of this research sought to confirm the involvement of noradrenergic pathways, using markers such as the secretion of salivary alpha-amylase (sAA) and pupil dilation (Burger et al., 2020). sAA is a digestive enzyme secreted by the salivary glands, whose secretion increases with the activation of the sympathetic ANS (Petrakova et al., 2015), while pupil size is a well-established marker of both tonic and phasic noradrenergic activity (Melnychuk et al., 2021). The reported effects have been mixed, with some studies showing promising effects (Giraudier et al., 2022; Lloyd et al., 2023; Sharon et al., 2021; Skora et al., 2024; Urbin et al., 2021; Ventura-Bort et al., 2018), while others reported null or inconclusive results (Burger et al., 2020; D’Agostini, Burger, Franssen, et al., 2023; D’Agostini et al., 2021, 2022; Keute et al., 2019). Given the mixed state of the literature, there is a present need for further research into the effects of taVNS on brain function and NA biomarkers, to elucidate the underlying mechanisms and promote therapeutic applications.

One of the most important functions of the LC-NA axis during wakefulness is optimizing arousal to promote sensory and attentional processing, learning and memory (Dahl et al., 2022). Under the assumption that taVNS may have beneficial effects on cognition via the LC-NA axis, a number of studies tested the effects of taVNS on attention (e.g. Maraver et al., 2020), memory (e.g. Mertens et al., 2020; Ventura‐Bort et al., 2021), cognitive control (e.g. De Smet et al., 2021) and learning (e.g. D’Agostini et al., 2021; Kühnel et al., 2020). Along with behavioral effects, several studies included neurophysiological measures to assess the effects of taVNS on cortical processing. The most prominent neurophysiological markers investigated have been the power of alpha oscillations, and the amplitude of P300. Alpha oscillations are a cortical oscillation of 8-13Hz, most prominently observed over parieto-occipital sites during rest (Klimesch et al., 2007). The fluctuations in alpha power have been linked to shifting attentional resources (Bauer et al., 2014; Klimesch, 2012; Rohenkohl & Nobre, 2011), and coordinating neural communications in the default-mode network (DMN (Clancy et al., 2022; Knyazev et al., 2011)). DMN activity is most apparent during rest and related to self-referential processing (Davey et al., 2016; van Buuren et al., 2010), and was previously shown to be modulated by noradrenergic transmission (Minzenberg et al., 2011). Research on alpha oscillations in the context of (ta)VNS tends to show a suppression of alpha oscillations by the stimulation (Lewine et al., 2019; Sharon et al., 2021; Wienke et al., 2023), aligning with the LC-NA hypothesis of enhanced attentional processing, though there have also been mixed and null findings (Lloyd et al., 2023). P300 is a long-latency event-related potential (ERP) manifesting as a positive deflection in the electroencephalogram (EEG), ~300ms after a salient stimulus, for example an oddball or a target (Polich, 1989; Yamaguchi & Knight, 1991). P300 has been source-localized to the temporo-parietal junction, where dense NA networks are located (Polich, 2007). Studies in iVNS have consistently reported increases in P300 as a marker of treatment success in patients (Neuhaus et al., 2007; Wostyn et al., 2017), whereas studies using taVNS in healthy individuals showed mixed results (D’Agostini, Burger, Jelinčić, et al., 2023; Giraudier et al., 2024; Ventura-Bort et al., 2018), calling into question the efficacy of (short-term) taVNS.

Previous studies probing the effects of taVNS on attention using EEG markers such as P300 have used external, namely visual and auditory stimuli to measure ERPs (D’Agostini, Burger, Jelinčić, et al., 2023; D’Agostini et al., 2022; Giraudier et al., 2024; Ventura-Bort et al., 2018). However, afferent vagal fibers are highly relevant to interoception – the processing and conscious experience of signals arising from *inside the body*, such as breathing, heartbeat, bladder distension and digestion (Ceunen et al., 2016; Khalsa & Lapidus, 2016). Accurate interoception is crucial to normal daily functioning, and disordered interoception is common in many prevalent chronic conditions, including chronic pain, migraine, irritable bowel disorders, and dyspnea (de Tommaso et al., 2003; Di Lernia et al., 2016; Köteles, 2021; Petersen et al., 2015; Van den Bergh et al., 2017). Surprisingly, there have only been two studies investigating the effects of taVNS on bodily-related processing, both using noxious laser pulses (Dumoulin et al., 2021; Laqua et al., 2014). A candidate interoceptive ERP that warrants investigation is the respiratory-related evoked potential (RREP), measured using brief inspiratory occlusions, which are detected by mechanoceptors in the thorax and propagated via vagal afferents through pontine nuclei, including NTS, and thalamic nuclei, terminating in the primary somatosensory cortices (Chan et al., 2022; Davenport et al., 2007; Davenport & Vovk, 2009). RREP consists of the early, sensory peaks (Nf/P1), the mid-latency N1 - a compound peak jointly influenced by sensory and attentional processes, and long-latency P2 and P3 representing later attentional and cognitive/emotional processing (von Leupoldt et al., 2010). In terms of morphology and cortical sources, RREP largely resembles the somatosensory evoked potential (SEP), which is elicited by electrical or mechanical stimulation of skin or peripheral nerves (Blom et al., 2012). Both RREP and SEP were shown to be affected by attentional and affective experimental manipulations, especially the N1 and P2 peaks (Jelinčić et al., 2022, 2024; Von Leupoldt et al., 2010), making them promising components to assess the potential enhancing effects of taVNS on attentional processing of bodily-related stimuli. Understanding the potential differences in how taVNS affects the neural processing of bodily-related relative to exteroceptive stimuli may help spearhead taVNS as a potential treatment option in patients suffering chronic bodily complaints.

Another interesting mechanism that has not received much attention in taVNS research is neural gating. Neural gating is a phenomenon of short-term suppression of cortical responses to a quick sequence of repetitive stimuli (Dalecki et al., 2011; Jelinčić, Torta, et al., 2021; Montoya et al., 2006; Patterson et al., 2008). It most reliably manifests in early and mid-latency peaks of the ERP, and has been extensively studied in patient populations as a symptom of cortical disinhibition. For example, neural gating was found to be reduced in disorders such as attention-deficit hyperactivity disorder (ADHD; Micoulaud-Franchi et al., 2016), Tourette’s syndrome (Morand-Beaulieu & Lavoie, 2019), and post-traumatic stress disorder (PTSD; Neylan et al., 1999), all of which are understood to arise at least in part through the dysfunction of the LC-NA axis (Alsene et al., 2006; Alsene & Bakshi, 2011). Neural gating is most often studied using paired auditory clicks, with most research focusing on the P50 component of the auditory evoked potential (AEP) (Patterson et al., 2008) and to a smaller extent the N1 component (Lijffijt et al., 2009). However, neural gating has been demonstrated for bodily-related ERPs as well, including RREP via paired occlusions (Chan & Davenport, 2008; Jelinčić, Torta, et al., 2021) and SEP via paired electrocutaneous stimuli (Cheng et al., 2016; Jelinčić, Torta, et al., 2021). There are two mechanisms by which taVNS could affect neural gating, both explainable by the LC-NA hypothesis: an increase in noradrenergic transmission could result in a general increase in cortical excitability (Capone et al., 2015), leading to a smaller suppression of the second stimulus (and thereby reduced neural gating). Alternatively, an increase in NA could promote neural gating via more efficient attentional processing, leading to a higher suppression of the second stimulus. Previous pharmacological studies found that increasing NA transmission in healthy adults reduced neural gating (Adler et al., 1994; Stevens et al., 1993), however two studies using non-invasive VNS found enhancement of neural gating (Lewine et al., 2019) or no effect (Mercante et al., 2023), respectively. Importantly, all the above mentioned studies investigated neural gating of auditory stimuli, with no studies addressing the potential effects of taVNS induced NA transmission on neural gating of respiratory or somatosensory sensations. Given that neural gating is thought to be partially elicited by bottom-up, sensory processes (Chan & Davenport, 2008; Edgar et al., 2005), and that it is known to relate to chronic conditions such as migraine (Hsiao et al., 2018), fibromyalgia (Montoya et al., 2006), and chronic obstructive pulmonary disease (Denutte et al., 2020), it is important to investigate whether any effects of taVNS on auditory gating generalize to the processing of bodily sensations.

In the current study, we investigated the effects of continuous short-term (45min) taVNS on peak amplitudes of early (P50), mid-latency (N1) and long-latency (P2) ERP components, as well as neural gating, in three sensory modalities: auditory, respiratory, and somatosensory. Very few studies thus far have investigated the effects of taVNS on cortical processing of bodily-related stimuli, and no studies have conducted a cross-modal investigation of these effects. We hypothesized increased ERP amplitudes in all modalities, given the findings of increased cortical excitability due to taVNS. We anticipated effects on neural gating, however did not hypothesize directional effects, given the lack of prior literature. Based on previous research, we also included measures of resting-state alpha oscillations, salivary alpha-amylase (sAA) and subjective experienced arousal. We hypothesized that taVNS, relative to Sham stimulation, would suppress alpha oscillations, and increase sAA and arousal.

# 2. Method

## 2.1 Participants

### 2.1.1 Power Analysis

Based on our previous research, which typically found small effects of experimental manipulations on ERP amplitudes and neural gating (Jelinčić et al., 2023, 2024), we powered the current study to detect a small to medium effect. The power was estimated given the sample size of *N* = 40, using R packages *lme4* (Bates et al., 2014) and *SIMR* (Green & MacLeod, 2016), which simulated 100 datasets with the specified underlying fixed and random effect structure using Monte Carlo simulations, and estimated average power to correctly reject the null-hypothesis. We specified the model with the following parameters: intercept of 0.7 (neural gating ratio of 70%), fixed estimate of *β* = 0.07 (7% increase or decrease in neural gating), and random intercept and residual variance of 0.16 and 0.09 respectively. The simulation estimated that the power to correctly reject the null-hypothesis given the model parameters at *N* = 40 was 0.95. The final target sample size was *N* = 45, to account for potential drop-out due to EEG/ERP measurement artifacts.

### 2.1.2 Demographics

45 participants (37 female) took part in the experiment. The mean age was 20 years (*SD*(44) = 3.0, range 17[[1]](#footnote-2)–30). Participants were recruited from the KU Leuven student population, provided written informed consent and received course credit or payment (10€/hour) for their participation. Participants were permitted to take part if they reported no (history of) chronic respiratory, cardiovascular, neurological, or psychiatric conditions, no chronic pain, no acute alcohol/drug intoxication, no habitual (self-)medication, no previous operations to the wrists, ears or head, if they were non-smokers, had normal or corrected-to-normal vision, normal hearing, no electronic implants, were not pregnant, and if they never participated in a two-session taVNS study before. Sufficient lung function was determined by standard spirometry and defined as forced expiratory volume in one second of at least 80% of the normative value given sex, age, and BMI (Miller et al., 2005).

All procedures were pre-approved by the Ethics Committee of the University hospital in Leuven (S66709), and the study was pre-registered at the Open Science Framework (Jelincic, 2023).

## 2.2 Experimental Set-Up

Unless specified otherwise, all the experimental events, including instructions, sensory stimulation, and questionnaires, were presented using PsychoPy, v.2023.1.2 (Peirce et al., 2019).

### 2.2.1 Transcutaneous auricular vagus nerve stimulation

Electrical stimulation of the ear was delivered and controlled using a constant current stimulator (DS5, Digitimer Ltd, Welwyn Garden City, UK) and a custom application in MATLAB (MathWorks Inc., Natick, MA, US). We employed two CE-certified titanium electrodes specifically designed for experimental research on transcutaneous vagus nerve stimulation (NEMOS®, Cerbomed GmbH, Erlangen, Germany) to ensure proper electrode placement (see Figure 1A). During the taVNS condition, the electrodes were positioned over the *cymba concha* of the left ear, a region of the outer ear innervated by afferent vagal fibers. In the Sham stimulation condition, the electrodes were attached to the center of the left earlobe, which is innervated by the great auricular nerve rather than the vagus nerve (Sclocco et al., 2019). Electroconductive cream (EC2+®, SOMNOmedics GmbH, Randersacker, Germany) was applied between the electrode and the skin—previously cleaned with alcohol—to ensure good contact and prevent excessive heat generation. An active Sham condition served as control, meaning the stimulation parameters were identical in both conditions, with the only difference being the stimulation site. Continuous bidirectional stimulation was administered at a frequency of 35Hz with a pulse width of 400μs. The stimulation intensity was individually calibrated to be above the perceptual threshold and below the pain threshold in both the taVNS and Sham conditions, with a minimum possible intensity of 0.5mA and a maximum of 4mA. This intensity was calibrated separately for the taVNS and Sham stimulation conditions, using a standard staircase procedure to determine perceptual and pain thresholds (see [2.3.1](#_2.3.1_First_session) for a detailed description of the calibration procedure). The average stimulation intensity was 1.33mA (*SD*(44) = 0.83) for the taVNS and 1.67mA (*SD*(44) = 0.81) for the Sham stimulation condition. To ensure that the specified current was delivered consistently, the difference between sent voltage and delivered current, as well as resistance, were continuously monitored using the in-house MATLAB application. In the rare cases (~10%) where the resistance would continue to increase throughout the experiment, the stimulation was stopped and the electrodes re-attached.

### 2.2.2 Arousal/NA markers

The concentration of salivary alpha-amylase (sAA) was measured at two time points during each session, once prior to stimulation, and once towards the end of the active stimulation period (see [2.3.1](#_2.3.1_First_session) for details on the collection of saliva samples). In addition to sAA, participants self-reported their subjective experienced arousal, using the Self-Assessment Manikin (SAM), a pictorial ten-point scale depicting a progressive increase from “0 - completely calm” to “9 - extremely excited” (Bradley & Lang, 1994).

### 2.2.3 Auditory stimuli

Auditory stimulation consisted of a series of paired auditory clicks, delivered at 440Hz, with the single-click duration of 10ms. The clicks were delivered through a speaker positioned 50cm behind the participant, while EEG was being recorded and taVNS/Sham was being administered. The sound intensity measured at the ear was 75dB. The first click was delivered manually by the experimenter, with the second following automatically 500ms later. The inter-pair interval was variable and controlled by the experimenter, with the average duration of approximately 10 seconds.

### 2.2.4 Respiratory stimuli

Respiratory stimulation consisted of delivering paired inspiratory occlusions during otherwise unobstructed breathing, while EEG was being recorded and taVNS/Sham was being administered. Participants wore a nose clip and breathed through a mouthpiece connected to a pneumotachograph via a two-way non-rebreathing valve (series 2700, Hans Rudolph Inc., Shawnee, USA). A custom occlusion valve and a trigger box (Aspire Products, Gainesville, USA) were attached to the inspiratory port of the non-rebreathing valve (Chan & Davenport, 2008). The experimenter manually operated the trigger box; at the start of every 2nd to 5th inhalation, compressed air was utilized to close the valve for 150ms, resulting in a brief inspiratory occlusion. Within the same inspiration, the second 150ms occlusion occurred automatically 500ms after the first. The pneumotachograph and a differential-pressure transducer (Hans Rudolph Inc., Shawnee, USA) enabled the recording of the inspiratory flow and mouth pressure. The signal was digitized and amplified using a physiological signal recording unit (MP160, BIOPAC Inc., Goleta, USA) and monitored and recorded using AcqKnowledge 5.0 (BIOPAC Inc., Goleta, USA). Incomplete occlusion pairs were excluded from EEG analysis without replacement. Please refer to Jelinčić et al. (2021) for a graphical representation and further detail.

### 2.2.5 Electrocutaneous stimuli

In the electrocutaneous blocks, brief paired electrical stimuli were delivered via a bipolar bar electrode attached to the participants’ posterior left wrist, 2cm above the pisiform bone. The electrode was filled with electroconductive gel (K-Y Gel, Reckitt plc, Slough, UK) to increase the precision of stimulus delivery and eliminate heat transfer. With the participants’ EEG being recorded and taVNS/Sham being delivered, a constant-current stimulator (DS7, Digitimer Ltd, Welwyn Garden City, UK) was used to send the square electrocutaneous pulses (pulse duration = 2ms, ISI = 500ms) (Jelinčić, Torta, et al., 2021). As in the other two sensory modalities, the first stimulus was administered manually by the experimenter, with the second stimulus following automatically after a 500ms interval. The amplitude of the stimulation was calibrated as five times the intensity that the participants could just perceive (perceptual threshold). The average stimulus amplitude across participants was 2.62mA (*SD*(44)=1.65).

### 2.2.6 EEG measurement

Participants’ EEG was measured during the sensory stimulation blocks and five minutes’ resting state pre- and post-stimulation, using a high-density 129-channel net (Philips Electrical Geodesics Inc., Eugene, USA). EEG was recorded at a sampling rate of 1kHz and was online referenced to the vertex electrode, with the channel impedances kept under 50kΩ, adhering to the manufacturer guidelines.

### 2.2.7 Adverse events

To assess whether any side-effects of taVNS lingered after the stimulation was turned off, participants filled in an adverse events form at the end of each session. The adverse events included pain, itching or burning at the stimulation site, headache, neck pain, limb numbness, nausea, drowsiness and a non-specific uncomfortable feeling. The occurrence and intensity of these events was assessed using a 6-point Likert scale with the descriptors *not at all, extremely limited, very limited, moderate, strong, very strong.*

## 2.3 Experimental Procedures

The study was designed as a two-session, single-blind crossover experiment. Each participant received both the active taVNS and active Sham stimulation, each in a separate session, with exactly 7 days in between the sessions. The assignment of stimulation type to the first and the second session was counterbalanced and randomly assigned to participants. To minimize the chance of the salivary alpha-amylase levels being confounded by circadian shifts, both sessions took place between 12PM and 8PM, at the same time for each participant. Participants were instructed to refrain from taking medication (apart from oral contraceptives) in the 24 hours preceding the experiment. They were also asked not to consume caffeinated beverages in the six hours preceding the experiment, to ensure arousal levels approaching each individual’s baseline. To ensure uncontaminated saliva samples, participants were asked to refrain from eating or chewing gum in the last hour before the experiment.

During all of the below described procedures, participants sat in a comfortable reclining chair, positioned 100cm away from a computer screen, which displayed instructions, rating scales and the fixation cross during the sensory stimulation blocks.

### 2.3.1 First session

The summary and timeline of all the experimental procedures can be seen in Figure 1B. Upon arriving to the lab, participants read and signed the informed consent forms, and were given time and opportunity to ask any questions regarding the experimental procedure. Thereafter, participants rinsed their mouth and provided the first saliva sample, by pooling the saliva under their tongue for one minute, and passively letting the pooled saliva flow into the test tube through the designated straw. This process was repeated three times. During the pooling of the saliva, participants were asked not to speak, swallow, or move their tongue. After the full sample was collected, participants rated their subjective experienced arousal via SAM. Participants then underwent the standard forced spirometry procedure, whereby their peak expiratory volume and flow were measured and compared to normative data. All included participants achieved at least 80% of the normed value for their age, sex and BMI over three attempts (Miller et al., 2005).

Following spirometry, participants’ head circumference and vertex point were determined, and the EEG net was placed for the first measurement of resting state EEG. Participants were instructed to lean back in the chair, relax and close their eyes, not to think of anything specific, and not to fall asleep. The resting state recording took five minutes.

Participants were then familiarized with the stimuli they would experience in the experiment: five example paired occlusions and five example paired auditory clicks were presented, and participants were instructed to provide the ratings of intensity and unpleasantness, using the modified Borg scale on the screen, ranging from 0 (not intense/unpleasant at all) to 10 (maximum intensity/unpleasantness). Participants were instructed to rate the stimuli they experienced in each block as a whole. Thereupon, participants’ perceptual threshold and individual intensity for paired electrocutaneous stimuli was determined: The bar electrode was mounted on the participant’s left wrist, and they were instructed to raise their right hand whenever they felt a single pulse of a very low intensity. The stimulation started from the pulse intensity of 0.2mA and was increased by 0.1mA until the participant raised their hand. Then, the intensity was lowered by 0.1mA, and ten consecutive pulses were given. If the participant indicated they felt at least five pulses, this intensity was taken as their perceptual threshold. If they did not feel five pulses, the intensity was raised by 0.1mA and the process repeated until five perceived pulses were achieved. The individual perceptual threshold was then multiplied by five to arrive at the intensity that would be used for the electrocutaneous pulses throughout the experiment. Participants then received five example paired stimuli at the calibrated intensity and were instructed on how to rate them on the Borg scale, following the same procedure as for the inspiratory occlusions and auditory stimuli.

After the participants were familiarized with the stimuli, the taVNS electrode was attached to their left *cymba concha* (in the active taVNS condition) or their earlobe (in the active Sham condition). The intensity of stimulation was individually calibrated (D’Agostini et al., 2022). First, the perceptual threshold was determined: participants received five seconds of stimulation, with the intensity starting at 0.2mA and increasing in steps of 0.1mA until the participant indicated that they perceived the stimulation. The individual intensity of stimulation was then determined using the simple staircase method. Participants were presented with a horizontal rating scale ranging from 0 (no sensation) to 100 (clearly painful), with a third anchor at 50 (moderately intense). They were instructed to rate each stimulation example on the scale, and were told that they should use the rating of 90 as the pain threshold. The stimulation intensity proceeded to rise in steps of 0.1mA, with participants rating each five-second instance of stimulation. When the participant indicated a rating of 90, the intensity was reduced in steps of 0.1mA until the participant rated the stimulation at 60. Then the intensity was again increased in steps of 0.1mA until the rating of 90, and subsequently decreased back to 60. The final intensity was determined as the average of the four intensities which were first rated as 80 on each of the four staircase runs, resulting in an intensity which was clearly perceptible but not painful. Participants were then presented with the final five seconds of stimulation at the calibrated intensity, and gave verbal consent to proceed with the experiment.

The stimulation was turned on and was delivered continuously for approximately 45 minutes. During this time, participants underwent six blocks of sensory stimulation, with two blocks of 22 paired stimuli for each of the three sensory modalities, as described in 2.2. The order of sensory modalities was counterbalanced across participants, and organized in an ABC–CBA fashion, to ensure that the average duration of stimulation up to each block was roughly the same (approximately 21 minutes) in all three modalities (see Figure 1). At the end of each block, participants were asked to rate the intensity and unpleasantness of the sensory stimuli (*not* taVNS) using modified Borg scales. After the third and the sixth block, participants were asked to rate the intensity of the ear stimulation (on the scale of 0 to 100; see the calibration section above) and the unpleasantness of the ear stimulation on the scale of -50 (extremely unpleasant) to 50 (extremely pleasant).

After the sixth sensory stimulation block and the final rating of ear stimulation, participants were asked to provide the second subjective experienced arousal rating using SAM, and the second saliva sample, which was collected in the same manner as the first. Stimulation was turned off, and participants underwent another five minutes’ resting state EEG recording with their eyes closed. Participants then filled in the adverse events checklist.

### 2.3.2 Second session

The second session proceeded similarly to the first, with the main difference being the auricular stimulation site. Participants once again signed the consent form and completed all of the pre-stimulation measures in the same order (see Figure 1), with the exception of spirometry, which was not performed in the second session under the assumption that participants’ lung function did not change in the seven intervening days. Familiarization with the sensory stimulation and ratings was also not performed in the second session, and neither was the calibration of the paired electrocutaneous stimuli, as the aim was to maintain the same stimulus intensity across b oth sessions. taVNS/Sham calibration was performed in the same manner as in the first session (see above). The active stimulation period proceeded identically to the first session, and the order of the sensory modalities was not changed from the first session. After the sixth block, post-stimulation measures were collected in the same manner as the first session. Additional to the second session was the manipulation blindness check – participants were asked to make an informed guess as to which of the two stimulation sites (*cymba concha*/earlobe) constituted the experimental and which the control condition, and were then debriefed as to the correct answer and the research aim and hypotheses. As in the first session, participants filled in the adverse events form at the end of the session.

A diagram of a device used to treat an ear

Description automatically generated with medium confidence

**Figure 1** A) The depiction of the electrode placement on the left ear for taVNS (left) and Sham stimulation (right). Created using Biorender.com B) Schematic depiction of the single-session protocol. Abbreviations: c.concha = cymba concha, SAM = Self-Assessment Manikin, PES = paired electrocutaneous stimuli

## 2.4 Data Analysis

### 2.4.1 Salivary alpha-amylase

The saliva samples were stored in test tubes labeled with consecutive three-digit codes (anonymously assigned to participants’ unique IDs and sessions/time points). The samples were kept frozen at -21°C. Upon the completion of data collection, the samples were sent for immunoassay analysis (Dresden LabService GmbH, Germany) to extract salivary alpha-amylase concentration (net sAA per milliliter of fluid - U/ml).

### 2.4.2 EEG analysis

All analyses of EEG data were performed using the MNE Python library (Gramfort et al., 2013).

#### 2.4.2.1 ERP analysis.

For the analysis of event-related potentials and neural gating, raw EEG data were downsampled to 500Hz and re-referenced to the average of all the channels. A copy of the raw data was then filtered for artifact rejection using a finite-impulse response (FIR) filter with a low cutoff frequency of 1 Hz, a high cutoff frequency of 30 Hz, with a length of 3.3s. The data were then visually inspected to identify noisy channels, which were interpolated using spherical splines. The number of interpolated channels was limited to 10%. The copy data were segmented into 4-second epochs locked to the delivery of the first stimulus in a pair [-2s,2s]. These epochs were then entered into an independent component analysis using the *fastica* algorithm (Hyvarinen, 1999) as implemented in the Python module *scikit-learn* (Pedregosa et al., 2011). Independent components capturing the activity related to eye blinks, eye movements, heartbeat, taVNS noise and channel noise/electrical artifacts were manually identified and marked for rejection. The original raw data were then filtered using a less-aggressive FIR filter with a low cutoff frequency of 0.3Hz and the length of 3.3s. A notch filter centered on 50Hz with a length of 10s was implemented to further suppress line noise. The rejected ICA components were regressed out of the original data, which were then segmented into the [-200ms,1000ms] epochs surrounding the delivery of each stimulus (first and second stimulus in each pair). The epochs were baseline corrected by the interval of [-130ms,-30ms] preceding the delivery of each stimulus, and averaged according to stimulus number (S1 = first stimulus in a pair, S2 = second stimulus in a pair). Prior to averaging, a copy of auditory stimulus epochs was created and additionally filtered using a FIR filter with a low cutoff frequency of 10Hz, for the measurement of the P50 component, to help distinguish it from the other early peaks (Patterson et al., 2008). Epochs with amplitude deviations of >200μV were automatically excluded. The average number of accepted trials per trial type is provided in the Supplement (Table S1). After averaging, the amplitudes of AEP P50 and AEP/RREP/SEP N1 were extracted for both the first stimulus (S1) and the second stimulus (S2), while the amplitudes of AEP/RREP/SEP P2 were extracted for S1 only. The P50 amplitudes were determined as the largest peak-to-peak positive deviations in the time window of [30ms,60ms] post-stimulus at a single centro-lateral channel. The N1 and P2 amplitudes were determined as the largest baseline-to-peak deviations in the time windows of [80ms,140ms] and [150ms,220ms] respectively. N1 and P2 amplitudes were identified at a hotspot cluster of three channels, as in our previous research (Jelinčić et al., 2022). For each participant and ERP component, the hotspot clusters were individually selected from the centro-lateral channels, based on the highest cluster activity. The N1 S2 peak was always identified from the same hotspot cluster as the N1 S1 peak. The neural gating of the P50 and N1 components was determined by calculating the average amplitude difference (S2-S1) (Chenivesse et al., 2014; Herzog, Sucec, Van Diest, Van den Bergh, et al., 2018) (see [2.4.3.2](#_2.4.3.2_Deviations_from) for the rationale on using this measure). During all of the above-described pre-processing and analysis steps, the researcher was blinded to the condition (taVNS or Sham) of the recording.

#### 2.4.2.2 Spectral power analysis.

To assess potential changes to spectral power dynamics in the alpha (8-13Hz) band, the raw resting state data were downsampled to 250Hz and cropped to the first four minutes of each resting state recording. The data were then re-referenced to the average of all electrodes and a notch filter centered at 50Hz with a length of 10s was applied to suppress line noise. The data were then segmented into epochs of equal length (4s) with a 0.75s overlap, and the epochs were detrended in a linear fashion. Bad channels and epochs were automatically identified using cross-validation and Random Sample Consensus, as implemented in the *autoreject* library (Jas et al., 2017) running in MNE-Python (Gramfort et al., 2013). The bad channels were interpolated, while the bad epochs were either repaired or rejected. The average number of accepted epochs per condition/time is provided in the Supplement (Table S2). Using the multitaper spectrum method, power-spectral density (PSD) in the alpha range (8-13Hz) was estimated across all epochs and channels. Average PSD was then extracted from parieto-occipital channels around Pz and Oz for each condition (taVNS/Sham) and time (pre-/post-stimulation).

### 2.4.3 Statistical analysis

All statistical analyses were performed using RStudio, version 2024.04.2 (RStudio Team, 2015) running the R version 4.3.1. The analyses were pre-registered at <https://osf.io/pv57c/>.

To test whether taVNS resulted in a relative increase of noradrenergic activity and a relative decrease in resting-state alpha power compared to Sham, we constructed linear mixed-effects models (LMEM) for sAA concentration, subjective experienced arousal, and alpha PSD, using the maximum-likelihood method within the *lme4* package (Bates et al., 2015). We entered *time* (levels: 0 – pre-stimulation, 1 – end-stimulation), *condition* (levels: 0 – Sham, 1 – taVNS), and the interaction of *time* and *condition* as fixed effects, with the participants’ individual intercepts as random effects, and no random slopes.

To test the effects of taVNS relative to Sham on the ERP amplitudes and neural gating, we constructed separate LMEM for AEP P50, AEP/RREP/SEP N1, AEP/RREP/SEP P2, AEP P50 gating and AEP/RREP/SEP N1 gating, with the fixed effect of *condition* and random individual intercepts. To assess whether neural gating occurred for all the studied ERPs, we performed a one-sample two-tailed t-test with alpha = .05 on the S2-S1 difference, against the *mu* = 0 (*H0*: S1 = S2).

To exploratorily assess any taVNS effects on perception, we constructed separate LMEM for intensity and unpleasantness of all three types of sensory stimulation (clicks, occlusions, and electrocutaneous stimuli), with the fixed effect of *condition* and random individual intercepts.

The significance of all the models was determined using the Satterthwaite’s degrees-of-freedom method as implemented in the package *lmertest* (Kuznetsova et al., 2017). Outcomes found to violate the LMEM assumption of homoscedasticity of residuals were transformed using the square root transformation or log transformation (ERP amplitudes), to improve the model fit.

#### 2.4.3.1 Data exclusion.

A number of data points were missing or excluded from the final LMEM analyses. The exclusions, along with the reasons for them, are listed below, in the order of studied outcomes. We did not interpolate the missing data. Participants with missing data in one or more of the outcomes were still included in the analysis of other outcomes (pairwise exclusion).

1. Due to issues with delivering ear stimulation (high impedance), one participant’s Sham condition was excluded from all analyses.
2. Due to non-compliance with instructions, three participants’ taVNS conditions and two participants’ Sham conditions were excluded from the sAA analysis. Due to sample contamination, one participant’s taVNS and one participant’s Sham condition had missing sAA values.
3. Due to an issue of PsychoPy crashing without saving the data, one participant’s pre-stimulation arousal rating and three participants’ post-stimulation arousal ratings in the taVNS condition were missing.
4. Due to non-compliance with instructions, one participant’s taVNS condition was excluded from the EEG analyses of auditory blocks. Three participants’ EEG data in the taVNS condition, and six participants’ data in the Sham condition were excluded from the EEG analyses of auditory blocks on account of noise.
5. Due to >50% incomplete occlusions, one participant’s taVNS condition, and three participants’ Sham conditions were excluded from the EEG analyses of respiratory blocks. Two participants’ respiratory blocks’ EEG data in the taVNS condition, and six participants’ data in the Sham condition were excluded on account of noise.
6. Due to issues with delivering electrocutaneous stimuli, one participant’s taVNS condition was excluded from the EEG analyses of somatosensory blocks. Three participant’s somatosensory blocks’ EEG data in the taVNS condition, and eight participants’ data in the Sham condition were excluded on account of noise.
7. Due to an issue of PsychoPy crashing without saving the data, four participants’ ratings (for sensory stimulation and ear stimulation) in the taVNS condition were missing.
8. Due to experimenter error, three participants’ manipulation checks were not collected.

#### 2.4.3.2 Deviations from the pre-registered analyses.

The pre-registered analyses for neural processing only concerned neural gating (as the topic of an over-arching project), however we have decided to additionally analyze the ERP amplitudes. This decision was made based on our recent research in which we found no effects of experimental manipulations on neural gating, but did find the effects on N1 and P2 amplitudes (Jelinčić et al., 2022, 2024). These analyses are considered confirmatory, given our directional hypothesis of taVNS > Sham for all the ERP amplitudes.

In addition, our previous research quantified N1 neural gating as the ratio of the S2 and S1 amplitudes, in accordance with most established research on the topic (Chan et al., 2012; Chan & Davenport, 2008; Montoya et al., 2006). However, this operationalization comes with the cost of having to drop data points where the S2/S1 ratio is negative (which results from one of the N1 amplitudes, usually S2, not reaching the negative amplitude range). In the pre-registration, we established that the ratio measure would be used in the current dataset as well, unless 20% or more data points in any of the three N1 gating ratio outcomes were negative. This cut-off was reached in the current dataset, with the highest percentage of negative gating ratios (for the AEP N1 gating ratio) of 31%. We therefore opted to measure neural gating as the S2-S1 difference for all the sensory modalities, which allowed us to retain all of the non-missing data points.

# 3. Results

All the below reported results are the output of linear mixed effects models (LMEM), unless specified otherwise. We report random effects and residual variance, as well as AIC and BIC criteria, in the Supplement, Tables S4-S6. In the LMEM tables, square-root-transformed outcomes are marked with a superscript SQRT, and log-transformed variables with a superscript LOG. In text, we report the fixed effect estimates () with 97.5% confidence intervals (*CI*), *p*-values (*lmertest* output) and the robust effect size index () (RESI; (Vandekar et al., 2020)). While they usually correspond, occasionallyRESI indicates the existence of an effect ( > 0.1) while *p-*values are larger than 0.05. These effects are described as “small and non-significant” in text. For one-sample t-tests, we report the *t*-statistics, degrees of freedom, *p*-values and Cohen’s *d* values (*dz*).

## 3.1 Markers of noradrenergic activation

### 3.1.1 Salivary alpha-amylase concentration

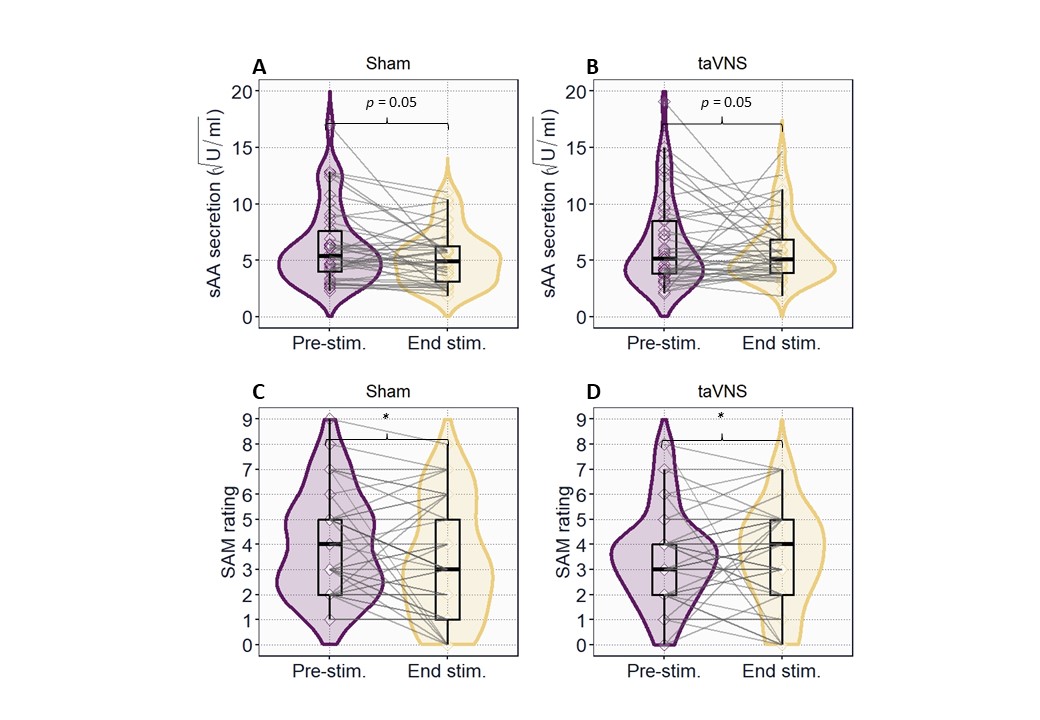
To test the effects of taVNS on the noradrenergic activation, salivary alpha amylase was measured before and after the stimulation on both days. For descriptive statistics, see Table 1. Results are shown graphically in Figure 2A/B. We observed a marginally significant decrease in sAA concentration after the stimulation ( = -0.76, *CI* = 1.50, *p* = 0.05, = 0.18). There was no significant effect of *condition* ( = 0.42, *CI* = 1.54, *p* = 0.29, = 0.07), and no significant interaction between *condition* and *time* ( = 0.48, *CI* = 2.98, *p* = 0.52, = 0.00) on log-transformed sAA values.

### 3.1.2 Subjective experienced arousal

Participants provided a rating of their subjective experienced arousal using SAM, pre- and post-stimulation on both days. For descriptive statistics, see Table 1. Results are shown graphically in Figure 2C/D. We observed no main effect of *time*, i.e. no significant change in subjective experienced arousal after the stimulation ( = 0.04, *CI* = 0.81, *p* = 0.85, = 0.00). There was no significant main effect of *condition* ( = -0.19, *CI* = 0.81, *p* = 0.36, = 0.00), however a significant interaction was found between *condition* and *time* ( = 0.88, *CI* = 1.59, *p* = 0.03, = 0.36) indicating the higher end-stimulation increase in arousal ratings in the taVNS condition.

**Table 1** Descriptive statistics for the NA markers: salivary alpha-amylase (sAA) concentration ( and subjective experienced arousal (Self-Assessment Manikin ratings)

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **taVNS** | | | | **Sham** | | | |
|  | **Pre-stimulation** | | **End-stimulation** | | **Pre-stimulation** | | **End-stimulation** | |
|  | sAA | Arousal | sAA | Arousal | sAA | Arousal | sAA | Arousal |
| *M* | 6.45 | 3.41 | 5.86 | 3.80 | 6.26 | 4.02 | 5.24 | 3.61 |
| *SD* | 3.92 | 2.27 | 2.84 | 1.74 | 3.45 | 2.03 | 2.42 | 1.86 |
| *N* | 41 | 44 | 41 | 41 | 42 | 44 | 42 | 44 |



**Figure 2** Graphical depiction of the results for NA markers. Violin plots with histograms and superimposed raw data for Sham stimulation (A) and taVNS (B), showing marginally significant effects of time but no condition effects on salivary alpha amylase (sAA) concentration. Violin plots with histograms and superimposed raw data for Sham stimulation (C) and taVNS (D), showing a significant interaction effect of time and condition on subjective experienced arousal, as measured via Self-Assessment Manikin (SAM) ratings.

## 3.2 ERP components and neural gating

### 3.2.1 Auditory evoked potentials

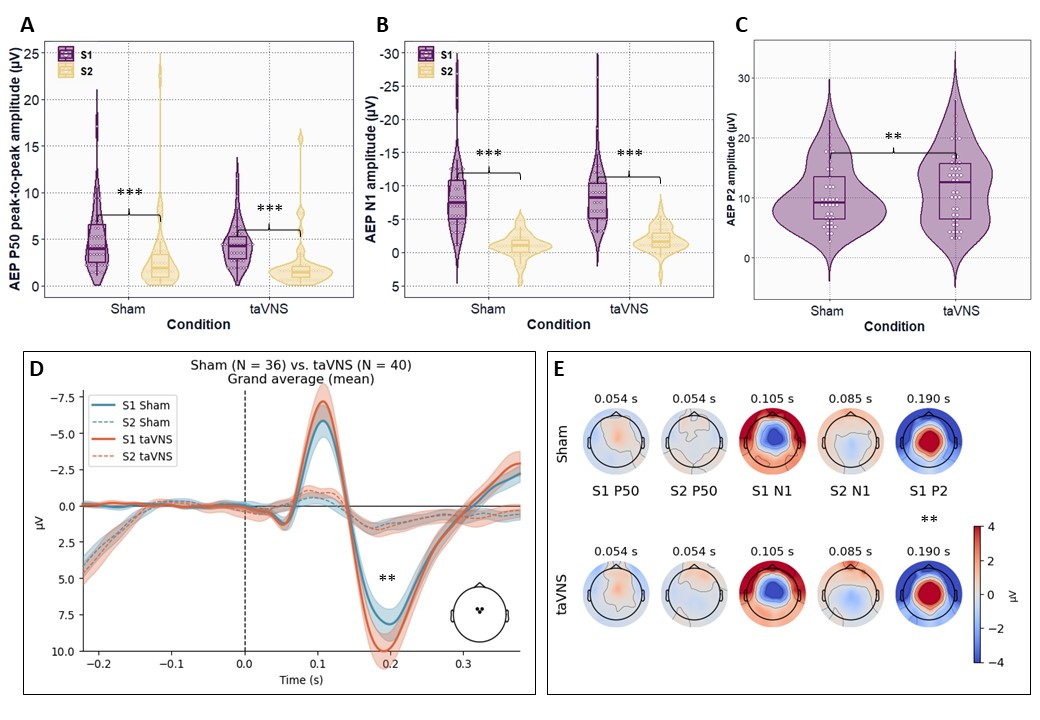
To test the effects of taVNS on AEP, we measured peak amplitudes for P50, N1 and P2 components in response to the first click in the pair, as well as neural gating as the difference between the peak-to-peak P50 to the second and the first click, and the difference of the baseline-to-peak N1 to the second and the first click. Two-sample paired t-tests showed a significantly decreased P50 peak-to-peak amplitude to the second click relative to the first click in the taVNS condition ( = -3.86, *df* = 76.79, *p* < 0.001, *dz* = -0.85) while the difference in the Sham condition was marginally significant ( =- 1.96, *df* = 70.98, *p* = 0.05, *dz* = -0.45). N1 amplitude to the second click relative to the first click was significantly decreased in both taVNS ( = 7.50, *df* = 47.93, *p* < 0.001, *dz* = 1.66) and Sham conditions ( = 7.67, *df* = 45.80, *p* < 0.001, *dz* = 1.76). For descriptive statistics, see Table 2, for graphical depiction, see Figure 3. While we observed no significant effect of *condition* on P50 amplitudes ( = -0.08, *CI* = 0.38, *p* = 0.42, = 0.00), or N1 amplitudes ( = -0.01, *CI* = 0.10, *p* = 0.60, = 0.00), we observed significantly increased P2 amplitudes in the taVNS condition ( = 1.96, *CI* = 3.02, *p* = 0.01, = 0.35). We found no effects of *condition* on the neural gating of the P50 ( = -0.02, *CI* = 0.28, *p* = 0.81, = 0.00) or the N1 components ( = -0.00, *CI* = 0.41, *p* = 0.96, = 0.00).

### 3.2.2 Respiratory-related evoked-potentials

To test the effects of taVNS on RREP, we measured peak amplitudes of N1 and P2 components in response to the first occlusion in the pair, as well as neural gating as the difference between the peak N1 response to the second and the first occlusion. Two-sample paired t-tests showed a significantly decreased N1 amplitude to the second occlusion in both taVNS ( = 8.41, *df* = 58.63, *p* < 0.001, *dz* = 1.12) and Sham conditions ( = 5.08, *df* = 63.56, *p* < 0.001, *dz* = 1.20). For descriptive statistics, see Table 3. We observed no significant effect of *condition* on N1 amplitudes ( = 0.06, *CI* = 0.32, *p* = 0.46, = 0.00), P2 amplitudes ( = -0.01, *CI* = 2.83, *p* = 0.98, = 0.00), or neural gating ( = 0.06, *CI* = 0.38, *p* = 0.50, = 0.00). For graphical depiction, see Figure 4.

### 3.2.3 Somatosensory evoked potentials

To test the effects of taVNS on SEP, we measured peak amplitudes for N1 and P2 components in response to the first stimulus in the pair, as well as neural gating as the difference between the peak N1 response to the second and the first stimulus. Descriptive statistics are found in Table 4, and the graphical depiction in Figure 5. Two-sample paired t-tests showed a significantly decreased N1 amplitude to the second stimulus relative to the first stimulus in both taVNS ( = 2.91, *df* = 67.35, *p* = 0.01, *dz* = 0.58) and Sham conditions ( = 2.65, *df* = 54.61, *p* = 0.01, *dz* = 0.63). While we observed no effect of *condition* on N1 amplitudes ( = -0.01, *CI* = 0.30, *p* = 0.86, = 0.00), there was a marginally significant moderate effect of condition on P2 amplitudes ( = 1.42, *CI* = 2.81, *p* = 0.05, = 0.26), which were increased in the taVNS condition. There was no significant effect on neural gating ( = -0.03, *CI* = 0.30, *p* = 0.67, = 0.00).

**Table 2** Descriptive statistics for the peak amplitudes of auditory evoked potentials and auditory neural gating (S2-S1). Abbreviations: S1/2 = firs/second click in the pair

**Figure 3** Graphical depiction of results for auditory evoked potentials (AEP). Violin plots with histograms and superimposed raw data for AEP P50 amplitudes (A) and AEP N1 amplitudes (B), showing no condition effects on peak amplitudes nor neural gating. Violin plots with histograms and superimposed raw data for AEP P2 amplitudes (C), showing a significant effect of condition. D) Grand-average AEP at central electrodes, with 0.8 confidence interval as the shaded area. E) Grand-average topography for each studied peak. Abbreviations: S1/2 = first/second stimulus in the pair

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | **S1 P50** | **S2 P50** | **P50 gating** | **S1 N1** | **S2 N1** | **N1 gating** | **S1 P2** |
| **taVNS** | *M* | 4.34 | 2.18 | 2.16 | -9.13 | -1.90 | 7.23 | 12.03 |
| *SD* | 2.26 | 2.78 | 3.04 | 5.88 | 1.86 | 5.66 | 6.22 |
| *N* | 41 | 41 | 41 | 41 | 41 | 41 | 41 |
| **Sham** | *M* | 5.11 | 3.28 | 1.84 | -8.07 | -0.99 | 7.08 | 10.23 |
| *SD* | 3.64 | 4.48 | 2.69 | 5.38 | 1.87 | 5.65 | 5.04 |
| *N* | 38 | 38 | 38 | 38 | 38 | 38 | 38 |

**Table 3** Descriptive statistics for the peak amplitudes of respiratory-related evoked potentials and respiratory neural gating (S2-S1). Abbreviations: S1/2 = first/second occlusion in the pair

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  | **S1 N1** | **S2 N1** | **N1 gating** | **S1 P2** |
| **taVNS** | *M* | -4.02 | -2.02 | 2.00 | 7.22 |
| *SD* | 3.82 | 2.30 | 2.83 | 5.06 |
| *N* | 42 | 42 | 42 | 42 |
| **Sham** | *M* | -4.43 | -2.34 | 2.09 | 5.99 |
| *SD* | 4.03 | 2.34 | 2.99 | 3.57 |
| *N* | 35 | 35 | 35 | 35 |

A close-up of a diagram

Description automatically generated

**Figure 4** Graphical depiction of results for respiratory-related evoked potentials (RREP). Violin plots with histograms and superimposed raw data for RREP N1 amplitudes (A), and RREP P2 amplitudes (B) showing no condition effects on peak amplitudes nor neural gating. C) Grand-average RREP at central electrodes, with 0.8 confidence interval as the shaded area. D) Grand-average topography for each studied peak. Abbreviations: S1/2 = first/second occlusion in the pair

***Table 4*** *Descriptive statistics for the peak amplitudes of somatosensory evoked potentials and somatosensory neural gating (S2-S1). Abbreviations: S1/2 = first/second stimulus in the pair*

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  | **S1 N1** | **S2 N1** | **N1 gating** | **S1 P2** |
| **taVNS** | *M* | -7.63 | -2.38 | 5.24 | 7.48 |
| *SD* | 3.58 | 1.78 | 3.41 | 4.82 |
| *N* | 41 | 41 | 41 | 41 |
| **Sham** | *M* | -7.70 | -3.10 | 4.61 | 7.55 |
| *SD* | 4.41 | 3.17 | 2.66 | 4.54 |
| *N* | 36 | 36 | 36 | 36 |

**A close-up of several graphs

Description automatically generated**

**Figure 5** Graphical depiction of results for somatosensory evoked potentials (SEP). Violin plots with histograms and superimposed raw data for N1 amplitudes (A), showing no condition effects on peak amplitudes nor neural gating. Violin plots with histograms and superimposed raw data for SEP P2 amplitudes (B), showing a marginally significant effect of condition. C) Grand-average SEP at centro-lateral channels, with 0.8 confidence interval as the shaded area. E) Grand-average topography for each studied peak. Abbreviations: S1/2 = first/second stimulus in the pair

## 3.3 Resting state alpha power

Extracted PSD values for the alpha band (8-13Hz) were log-transformed and compared between pre- and post-stimulation on both days. For descriptive statistics, see Table 5. Results are depicted graphically in the Supplement (Figure S1). We observed a significant main effect of *time*, with increased alpha PSD after stimulation in both conditions ( = 1.00, *CI* = 0.92, *p* < 0.001, = 0.77), but found no significant effect of *condition* ( = 0.12, *CI* = 1.01, *p* = 0.65, = 0.00), and no interaction between *condition* and *time* ( = -0.07, *CI* = 1.84, *p* = 0.89, = 0.00).

**Table 5** Descriptive statistics for the log-transformed alpha (8-13Hz) power-spectral density (PSD) during resting state

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **taVNS** | | **Sham** | |
|  | **Pre-stimulation**  **alpha PSD** | **Post-stimulation alpha PSD** | **Pre-stimulation alpha PSD** | **Post-stimulation alpha PSD** |
| *M* | -32.15 | -31.43 | -31.90 | -31.18 |
| *SD* | 5.97 | 5.56 | 5.03 | 5.51 |
| *N* | 36 | 39 | 39 | 37 |

## 3.4 Exploratory analyses

### 3.4.1 Perception of sensory stimuli

To explore the effects of taVNS on self-reported intensity and unpleasantness of sensory stimuli, we compared the ratings during taVNS and Sham conditions. Descriptive statistics are detailed in Table 6. Results are depicted graphically in the Supplement (Figure S2). We found a small and non-significant main effect of *condition* on the square-root-transformed intensity ratings of auditory clicks ( = 0.13, *CI* = 0.33, *p* = 0.13, = 0.19, but not unpleasantness: ( = -0.06, *CI* = 0.34, *p* = 0.49, = 0.00). Similarly, we found a small and non-significant main effect of *condition* on the square-root-transformed intensity ratings of electrocutaneous stimuli ( = 0.08, *CI* = 0.23, *p* = 0.18, = 0.13), but not unpleasantness: ( = 0.04, *CI* = 0.22, *p* = 0.48, = 0.00). We found no effects of *condition* on the perception of inspiratory occlusions (intensity: = 0.11, *CI* = 0.79, *p* = 0.57, = 0.00; unpleasantness: = 0.05, *CI* = 0.74, *p* = 0.78, = 0.00).

**Table 6** Descriptive statistics for the self-reported intensity and unpleasantness of sensory stimulation, as measured using modified Borg scale ratings (0-10)

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | **Auditory blocks** | | **Respiratory blocks** | | **Somatosensory blocks** | |
|  |  | **Intensity** | **Unpleasant-ness** | **Intensity** | **Unpleasant-ness** | **Intensity** | **Unpleasant-ness** |
| **taVNS** | *M* | 2.96 | 2.53 | 3.31 | 3.55 | 2.15 | 2.18 |
| *SD* | 1.86 | 1.76 | 1.98 | 2.07 | 1.46 | 1.72 |
| *N* | 41 | 41 | 41 | 41 | 41 | 41 |
| **Sham** | *M* | 2.60 | 2.75 | 3.12 | 3.35 | 2.02 | 2.02 |
| *SD* | 1.90 | 2.00 | 2.07 | 2.11 | 1.76 | 1.67 |
| *N* | 44 | 44 | 44 | 44 | 44 | 44 |

### 3.4.2 Stimulation perception, side-effects and manipulation blindness

During two rating instances (mid-stimulation and end-stimulation), participants indicated a mean perceived intensity of 21.17 (*SD*(41) = 20.65) for taVNS and 19.93 (*SD*(44) = 18.67) for Sham. Participants indicated on average that taVNS was slightly unpleasant, with a mean rating of -4.34 (*SD*(41) = 16.71), while Sham stimulation was on average slightly pleasant, with a mean rating of 2.73 (*SD*(44) = 19.75). The model comparing taVNS and Sham on log-transformed intensity ratings revealed no significant effect of *condition* ( = 0.12, *CI* = 0.20, *p* = 0.54, = 0.00), while the model for log-transformed unpleasantness ratings revealed a significant effect ( = -0.23, *CI* = 0.10, *p* = 0.03, = 0.29)*.*

Descriptive statistics and analyses for the adverse side-effects are reported in the Supplement, in Tables S3 and S7, respectively. Most participants reported at least one side-effect, in a large majority of cases to an ‘extremely limited’ (1) or ‘very limited’ (2) extent. Generalized linear mixed effects models, using a Poisson distribution for zero-inflated data, showed that participants reported significantly more instances of pain ( = 0.68, *CI* = 0.31, *p* = 0.03), tingling ( = 0.59, *CI* = 0.21, *p* = 0.004), and burning ( = 0.90, *CI* = 0.43, *p* = 0.04) after taVNS compared to Sham. The models for other side-effects revealed no significant differences.

When asked to speculate which of the two ear stimulation sites constituted the experimental condition (manipulation blindness check), 21 of the participants guessed *cymba concha,* and 21 the earlobe, an even split suggesting a successful single-blind manipulation.

# 4. Discussion

The current study investigated physiological and neurophysiological markers of transcutaneous auricular vagus nerve stimulation, as well as its effects on neural processing of sensory stimulation across three modalities (auditory, respiratory, and somatosensory). Motivated by the current literature reporting mostly mixed findings and single-domain investigations, we conceived this systematic cross-modal study in an attempt to clarify the mechanism of taVNS, as the three chosen modalities arise from discrete bottom-up pathways with different levels of vagal afferent involvement.

## 4.1 taVNS increased subjective experienced arousal, but did not significantly affect sAA concentration or alpha oscillations

Our results show that participants reported higher post-stimulation arousal in the taVNS condition relative to the active Sham condition. This finding supports the current understanding that taVNS increases arousal, likely by way of the LC-NA axis (Urbin et al., 2021). However, we found no evidence that salivary alpha-amylase concentration was significantly increased after taVNS relative to Sham. This finding counters the LC-NA hypothesis, as sympathetic activation (partly indexed by sAA) and noradrenergic activation (NA) tend to be strongly related (Ali & Nater, 2020), and are increased by the same pharmacological interventions (Warren et al., 2017). It was suggested in a recent mega-analysis that the taVNS effects on sAA are relatively small, which in addition to small sample sizes and other methodological differences between studies could contribute to null findings (Giraudier et al., 2022). A further possible explanation for these and previous null findings is that placing the electrodes at the earlobe may also lead to brainstem activation via the greater auricular nerve (GAN), with carryover effects to the relevant nuclei (NTS, LC) resulting in smaller group-level effects (Keute et al., 2018; Sclocco et al., 2019). It is also important to note that the measure of sAA *concentration* is not an ideal index of sympathetic activity, as it depends on salivary flow rate, which is modulated by the parasympathetic ANS (Burger et al., 2020).

Similarly, we found no evidence that taVNS, compared to Sham, suppresses spontaneous alpha oscillations, in contrast to previous reports (Sharon et al., 2021; Wienke et al., 2023). We found a main effect showing that the relative power of alpha oscillations increased after stimulation, however we found no effect of stimulation type or an interaction effect. Since alpha oscillations synchronize (increase in power) during relaxed and tired states (Klimesch et al., 1998), the main effect likely stems from a general fatigue induced by the experimental procedure, given that all the sessions lasted two hours and took place in the afternoon. Another possible explanation is that participants were experiencing acute relief and positive affect during the second resting-state measurement, as the ear stimulation was turned off immediately prior to it, and alpha synchronization has previously been linked to positive affect (Yu et al., 2011). This could also explain the absent interaction effect, given that taVNS was experienced as more unpleasant than Sham stimulation, which could have prompted stronger positive affect and alpha synchronization upon it being turned off, potentially masking any suppressive effects of the LC-NA axis. To address this issue, future studies investigating resting-state alpha dynamics should introduce more measurement points, to assess the changes in alpha dynamics during a longer post-stimulation period, as opposed to only immediately after the stimulation is turned off. One such previous study using transcutaneous cervical VNS, and taking multiple post-stimulation measurements, found suppressive effects on low frequencies (including alpha) lasting for up to two hours post-stimulation (Lewine et al., 2019). Another reason for the lack of an interaction may be our use of continuous stimulation and measuring resting-state dynamics, while Sharon and colleagues used short stimulation bursts, and measured event-related alpha dynamics in response to the bursts (Sharon et al., 2021). A recent study found that event-related pupil responses were modulated by taVNS only when higher-dose short bursts were employed (D’Agostini, Burger, Franssen, et al., 2023), indicating that continuous (tonic) stimulation may not be the most effective manner of modulating phasic LC-NA activity as measured by pupil dilation and alpha oscillations. It is, however, also important to note a recent non-replication of the alpha oscillation finding of Sharon et al. (Lloyd et al., 2023), which shows that these effects may not be very stable. In relation to this, the power of alpha oscillations is influenced by a plethora of neural and cognitive processes (Bazanova & Vernon, 2014; Jelinčić, Van Diest, et al., 2021), making it a highly non-specific outcome, and therefore likely not the most appropriate marker for the effects of taVNS on neural processing and the LC-NA axis.

## 4.2 ERP effects manifested in long-latency peaks in response to auditory and electrocutaneous stimuli

The current results showed taVNS effects on the peak amplitudes of AEP and SEP P2 components, but no effects on the earlier N1 or P50 peak amplitudes. As a long-latency component, P2 is known to be affected by attentional manipulations (Jelinčić et al., 2024; Lijffijt et al., 2009). The current results thus indicate that taVNS likely exerts an influence on central attentional and cognitive processing, independent of the modality-specific sensory processing, which is consistent with the current understanding based on the LC-NA hypothesis (Farmer et al., 2021; Ventura-Bort et al., 2018), and previous research (Giraudier et al., 2024; Lewine et al., 2019; Obst et al., 2020; Ventura-Bort et al., 2018). Apart from the central working mechanism of taVNS, another explanation for the absent effects on P50 and N1 is that these components are intensity-dependent (Klostermann et al., 2009; Lewine et al., 2019; Linka et al., 2007). In the current study, we aimed for all the stimuli to be mild, but noticeable, however it is possible that this intensity range is not an optimal one to show modulations by taVNS. Future studies could probe the effects of intensity by employing (sub)liminal in addition to supraliminal stimuli in all modalities, or assessing stimulus discrimination accuracy (Jigo et al., 2024).

We found no effects on any components of the respiratory-related evoked potential (RREP), a highly surprising finding given the direct involvement of vagal afferents in the processing of inspiratory occlusions (Davenport & Vovk, 2009), and known cross-communication between the auricular and laryngeal afferent vagal branches (Ryan et al., 2014). The current results therefore suggest that taVNS is more successful at modulating cortical responses to exteroceptive auditory stimuli and proximally exteroceptive somatosensory stimuli than to vagally mediated interoceptive stimuli. There are several potential explanations for this surprising finding. Since inspiratory occlusions stimulate thoracic tissue and muscles, thereby already activating the afferent vagal fibers, it is possible that additional activation via taVNS would not have had a significant contribution (i.e. the ceiling effect). Related to this, since participants experienced respiratory stimuli as more intense and unpleasant relative to auditory and electrocutaneous stimuli (see [Supplement, Figure S2](#_Figure_S2:_Self-reported)), the respiratory stimuli could have attracted attention in both conditions, negating potential stimulation effects. Previous studies investigating the effects of taVNS on experimentally delivered nociceptive stimuli (which are highly salient) also reported null effects, both in terms of subjective experience and laser-evoked potentials (Dumoulin et al., 2021; Laqua et al., 2014). It is also possible that the observed increase in P2 in AEP and SEP was due to diminished habituation, and that habituation was slower to develop in RREP, leading to no observable differences between conditions. This explanation is supported by our recent research, showing that both SEP and RREP amplitudes exhibit habituation, but that SEP amplitudes may be more susceptible relative to RREP amplitudes (Jelinčić et al., 2024). ERP methodology, being reliant on averaging, does not allow for the assessment of the habituation time-course. Future studies should therefore consider using single-trial or time-frequency EEG analyses, which would allow for a more sensitive estimation of habituation effects during continuous stimulation (Bauer et al., 2014; Hu et al., 2014). Finally, since AEP and SEP are evoked by precisely delivered stimuli, they tend to have a higher signal-to-noise ratio (SNR). In comparison, inspiratory occlusions are induced by the mechanical closure of the respiratory valve, which is less precise, in addition to further possible confounds from facial muscle artifacts and respiration. It is possible that the higher SNR allowed the small effects of condition to emerge in SEP and AEP, which were not evident in RREP.

## 4.3 No effects of taVNS on neural gating in either modality

We observed no effects on neural gating in either modality in the current study. While we did not hypothesize directional effects (see [Introduction](#_1._Introduction)), we expected to see significant differences between taVNS and Sham stimulation conditions in neural gating, given the previous pharmacological research showing interactions of neural gating with the LC-NA axis (Adler et al., 1994; Logemann et al., 2013; Stevens et al., 1993), and previous research reporting prolonged enhancing effects of nVNS on neural gating (Lewine et al., 2019). One likely explanation for these findings is that the large proportion of uninterpretable data (negative gating ratios) required us to use a difference measure of neural gating (S2-S1) as opposed to the ratio measure (S2/S1) which was used in all the above-referenced research. While the difference measure is a valid measure of neural gating, it is strongly biased towards the response to the first stimulus (S1; Jelinčić et al., 2021). Since we observed no condition effects in the S1 P50 nor S1 N1 in any of the modalities, it is not surprising that no neural gating effects were found. Furthermore, as discussed in our previous research (Jelinčić et al., 2023, 2024), ERP averaging is likely to introduce significant distortion of the S2 peaks due to small latency shifts on individual trials, leading to reduced power to find meaningful effects. There is at present no satisfactory solution to this, though research on neural gating using more sensitive time-frequency measures has become more frequent in the past years (Liang et al., 2023; Nguyen et al., 2020; Wiesman et al., 2017) and will likely supplement ERP measures in future research.

## 4.4 Exploratory findings

Our exploratory analyses uncovered no significant effects of stimulation condition on self-reported intensity or unpleasantness of any of the sensory stimuli (clicks, occlusions, electrocutaneous pulses). We found small effects (which were not significant) of increased self-reported intensity of auditory clicks and electrocutaneous stimuli during taVNS, which correspond to the increased P2 amplitudes we observed. We observed no effects on respiratory perception, which was somewhat unusual, given that respiratory perception tends to be strongly modulated by affective experimental manipulations which increase arousal (Bogaerts et al., 2019; Herzog, Sucec, Van Diest, Van Den Bergh, et al., 2018; Jelinčić et al., 2022, 2024). However, the small or null effects are not entirely unexpected, given that previous taVNS studies using nociceptive stimuli similarly found no effects on self-report (Dumoulin et al., 2021; Laqua et al., 2014). As posited above ([4.2](#_4.2_ERP_effects)), it is possible that the respiratory stimuli were too salient for their perception to be modulated by the small increase in the LC-NA axis activation. However, reducing the intensity of the stimuli would likely yield null effects due to range restriction in ratings, an issue we already discussed in our previous research on bodily perception in healthy volunteers (Jelinčić et al., 2023). These results call into question the efficacy of short-term taVNS in modulating bodily and symptom perception, however further clinical research in patient populations is needed to probe the potential utility of long-term, clinically administered taVNS.

Our exploratory probing of the subjective experience and safety of taVNS showed that participants experienced both taVNS and Sham stimulation as largely mild and tolerable. Side-effects such as pain, tingling, and itching were seldom reported, and were largely mild in intensity. We found, however, that even though both types of stimulation were perceived as neutral on average, taVNS was experienced to be more unpleasant than Sham, and was associated with a higher incidence of reported side-effects. This higher intensity and unpleasantness of taVNS relative to Sham has also been reported in previous taVNS research (D’Agostini, Burger, Franssen, et al., 2023; Keute et al., 2018), adding to the calls to reconsider earlobe as the optimal control stimulation site.

## 4.5 Limitations and future directions

There are several limitations to the current study that could have influenced the results and subsequent interpretations.

Firstly, our statistical power to detect ERP and neural gating effects was reduced by the exclusion of a number of data points due to measurement noise. The noise primarily arose from the electrical interference by the taVNS electrode, which introduced a strong 35Hz signal. Based on our previous research (D’Agostini, Burger, Jelinčić, et al., 2023), we anticipated this issue and delivered taVNS at 35Hz, rather than the more common 25Hz (Farmer et al., 2021), which would have required more aggressive low-pass filtering. While in most participants, the 35Hz interference was captured by the ICA and successfully removed, in several cases this was not possible without removing meaningful signal. Furthermore, the noise introduced bias, as it was not equally distributed across conditions – the interference was (up to three times) more likely in the active Sham condition. The likely reason for this was the placement of the electrode at the center of the left earlobe, which allowed for occasional physical contact between the electrode and the channels placed around the left ear in our high-density EEG set-up. In future studies, this issue could be mitigated by changing the Sham stimulation site, or using a non-active Sham condition. Another option is delivering short taVNS bursts followed by sensory stimulation, instead of continuously delivering taVNS concurrent with sensory stimulation (see [4.1](#_4.1_taVNS_increased)). There is also a possibility to further increase the frequency of taVNS, as previous studies showed that frequencies of up to 100Hz are safe and tolerated by participants (Lewine et al., 2019; Sclocco et al., 2020), or to use less dense EEG set-ups to avoid the contamination of signal by noise. Finally, future taVNS-ERP studies should consider it important to recruit large samples to ensure the statistical power necessary to detect small ERP effects, and to allow for more sophisticated analyses (for example, partial mediation analysis to assess whether subjective experienced arousal or sAA concentration mediate the taVNS – ERP relationships).

Related to the above mentioned issue of bias, participants did not experience taVNS and Sham stimulation equally. The calibration procedure aimed to ensure that taVNS and Sham stimulation felt equally intense to participants, however ratings given at the halfway point and towards the end of the stimulation indicated that participants experienced taVNS as more unpleasant relative to Sham. When asked about any lingering side-effects, participants reported higher incidence of pain, burning and tingling after taVNS compared to Sham. Given that pain in particular is an arousing sensation, this difference in subjective experience could have contributed to the observed effects in subjective experienced arousal. However, given that 66% of participants did not report any pain in either condition, and no participants reported pain higher than “very limited”, it is unlikely that this difference solely accounted for the observed effect. Nevertheless, it also points to the fact that earlobe may not be the optimal control stimulation site in terms of subjective experience (Keute et al., 2018). Future studies could investigate this by introducing a third crossover session, with inactive stimulation as the additional control condition, as done in previous studies (Janner et al., 2018).

Importantly, our sample was heavily skewed towards young, female participants, posing issues for the generalizability of the results. This skew could be especially relevant for the perception and neural processing of sensory stimulation, as previous studies found that taVNS (compared to Sham) was more successful in modulating pain responses in men (Janner et al., 2018). Future studies should aim at recruiting gender-balanced samples.

In conclusion, the current study provides limited support for the LC-NA hypothesis of taVNS, showing enhanced long-latency ERP amplitudes and subjective experienced arousal. However, we found no effects of taVNS on salivary alpha-amylase and spontaneous alpha oscillations, which further adds to the mixed findings in the experimental taVNS research. This complex pattern of findings indicates that the current experimental taVNS protocols can be further optimized, both in terms of stimulation parameters and the control condition, in order to elucidate the mechanism underlying therapeutic effects of taVNS.

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# Author Contributions

* V.J. acquired funding, developed the research questions, pre-registered and set up the experiment, collected the data, analyzed the data, wrote and edited the manuscript.
* M.D. acquired funding, set-up the experiment, analyzed the data, wrote and edited the manuscript.
* C.V-B. developed the research questions, supervised the research, and edited the manuscript.
* L.C. collected the data, analyzed the data, and edited the manuscript.
* E.G. collected the data, analyzed the data, and edited the manuscript.
* M.W. developed the research questions, supervised the research, and edited the manuscript.
* D.M.T. acquired funding, supervised the research, and edited the manuscript.
* I.VD. acquired funding, developed the research questions, supervised the research, and edited the manuscript.
* A.vL. acquired funding, developed the research questions, supervised the research, wrote and edited the manuscript.

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1. 17-year-olds were able to participate without parental consent, if they were 1st year bachelor students and if participation in experimental research formed a requirement in their course syllabus. [↑](#footnote-ref-2)