Tonic and phasic transcutaneous auricular Vagus Nerve Stimulation both evoke rapid and transient pupil dilation.

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Abstract

Transcutaneous auricular vagus nerve stimulation (tVNS or taVNS) is a non-invasive method of electrical stimulation of the afferent branch of the vagus nerve, suggested to drive changes in putative physiological markers of noradrenergic activity, including pupil dilation. However, it is unknown whether taVNS can map onto the phasic and tonic modes of noradrenergic activity. The effects of taVNS on pupil dilation in humans are inconsistent, largely due to differences in stimulation protocols. Here, we addressed these issues by investigating pupil dilation under phasic (1 s) and tonic (30 s) stimulation, in a single-blind, sham-controlled, within-subject cross-over design, in the absence of a behavioural task. Phasic taVNS induced a rapid increase in pupil size over baseline, significantly greater than under sham stimulation, which rapidly declined after stimulation offset. The 30 s tonic taVNS induced a similarly rapid (and larger than sham) increase in pupil size over baseline, returning to baseline within 5 s, despite the ongoing stimulation. Thus, both active and sham tonic modes closely resembled the phasic effect. There were no differences in tonic baseline pupil size, and no sustained effects of stimulation on tonic baseline pupil size. This suggests that both phasic- and tonic-like taVNS under the standard stimulation parameters may modulate the noradrenergic system in the phasic mode only, as indexed by evoked pupil dilation, over and above somatosensory effects. This result sheds light on the temporal profile of phasic and tonic stimulation, with implications for their applicability in further research.

Introduction

Transcutaneous auricular vagus nerve stimulation (tVNS or taVNS) is a non-invasive method of electrical stimulation of the afferent branch of the vagus nerve, delivered transcutaneously by placing the electrodes in the cymba conchae of the ear, which is innervated by the auricular branch of the vagus nerve (ABVN) (ref). Recent evidence suggests that taVNS can serve as a tool for modulating noradrenaline (NE) levels (Raedt et al., 2011), spurring promising results in treatment of pharmaco-resistant depression and epilepsy (Cristancho et al., 2011; Grimonprez et al., 2015; Morris et al., 2013), and indicating that it may be used in a task-dependent manner to probe noradrenergic influences on human behaviour (Ludwig et al., 2021; Villani et al., 2022). However, the effect of taVNS on markers of noradrenergic activity is still unclear, with different studies yielding an inconsistent pattern of results. This is likely a consequence of the variety of combinations of stimulation parameters that have been used (particularly intensity, pulse width, frequency, duration of stimulation) (D'Agostini et al., 2023; Ludwig et al., 2021). Given the limited understanding of the precise mechanism of action, a clearer mapping of taVNS-driven noradrenergic activity is imperative.

The afferent vagus nerve has highly diffuse targets in the brain, complicating the understanding of the neuromodulatory mechanisms of taVNS. The nerve terminates at the nucleus of the solitary tract (NTS), projecting to the parabrachial nucleus, locus coeruleus (LC; the primary noradrenergic (NE) release site), as well as the dorsal raphe nucleus (the primary source of serotonin) in the brainstem, and further to the periaqueductal grey, thalamus, nucleus accumbens, amygdala, insula, and hippocampus (Butt et al., 2020; Ruggiero et al., 2000; Sawchenko, 1983). Those targets have been found to be activated by taVNS in sham-controlled human neuroimaging studies (Assenza et al., 2017; Badran et al., 2018; Borgmann et al., 2021; Butt et al., 2020; Frangos et al., 2015; Yakunina et al., 2017).

Stimulation with implanted VNS (iVNS) in rodents has been shown to robustly elicit LC firing and increase noradrenergic discharge, in a dose-dependent manner (Collins et al., 2021; Hulsey et al., 2017; Raedt et al., 2011; Roosevelt et al., 2006). Two modes of LC-NE firing patterns have been described: the tonic mode, characterised by a continuous, low-frequency firing pattern, and phasic mode, characterised by short bursts at higher frequencies (Aston-Jones & Cohen, 2005). It is currently unclear whether and to what extent taVNS modes and parameters can map onto the two modes of LC-NE discharge. In addition, the picture is obscured by inconsistencies in stimulation protocols and the resulting biomarkers (see review by Ludwig et al., 2021).

In the current study, we focus on pupil diameter as a marker of LC-NE activity. Pupil dilation is sympathetically driven, primarily through activation of the LC, thought to increase activity in the dilator muscle while inhibiting activity in the iris sphincter (Joshi et al., 2016; Reimer et al., 2016;

Samuels & Szabadi, 2008). Using pupil size as a putative marker of noradrenergic activity in humans (Gilzenrat et al., 2010; Murphy et al., 2014), taVNS applied at the standard clinical duty protocol of 30 s on/30 s off typically results in an absence of a tonic change in pupil size (Borges et al., 2021; Burger et al., 2020; D'Agostini et al., 2021, 2022; Keute et al., 2019; Warren et al., 2019; see Table 1), although contrary results have been reported both for taVNS (Capone et al., 2021) and human iVNS (Desbeaumes Jodoin et al., 2015). In contrast, in more recent studies with short, phasic-like (600ms - 2 min) stimulation pulses, taVNS has been found to elicit a rapid pupil dilation, peaking and returning to baseline within a few seconds from stimulation onset (D'Agostini et al., 2023; Lloyd et al., 2023; Sharon et al., 2021; Urbin et al., 2021). A comparable effect has been found in rodents with iVNS (Collins et al., 2021; Hulsey et al., 2017; Mridha et al., 2021). Together, those results suggest that stimulating the LC through the afferent vagal projections might induce a phasic noradrenergic discharge reflected in rapid and short-lived pupil dilation. Nonetheless, the temporal profiles of tonic and phasic-like taVNS under matched parameters have not yet been compared.

Here, we set out to investigate pupil dilation under phasic (1 s) and tonic (30 s) stimulation, in a single-blind, sham-controlled, within-subject cross-over design, in the absence of a behavioural task. To our knowledge, the present study is the first to compare phasic and tonic stimulation protocols with matched parameters in a within-subject design at rest, thus elucidating the temporal profiles of pupil diameter changes under both stimulation modes. Our secondary motivation was to use pulses as short as technically possible in the phasic condition, in order to explore their efficacy as a phasic noradrenergic manipulation, which could be used in behavioural tasks. We expected to replicate the previous findings that short-burst taVNS evoke a phasic event-related response. Furthermore, if the tonic stimulation protocol elevates NE levels reflected in baseline pupil values, we expected to observe larger tonic baseline pupil values for active vs sham stimulation. Finally, if tonic stimulation elicits a short-lived event-related response which drops quickly after peaking despite ongoing stimulation, as suggested by Hulsey et al (2017), we hypothesised tonic stimulation to resemble the temporal profile of phasic stimulation. This approach can shed light on the efficiency of taVNS as a noradrenergic modulatory tool, as well as its potential for use in an event-related manner for behavioural tasks.

Table 1. Overview of past evidence on the effects of taVNS on pupil diameter (PD) across studies investigating phasic-like (brief, 0.6-5 s) stimulation and prolonged, tonic-like (30s ON/OFF or continuous) stimulation.

Stimulation mode		**Significant effects**		**No significant effects**	
		Event-related phasic analysis	**PD baseline analysis**	**Event-related phasic analysis**	**PD baseline analysis**
Phasic	**D'Agostini et al (2023)**	5 s ON/15 s OFF+ 3–5 s OFF **Resting**			
	Lloyd et al (2023) replication of Sharon et al (2021)	3.4 s ON / 25 OFF **Resting**			10-s pre-stimulation baseline
	Sharon et al (2021)	3.4 s ON / 25 OFF **Resting**			10-s pre-stimulation baseline
	Urbin et al (2021)	0.6 s ON/ 3 OFF + 6.4 OFF **Resting** (canal, concha, earlobe)			1-s pre-stimulus baseline
	Villani et al (2023)	3 s ON/ (min.) 9 s OFF **Oddball task**: target vs standard (tragus, earlobe)			
30s ON/OFF or continuous	**Borges et al (2021)**			**Continuous** 4 min Flanker task: post-error slowing	Pre-stimulus baseline PD (200 ms)
	Burger et al. (2020) Study, 1, 2, and 3			**30 s ON/OFF** Attentional Blink task (Study 2 and 3)	Resting measurement (2 min): pre-stimulation, at 40 min, at 80 min
	Capone et al (2021)		**Continuous** stimulation: (2 min) Sig. effect in: left eye, 2 mA intensity, scotopic illuminance		No significant effects for: right eye; photopic and mesopic luminance; scotopic illuminance and left eye at 0.5, 1, 3 mA stimulation intensity
	D'Agostini et al (2021)				**30 s ON/OFF** Resting measurement (3x 2 min) pre- and post-stimulation

Table 1 (continued)

D'Agostini et al (2022)	**Continuous** 10 min before and throughout the task Oddball task: standard, novelty, target	Pre-stimulus baseline PD in the oddball task Resting measurement (2 min) pre - vs. post-stimulation
Keute et al (2019)	**Continuous (13 min)**: Oddball task: target vs. standard stimuli	Resting measurement (1 min) at the beginning and the end of the session First five minutes of stimulation, without a task)
Warren et al (2019) Experiment 2		**30 s ON/OFF** Resting measurement (3x): pre-stimulation, at 50 min, at 70 min

Method

Participants

61 participants (38 females, 2 other/prefer not to say; M_{AGE} = 22.3, range = 18-34, age was missing for 4) were recruited to participate among the student population of Heinrich Heine University Düsseldorf. Participants were native German speakers, or spoke fluent or native English. Sample size of 52 was determined to be necessary for obtaining a small-to-medium effect size with 80% power (computed with G*Power (Faul et al., 2007) for the Active v Sham comparison), but extra participants were collected in order to account for possible drop-outs or exclusions. Three participants failed to complete one or both sessions, yielding 58 analysable participants. Participants were compensated at the rate of 10 EUR/h, with a 10 EUR bonus for attending both testing sessions (50 EUR total).

All participants had normal or corrected-to-normal vision. Participants reported no current or history of neurological or psychiatric disease, cardiovascular disease, arrhythmia, seizures, hypertension, head injury, hearing problems, vision problems, mobility problems, thyroid dysfunction, diabetes, asthma, epilepsy, glaucoma, haemophilia, impaired liver or kidney function, and no substance abuse. They had no cochlear implants, cardiac pacemakers, implanted vagus nerve stimulators (VNS), or cerebral shunts, and reported no current sores, dryness, or skin disorders (e.g. eczema) on the ears. They were required to take no prescription medication, centrally-acting or known to increase seizure risk (e.g. buproprion, neuroleptics, albuterol, theophylline, antidepressants,

thyroid medication, or stimulants). The study was approved by the Ethics Committee of the Medical Department at the HHU (ref. 2021-1754_1).

The study was preregistered on OSF at https://osf.io/6gsfp.

Stimuli and Equipment

The experiment was conducted in Matlab (version; Mathworks), running Psychtoolbox (Brainard, 1997). The task was presented on a 24 inch Asus PG248Q monitor (1440 by 900 resolution). The only stimulus used was a single black-and-white fixation point combining a bullseye and cross-hair (0.6 x 0.2 degrees of visual angle), generated following Thaler et al., 2013 recommendation to ensure stable central fixation and minimise eye movements. The fixation point was presented on a grey screen (RGB 100,100,100). The task code can be found at https://osf.io/v2u9s/ .

Eyetracking: Throughout the task, participant's pupil size, as well as horizontal and vertical coordinates of eye position from both eyes were recorded with an Eyelink1000 Plus (SR Research Ltd., Ontario, Canada) eye tracker. Data were sampled at 500 Hz (note that in 25% of participants data were sampled at a higher rate and downsampled prior to pre-processing), at 75% (in the majority of participants) or 100% illuminator power. Participants' heads were maintained in a stable position using a chinrest placed 78 cm from the screen, in a room with constant, dim, ambient light. No images of the pupils were collected.

taVNS: A NEMOS® NextGen (tVNS Technologies, Erlangen, Germany) taVNS device with legacy electrodes was used to stimulate the left auricular branch of the vagus nerve (ABVN). Two titanium electrodes, covered in a conductive pad and coated in conductive cream, were placed in the cymba conchae of the left ear for active stimulation, or on the left earlobe for sham stimulation. Particularly in the sham position, where electrode-skin contact was more challenging to maintain, electrodes were kept in place with medical tape and a headband, if needed (see Fig.1). Electrical stimulation was delivered as a biphasic square wave, with impulse frequency of 25 Hz and pulse width of 250 µs.

Stimulation intensity was calibrated individually for every participant with a work-up procedure to find a clearly perceptible, but not painful, sensation, within the range of 1-5 mA. In the calibration procedure, stimulation was manually started at the intensity of 1 mA, and increased in steps of 0.2 mA. After each stepwise increase, participants were asked to report their experience on a visual analogue scale from 1 ("I don't feel it at all/Ich fuehle nichts") to 5 ("schmerzhaft"/"painful"). The process continued until participants reached the rating of 5, in which case intensity was decreased to a level rated as 4 ("perceptible but not painful"). If the decreased intensity was rated as lower than

4, the stepwise increase again continued to a level rated as 5, in which case it was reduced until participants reported a sensation corresponding to level 4.

During the task, stimulation commands were sent to the NEMOS® device using a custom tVNS Technologies app (tVNS Manager, version 0.9.3) via a Bluetooth connection. The timings of stimulation onsets and offsets were controlled automatically from the Psychtoolbox/Matlab task by sending http POST requests to tVNS Manager, and were recorded in the Eyelink data file. Stimulation duration depended on the phasic or tonic condition (see *Procedure),* and was applied with immediate full-intensity onset (without ramp-up), which was critical for maintaining the 1 s stimulation duration. It also allowed for precise time-stamping of stimulation onsets.

Procedure

The study was conducted as a within-subjects, cross-over, single-blind design. Participants completed two sessions, one for the tonic and phasic conditions, scheduled at the same time of the day at least a week apart. The order of the conditions and the order of the stimulation modes within each condition were counterbalanced across participants. The second session additionally included a 5-min block of resting state pupillometry prior to the stimulated blocks, in order to assess the baseline pupil size at rest without any stimulation applied, while minimising the possibility of stimulation affecting the resting pupil size. See Fig. 1 for an illustration of the design.

In every session, participants were seated in a windowless, dimly-lit room with constant luminance. The first session began with the placement of the conductive gel or cream-coated electrode in the active position for intensity calibration. After a perceptible, but not painful, stimulation intensity level was established (see previous paragraph), participants were seated 78 cm away from the screen, with their heads placed in a chinrest. After the instructions were delivered, each block began with a 9-point eye-tracker calibration. Subsequently, participants were asked to fixate on a black-and-white fixation point, presented continuously, without offset, in the centre of a grey screen. Each trial in each block began with a 10 s baseline period (without stimulation), after which stimulation was applied in the corresponding condition/mode setting (see below). In the second session, a 5- min resting block was recorded. Afterwards, the electrodes were placed in the first location to be stimulated, and the intensity was gradually brought to the level determined in the first session, unless rated as painful earlier, in which case the new intensity level was maintained.

Phasic condition: The phasic condition followed the design of Sharon et al (2021), with the stimulation duration reduced to 1 s. Eight blocks of 11 30 s-long trials were conducted, four blocks (44 trials) in the Active mode, and four blocks (44 trials) in the Sham mode (mode order was counterbalanced across participants). The first trial in each block started with a 10 s baseline period

without stimulation, followed by 1 s ON-stimulation, followed by 29s OFF-stimulation. Subsequent trials in a block were separated by a jittered 800-1200ms intertrial interval.

Tonic condition: The tonic condition followed the classic taVNS duty cycle, 30-s on period, followed by a 30-s off period. Eight blocks of 6 60 s long trials were conducted, four blocks (24 trials) in the Active mode, and four blocks (24 trials) in the Sham mode. The first trial in each block started with a 10 s baseline period without stimulation, followed by 30 s ON-stimulation, followed by 30 s OFF-stimulation. Subsequent trials in a block were separated by a jittered 800-1200ms intertrial interval. Note that the reduced trial number compared to the phasic condition arose due to the doubled duration of each tonic trial in the 30 s on/off duty cycle.

Resting state measurement: The resting state measurement consisted of a single block of 11 30 s-long trials, without stimulation. Again, the first trial started with a 10 s baseline period. Subsequent trials were separated by a jittered 800-1200ms interval.

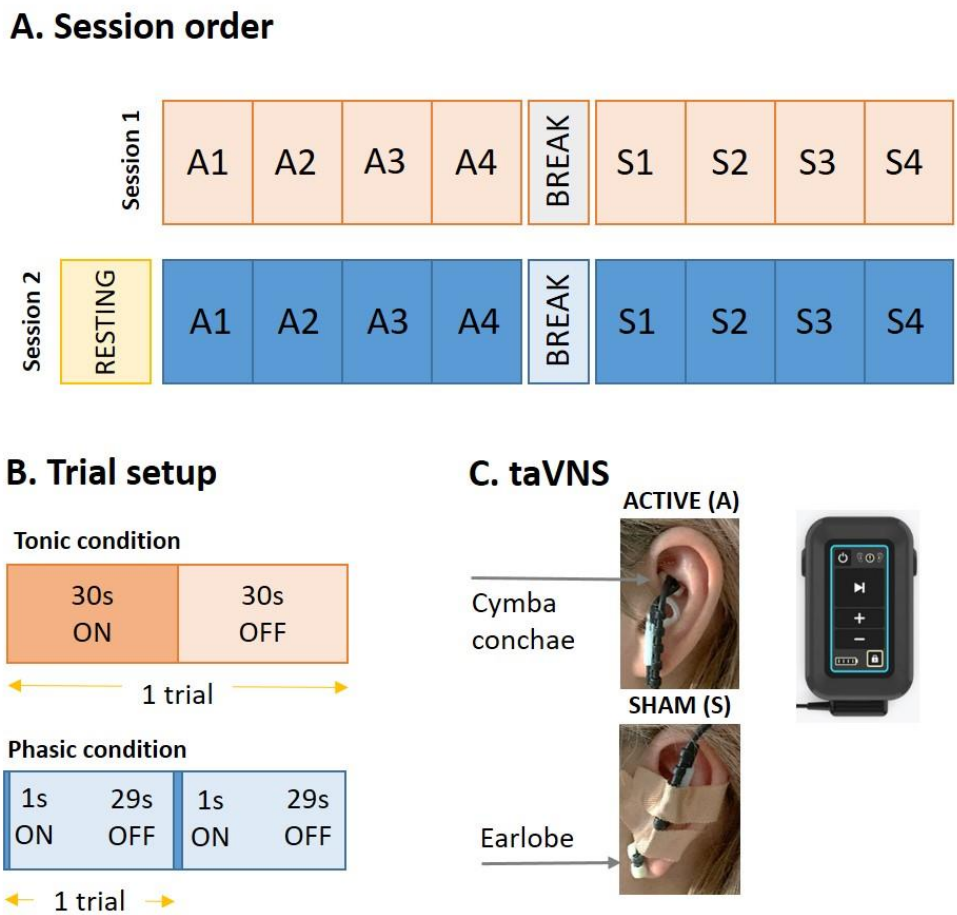

Figure 1. **A:** Example of session order and stimulation mode order in the sessions. Both the session order (e.g. phasic in session 1, tonic in session 2) and stimulation mode order (Active (A) first or Sham (S) first) were counterbalanced across subjects. **B:** Trial set-up. In the tonic condition, stimulation was delivered for 30 s (in both A and S modes), followed by 30 s off-stimulation, resulting in six 60 s long trials in each of the four blocks. In the phasic condition, stimulation was delivered for 1 s (in both A

and S modes), followed by 29s off-stimulation, resulting in 11 30 s long trials in each of the four blocks. **C:** Stimulation locations on the left ear for Active (electrodes placed in the cymba conchae) and Sham (electrodes placed on the earlobe and secured with medical tape) modes (photo credit: authors). taVNS NEMOS® NextGen device (image reproduced with permission by tVNS Technologies, Erlangen, Germany).

Hypotheses

Phasic

If phasic taVNS results in a phasic activation of the LC-NE system, we made a (pre-registered) prediction that baseline-corrected event-related pupil diameter increases following the Active ON stimulation, returning to baseline 4-8s into the off-stimulation period (following Sharon et al., 2021). Pupil diameter is predicted to be larger under Active stimulation than under Sham.

Tonic

If tonic taVNS results in a tonic activation of the LC-NE system, we made a number of pre-registered predictions:

a. *Active v Sham, 60 s period:* Averaged baseline pupil size is larger in the entire Active condition than in Sham.
b. *Active v Sham, ON periods:* Averaged baseline pupil size is larger during Active ON period than during Sham ON period.
c. *Sham-on v resting:* There should be no significant difference in baseline pupil size in Sham ON period and resting trials.

The following were formulated as research questions without directional predictions for the tonic stimulation condition:

d. *Active ON v Active OFF:* Is the effect of Active stimulation on pupil size sustained from the ON to OFF periods?
e. *Active OFF v Sham OFF:* Does the Active OFF period present a larger pupil size than Sham OFF period?
f. *Phasic-like activation, Active v Sham:* Does the onset of tonic stimulation present a phasic-like initial increase?

Data Preparation and Analysis

Data processing

Pupil diameter data recorded from both eyes were processed using Pupillometry Pipeliner (PuPL version 2.1.0; Kinley & Levy, 2022), running on Matlab. In the first step, data recorded as area were converted to diameter. Data recorded at higher sampling frequency than 500 Hz were

downsampled to 500 Hz. All data were smoothed with a 10 Hz moving mean window filter. Subsequently, blinks were detected using a velocity profile method (Maathot, 2013). Blinks were padded with 100 ms period pre- and post-blink, and linearly interpolated (the duration of the interpolated segment was maximum 1000 ms). Concatenated data were then segmented into epochs, depending on the condition to be analysed (phasic, tonic, and phasic-like tonic).

For the analysis of the phasic condition, data were segmented into epochs of 10 s before and 11 s after the stimulation onset. They were baseline corrected using the mean of the 10 s pre-stimulus period, and expressed as percent change from the baseline (Winn, Wendt, Koelewijn, & Kuchinsky, 2018).

For the analysis of the tonic condition, the data (with no baseline correction or transformation applied) were segmented into epochs of 30 s duration, starting from the stimulation onsets and offsets (ON periods and OFF periods respectively, see Fig. 1B). For the phasic-like analysis of the tonic condition, epochs of 10 s before and 30 s after the stimulation onset were extracted, baseline corrected, and expressed as percent change from the baseline, following the analysis of the phasic condition proper.

In all analyses, epochs in which 30% or more of data were missing were excluded. Additionally, data with the largest amounts of blinks were excluded (the threshold was determined based on the visual inspection of the data). The mean proportion of rejected epochs was 2.29% in tonic (range: 0-16% rejected), and 3.66% in phasic (range: 0-20% rejected)[1] .

Exclusion criteria

Participants with fewer than 50% of trials retained after blink correction and artefact rejection were flagged for removal – no such participants were present.

In the phasic condition, six subjects (out of the 58 analysable) were excluded: one could not tolerate stimulation intensity at the minimum predefined level (1 mA), and five were excluded due to either not feeling the stimulation on over 50% of blocks across the entire condition, or received a highly inconsistent stimulation intensity within any mode (e.g. stimulation being changed multiple times or by over 1mA within the condition, due to changes in pain threshold), yielding a sample of 52.

In the tonic condition, three subjects were excluded: one could not tolerate stimulation intensity at the minimum predefined level (1 mA), and two were excluded due to the criteria defined above, yielding a sample of 55.

[1] Note that two preprocessing steps diverge from the pre-registered ones: choice of the filter window, and the criteria for epoch rejection. These were motivated by the availability of processing options in the PuPl tool, and are compatible in terms of reduction of high-frequency noise, and artefact rejection.

Phasic analyses

The pre-registered analysis on the time-course of each trial was conducted with R Studio (R Core Team, 2021; RStudio Team, 2021). To compare the magnitude of pupil dilation (in percentage change over baseline) in Active versus Sham, mean pupil size values per participant per condition at each sampling point between 10 s before stimulation onset to 11 s post-stimulation were entered into paired Wilcoxon Signed Rank tests. The resulting p-values were corrected for false discovery rate (FDR) using the standard Benjamini-Hochberg (BH; Benjamini & Hochberg, 1995) method, using the *p.adjust* function from core R stats package[2].

Tonic analyses

For the pre-registered (and exploratory where stated) tonic comparisons, pupil sizes in arbitrary units (not baseline-corrected) were averaged into scalar values per participant per period of interest. All analyses were performed in JASP (v0.17.3; JASP Team, 2023), with default priors for the Bayes factors. For the pre-registered analysis of whether the onset of tonic stimulation causes a phasic-like initial increase in pupil size, the analysis proceeded in an identical fashion to the phasic analysis above, with mean pupil size values per participant per condition at each sampling point between 10 s before stimulation onset to 30 s post-stimulation onset (i.e. the entire ON period).

Resting analyses

For the pre-registered (and exploratory where stated) resting measurement (no stimulation applied) comparisons to tonic condition, pupil sizes in arbitrary units (not baseline-corrected) were averaged into scalar values per participant. All analyses were performed in JASP with default priors for the Bayes factors.

Results

Intensity control

We successfully maintained the same intensity for both the phasic and tonic conditions for Active stimulation in 35 participants, and for Sham stimulation in 31 participants. In both conditions, the range of intensities was 1-5 mA.

During stimulation in the phasic condition, intensity in the Active mode (M_{ACTIVE} = 2.72 mA, *SD* = 1.32) was not significantly different from intensity in the Sham mode (M_{SHAM} = 2.72 mA, *SD* = 1.31;

[2] Note that this deviates from our preregistered Benjamini-Yekutieli (Benjamini & Yekutieli, 2001) method for FDR correction. Two studies published after our pre-registration (D'Agostini et al., 2023, and Lloyd et al. 2023), which replicated Sharon et al (2021) findings, a used BH correction. Thus, we have opted for this correction in view of the recent publications.

W = 32.50, p = 0.645, BF_{10} = 0.15). We successfully kept the intensity identical between Active and Sham modes in 41/52 subjects (in 11 participants, intensity increase or reduction was necessary once the stimulation location changed). For those participants for whom the intensity needed to be adjusted within the block (3 participants), there was also no difference between the Active and Sham modes ($M_{\Delta ACTIVE}$ = 2.70 mA, SD = 1.34; $M_{\Delta SHAM}$ = 2.71 mA, SD = 1.32; W = 30.50, p = 0.789, BF_{10} = 0.17). 32 participants perceived Active stimulation as stronger, while 18 perceived Sham as stronger; two reported no difference.

During stimulation in the tonic condition, intensity in the Active mode (M_{ACTIVE} = 2.68 mA, SD = 1.23) was not significantly different from intensity in the Sham mode (M_{SHAM} = 2.64 mA, SD = 1.22; W = 49.00, p = 0.164, BF_{10} = 0.38). We successfully kept the intensity identical between Active and Sham modes in 43/55 subjects (in 12 subjects, intensity increase or reduction was once the stimulation location changed). For those participants for whom the intensity needed to be adjusted within the block (3 participants), there was also no difference between the Active and Sham modes ($M_{\Delta ACTIVE}$ = 2.67 mA, SD = 1.23; $M_{\Delta SHAM}$ = 2.64 mA, SD = 1.12; W = 45.50, p = 0.281, BF_{10} = 0.27). 32 participants perceived Active stimulation as stronger, while 21 perceived Sham as stronger; two reported no difference.

Phasic

Pupil dilation was observed in both Active and Sham stimulation modes. Active taVNS led to a rapid pupil dilation reaching its half-maximum (2.29% over baseline) at 0.57 s after stimulation onset, peaking (4.57% over baseline) at 1.43 s, and returning back to half-maximum at 2.51 s. Pupil size returned to its average baseline value at 4.91 s. Sham taVNS provoked a similarly rapid, but smaller in magnitude, pupil dilation reaching its half-maximum (1.21% over baseline) at 0.62 s after stimulation onset, peaking (2.41% over baseline) at 1.46s, and returning back to half-maximum at 2.47 s. Pupil size returned to its average baseline value at 4.63 s. Pupil dilation was significantly greater under Active taVNS stimulation than under Sham stimulation in the time range between 0.442 – 2.034 s post stimulation onset (ps < 0.05, paired Wilcoxon signed rank tests, FDR-BH-corrected; Fig. 2A). Average pupil dilation during the significant timeframe was 3.48% (SE = 0.02) for Active, 1.78% (SE = 0.01) for Sham. There was no difference in the baseline values between Active and Sham conditions (ps > 0.05, paired Wilcoxon signed rank test, FDR-BH-corrected; see Fig. 2A).

To further quantify the difference in pupil size between the conditions, we isolated the window of full duration at half-maximum (FDHM) from a grand average of Active and Sham time-courses (exploratory; following Sharon et al., 2021). The pupil dilation values for active (Mdn = 2.70%, SE = 0.44, M = 3.44%) and sham (Mdn = 0.99%, SE = 0.39, M = 1.78%) were then entered into a Bayesian

(default prior) paired Wilcoxon Signed Rank test in JASP. In the resulting FDHM of 0.59 – 2.50 s, active taVNS led to a significantly higher pupil dilation than sham taVNS (W = 1053, $p < .001$, $BF_{10} = 45.50$; Fig. 2B). Note that after removing two participants with the largest (over 10%) dilation, the difference between Active vs. Sham taVNS remained significant (W = 950.00, $p = .003$, $BF_{10} = 36.30$).

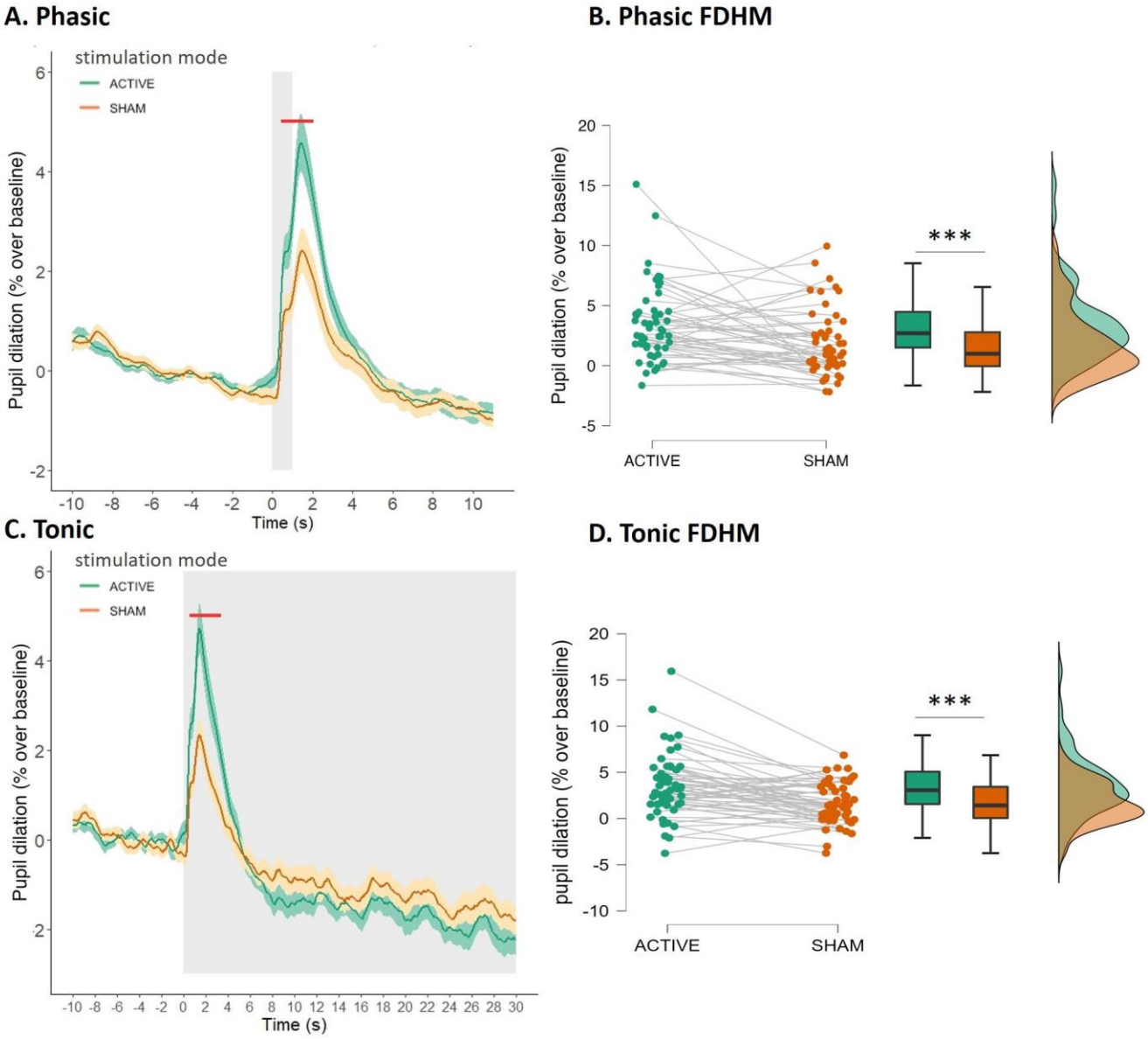

Figure 2. A/C: Pupil dilation (percentage change, baseline-corrected) in the phasic (A) and tonic (C) conditions, relative to a 10 s baseline before stimulation onset. Solid lines represent mean values. Ribbons represent SEM. The grey shading indicates stimulation duration (phasic: 1 s; tonic: 30 s, onsets without ramp-up). The short red lines indicate the period of significant difference between the values for active and sham taVNS (phasic: starting at 0.44 s post-stimulation onset, and ending at 2.03 s post-stimulation onset; tonic: starting at 0.55 s post-stimulation onset, and ending at 3.36 s post-stimulation onset). **B/D:** Average pupil dilation (percentage change over baseline) for active and sham taVNS, during the full duration at half maximum, in the phasic (B) and tonic (D) conditions. In the phasic condition, 35 participants had a higher response in active stimulation mode than in sham; 17

had a higher response in sham. In the tonic condition, 42 participants had a higher response in active stimulation mode than in sham; 13 had a higher response in sham.

Tonic: Phasic-like activation

Pupil dilation was observed in both Active and Sham stimulation modes. Active taVNS led to rapid pupil dilation reaching its half-maximum (2.34% over baseline) at 0.55s after stimulation onset, peaking (4.67% over baseline) at 1.45s, and returning back to half-maximum at 3.04s. Pupil size returned to its average baseline value at 5.00 s. Sham taVNS provoked a similarly rapid, but smaller in magnitude, pupil dilation reaching its half-maximum (1.17% over baseline) at 0.58s after stimulation onset, peaking (2.34% over baseline) at 1.44s, and returning back to half-maximum at 2.66s. Pupil size returned to its average baseline value at 4.67 s. Pupil dilation was significantly greater under active taVNS stimulation than under sham stimulation in the time range between 0.55 – 3.36s post stimulation onset (*ps* < 0.05, paired Wilcoxon signed rank tests at each sampling point from -10 s to 30 s post-stimulation onset, FDR-BH-corrected; Fig.2C). Average pupil dilation during the significant timeframe was 3.28% (*SE* = 0.01) for active, 1.52% (*SE* = 0.01) for sham. There was no difference in the baseline values between active and sham conditions (*ps* > 0.05, FDR-BH-corrected; Fig.2C). Early in the stimulation period, both active and sham time-courses strongly resembled the time-courses of the phasic condition, albeit peaking later, and continuing slightly longer.

To compare the average difference in pupil size between the conditions, we isolated the window of FDHM from a grand average of active and sham time-courses. The median pupil dilation values for active (*Mdn* = 3.07%, *SE* = 0.46, *M* = 3.49%) and sham (*Mdn* =1.42%, *SE* = 0.30, *M* = 1.64%) were then entered into a Bayesian (default prior) paired Wilcoxon Signed Rank test in JASP. In the resulting FDHM of 0.56 – 2.93s, active taVNS led to significantly higher pupil dilation than sham taVNS (W = 1232.00, p < .001, BF_{10} = 599.56; see Fig.2D). Note that after removing two participants with the largest (over 10%) dilation, the difference between active vs. sham taVNS remained significant (W = 1123.00, *p* < .001, BF_{10} = 212.08).

Tonic

Active v Sham, 60 s period (hypothesis a.): Averaged pupil size from the 60-s period following the onset of Active stimulation (i.e., collapsing 30 s ON and 30 s OFF periods) was compared with the pupil size in the 60-s Sham period with a paired Bayesian Wilcoxon signed-rank test. Mean pupil size values in the entire 60-s period were slightly lower for Active (*M* = 4677.83, *SE* = 75.03) than for Sham (*M* = 4688.63, *SE* = 72.61), and the difference was not significant (W = 732.00, *p* = 0.753; BF_{10} = 0.17; Fig. 3).

Active v Sham, 45s period (exploratory): In order to isolate potential sustained shifts in pupil diameter, and to account for phasic-like pupil dilation peaking rapidly after the onset of the stimulation, and returning to the baseline before 15 s (see Fig. 3A), we then compared averaged pupil sizes between Active and Sham modes discounting the first 15 s of ON stimulation (resulting in a 45s period) with a paired Bayesian Wilcoxon test. Here, similarly to above, no difference in pupil size between Active (M = 4655.70, SE = 75.27) and Sham (M = 4674.27, SE = 72.56; W = 711.00, p = 0.624; BF_{10} = 0.19) was observed.

Active v Sham, ON periods (hyp. b.): Averaged pupil size in the 30 s ON period of the active mode was compared to the 30 s ON period of the sham mode with a paired Bayesian Wilcoxon test. There was no difference pupil size between Active (M = 4724.11, SE = 75.68) and Sham (M = 4730.60, SE = 73.03; W = 736.00, p = 0.779; BF_{10} = 0.16).

Active ON v Active OFF (hyp. d.): Averaged pupil size in the 30 s Active ON period was compared with averaged pupil size in the 30 s Active OFF period, to assess whether the effect of taVNS would be sustained from the ON to OFF period. In the paired Bayesian Wilcoxon test, pupil size in Active ON (M = 4724.11, SE = 75.67) was significantly larger than pupil size in Active OFF (M = 4631.55, SE = 74.66; W = 1512.00, $p < .001$, BF_{10} = 59876.90), suggesting that there was a decrease in tonic pupil size, rather than a sustained effect of Active stimulation from the ON to OFF period.

Sham ON v Sham OFF (exploratory): Averaged pupil size in the 30 s Sham ON period was compared with averaged pupil sizes in the 30 s Sham OFF period. In the paired Bayesian Wilcoxon test, pupil size in Sham ON (M = 4730.60, SE = 73.03) was significantly larger than pupil size in Sham OFF (M = 4646.65, SE = 72.45; W = 1505.00, $p < .001$, BF_{10} = 7808.77), suggesting that, similarly to Active ON vs Active OFF comparison, there was a decrease in tonic pupil size, rather than a sustained effect of Sham stimulation the ON to OFF period.

Active OFF v Sham OFF (hyp. e.): Averaged pupil size in the 30 s Active OFF period was compared with averaged pupil size in the same period under Sham. In the paired Bayesian Wilcoxon test, there was no difference in pupil size values between Active OFF (M = 4631.55, SE = 74.66) and Sham OFF (M = 4646.65, SE = 72.45; W = 738.00, p = 0.792, BF_{10} = 0.17).

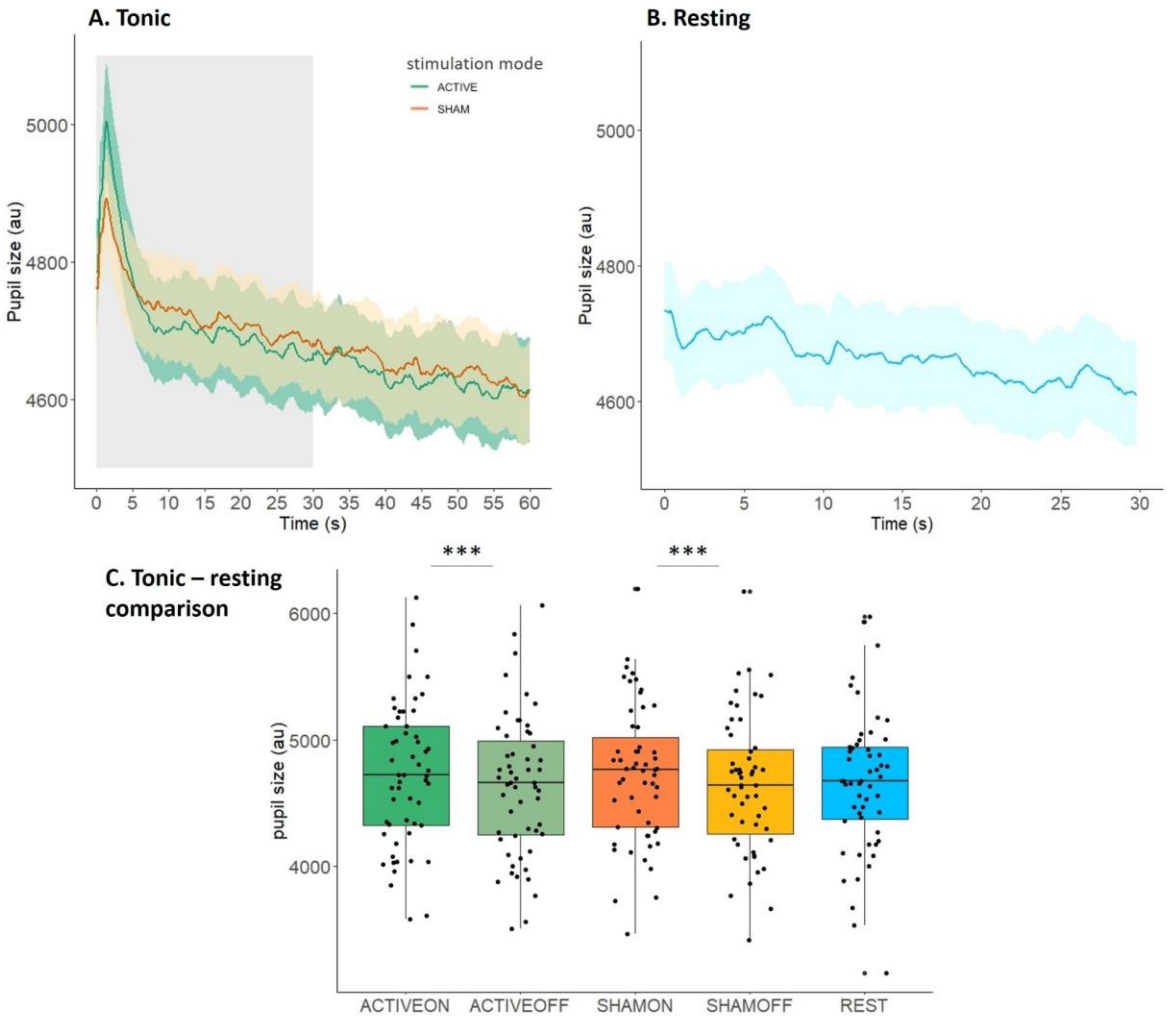

Figure 3. A: Tonic condition, averaged tonic baseline pupil sizes (non-corrected) across the entire 60-s trial span, for Active and Sham. The grey shading indicates stimulation duration (30 s, onset without ramp-up). **B:** Resting pupil measurement (no stimulation applied), averaged pupil size (non-corrected) across the 30-s blocks. **C:** Comparison of pupil sizes under tonic stimulation in Active (ON/OFF) and Sham (ON/OFF) modes, and the resting measurement.

Resting pupil size

Sham ON v Resting (hyp. c.): Averaged pupil size in the 30 s Sham ON period was compared with the average pupil size in the 30 s resting period. In the paired Bayesian Wilcoxon test, there was no difference in pupil size between Sham ON (*M* = 4730.96, *SE* = 74.05) and resting (*M* = 4662.85, *SE* = 74.01; W = 585.00, *p* = 0.122, BF_{10} = 0.40), with Bayes factor providing weak support for the absence of a difference.

Active ON v Resting (exploratory): Averaged pupil size in the 30 s Active ON period was compared with the average pupil size in the 30 s resting period. In the paired Bayesian Wilcoxon test, there was no difference in pupil size between Active ON (*M* = 4724.55, *SE* = 75.67) and resting (*M* = 4662.85, *SE* = 74.01; W = 602.00, *p* = 0.160, BF_{10} = 0.35), with Bayes factor providing weak support for

the absence of a difference. In conclusion, mean pupil size values under any kind of stimulation did not differ from resting pupil size values.

Discussion

A better understanding of noradrenergic effects of taVNS is essential for refining its applications as a therapeutic tool, as well as its efficiency as an experimental manipulation to probe neuromodulatory influences on human behaviour. In this study, we compared the effects of phasic-like and tonic-like taVNS on pupil size, a putative biomarker associated with LC-NE activity, in a single, sham-controlled within-subject design at rest – to our knowledge, the first such investigation.

In the phasic (1 s) stimulation condition, we found that active taVNS applied to left cymba conchae induced a rapid increase in pupil size over baseline while stimulation was being delivered, peaking soon after stimulation offset, and rapidly declining within two seconds. The magnitude of this increase was significantly larger than sham stimulation to the earlobe. This result corroborates recent findings that relatively brief taVNS pulses are able to elicit pupil dilation in humans (D'Agostini et al., 2023; Lloyd et al., 2023; Sharon et al., 2021; Urbin et al., 2021), as well as the rodent literature showing a comparable effect of iVNS (Collins et al., 2021; Hulsey et al., 2017; Mridha et al., 2021). Hence, our result strengthens the claim that taVNS pulses as brief as 1 s might modulate the noradrenergic system in the phasic mode, as indexed by evoked pupil dilation.

In the tonic (30 s on/30 s off standard duty cycle) condition, matched for location and intensity, active taVNS induced a similarly rapid increase in pupil size over baseline, which was significantly larger than sham stimulation (phasic-like analysis). Importantly, however, the pupil size rapidly declined back to baseline within 5s into the ongoing stimulation, in both active and sham modes. The time course of the peaking pupil dilation resembled the rapid onset observed in phasic stimulation, but the dilation in the tonic condition continued for over a second longer than in the phasic condition before returning to baseline (while stimulation was still being delivered). We further unpacked the tonic effects with a preregistered battery of comparisons on Active-Sham mean pupil size values in the ON and OFF periods. Tonic baseline pupil sizes in the whole 30-s ON period, as well as in the OFF period, did not significantly differ between Active and Sham stimulation. For both Active and Sham stimulation, pupil sizes in the ON period were significantly higher than in the OFF period, demonstrating that the increase in pupil size following stimulation was not sustained. Our analysis approach, uncovering the transient event-related effect in the tonic stimulation condition, may explain why the pre- vs post-stimulation comparisons of pupil size, or comparisons performed on binned time points, often applied to study the tonic effects of stimulation, failed to find evidence of taVNS modulation of pupil size (Borges et al., 2021; Burger et al., 2020; D'Agostini et al., 2021, 2022; Keute et al., 2019; Warren et al., 2019). Overall, this result suggests that prolonged taVNS under the

standard set of parameters does not seem to modulate pupil size in a sustained manner – instead, it presents a phasic-like transient response (Hulsey et al., 2017).

This result raises the question of why tonic stimulation would induce only a short-lived pupil dilation, rather than a sustained effect. Recently, D'Agostini et al., 2023 speculated that longer stimulation durations could elevate tonic noradrenergic firing, obscuring phasic pupil responses or phasic LC activity more broadly (Aston-Jones & Cohen, 2005). However, in our study, rapid phasic responses at the onset of the tonic stimulation were clearly present. We also did not observe any evidence of an elevated tonic baseline - in fact, in addition to the decrease in tonic baseline from the ON period to OFF period, we observed a steady decrease as the block progresses, under both Active and Sham stimulation modes (see Supplementary Materials; note that a decrease from the beginning to the end of the task was also observed by Keute et al., 2019, while an opposite effect was found by Warren et al., 2019). Similar decrease in pupil size over time has been reported previously over the course of longer trials (Zhao et al., 2019), entire tasks (Unsworth et al., 2019), and over the course of resting measurements (Burger et al., 2020; Tsukahara et al., 2016). This decrease has been associated with fatigue and reduction in vigilance (Hopstaken et al., 2015; Martin et al., 2022). Nonetheless, it may be argued that the decline in pupil size throughout the block may have obscured potential fine-grained tonic pupil effects induced by stimulation. Although our analyses were not able to capture this, this notion merits further study, as the effect may exist on a different time-scale than captured by our analyses. Finally, it is plausible that pupil dilation may be a better suited readout of phasic, rather than tonic, LC activity (Ludwig et al., 2021).

Another possible reason behind the absence of a sustained effect of tonic stimulation on the tonic baseline pupil size could be parameter choice. Firstly, it is possible that the stimulation session (24 minutes per Active/Sham mode) may have been too short to observe tonic pupil effects. Previous studies exploring tonic stimulation in humans typically use slightly longer durations (e.g. 40-55 min in D'Agostini et al., 2021). However, as described earlier, they also failed to observe the effect of prolonged stimulation on pupil size (in addition to other noradrenergic biomarkers, such as salivary alpha amylase). It could be that the effects of stimulation manifest under durations of hours or days, with compound effects of multiple stimulation sessions. Indeed, continuous 30 s on/ 5 min off iVNS in rodents delivered for 14 days showed increases in NE in the prefrontal cortex and hippocampus (Manta et al., 2013). Elsewhere, one hour iVNS in rodents delivered at regular intervals showed a systematic increase in extracellular NE levels, which returned to baseline when not stimulated (Roosevelt et al., 2006). It has been proposed that such noradrenergic modulations drive the clinical effects of chronic VNS as an adjunct treatment for depression and epilepsy in humans (Cristancho et

al., 2011; Grimonprez et al., 2015; Morris et al., 2013). Consequently, short-duration tonic taVNS might not evoke detectable shifts in the tonic LC-NE , or detectable readouts in baseline pupil size.

Secondly, it is plausible that the stimulation frequency of 25 Hz (NEMOS® default) applied in our and similar studies was well suited for a phasic effect, but ill-suited for a sustained tonic effect. In mice, increasing pulse frequencies affected the timing of LC activity, with greater increases in LC firing over a short period of time (Hulsey et al., 2017). This opens up a suggestion that tonic effects on pupil size may still be found with lower frequency of stimulation over a longer period, matched to the 0.5-5 Hz frequency of tonic LC firing (Aston Jones & Cohen, 2005; Berridge & Waterhouse, 2003; Florin-Lechner et al. 2016; Hulsey et al., 2017). This possibility remains to be investigated. Here, we varied only the duration of stimulation (1 s vs 30 s) while keeping the other parameters constant on their standard settings (25 Hz frequency, 250 µs pulse width; notably, the intensities were matched between tonic and phasic conditions within participants as closely as possible, an issue we return to). This bolsters the notion that the interaction of stimulation duration with frequency might be an important factor in targeting the LC-NE system, with the standard setting combination potentially permitting only rapid but short-lived, phasic effects, but not sustained, tonic effects. This could be particularly important for applying phasic taVNS during behavioural tasks – although our study cannot provide a comment on the interaction between phasic stimulation and stimulus-evoked phasic responses.

Finally, it has been noted that general neuromodulatory and psychological effects of taVNS might depend on intensity in a dose-dependent manner. LC-NE activity readouts appear to increase monotonically with increased stimulation intensity (D'Agostini et al., 2023; Hulsey et al., 2016), and there might be an intensity threshold under which LC-NE is not sufficiently activated (Helmers et al., 2012 propose a minimum range of 0.75-1.75 mA for iVNS, which is likely to be higher for taVNS due to skin impedance and subcutaneous tissue affecting current flow; Ludwig et al., 2021). A systematic manipulation of total charge per pulse (intensity*pulse width) revealed a dose-dependent effect of taVNS on pupil dilation, which was stronger in active than in sham stimulation, in a study using 5-s stimulation (D'Agostini et al., 2023). Intensity effects could also be confounded by individual differences in perceptual thresholds, and interactions with other parameters. For instance, it has been noted that perceptual thresholds may decrease with increasing pulse width (Badran et al., 2019; note tragus stimulation). Current understanding of the interaction of objective intensity, subjective sensation, and the resulting outcomes remains limited (see Ludwig et al., 2021, for a review).

It is also plausible that the phasic pupil response in both phasic-proper and at the onset of tonic stimulation reflects not taVNS-mediated noradrenergic activity, but rather surprise or a response to a sudden intense sensation caused by the stimulation, similar to a typical stimulus-evoked phasic

dilation. Indeed, somatosensory stimulation consistently evokes phasic pupil dilation (Gusso et al., 2021; Joshi et al., 2016; Nieuwenhuis et al., 2011), which has been proposed to reflect an orienting response, a suite of central and peripheral processes facilitating the processing of novelty or threat (Sokolov, 1963). The orienting response itself has been proposed to rely on the LC-NE system (Bouret & Sara, 2005; Nieuwenhuis et al., 2011; Sara & Bouret, 2012), with the phasic responses observable at moderate levels of tonic discharge indicative of task responsiveness or engagement, in line with the Adaptive Gain Theory (Aston-Jones & Cohen, 2005). Our observation that both the phasic and tonic taVNS, in both Active and Sham modes, elicit a rapid, short-lived phasic pupil response would support the interpretation that pupil dilation could have been partly a reflection of orienting to a sudden somatosensory stimulus, regardless of stimulation location. However, the significantly higher pupil dilation in the Active mode in comparison to Sham suggests that taVNS may still be able to induce phasic LC-NE activity over and above the orienting or somatosensory effect.

Another possibility, related to the above, is that the orienting or somatosensory effects interact with perceived intensity, visible as Active-Sham differences in stimulation-evoked pupil response. In this study, the objective intensity levels were matched as closely as possible, in an effort to keep the intensity comparable between the conditions and modes. In this vein, stimulation intensity was established for the Active mode with a subjective perception scale, and re-adjusted should participants perceive it to be either painful or poorly detectable in the Sham mode, but it was not established anew for Sham. This may have had the unintended consequence of producing a higher perceived intensity of Active stimulation as compared to Sham, thus driving a larger orienting or somatosensory effect under Active stimulation. However, in recent studies, independently established Sham intensity was typically higher than in Active mode, still leading to the same direction of the effect as found here (Lloyd et al., 2023; Sharon et al., 2021). In addition, dose-dependent effects of intensity were found to be stronger for Active stimulation mode than for Sham (D'Agostini et al., 2023). Therefore, we consider the likelihood of perceived intensity entirely driving the observed effect small.

Despite taVNS driving the robust phasic pupil dilation, considered chiefly a noradrenergic biomarker, a considerable limitation to note is that taVNS is not a targeted tool - stimulation of the vagus nerve also activates other neurotransmitter systems, with substantial neural interconnections, including the GABAergic (Dorr & Debonnel, 2006; Ben-Menachem et al., 1995; Capone et al., 2015), serotonergic (Dorr & Debonnel, 2006), and cholinergic (Mridha et al., 2021) systems. However, it has been noted that VNS-driven increase in LC firing occurs earlier than the increase in dorsal raphe (main source of serotonin) firing, which has been interpreted as serotonergic effects being secondary to the noradrenergic ones (Dorr & Debonell, 2006). Indeed, the LC receives direct projections from the NTS, the central target of the vagus nerve (Butt et al., 2020; Ruggiero et al., 2000; Sawchenko, 1983).

Moreover, lesions to the LC abolish the noradrenergically-modulated antidepressant effects of VNS in rodents (Grimonprez et al., 2015; Nemeroff et al., 2006).

Similarly, despite ample evidence that LC activity is reflected in pupil size changes (de Gee et al., 2021; Gilzenrat et al., 2010; Joshi et al., 2016; Murphy et al., 2014; Reimer et al., 2016; Samuels & Szabadi, 2008), one cannot isolate the noradrenergic pathway as a sole contributor to pupil size changes. Not only noradrenergic, but also cholinergic axons have been shown to be involved in pupil size control (Reimer et al., 2016), and iVNS-driven pupil dilation in rodents has been associated with cholinergic activity (Mridha et al., 2021). However, noradrenergic activity is considered to be closely associated with rapid, phasic changes in pupil size, while cholinergic activity is linked to tonic pupil changes at larger timescales, e.g. during locomotion (Reimer et al., 2016). Recently, transient pupil size changes have also been linked to serotonin release, particularly under conditions of low uncertainty (Cazettes et al., 2021). This has been interpreted as reflecting prediction error or surprise, a signal also attributed to LC-NE (Dayan & Yu, 2006; de Gee et al., 2021), thus potentially implicating both systems in phasic (stimulus- or task-related) control of pupil size.

To conclude, in this study, we compared the effects of phasic-like (1 s) and tonic-like (30 s ON/OFF) taVNS on pupil size, a putative biomarker associated with LC-NE activity, in a single, sham-controlled within-subject design at rest – to our knowledge, the first such investigation. We show that 1 s taVNS to the left cymba conchae induced a rapid and transient increase in pupil size over baseline during stimulation, which was significantly larger than under sham stimulation to the earlobe. This suggests that brief taVNS pulses may modulate the noradrenergic system in the phasic mode, as indexed by evoked pupil dilation, over and above any potential somatosensory effect. Importantly, 30 s tonic taVNS induced a similarly rapid increase in event-related pupil size over baseline, significantly larger than under sham stimulation, resembling the transient phasic effect. No sustained effects on tonic baseline pupil size were observed. This result sheds light on the temporal profile of phasic and tonic stimulation, with implications for their applicability in further research. It suggests that the standard stimulation parameters may be better suited for phasic, rather than tonic, modulation of the noradrenergic system, and addresses the inconsistent findings in the field.

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Conflict of Interest Statement

The authors declare no conflicts of interest.

CRediT Author Statement

LS: Conceptualisation, Methodology, Software, Investigation, Data Curation, Formal Analysis, Visualisation, Writing: Original draft, Writing: Review & editing. **AM:** Conceptualisation, Methodology, Software, Investigation, Data Curation, Formal Analysis, Writing: Original draft, Writing: Review & editing. **GJ:** Conceptualisation, Funding Acquisition, Resources, Writing: Review & editing.

References:

Assenza, G., Campana, C., Colicchio, G., Tombini, M., Assenza, F., Di Pino, G., & Di Lazzaro, V. (2017). Transcutaneous and invasive vagal nerve stimulations engage the same neural pathways: In-vivo human evidence. *Brain Stimulation*, *10*, 853–854. https://doi.org/10.1016/j.brs.2017.03.005

Aston-Jones, G., & Cohen, J. D. (2005). AN INTEGRATIVE THEORY OF LOCUS COERULEUS-NOREPINEPHRINE FUNCTION: Adaptive Gain and Optimal Performance. *Annual Review of Neuroscience*, *28*(1), 403–450. https://doi.org/10.1146/annurev.neuro.28.061604.135709

Badran, B. W., Dowdle, L. T., Mithoefer, O. J., Labate, N. T., Coatsworth, J., Brown, J. C., Devries, W. H., Austelle, C. W., Mcteague, L. M., George, M. S., States, U., States, U., Ground, A. P., States, U., States, U., States, U., & States, U. (2018). Neurophysiologic effects of transcutaneous auricular vagus nerve stimulation (taVNS) via electrical stimulation of the tragus: A concurrent taVNS/fMRI study and review. *Brain Stimulation*, *11*(3), 492–500. https://doi.org/10.1016/j.brs.2017.12.009.

Benjamini, Y., & Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society. Series B (Methodological)*, *57*(1), 289–300.

Benjamini, Y., & Yekutieli, D. (2001). The control of the false discovery rate in multiple testing under dependency. *The Annals of Statistics*, *29*(4), 1165–1188.

Borges, U., Pfannenstiel, M., Tsukahara, J., Laborde, S., Klatt, S., & Raab, M. (2021). Transcutaneous vagus nerve stimulation via tragus or cymba conchae: Are its psychophysiological effects dependent on the stimulation area? *International Journal of Psychophysiology*, *161*, 64–75. https://doi.org/10.1016/j.ijpsycho.2021.01.003

Borgmann, D., Rigoux, L., Kuzmanovic, B., Edwin Thanarajah, S., Münte, T. F., Fenselau, H., & Tittgemeyer, M. (2021). Technical Note: Modulation of fMRI brainstem responses by transcutaneous vagus nerve stimulation. *NeuroImage*, *244*, 118566. https://doi.org/10.1016/J.NEUROIMAGE.2021.118566

Bouret, S., & Sara, S. J. (2005). Network reset: A simplified overarching theory of locus coeruleus noradrenaline function. *Trends in Neurosciences*, *28*(11), 574–582. https://doi.org/10.1016/J.TINS.2005.09.002

Brainard, D. H. (1997). The Psychophysics Toolbox. *Spatial Vision*, *10*(4), 433–436.

Burger, A. M., Van der Does, W., Brosschot, J. F., & Verkuil, B. (2020). From ear to eye? No effect of transcutaneous vagus nerve stimulation on human pupil dilation: A report of three studies. *Biological Psychology*, *152*, 107863. https://doi.org/10.1016/J.BIOPSYCHO.2020.107863

Butt, M. F., Albusoda, A., Farmer, A. D., & Aziz, Q. (2020). The anatomical basis for transcutaneous auricular vagus nerve stimulation. *Journal of Anatomy*, *236*(4), 588–611. https://doi.org/10.1111/JOA.13122

Capone, F., Motolese, F., Di Zazzo, A., Antonini, M., Magliozzi, A., Rossi, M., Marano, M., Pilato, F., Musumeci, G., Coassin, M., & Di Lazzaro, V. (2021). The effects of transcutaneous auricular vagal nerve stimulation on pupil size. *Clinical Neurophysiology*, *132*(8), 1859–1865. https://doi.org/10.1016/J.CLINPH.2021.05.014

Cazettes, F., Reato, D., Morais, J. P., Renart, A., & Mainen, Z. F. (2021). Phasic Activation of Dorsal Raphe Serotonergic Neurons Increases Pupil Size. *Current Biology*, *31*(1), 192-197.e4. https://doi.org/10.1016/j.cub.2020.09.090

Collins, L., Boddington, L., Steffan, P. J., & McCormick, D. (2021). Vagus nerve stimulation induces widespread cortical and behavioral activation. *Current Biology*, *31*(10), 2088-2098.e3. https://doi.org/10.1016/J.CUB.2021.02.049

Cristancho, P., Cristancho, M. A., Baltuch, G. H., Thase, M. E., & O†Reardon, J. P. (2011). Effectiveness and Safety of Vagus Nerve Stimulation for Severe Treatment-Resistant Major Depression in Clinical Practice After FDA Approval: Outcomes at 1 Year. *The Journal of Clinical Psychiatry*, *72*(10), 5594. https://doi.org/10.4088/JCP.09m05888blu

D'Agostini, M., Burger, A. M., Franssen, M., Claes, N., Weymar, M., Leupoldt, A., & Van Diest, I. (2021). Effects of transcutaneous auricular vagus nerve stimulation on reversal learning, tonic pupil size, salivary alpha-amylase, and cortisol. *Psychophysiology*, 1–20. https://doi.org/10.1111/PSYP.13885

D'Agostini, M., Burger, A. M., Franssen, M., Perkovic, A., Claes, S., von Leupoldt, A., Murphy, P. R., & Van Diest, I. (2023). Short bursts of transcutaneous auricular vagus nerve stimulation enhance evoked pupil dilation as a function of stimulation parameters. *Cortex*, *159*, 233–253. https://doi.org/10.1016/j.cortex.2022.11.012

D'Agostini, M., Burger, A. M., Villca Ponce, G., Claes, S., von Leupoldt, A., & Van Diest, I. (2022). No evidence for a modulating effect of continuous transcutaneous auricular vagus nerve stimulation on markers of noradrenergic activity. *Psychophysiology*, *59*(4), e13984. https://doi.org/10.1111/psyp.13984

Dayan, P., & Yu, A. J. (2006). Phasic norepinephrine: A neural interrupt signal for unexpected events. *Network: Computation in Neural Systems*, *17*, 335–350. https://doi.org/10.1080/09548980601004024

de Gee, J. W., Correa, C. M. C., Weaver, M., Donner, T. H., & van Gaal, S. (2021). Pupil Dilation and the Slow Wave ERP Reflect Surprise about Choice Outcome Resulting from Intrinsic Variability in Decision Confidence. *Cerebral Cortex*, *31*(7), 3565–3578. https://doi.org/10.1093/cercor/bhab032

Desbeaumes Jodoin, V., Lespérance, P., Nguyen, D. K., Fournier-Gosselin, M. P., & Richer, F. (2015). Effects of vagus nerve stimulation on pupillary function. *International Journal of Psychophysiology*, *98*(3), 455–459. https://doi.org/10.1016/J.IJPSYCHO.2015.10.001

Dorr, A. E., & Debonnel, G. (2006). Effect of vagus nerve stimulation on serotonergic and noradrenergic transmission. *Journal of Pharmacology and Experimental Therapeutics*, *318*(2), 890–898. https://doi.org/10.1124/JPET.106.104166

Faul, F., Erdfelder, E., Lang, A.-G., & Buchner, A. (2007). G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behavior Research Methods*, *39*(2), 175–191. https://doi.org/10.3758/bf03193146

Frangos, E., Ellrich, J., & Komisaruk, B. R. (2015). Non-invasive Access to the Vagus Nerve Central Projections via Electrical Stimulation of the External Ear: FMRI Evidence in Humans. *Brain Stimulation*, *8*(3), 624–636. https://doi.org/10.1016/j.brs.2014.11.018

Gilzenrat, M. S., Nieuwenhuis, S., Jepma, M., & Cohen, J. D. (2010). Pupil diameter tracks changes in control state predicted by the adaptive gain theory of locus coeruleus function. *Cognitive, Affective and Behavioral Neuroscience*, *10*(2), 252–269. https://doi.org/10.3758/CABN.10.2.252

Grimonprez, A., Raedt, R., Portelli, J., Dauwe, I., Larsen, L. E., Bouckaert, C., Delbeke, J., Carrette, E., Meurs, A., De Herdt, V., Boon, P., & Vonck, K. (2015). The antidepressant-like effect of vagus nerve stimulation is mediated through the locus coeruleus. *Journal of Psychiatric Research*, *68*, 1–7. https://doi.org/10.1016/J.JPSYCHIRES.2015.05.002

Gusso, M. de M., Serur, G., & Nohama, P. (2021). Pupil Reactions to Tactile Stimulation: A Systematic Review. *Frontiers in Neuroscience*, *15*. https://doi.org/10.3389/fnins.2021.610841

Helmers, S. L., Begnaud, J., Cowley, A., Corwin, H. M., Edwards, J. C., Holder, D. L., Kostov, H., Larsson, P. G., Levisohn, P. M., De Menezes, M. S., Stefan, H., & Labiner, D. M. (2012). Application of a computational model of vagus nerve stimulation. *Acta Neurologica Scandinavica*, *126*(5), 336–343. https://doi.org/10.1111/j.1600-0404.2012.01656.x

Hopstaken, J. F., van der Linden, D., Bakker, A. B., & Kompier, M. A. J. (2015). The window of my eyes: Task disengagement and mental fatigue covary with pupil dynamics. *Biological Psychology*, *110*, 100–106. https://doi.org/10.1016/j.biopsycho.2015.06.013

Hulsey, D. R., Riley, J. R., Loerwald, K. W., Rennaker, R. L., Kilgard, M. P., & Hays, S. A. (2017). Parametric characterization of neural activity in the locus coeruleus in response to vagus nerve stimulation. *Experimental Neurology*, *289*, 21–30. https://doi.org/10.1016/J.EXPNEUROL.2016.12.005

Joshi, S., Li, Y., Kalwani, R. M., & Gold, J. I. (2016). Relationships between Pupil Diameter and Neuronal Activity in the Locus Coeruleus, Colliculi, and Cingulate Cortex. *Neuron*, *89*(1), 221–234. https://doi.org/10.1016/j.neuron.2015.11.028

Keute, M., Demirezen, M., Graf, A., Mueller, N. G., & Zaehle, T. (2019). No modulation of pupil size and event-related pupil response by transcutaneous auricular vagus nerve stimulation (taVNS). *Scientific Reports*, *9*(1), 11452. https://doi.org/10.1038/s41598-019-47961-4

Lloyd, B., Wurm, F., de Kleijn, R., & Nieuwenhuis, S. (2023). Short-term transcutaneous vagus nerve stimulation increases pupil size but does not affect EEG alpha power: A replication of Sharon et al. (2021). *Brain Stimulation*, *16*(4), 1001–1008. https://doi.org/10.1016/j.brs.2023.06.010

Ludwig, M., Wienke, C., Betts, M. J., Zaehle, T., & Hämmerer, D. (2021). Current challenges in reliably targeting the noradrenergic locus coeruleus using transcutaneous auricular vagus nerve stimulation (taVNS). *Autonomic Neuroscience*, *236*, 102900. https://doi.org/10.1016/J.AUTNEU.2021.102900

Manta, S., El Mansari, M., Debonnel, G., & Blier, P. (2013). Electrophysiological and neurochemical effects of long-term vagus nerve stimulation on the rat monoaminergic systems. *International Journal of Neuropsychopharmacology*, *16*(2), 459–470. https://doi.org/10.1017/S1461145712000387

Martin, J. T., Whittaker, A. H., & Johnston, S. J. (2022). Pupillometry and the vigilance decrement: Task-evoked but not baseline pupil measures reflect declining performance in visual vigilance tasks. *European Journal of Neuroscience*, *55*(3), 778–799. https://doi.org/10.1111/ejn.15585

Morris, G. L., Gloss, D., Buchhalter, J., Mack, K. J., Nickels, K., & Harden, C. (2013). Evidence-based guideline update: Vagus nerve stimulation for the treatment of epilepsy: Report of the Guideline Development Subcommittee of the American Academy of Neurology. *Neurology*, *81*(16), 1453–1459. https://doi.org/10.1212/WNL.0b013e3182a393d1

Mridha, Z., Gee, J. W. de, Shi, Y., Alkashgari, R., Williams, J., Suminski, A., Ward, M. P., Zhang, W., & McGinley, M. J. (2021). Graded recruitment of pupil-linked neuromodulation by parametric stimulation of the vagus nerve. *Nature Communications*, *12*(1), 1–14. https://doi.org/10.1038/S41467-021-21730-2

Murphy, P. R., O'Connell, R. G., O'Sullivan, M., Robertson, I. H., & Balsters, J. H. (2014). Pupil diameter covaries with BOLD activity in human locus coeruleus. *Human Brain Mapping*, *35*(8), 4140–4154. https://doi.org/10.1002/hbm.22466

Nemeroff, C. B., Mayberg, H. S., Krahl, S. E., McNamara, J., Frazer, A., Henry, T. R., George, M. S., Charney, D. S., & Brannan, S. K. (2006). VNS therapy in treatment-resistant depression: Clinical evidence and putative neurobiological mechanisms. *Neuropsychopharmacology*, *31*(7), 1345–1355. https://doi.org/10.1038/sj.npp.1301082

NEMOS® NextGen (2021) transcutaneous auricular vagus nerve stimulator, Research Version, tVNS Technologies, Erlangen, Germany. https://t-vns.com/en/?lang=en

Nieuwenhuis, S., De Geus, E. J., & Aston-Jones, G. (2011). The anatomical and functional relationship between the P3 and autonomic components of the orienting response. *Psychophysiology*, *48*(2), 162–175. https://doi.org/10.1111/j.1469-8986.2010.01057.x

Raedt, R., Clinckers, R., Mollet, L., Vonck, K., El Tahry, R., Wyckhuys, T., De Herdt, V., Carrette, E., Wadman, W., Michotte, Y., Smolders, I., Boon, P., & Meurs, A. (2011). Increased hippocampal noradrenaline is a biomarker for efficacy of vagus nerve stimulation in a limbic seizure model. *Journal of Neurochemistry*, *117*(3), 461–469. https://doi.org/10.1111/j.1471-4159.2011.07214.x

Reimer, J., McGinley, M. J., Liu, Y., Rodenkirch, C., Wang, Q., McCormick, D. A., & Tolias, A. S. (2016). Pupil fluctuations track rapid changes in adrenergic and cholinergic activity in cortex. *Nature Communications*, *7*, 13289. https://doi.org/10.1038/ncomms13289

Roosevelt, R. W., Smith, D. C., Clough, R. W., Jensen, R. A., & Browning, R. A. (2006). Increased extracellular concentrations of norepinephrine in cortex and hippocampus following vagus nerve stimulation in the rat. *Brain Research*, *1119*(1), 124–132. https://doi.org/10.1016/j.brainres.2006.08.048

Ruggiero, D. A., Underwood, M. D., Mann, J. J., Anwar, M., & Arango, V. (2000). The human nucleus of the solitary tract: Visceral pathways revealed with an "in vitro" postmortem tracing method. *Journal of the Autonomic Nervous System*, *79*(2), 181–190. https://doi.org/10.1016/S0165-1838(99)00097-1

Samuels, E. R., & Szabadi, E. (2008). Functional neuroanatomy of the noradrenergic locus coeruleus: its role in regulation of arousal and autonomic function part I: Principles of functional organisation. *Current Neuropharmacology, 6,* 235-253. https://doi.org/10.2174/157015908785777229

Sara, S. J., & Bouret, S. (2012). Orienting and Reorienting: The Locus Coeruleus Mediates Cognition through Arousal. *Neuron*, *76*(1), 130–141. https://doi.org/10.1016/j.neuron.2012.09.011

Sawchenko, P. E. (1983). Central connections of the sensory and motor nuclei of the vagus nerve. *Journal of the Autonomic Nervous System*, *9*(1), 13–26. https://doi.org/10.1016/0165-1838(83)90129-7

Sharon, O., Fahoum, F., & Nir, Y. (2021). Transcutaneous Vagus Nerve Stimulation in humans induces pupil dilation and attenuates alpha oscillations. *The Journal of Neuroscience*, *41*(2), 320–330. https://doi.org/10.1523/JNEUROSCI.1361-20.2020

Sokolov, E. N. (1963). Higher nervous functions: The orienting reflex. *Annual Review of Physiology*, *25*, 545–580.

Thaler, L., Schütz, A. C., Goodale, M. A., & Gegenfurtner, K. R. (2013). What is the best fixation target? The effect of target shape on stability of fixational eye movements. *Vision Research*, *76*, 31–42. https://doi.org/10.1016/J.VISRES.2012.10.012

Tsukahara, J. S., Harrison, T. L., & Engle, R. W. (2016). The relationship between baseline pupil size and intelligence. *Cognitive Psychology*, *91*, 109–123. https://doi.org/10.1016/j.cogpsych.2016.10.001

Unsworth, N., Robison, M. K., & Miller, A. L. (2019). Individual differences in baseline oculometrics: Examining variation in baseline pupil diameter, spontaneous eye blink rate, and fixation stability. *Cognitive, Affective, & Behavioral Neuroscience*, *19*(4), 1074–1093. https://doi.org/10.3758/s13415-019-00709-z

Urbin, M. A., Lafe, C. W., Simpson, T. W., Wittenberg, G. F., Chandrasekaran, B., & Weber, D. J. (2021). Electrical stimulation of the external ear acutely activates noradrenergic mechanisms in humans. *Brain Stimulation*, *14*(4), 990–1001. https://doi.org/10.1016/J.BRS.2021.06.002

Villani, V., Finotti, G., Di Lernia, D., Tsakiris, M., & Azevedo, R. T. (2022). Event-related transcutaneous vagus nerve stimulation modulates behaviour and pupillary responses during an auditory oddball task. *Psychoneuroendocrinology*, *140*(March), 105719. https://doi.org/10.1016/j.psyneuen.2022.105719

Warren, C. M., Tona, K. D., Ouwerkerk, L., van Paridon, J., Poletiek, F., van Steenbergen, H., Bosch, J. A., & Nieuwenhuis, S. (2019). 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. *Brain Stimulation*, *12*(3), 635–642. https://doi.org/10.1016/J.BRS.2018.12.224

Yakunina, N., Kim, S. S., & Nam, E.-C. (2017). Optimization of Transcutaneous Vagus Nerve Stimulation Using Functional MRI. *Neuromodulation: Technology at the Neural Interface*, *20*(3), 290–300. https://doi.org/10.1111/NER.12541