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Three Hundred Hertz Transcutaneous Auricular Vagus Nerve Stimulation (taVNS) Impacts Pupil Size Non-Linearly as a Function of Intensity

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ABSTRACT

Transcutaneous auricular vagus nerve stimulation (taVNS) is a neuromodulatory technique that may have numerous potential health and human performance benefits. However, optimal stimulation parameters for maximizing taVNS efficacy are unknown. Progress is impeded by disagreement on the identification of a biomarker that reliably indexes activation of neuromodulatory systems targeted by taVNS, including the locus coeruleus-norepinephrine (LC-NE) system. Pupil size varies with LC-NE activity and is one potential taVNS biomarker that has shown inconsistent sensitivity to taVNS in prior studies. The present study examined the relationship between pupil size and taVNS using stimulation parameters that have shown promising behavioral effects in prior studies but have received comparatively little attention. Participants received trains of 50 μs taVNS pulses delivered continuously below perceptual threshold at 300 Hz to the left external acoustic meatus (EAM) while pupil size was recorded during a pupillary light reflex task. Analysis of pupil size using generalized additive mixed modeling (GAMM) revealed a non-linear relationship between taVNS intensity and pupil diameter. Active taVNS increased pupil size during stimulation for participants who received taVNS between 2 and approximately 4.8 mA, but not for participants who received higher-intensity taVNS (up to 8.1 mA). In addition, taVNS effects persisted in subsequent blocks, mitigating decreases in pupil size over the course of the task. These findings suggest 300 Hz taVNS activates the LC-NE system when applied to the EAM, but its effects may be counteracted at higher intensities.

1 | Introduction

Transcutaneous auricular vagus nerve stimulation (taVNS) is a promising, non-invasive technique for inducing broad neuromodulatory changes throughout the brain by applying weak electrical current to parts of the outer ear that receive vagal innervation. taVNS is a lower-risk and lower-cost alternative to invasive (implanted) vagus nerve stimulation (iVNS), which has several FDA-approved applications but requires surgery and is limited to patient populations. Research on taVNS has increased markedly in recent years due to numerous potential health and human performance applications, including but not limited to treatment for epilepsy (Bauer et al. 2016; Lampros et al. 2021), depression (Hein et al. 2013; Kong et al. 2018; Rong et al. 2016), anxiety (Burger et al. 2016, 2019), posttraumatic stress disorder (Lamb et al. 2017), chronic pain

(Busch et al. 2013; Farmer et al. 2020), migraine (Barbanti et al. 2015; Straube et al. 2015), cluster headache (Gaul et al. 2016), tinnitus (Hyvärinen et al. 2015; Kreuzer et al. 2012; Yakunina and Nam 2021), and schizophrenia (Hasan et al. 2015); as well as improving upper limb motor rehabilitation (Wu et al. 2020) and infant oral feeding (Badran et al. 2020); see Verma et al. (2021) for a systematic review and discussion of study limitations. In healthy adults, taVNS has also been linked to short-term cognitive benefits in learning and memory (Giraudier, Ventura-Bort, and Weymar 2020; Jacobs et al. 2015; Llanos et al. 2020; McHaney et al. 2023; Pandža et al. 2020; Phillips et al. 2021), executive function (Ridgewell et al. 2021), as well as improved mood and reduced anxiety (Calloway et al. 2020).

Despite its promise, use of taVNS outside of the laboratory is hindered by the lack of a reliable method for titrating stimulation to achieve the desired neuromodulatory effects. Studies of iVNS parametric manipulations indicate that its efficacy depends on stimulation parameters, including but not limited to intensity, pulse width, and frequency (Hulsey et al. 2017; Mridha et al. 2021). Moderate-intensity stimulation (0.4–0.8 mA, depending on the study) has been shown to optimize the neuroplastic (Borland et al. 2016; Morrison et al. 2019, 2020) and behavioral effects (Pruitt et al. 2021) of iVNS in animal models (for a recent summary, see Hays, Rennaker, and Kilgard 2023). In humans, the effect of iVNS on cognition has shown a similar intensity-dependent relationship (Clark et al. 1999; Vonck et al. 2014). Importantly, in both humans and animal models, iVNS delivered outside of a narrow intensity range does not enhance desired neural or behavioral outcomes (Clark et al. 1999; Morrison et al. 2019, 2020). In some cases, higher intensities may even have a negative effect (Clark et al. 1999; Helmstaedter, Hoppe, and Elger 2001; Morrison et al. 2021). Efficacy of taVNS is likely to similarly vary as a function of intensity given that common mechanisms are hypothesized for both techniques (more on this below).

Parametric studies of taVNS are necessary to establish optimal stimulation parameters; however, this endeavor is complicated by the fact that it is not possible to directly measure the amount of electrical current that reaches the auricular branch of the vagus nerve (ABVN) from stimulating electrodes placed on the skin of the outer ear. Further, estimating the amount of charge reaching vagal afferents from the stimulating electrode is not possible due to individual differences in skin impedance and cutaneous distribution among regions of the outer ear that are thought to receive vagal innervation (Butt et al. 2020; Ludwig et al. 2021; Yap et al. 2020). Titrating taVNS parameters to optimize its effectiveness across individuals thus requires a biomarker that reliably indexes activation of the hypothesized target neuromodulatory systems (Burger, D'Agostini, et al. 2020). The present study contributes to this endeavor by showing a non-linear relationship between pupil dilation—one potential taVNS biomarker that has shown inconsistent prior results—and high-frequency taVNS intensity.

1.1 | Purported Mechanisms of Action

The taVNS mechanism of action is thought to be like that of iVNS, which involves triggering the broad release of neurotransmitters including norepinephrine (NE; Dorr and Debonnel 2006; Hassert, Miyashita, and Williams 2004; Hulsey et al. 2019; Manta et al. 2009; Roosevelt et al. 2006), acetylcholine (ACh; Hulsey et al. 2016; Mridha et al. 2021), and serotonin (5-HT; Dorr and Debonnel 2006; Hulsey et al. 2019; Manta et al. 2009). This occurs via the relay of sensory information from the auricular branch of the vagus nerve (ABVN) by the nucleus of the solitary tract (NTS) to the locus coeruleus (LC) and its projections to the raphe nuclei and throughout the cortex (Badran et al. 2018; Ruffoli et al. 2011; Sclocco et al. 2019); for a recent review, see Butt et al. (2020). Given the hypothesized central role of the LC-NE system in driving taVNS effects, several biomarkers that reflect LC-NE activity have been assessed for their sensitivity to taVNS, including concentrations of salivary alpha amylase (sAA) and cortisol; pupil size (including short-lived changes time-locked to stimulation or task events that reflect phasic LC activity, and more sustained changes that reflect LC tonic activity); spectral power of the electroencephalogram (EEG); intensity and latency of the P3 component of the event-related potential (ERP); heart rate variability; respiratory rate; and salivary flow rate (for a review, see Burger, D'Agostini, et al. 2020). Of these measures, using pupillometry to assess taVNS efficacy is of particular interest because changes in pupil size show a relatively tight temporal coupling to fluctuations in NE as well as ACh activity (Aston-Jones and Cohen 2005; Joshi et al. 2016; Murphy et al. 2014; Reimer et al. 2016; Samuels and Szabadi 2008b) and can be easily measured and analyzed in near real time at the level of the individual at relatively low cost.

Direct stimulation of the LC has been shown to cause pupil dilation in animal models (Breton-Provencher and Sur 2019; Joshi et al. 2016; Liu et al. 2017; Megemont, McBurney-Lin, and Yang 2022; Reimer et al. 2016). Crucially, direct stimulation of vagal afferents via *iVNS* has shown similar causal effects on pupil dilation in patients (Desbeaumes Jodoin et al. 2015; but cf. Schevernels et al. 2016) and in animal models (Bianca and Komisaruk 2007; Mridha et al. 2021), demonstrating that the impact of electrical vagus stimulation on LC activity can in principle be measured in real time via changes in pupil size. However, the impact of *taVNS* on pupillary measures has been inconsistent (Burger, D'Agostini, et al. 2020). Pupil size is affected by multiple neural circuits, and the relationship between pupil dilation and the neuromodulators that influence these circuits has not been fully characterized (Joshi et al. 2016). Inconsistent effects of taVNS on pupil size are due in part to uncertainty about the underlying physiological mechanisms and how they may be influenced by specific taVNS parameters. In line with the current state of knowledge, we present changes in pupil size due to VNS as primarily reflecting fluctuations in LC activity, but we acknowledge that this relationship only partially reflects the activity of neuromodulatory systems that may or may not be impacted by vagal stimulation. Identifying a biomarker that reliably indexes taVNS is necessary, but it is only a first step in establishing links between taVNS and specific neuromodulatory changes.

1.2 | Overview of Previous Studies of the Impact of taVNS on the Pupil Response

To date, 16 studies have been published examining the effects of taVNS on the size and timing of the pupil response.

These studies, summarized in Table 1, have targeted three regions of the external ear known to receive vagal innervation: the concha, tragus, and external acoustic meatus (EAM; Butt et al. 2020). Thirteen of the 16 published studies targeted the concha with 200–300 μs taVNS pulses delivered at 25–30 Hz (Borges et al. 2021; Burger, Van der Does, et al. 2020; D'Agostini et al. 2021, 2022, 2023; Keute et al. 2019; Lloyd et al. 2023; McHaney et al. 2023; Sharon, Fahoum, and Nir 2021; Skora, Marzecová, and Jocham 2024; Urbin et al. 2021; Warren et al. 2019; Wienke et al. 2023). Of these studies, two also tested parameters that deviated from these ranges: Urbin et al. (2021) included 300 Hz taVNS and D'Agostini et al. (2023) included a 400 μs pulse-width condition. Across these studies, taVNS intensity and titration method varied. Four studies tested fixed taVNS intensity at 0.2 and 0.5 mA (D'Agostini et al. 2023), 0.5 mA (Burger, Van der Does, et al. 2020; Warren et al. 2019), and 3.0 mA (Keute et al. 2019). Five studies tested taVNS delivered just below pain threshold, with reported intensities ranging from 0.25 to 5.0 mA (D'Agostini et al. 2021, 2022, 2023; Lloyd et al. 2023; Sharon, Fahoum, and Nir 2021). The remaining five studies tested taVNS intensities below, at, or above perceptual threshold (but below pain threshold), with reported values ranging from 0.08 to 4.4 mA (Borges et al. 2021; McHaney et al. 2023; Skora, Marzecová, and Jocham 2024; Urbin et al. 2021; Wienke et al. 2023).

Only six of the 13 studies targeting the concha noted an effect of taVNS on pupil size. In five studies, taVNS increased the amplitude of short-lived pupil dilation that was time-locked to brief trains (0.6–5 s) of taVNS compared to a control condition (D'Agostini et al. 2023; Lloyd et al. 2023; Sharon, Fahoum, and Nir 2021; Skora, Marzecová, and Jocham 2024; Urbin et al. 2021). Participants in these studies were not engaged in a task during pupil measurement; therefore, the difference in pupil dilation can be attributed to taVNS itself and can be characterized as a stimulation-evoked pupillary response (SEPR). This is conceptually distinct from a task-evoked pupillary response (TEPR), which reflects the cognitive processing involved in performing an active task. TEPR tasks have included a range of sensory and cognitive demands, such as the identification of infrequently occurring target stimuli in an active oddball task (D'Agostini et al. 2022; Keute et al. 2019), detection of a target occurring after a similar nontarget in an attentional blink task (Burger, Van der Does, et al. 2020), cognitive control tasks such as the Flanker task (Borges et al. 2021) or Stroop task (Wienke et al. 2023), as well as tasks involving the recognition of nonnative speech sounds (McHaney et al. 2023). In one study, longer trains (30 s) of taVNS also elicited a short-lived increase in pupil size, which resembled the SEPR elicited by shorter taVNS trains in the same study (Skora, Marzecová, and Jocham 2024). In the sixth study, taVNS increased TEPR amplitude during an active task and reduced constriction during a pupillary light reflex task (Wienke et al. 2023).

Two studies measured pupillary responses to taVNS applied to the tragus (Borges et al. 2021; Villani et al. 2022). Only Villani et al. (2022) found an effect of taVNS on TEPR amplitude, though opposite from the expected direction. In that study, 500 μs taVNS pulses were delivered at 25 Hz just below perceptual threshold for 3 s around stimulus onset in an auditory oddball task. The effects of taVNS were largely null, only decreasing TEPR peak amplitude for trials with low baseline pupil size determined by a median split.

Three studies have measured pupillary responses to taVNS applied to the EAM. All three studies found an effect of taVNS on pupil size. In Capone et al. (2021), 300 μs taVNS pulses delivered at 20 Hz continuously while pupil size was measured elicited larger resting-state pupil size in the left eye (but not the right eye), but only for 2.0 mA taVNS during low illuminance. No taVNS-related effects were observed under medium or high illuminance for any taVNS intensity (0, 0.5, 1.0, or 3.0 mA). Urbin et al. (2021) directly compared taVNS rate (25 versus 300 Hz), stimulation site (concha vs. EAM), and intensity (0%, 80%, 100%, 150%, and 200% of perceptual threshold). Increasing taVNS intensity generally increased amplitude and decreased onset and peak latency of the SEPR time-locked to ~0.6 s taVNS trains, with the strongest effects observed for 300 Hz taVNS delivered to the EAM. taVNS delivered to the EAM at and above perceptual threshold increased pupil size over earlobe (active sham) stimulation and increased pupil diameter over concha stimulation at 150% of perceptual threshold. The area under the curve between pupil response onset and peak also showed that 300 Hz taVNS applied to the EAM elicited larger pupil responses than 25 Hz taVNS delivered to the same location and 300 Hz taVNS delivered to the concha or earlobe. Pupil response onset latency was also shorter for 300 Hz versus 25 Hz taVNS across sites, and onset latency was shorter for EAM than concha.

Pandža et al. (2020) examined behavioral and pupillary effects of delivering 50 μs taVNS pulses to the EAM at 300 Hz and 0.2 mA below perceptual threshold during a foreign language learning study. Behavioral improvements and electrophysiological changes (reported in Phillips et al. 2021) were observed for participants who received active taVNS (both 0.5 s taVNS trains time-locked to task stimuli and 10 min taVNS trains administered before tasks), and these changes coincided with distinct changes in the TEPR across two training days. However, given the nature of the training task, the TEPR changes in Pandža et al. (2020) were interpreted as reflecting between-group differences in the allocation of effort during learning, rather than reflecting the direct effect of taVNS on the LC-NE system.

That many studies to date have found no effect of taVNS on pupil size has led to questioning whether taVNS can actually modulate LC activity (Burger and Verkuil 2018). However, there are at least three factors that may contribute to the absence of taVNS effects on pupil size in previous work that do not necessarily depend on taVNS failing to activate the LC. The first pertains to taVNS parameters and how taVNS-related changes in pupil size are quantified. The taVNS parameters most often used in previous studies may not drive sufficient change in LC activity required for coupling taVNS and pupil dilation, given recent evidence that pupil dilation may not accurately track LC activity below a certain LC firing rate (Megemont, McBurney-Lin, and Yang 2022). Short-lived pupil dilation time-locked to an event—taVNS in SEPR, a task stimulus in TEPR—has been interpreted to reflect changes in phasic LC activity, while longer-lasting changes in pupil size during rest are hypothesized to reflect tonic LC activity (e.g., Burger, D'Agostini, et al. 2020; Burger, Van der Does, et al. 2020; Capone et al. 2021; Skora,

TABLE 1 | Summary of taVNS studies measuring pupillary response.

Citation	Sub-study	taVNS location	Pulse width (μs)	Rate (Hz)	Intensity (mA)	Mean (SD) intensity (mA)	Intensity titration method	taVNS timing and duration	*N*	Eye analyzed	Task during pupil measurement	Pupillary index	Significant effect
Warren et al. (2019)		Cymba concha	200–300	25	0.5	0.5 (0)	Fixed	30 s on/off during tasks (75 min)	25	NR	No task (fixation)	Resting state	NA
Keute et al. (2019)		Cymba concha	200	25	3.0	3.0 (0)	Fixed	continuous during tasks (~18 min)	33	Right	Active oddball; no task (fixation)	TEPR; resting state	NA
Burger, Van der Does, et al. (2020)	1	Cymba concha	250	25	0.5	0.5 (0)	Fixed	30 s on/off during tasks (80 min)	94	NR	No task (fixation)	Resting state	NA
	2	Cymba concha	250	25	0.5	0.5 (0)	Fixed	30 s on/off during tasks (32 min)	30	NR	Attentional blink; no task (fixation)	TEPR; resting state	NA
	3	Cymba concha	250	25	0.5	0.5 (0)	Fixed	30 s on/off during tasks (32 min)	80	NR	Attentional blink; no task (fixation)	TEPR; resting state	NA
Pandža et al. (2020)		**EAM**	**50**	**300**	**2.0–10.0**	**NR**	**0.2 mA< perceptual threshold**	**continuous before task (10 min × 3); 0.5 s per trial**	**69**	**Right**	**Nonnative speech perception**	**TEPR**	**Amplitude, latency, duration**
Sharon, Fahoum, and Nir (2021)	**1**	**Cymba concha**	**200–300**	**25**	**≤ 5.0**	**2.2 (0.24)**	**Just below pain threshold (max 5 mA)**	**3.4 s per trial**	**24**	**Dominant eye**	**No task (fixation)**	**SEPR**	**Amplitude**
	Pilot	Cymba concha	200–300	25	≤ 5.0	NR	Just below pain threshold	30 s on/off	29	Dominant eye	no task (fixation)	Resting state	NA

(Continues)

TABLE 1 | (Continued)

Citation	Sub-study	taVNS location	Pulse width (μs)	Rate (Hz)	Intensity (mA)	Mean (SD) intensity (mA)	Intensity titration method	taVNS timing and duration	*N*	Eye analyzed	Task during pupil measurement	Pupillary index	Significant effect
Borges et al. (2021)		Cymba concha	200–300	25	NR	0.94 (0.57)	Halfway between perceptual intensity and max comfortable intensity	continuous during task (45 min)	42	Right	Modified Flanker task	TEPR; resting state	NA
		Tragus	200–300	25	NR	2.18 (0.69)	Halfway between perceptual intensity and max comfortable intensity	continuous during task (45 min)	42	Right	Modified Flanker task	TEPR; resting state	NA
Capone et al. (2021)		**EAM**	**300**	**20**	**0.5–3.0**	—	**Fixed; 0.5, 1, 2, 3 mA**	**continuous during each 2 min block (×8)**	**19**	**Binocular**	**No task (fixation)**	**Resting state**	**Diameter**
Urbin et al. (2021)		**Concha**	**250**	**25, 300**	**0.08–4.4**	**0.61 (0.08)**	**80%, 100%, 150%, 200% of perceptual threshold**	**~0.6 s per trial**	**19**	**Left**	**No task (fixation)**	**SEPR**	**Amplitude, latency**
		EAM	**250**	**25, 300**	**0.08–4.4**	**1.04 (0.1)**	**80%, 100%, 150%, 200% of perceptual threshold**	**~0.6 s per trial**	**19**	**Left**	**No task (fixation)**	**SEPR**	**Amplitude, latency, AuC**
D'Agostini et al. (2021)		Cymba concha	250	25	0.25–0.5	0.49 (0.05)	Highest non-painful intensity up to 0.5 mA	30 s on/off (40 min)	71	Left	no task (fixation)	Resting state	NA
D'Agostini et al. (2022)		Cymba concha	250	25	0.4–4.0	1.58 (0.85)	Just below pain threshold	continuous during task (50–55 min)	40	Right	Active oddball; no task (fixation)	TEPR; resting state	NA
Villani et al. (2022)		**Tragus**	**500**	**25**	**NR**	**0.38 (0.25)**	**Just below perceptual threshold**	**0.55 s per trial**	**36**	**NR**	**Active oddball**	**TEPR**	**Amplitude**
McHaney et al. (2023)		Cymba concha & cymba cavum	250	30	0.1–1.5	0.71 (0.42)	0.2 mA under perceptual threshold	3 s per trial	38	Left	Nonnative speech category learning	TEPR	NA

(Continues)

TABLE 1 | (Continued)

Citation	Sub-study	taVNS location	Pulse width (μs)	Rate (Hz)	Intensity (mA)	Mean (SD) intensity (mA)	Intensity titration method	taVNS timing and duration	*N*	Eye analyzed	Task during pupil measurement	Pupillary index	Significant effect
D'Agostini et al. (2023)		**Cymba concha**	**200–400**	**25**	**0.2–4.0**	**1.19 (0.65)**	**Maximum intensity below pain threshold for the highest pulse width setting (400 μs)**	**5 s per trial**	**43**	**Left**	**No task (fixation)**	**SEPR**	**Amplitude, temporal derivative**
Lloyd et al. (2023)		**Cymba concha**	**200–300**	**25**	**≤5.0**	**2.3 (1.3 SEM)**	**Just below painful**	**3.4 s per trial**	**29**	**Dominant eye**	**No task (fixation)**	**SEPR**	**Amplitude**
Wienke et al. (2023)		**Cymba concha**	**200**	**30**	**≤2.0**	**1.91 (0.23)**	**Maximum intensity without discomfort**	**0.5 s per trial**	**24**	**Left**	**Pupillary light reflex task**	**Pupillary light reflex**	**Amplitude, latency**
		Cymba concha	**200**	**30**	**≤2.0**	**1.91 (0.23)**	**Maximum intensity without discomfort**	**0.5 s per trial**	**24**	**Left**	**Emotional Stroop task**	**TEPR**	**Amplitude**
Skora, Marzecová, and Jocham (2024)		**Cymba concha**	**250**	**25**	**1.0–5.0**	**2.72 (1.33)**	**Clearly perceptible, but not painful sensation**	**1 s per trial**	**52**	**Binocular**	**No task (fixation)**	**SEPR**	**Amplitude**
		Cymba concha	**250**	**25**	**1.0–5.0**	**2.68 (1.23)**	**Clearly perceptible, but not painful sensation**	**30 s per trial**	**55**	**Binocular**	**No task (fixation)**	**SEPR**	**Amplitude**

Note: Studies that report significant pupillary effects due to taVNS are highlighted in bold.
Abbreviations: AuC = area under the curve, NA = not applicable, NR = not reported, SEM = standard error of the mean, SEPR = stimulation-evoked pupillary response, TEPR = task-evoked pupillary response.

Marzecová, and Jocham 2024). The general trend visible in Table 1 of longer trains of taVNS administered over many minutes failing to elicit changes in TEPR or resting-state pupil size versus short taVNS trains (5 s or less) fairly consistently eliciting SEPRs may also suggests that effective modulation of tonic LC activity (via longer trains) and phasic LC activity (via short time-locked trains) may require different taVNS parameters. The second pertains to statistical analysis. Inconsistencies in the amount of charge reaching vagal afferents across participants within a study due to individual differences in skin impedance, discomfort tolerance (when suprathreshold stimulation is used), electrode placement, and cutaneous distribution of the ABVN may reduce the power to link fixed taVNS intensities to outcome measures. In studies where taVNS intensity is titrated individually based on sensory percepts, the relationship between taVNS intensity and resulting physiological changes across participants may be nonmonotonic. Such a relationship may not be captured in linear analyses. The third pertains to experimental design. Characterizing the relationship between taVNS and TEPR measures during a task where learning may occur poses potential problems. Pairing taVNS with cognitive training is likely to reduce the difficulty or amount of cognitive effort required to sustain levels of task performance potentially by facilitating the neural mechanisms that underpin learning or by modulating arousal state. Pupil size is known to reflect cognitive load, with less difficult or effortful tasks eliciting smaller task-evoked pupil responses (Beatty 1982). Thus, the effect of taVNS reducing the cognitive effort required during task performance may counteract expected increases in TEPR intensity due to taVNS modulating LC activity.

1.3 | Goals of the Current Study

With the goal of further evaluating the use of pupil dilation as a physiological biomarker of taVNS-related changes in LC-NE activity, the present study used a single-blind, sham-controlled, within-subjects experimental design to measure taVNS-related changes in pupil diameter during a pupillary light reflex task, similar to that used in a prior study that shows pupillary response to iVNS (Desbeaumes Jodoin et al. 2015). We selected this task in order to replicate methods that had shown an effect of VNS on pupil size as closely as possible. We addressed each of the potential issues noted above by using taVNS parameters that have shown a more robust pupillary response in prior studies (300 Hz applied to the EAM), measuring pupil changes during a passive task, and analyzing the effect of individually-titrated taVNS intensity using generalized additive mixed models (GAMMs; Wood 2006). GAMMs allow for the characterization of non-linear effects of taVNS intensity on the size and timing of pupillary responses while reducing Type 1 error by simultaneously modeling participant-level random effects and accounting for the high degree of autocorrelation in pupil data (van Rij et al. 2019).

In addition to addressing the three potential issues noted above, the features of taVNS used in the present study were selected to address knowledge gaps regarding optimal taVNS intensity, frequency, pulse width, and stimulation location. Note that this study is not an exploration of taVNS parameter manipulations within participants. Rather, it aims to characterize the relationship between pupil size and a single taVNS intensity across participants. However, by testing taVNS parameters that have received comparatively little attention, such as delivering a higher stimulation rate (300 Hz) and shorter pulse width (50 μs) to the EAM, and by exploring the effect of taVNS across a range of intensities below perceptual threshold, the present study makes a unique contribution to our understanding of the range of potential taVNS parameters that may be effective in achieving desired neuromodulatory outcomes. Based on the results of Desbeaumes Jodoin et al. (2015), we hypothesized an overall increase in pupil size during trials during which participants received active taVNS compared to pupil size when they received passive sham taVNS; we did not hypothesize that active taVNS would have differential effects on components of the pupillary light reflex that reflect sympathetic versus parasympathetic influences (Samuels and Szabadi 2008b).

2 | Method

This study was approved by the Institutional Review Board (IRB) of the University of Maryland and the U.S. Department of Navy Human Research Protection Program.

2.1 | Participants

One hundred and twelve healthy adults consented to participate in this study. Participants were 18–31 years old ($M = 20.46$, $SD = 2.49$), and 63.5% identified as female. The relevant task was administered as part of a larger, multisession study assessing the influence of taVNS on Mandarin lexical tone learning. Sample size was determined based on the expected effect sizes for the behavioral learning tasks administered in subsequent sessions, based in previous work by the authors (Pandža et al. 2020; Phillips et al. 2021). Data collection for this study began in 2019. At that time, no study reporting changes in pupil size due to taVNS had been published, which precluded determining a sample size based on similar prior studies. Since then, all of the studies that have reported significant effects of taVNS on pupil size all involved smaller samples than that of the present study (see Table 1), indicating that the effects of taVNS on pupil size can be observed with much smaller samples than that of the present study.

Participants enrolled in this study reported having normal or corrected-to-normal vision, normal hearing, and no ear canal blockage. Individuals were excluded from participating if they were pregnant or nursing, or if they reported a history of any of the following: learning disabilities, neurological, neuropsychiatric, or psychiatric disorders; ocular disorders affecting pupillometry (e.g., cataract, nystagmus, amblyopia); recently receiving VNS/TENS outside of lab protocols; electroshock therapy for depression; taVNS contraindications including cardiac or vascular disease, diabetes, epilepsy, fainting; head or face injuries, pain, or pain disorders; implanted metallic or electronic devices, non-removable metal piercings around the face; heart attack, stroke/TIA, congestive heart failure, coronary heart disease, peripheral vascular disease, angina pectoris, irregular heartbeat or arrhythmia; concussion or loss of consciousness for more than 10 min; recent hospitalization; taking psychoactive

medications or medications to treat taVNS contraindications within 2 months.

2.2 | Pupillary Light Reflex Task

The experimental task administered in this study was designed to closely follow the pupillary light reflex task in Desbeaumes Jodoin et al. (2015) used to measure changes in pupil size during iVNS in patients with refractory epilepsy or depression. The purpose of this task is to trigger constriction of the pupil by abruptly increasing the amount of light that reaches the retina by quickly increasing the luminance of a visual stimulus. In principle, eliciting the pupillary light reflex is not required in order to observe changes in pupil size due to vagal stimulation. However, this task design provides an opportunity to tease apart sympathetic versus parasympathetic influences of vagal stimulation via the indirect influence of inhibitory and excitatory inputs from the LC on pupil size. For example, increases in pupil size during the prestimulus baseline period of this task would reflect increased sympathetic excitatory input to the superior cervical ganglion (SCG; Mridha et al. 2021) whereas attenuation of the pupil constriction triggered by a luminance increase would reflect inhibition of parasympathetic activity of the Edinger-Westphal nucleus (EWN; Samuels and Szabadi 2008a). Certain features of the pupillary light reflex are also selectively sensitive to sympathetic versus parasympathetic activity, such as maximum constriction velocity or acceleration (selectively sensitive to parasympathetic activity) and 75% amplitude recovery time or recovery amplitude after 2.4 s (selectively sensitive to sympathetic activity). See Desbeaumes Jodoin et al. (2015) for further details.

The aim of the present study was to provide an initial indication of whether taVNS could modulate resting-state pupil size. We did not have specific predictions regarding potential differential effects of taVNS on sympathetic versus parasympathetic activity; therefore, it was not strictly necessary to utilize a pupillary light reflex task in the present study. However, when the present study was designed and data collection began in 2019, Desbeaumes Jodoin et al. (2015) was the only published study known to the authors in which vagus stimulation (in that case iVNS) had been found to modulate resting-state pupil size. Therefore, we chose to replicate the pupillary light reflex task used in Desbeaumes Jodoin et al. (2015) as closely as possible, with few necessary changes noted below.

The sequence and timing of task events, including visual stimuli and taVNS during each trial, are shown in Figure 1.

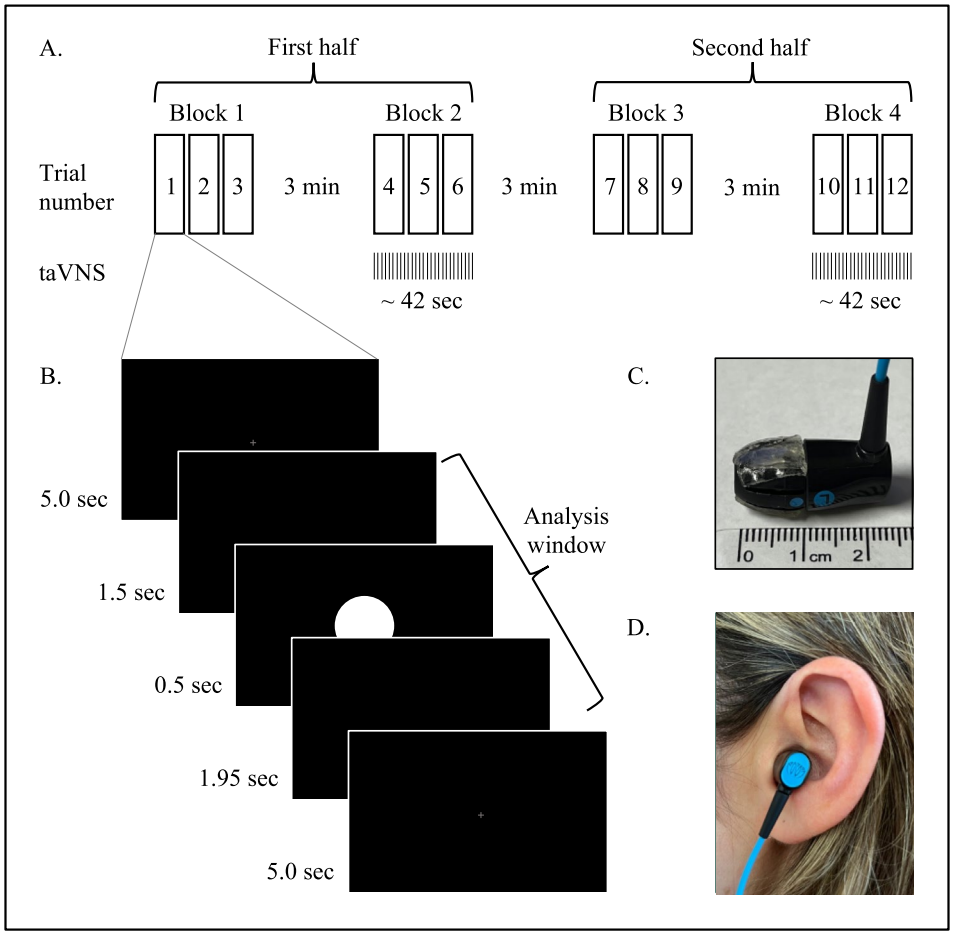

FIGURE 1 | Pupillary light reflex sequence and timing of events (A) across trials and (B) within each trial, with analysis window noted. (C) Modified earbuds used to deliver taVNS to the left EAM. Note, conductive gel was placed on the top (not shown) and bottom (shown) of the earbud, over the conductive surfaces. (D) Position of the modified earbud within the left EAM.

The pupillary light reflex task consisted of 12 trials split into four blocks of three trials each. Each block lasted about 42 s with a three-minute rest period between blocks. Each trial began with a 1 cm gray fixation cross shown centered over a black background on the participant computer display (Dell 24-in. LED monitor). The fixation cross was visible for 5 s, after which the fixation cross disappeared and the black background remained unchanged for 1.5 s. Next, a white 10 cm diameter disk appeared over the black background in the center of the display. After 0.5 s, the disk disappeared and the black background was displayed for 1.95 s, followed by the presentation of the gray fixation cross in the center of the screen for another 5 s. All participants received passive sham taVNS during the first and third blocks and active taVNS continuously throughout the second and fourth blocks. In each active taVNS block, stimulation began immediately prior to the onset of the first trial and ended immediately following the offset of the third trial, with a total duration of approximately 42 s per block. No stimulation was delivered during the three-minute rest period between blocks.

The fixed block order in the present study differed from the randomized block ordering used in Desbeaumes Jodoin et al. (2015). This was intentional to permit inclusion of trial number as a covariate in statistical modeling. The total duration of stimulation across each block was also longer in the present study (42 s) compared to the 30 s of stimulation per block in Desbeaumes Jodoin et al. (2015). This was the result of inserting a 5 s fixation screen before each trial in the present task. Participants in Desbeaumes Jodoin et al. (2015) received 30 s trains of stimulation that were automatically triggered from an implanted pulse generator, with 3–5 min rest periods between each 30 s train of VNS.

2.3 | taVNS Parameters and Titration

A Digitimer DS8R Biphasic Constant Current Stimulator (DS8R; Digitimer North America LLC, Fort Lauderdale, FL) generated the taVNS. Each stimulus train consisted of 50 μs biphasic square pulses with 350 microsecond interphase dwell and 100% recovery phase ratio delivered at 300 Hz. We delivered continuous taVNS at 300 Hz, rather than the 25–30 Hz rate standardly used in taVNS studies, because of evidence suggesting it was better tolerated by participants (Tyler et al. 2019) and positive effects of these taVNS parameters were found for behavioral, pupillary, and ERP results in a previous study conducted by the authors (Pandža et al. 2020; Phillips et al. 2021). The DS8R output a single stimulus when triggered via a TTL pulse over a BNC jack, which originated from a custom-programmed Arduino UNO board controlled by the experiment computer via USB. The experiment software controlled the 300 Hz pulse delivery rate via the USB connection and also controlled the DS8R's current intensity via a DS8R API over a secondary USB connection.

A set of modified Neuvana earbuds (previously Nervana; Neuvana LLC, Deerfield Beach, FL) was used to deliver taVNS via a 2.5 mm electrode plug connected to the DS8R and also delivered auditory stimuli via a 3.5 mm audio plug connected to the experiment computer. The earbuds contained two electrodes on the left earbud, positioned to stimulate the ABVN from within the EAM. The modification involved replacing a small portion of the silicone earbud tip over each electrode with Axelgaard AG735 and/or AG2550 hydrogel (Axelgaard Manufacturing Co. Ltd.) and affixing a small patch (approximately 0.5–1 cm^2) of the same hydrogel on the surface of the earbud tip over each replaced section (see Figure 1). Hydrogel was used as the transmission medium to maintain consistent connectivity throughout the experiment. The earbud was inserted into the participant's left ear until no hydrogel was visible outside of the EAM. The angle of the earbud was such that the hydrogel patches provided contact between the stimulating electrode and relatively large areas of the inferoanterior and posterosuperior walls of the EAM (see Figure 1). Following testing, placement of the earbud was visually inspected to ensure that it had not moved during testing. During testing, consistent contact between the electrode and the EAM was ensured by monitoring the DS8R compliance warning indicator that indicates when the resistance in the skin or electrode is too high for the DS8R to deliver the specified current. This indicator was not activated during testing for any participant, indicating that the DS8R was able to consistently deliver the specified current.

Each participant's taVNS perceptual threshold was determined using the titration procedure described in Pandža et al. (2020). Briefly, 2 s tVNS pulse trains were delivered at random intervals from 1 to 3 s, starting at 2 mA and increasing in 0.5 mA steps to a maximum of 10 mA. When participants pressed a button to indicate that they felt stimulation, taVNS intensity was reduced by 1.0 mA (to a minimum of 2.0 mA), and the staircase procedure was repeated in 0.1 mA steps until participants again pressed a button to indicate they felt stimulation. The intensity when the participants pressed the button the second time was taken as the perceptual threshold.

During the task, taVNS was delivered 0.2 mA below perceptual threshold to maintain participant blindness to the taVNS condition (active versus sham) during each experimental block. Passive sham taVNS was used as the control condition in this study. During sham taVNS blocks, no current was delivered to the stimulating (left) earbud. The use of passive sham taVNS as the control condition was deemed appropriate for this study because active taVNS was delivered below perceptual threshold. The passive and sham control taVNS conditions in this study had identical characteristics (same location of stimulating earbud, same sensory profile [no feeling of stimulation]), which is ideal for blinding participants to taVNS condition. Because participants could not feel the stimulation, it was not necessary to stimulate a different region of the ear (e.g., the earlobe) in order to control for changes in arousal or anticipation that occur when taVNS is delivered above perceptual threshold.

Two-second trains of taVNS were used to establish the perceptual threshold for each participant because it was not feasible to complete the titration procedure using taVNS trains that were more comparable in length to the 42 s taVNS trains that were administered during the pupillary light reflex task. Prior work by the authors indicated that determining perceptual threshold using 2 s trains of taVNS was viable for subsequently delivering

much longer (10-min) taVNS trains below perceptual threshold (Pandža et al. 2020). In this prior work and in the present study, taVNS was administered with biphasic waves with a 100% recovery phase ratio to prevent charge buildup under the electrodes, which may alert participants to when they are receiving active taVNS.

2.4 | Procedure

For all but one participant included in the analysis, the pupillary light reflex task was administered at the end of the first testing session of the larger study and was participants' first encounter with taVNS in this study.[1] The session lasted approximately 2 h. Before the pupillary light reflex task, each participant provided informed consent and completed several questionnaires and experimental tasks to assess Mandarin lexical tone identification and categorization ability, in line with the aims of the larger study. Participants completed the pupillary light reflex task seated in front of the computer display positioned approximately 65 cm from participants' eyes, with their heads stabilized using a chinrest. Pupil size (in mm) and gaze position were recorded with a Tobii X120 eye tracker affixed below the display, with pupil variables sampled at 40 Hz and gaze variables sampled at 120 Hz. Visual stimuli, taVNS delivery, and recording of eye data were all controlled using a custom script developed using PsychoPy (v. 3.1.5; Peirce et al. 2019).

At the beginning of the task, participants read task instructions on the display and then completed the taVNS threshold procedure described above, followed by a nine-point eye-tracker calibration. The task instructions informed participants that they would see a series of shapes flash on the screen while their pupils are recorded and that they may receive taVNS at different points during the task. Participants were instructed to keep their chin in the chinrest throughout the task, focus on the center of the screen, limit their blinks only to when the fixation cross was on screen, and to avoid other movements.

In addition to delivering taVNS below perceptual threshold, potential sound artifacts that occasionally occurred during taVNS due to leakage of current between the taVNS and audio lines within the stimulating earbud were masked to ensure that participants were unaware of when they were receiving taVNS during the task. To mask potential sound artifacts, all participants heard a pink noise sound mask described in Phillips et al. (2021) continuously during any period when taVNS was possible, including titration and each task block.

At the onset of the study, the testing booth was illuminated by a single lamp positioned behind the participant, resulting in a luminance of 40 lx as measured at the participant's eye (i.e., at the chinrest facing the screen). A check of data quality performed halfway through data collection indicated noisier-than-expected pupil tracking. It was determined that this was due to insufficient ambient luminance. As a result, the overhead light inside of the booth was turned on during the task for the remainder of participants, increasing luminance to 159 lx as measured at the participant's eye (see Supporting Information for an overview of excluded trials before vs. after the change in luminance).

2.5 | Pupillometry Pre-Processing

Prior to analysis, pupillary data for left and right eyes were pre-processed using a custom script in R (v. 4.2; R Core Team 2022) based on recommendations of Kret and Sjak-Shie (2019). For each eye, pupillary data were first upsampled to the 120 Hz gaze sampling rate by linearly interpolating between valid pupil samples. This was done to improve correction of artifacts described below. Next, pupil samples were marked as invalid and removed if the difference in diameter between that sample and either neighboring sample exceeded ten times the sample-to-sample median absolute deviation across the entire trial. After removing invalid samples, contiguous segments of invalid pupil samples exceeding 75 ms were marked as blinks, and samples during the 50 ms preceding and following blinks were removed due to apparent rapid changes in pupil size during these segments due to partial occlusion of the pupil by the eyelid.

Samples that were missing across both eyes were then replaced using linear interpolation, and a smoothed vector of pupil data was created using a 125 ms symmetrical moving window average. Next, pupil samples were marked as invalid and removed if the difference in diameter between that sample and the corresponding sample in the smoothed vector exceeded 0.5 times the median absolute deviation between pupil samples and their corresponding smoothed values across the entire trial. The process of linear interpolation, smoothing, and removal of outliers was then repeated a second time. The deviation multipliers used in pre-processing were determined empirically as recommended in Kret and Sjak-Shie (2019) as those that removed obvious outlier samples without resulting in excessive removal of data. Pupil samples that were missing from one eye only were replaced based on the value of the other eye and model coefficients from regressing each eye onto the other. Finally, left and right pupil diameter was averaged at each sample, remaining missing values were linearly interpolated, and the averaged pupil vector was smoothed using a 125 ms symmetrical moving window average.

The pupillary analysis window spanned the 3.95 s comprising the white disk and the preceding and following blank screens. Pupil diameter during the blank screens before and after stimulus that elicited the pupillary light reflex was modeled in order to permit identification of taVNS effects during both the resting state and during the pupillary light reflex, which may be differentially affected by increases in LC activity resulting from taVNS (Samuels and Szabadi 2008b).

Of the 112 consented participants, five were dismissed after determining during screening that they did not meet eligibility criteria for the larger study. One additional participant was dismissed because they were able to feel the taVNS at the minimum titration intensity of 2.2 mA, and two additional participants withdrew during taVNS titration. Data for an additional two participants was missing due to a technical error, leaving 102 participants with data. Trials in which more than one-third of samples during the analysis window were missing for both eyes

prior to interpolation were excluded (396 of the 1224 possible trials, or 28.13%). As a result, data for 6 participants were excluded entirely. An additional 6 participants who did not have at least one trial in an active taVNS block and a sham taVNS block were also excluded. The final dataset consisted of 822 trials across 90 participants. Of the 612 possible trials per taVNS condition, 393 sham trials remained (35.78% excluded; average of 2.14 trials per participant) and 429 active trials remained (29.90% excluded; average of 1.79 per participant).

2.6 | Analysis Approach

The aim of the present study was to provide an initial indication of whether taVNS could modulate resting-state pupil size. For this reason, rather than extracting and analyzing specific features of the pupillary light reflex that selectively reflect sympathetic versus parasympathetic activity as in Desbeaumes Jodoin et al. (2015), we analyzed trial-level vectors of pupil diameter (averaged between eyes, interpolated, and smoothed) that spanned the entire 3.95 s analysis window (475 samples each) consisting of the visual stimulus designed to elicit the pupillary light reflex as well as resting periods before and after. Because we were interested in differences in pupil size during the baseline period prior to the visual stimulus, actual pupil size was analyzed; no baseline correction or normalization was performed. Individual differences in pupil size were controlled for by using a within-subjects factor design. These data were analyzed using a generalized additive mixed model (GAMM). GAMMs are designed to fit non-linear trends in time-series data, such as the pupillary response, and have added benefits over other forms of time-series analysis (e.g., growth curve analysis), such as the ability to account for autocorrelation to reduce Type I error rates (van Rij et al. 2019) and the fact that no a priori assumptions about the shape of the curve are required.

All models were fit in R (v. 4.4.1; R Core Team 2024) using the bam function in the mgcv package (v. 1.9–1; Wood 2003, 2011, 2017). Model criticism, testing, and visualization were conducted using the itsadug package (v. 2.4.1; van Rij et al. 2022) and the mgcViz package (v. 0.1.11; Fasiolo et al. 2020). The model-predicted pupil diameter over the course of each trial by the ordered factor variables representing taVNS condition (*IsActive* [sham = 0; active = 1]), experiment half (*IsSecondHalf* [first half = 0; second half = 1]), and their interaction with taVNS intensity (continuous variable). Ordered factor variables were used to estimate differences between specific conditions similar to linear regression but implemented within the GAMM framework. These ordered factor variables were specified in both the parametric and smooth terms. Parametric terms estimate the overall difference in pupil diameter (i.e., irrespective of time) across conditions, while smooth terms estimate the non-linear trajectory of pupil size across conditions as a function of time. The smooth terms for the experiment half and taVNS condition were interacted with taVNS intensity to model dependencies between differences in pupil size due to taVNS and taVNS intensity level.

A term for experiment half was included to model potential changes in pupil size that persist after delivery of active taVNS during the second block. Following our hypothesis, we expected that pupil size would increase during block 2, in which participants received active taVNS for the first time, but we were not sure how long the increase in pupil size would last, given that the longevity of pupil dilation due to taVNS is unknown. Experiment half was modeled in the GAMM to capture a potential sustained overall increase in pupil size during the second half of the experiment (blocks 3 and 4) due to receiving active taVNS for the first time in block 2.

The formula for the GAMM is given in the Table 2 note. The model was specified with a "reference smooth" (te(time, taVNS intensity, $k = 20$)) which estimates the pupillary response for the reference level (in this case, sham taVNS, first half) by omitting the factor specified in the "by" argument of other terms that are otherwise identical. The reference smooth is analogous to the intercept in the summary of a traditional linear model. The smooth terms in the model that include a "by" term (te(time, taVNS intensity, by = IsActive, $k = 20$); te(time, taVNS intensity, by = IsSecondHalf, $k = 20$)) are referred to as "difference smooths". These estimate the difference between the reference smooth and the condition specified in the "by" argument of the smooth term. Difference smooths are analogous to the estimates given below the intercept in the summary of a traditional linear model. For example, the ordered factor variable "IsActive" has a value of 1 for all data points in the active taVNS trials and 0 for all data points in the sham taVNS trials. If this were the only term in the model, the reference smooth would then estimate the pupillary response for the sham taVNS condition while the "IsActive" difference smooth would estimate the *difference* between the sham and active taVNS conditions. The associated *p*-value thus indicates if this difference is significantly different from zero. The addition of other ordered factor terms allows for specific contrasts to be represented, including interactions that estimate the difference conditions across the levels of some other predictor variable.

In the present study, the smooth terms were specified as tensor product interactions (for further details and a similar use of tensor product interactions, see Johns et al. 2024). Smooth terms denoted with *s* model the dependent variable as a function of one or more continuous independent variables with the same scale; smooth terms denoted with *te* model the dependent variable as a function of two or more continuous independent variables with different scales, as a separate penalty is applied to each variable (Wood 2017, 325–328). In this case, pupil diameter was modeled simultaneously as a function of time with units in sec and taVNS stimulation intensity with units in mA. The model also included what Sóskuthy (2021) refers to as "random reference/difference smooths" (s(time, subject, bs = "fs", $m = 1$, $k = 5$); s(time, subject, by = IsActive, bs = "fs", $m = 1$, $k = 5$); s(time, subject, by = IsSecondHalf, bs = "fs", $m = 1$, $k = 5$)), which are one way of modeling non-linear random effects. These random smooths use the same ordered factor variables specified in the tensor product interactions: the random reference smooth can be thought of as non-linear by-subject differences at the reference level (analogous to random intercepts), while the random difference smooths represent non-linear by-subject differences between conditions (analogous to random slopes). Lastly, two

TABLE 2 | GAMM summary.

Parametric coefficients	Estimate	SE	*t*-value	*p*
(Intercept)	4.436	0.106	42.056	<0.001
IsActive	0.229	0.044	5.165	<0.001
IsSecondHalf	0.591	0.080	7.366	<0.001
Smooth terms	**EDF**	**Ref. DF**	***F*-value**	***p***
s(trial number)	7.356	7.872	84.867	<0.001
s(x-position, y-position)	2.004	2.008	1.632	0.195
te(time, taVNS intensity)	188.997	214.451	51.313	<0.001
te(time, taVNS intensity):IsActive	226.367	285.039	2.231	<0.001
te(time, taVNS intensity):IsSecondHalf	152.860	209.123	1.544	<0.001
Random smooth terms	**EDF**	**Ref. DF**	***F*-value**	***p***
s(time, subject)	379.837	443.000	31.436	<0.001
s(time, subject):IsActive	232.775	438.000	2.725	<0.001
s(time, subject):IsSecondHalf	272.791	425.000	4.577	<0.001

Note: Model formula: pupil diameter ~ IsActive + IsSecondHalf + s(trial number) + s(x-position, y-position) + te(time, taVNS intensity, $k = 20$) + te(time, taVNS intensity, by = IsActive, $k = 20$) + te(time, taVNS intensity, by = IsSecondHalf, $k = 20$) + s(time, subject, bs = "fs", $m = 1$, $k = 5$) + s(time, subject, by = IsActive, bs = "fs", $m = 1$, $k = 5$) + s(time, subject, by = IsSecondHalf, bs = "fs", $m = 1$, $k = 5$). Adjusted $R^2 = 0.931$, Deviance Explained = 81.2%, fREML = 9413.8, $n = 122{,}138$.
Abbreviations: EDF = effective degrees of freedom, Ref. DF = reference degrees of freedom.

additional smooth terms were included in the model: a smooth for overall trial number (1–12; s(trial number)) to account for the expected (potentially non-linear) decrease in pupil size across the course of the experiment, which commonly occurs in experiments and is thought to reflect general fatigue (McLaughlin et al. 2023); and a smooth for x- and y-gaze position (s(x-position, y-position)), to account for systematic artifactual changes in pupil size due to changes in gaze position (Brisson et al. 2013; Gagl, Hawelka, and Hutzler 2011). For all smooth terms, the maximum number of knots representing the maximum number of basis functions used to estimate the smooth was specified (tensor product interactions: 20; random smooths: 5; smooth for overall trial: 10; smooth for gaze position: 30).

The model building procedure followed recommended practices for GAMMs (van Rij et al. 2019). First, a maximal model was fit with all parametric and smooth terms with the ordered factor variables of the taVNS condition, experiment half, and their interaction. The model was specified to use a scaled-*t* distribution, as this distribution has been shown to best represent pupillometry data (van Rij et al. 2019). This model was then used to estimate the autocorrelation parameter *rho*, and the model was then refit including an embedded autoregressive model using this *rho* value. Autocorrelation was assessed using the acf_resid function in the itsadug package and was found to be sufficiently low (autocorrelation at lag 1: 0.33). Both the parametric and smooth terms representing the interaction between the taVNS condition and experiment half were non-significant ($p = 0.249$ and $p = 0.916$, respectively); as such, they were excluded from the model, and the model was re-fit in order to simplify the interpretation of the main effects of taVNS condition and experiment half. Autocorrelation was assessed again and was determined to be sufficiently low (autocorrelation at lag 1: 0.317). Model diagnostics were performed using the check.gamViz function in the mgcViz package: visual inspection of the residuals suggested that the scaled-*t* distribution was appropriate and that the maximum number of knots for each smooth term was sufficiently high as to appropriately estimate all smooths. The full R script and the accompanying R workspace are available online at https://osf.io/8p47b/ and contain further information about the model fitting procedure and model diagnostics. Likewise, information about the maximal model, a detailed summary of all terms in the model, as well as plots and summaries of the model diagnostics, can be found in the Supporting Information. For smooth terms, an alpha level of 0.05 was used to determine significance.

3 | Results

3.1 | taVNS Intensity

For the $N = 90$ participants included in the analyses, taVNS intensity (0.2 mA below perceptual threshold) ranged from 2.0 to 8.1 mA (M (SD) = 3.63 (1.37)). The distribution of taVNS intensity is shown in Figure 3A. taVNS intensity was compared between participants who completed the task before and after the change in ambient lighting halfway through data collection to ensure that there was no relationship between taVNS intensity and light level, which may confound the results. Welch's independent t-test confirms that taVNS intensity does not differ between participants who completed the task under lower ambient luminance (M (SD) = 3.45 (1.20) mA) and participants who competed the task under higher ambient luminance conditions (M (SD) = 3.87 (1.56) mA) participants, $t(66.76) = -1.36$, $p = 0.176$. Further, a two-sample Kolmogorov–Smirnov test also indicates

that the distributions of taVNS intensity do not differ between these participant groups ($D = 0.18$, $p = 0.359$).

3.2 | Pupil Diameter

The GAMM was constructed to determine (1) whether pupil diameter during any part of the pupillary light reflex task differed between blocks during which active versus sham taVNS was continuously delivered, (2) whether any taVNS-block-level differences were modulated by taVNS intensity, and (3) whether any changes in pupil size during active taVNS persisted beyond the stimulating period. In the initial model, the highest-order interaction (trial time by taVNS intensity by taVNS condition by experiment half) was not significant. To facilitate interpretation, this term was removed and the GAMM was re-fit to the data. Ambient light level (lower vs. higher) was also not included in the model, following analyses that showed inclusion of a parametric term for ambient light level resulted in almost no difference in model-estimated effects of interest. A summary of this model is available in the Supporting Information. The summary of the final GAMM is shown in Table 2. Model-predicted values for pupil diameter as a function of trial time and taVNS intensity are plotted for each block in Figure 2.

For each significant smooth, model-predicted values are plotted to allow for interpretation, as is standard practice in GAMMs analysis (Wieling 2018). Smooth terms were plotted using the l_fitLine and l_ciLine functions in the mgcViz package (Figure 3B) and the plot_smooth function in the itsadug package (Figure 5); tensor product interaction heatmaps were plotted using the fvisgam function in the itsadug package (Figure 2); and difference heatmaps were plotted using the plot_diff2 function in the itsadug package (Figures 4 and 5). The significant smooth *s(trial number)* indicates that pupil diameter varied as a function of trial number ($p < 0.001$). Model-predicted values for this effect are plotted in Figure 3B. This plot indicates a fairly linear decrease in pupil diameter across the 12 trials in the task. When accounting for this overall decrease, however, the significant parametric term *IsSecondHalf* indicates that the decrease in pupil size was significantly weaker than expected during the second half of the task compared to the first half (*Est.* $= 0.591$, $p < 0.001$). In other words, the change in pupil size from the first to the second half of the experiment persists beyond the overall decrease in pupil size across the task once the latter has been taken into account. Also, the difference between task halves was not consistent across taVNS intensities, indicated by the significant tensor product smooth *te(time, taVNS threshold):IsSecondHalf* ($p < 0.001$). Visual inspection of this tensor product, plotted in Figure 4, reveals that pupil diameter during the second half of the task was larger than expected across the analysis window for all participants except those who received taVNS between approximately 6.0–7.0 mA. For these participants, the effect was significant only after the first 500 ms of the analysis window.

The parametric term *IsActive* in Table 2 indicates that pupil diameter across the analysis window was significantly larger for active versus sham taVNS blocks (*Est.* $= 0.229$, $p < 0.001$). The significant tensor product smooth *te(time, taVNS threshold):IsActive* further indicates that this effect varied as a function of taVNS intensity ($p < 0.001$). To interpret this effect, this tensor product is plotted in Figure 5 and includes fitted smooths at the 25th, 50th, and 75th percentile taVNS intensity values. Highlighted regions in the heatmap in Figure 5 indicate regions where the model-estimated 95% confidence interval does not overlap with zero, indicating a significant difference between the active and taVNS conditions. This plot reveals that the increase in pupil diameter for active versus sham taVNS was significant for participants with taVNS intensities between 2 and ~4.8 mA, but not for participants with higher taVNS intensities, specifically between ~4.9 and 8.1 mA.

4 | Discussion

The present study analyzed the effects of taVNS on pupil diameter during a pupillary light reflex task in order to further evaluate the use of pupil dilation as a biomarker of taVNS-related changes in LC activity. The main result of this study indicates that delivering sub-perceptual threshold taVNS (50 μA pulses at 300 Hz) to the left EAM temporarily increased pupil size in individuals receiving intensities between 2.0 and ~4.8 mA, but not in those receiving higher intensities (up to 8.1 mA). A secondary finding was that taVNS effects on pupil dilation persisted across experimental blocks and this effect was relatively consistent across taVNS intensities.

4.1 | Transient Effects of taVNS on Pupil Size

The model-estimated effect of taVNS on pupil diameter during stimulation in the present study was an increase of 0.229 mm. This effect is very close to the average increase in resting pupil diameter of 0.23 mm elicited by iVNS using a nearly identical task in Desbeaumes Jodoin et al. (2015). Thus, our findings for taVNS appear to replicate and extend the effect of iVNS on pupil size found in Desbeaumes Jodoin et al. (2015) and the effects of taVNS on pupil size in Wienke et al. (2023), albeit with different VNS stimulation parameters. Our finding of increased pupil size with active taVNS across the entirety of the analysis window generally align with those reported in Wienke et al. (2023), in which 200 μs taVNS pulse delivered at 30 Hz at or just below 2.0 mA to the cymba concha attenuated pupil constriction during most of the analysis window (100–1200 ms, 1900–7000 ms) following onset of stimulus eliciting the light reflex, although Wienke et al. (2023) crucially differed from the present study in that taVNS was delivered for only 500 ms starting at the onset of the light reflex stimulus. In Desbeaumes Jodoin et al. (2015), 250–500 μs iVNS pulses were delivered at 20–30 Hz between 0.25 and 2.25 mA. However, it should be noted that the significant effect of iVNS found in Desbeaumes Jodoin et al. (2015) was based on resting pupil diameter during the 1 s prestimulus baseline period preceding presentation of the white disk, although the plot of the effect in the paper suggests it is maintained over the entire 3.95 s trial (Desbeaumes Jodoin et al. 2015, Figure 1). We did not isolate the prestimulus period in the analysis of our pupillary data. Instead, we modeled the entire pupil time series in order to characterize taVNS effects over the course of the pupillary light reflex and the preceding resting-state period.

The model-estimated interaction between taVNS intensity and condition (active vs. sham) also suggests a non-linear effect of

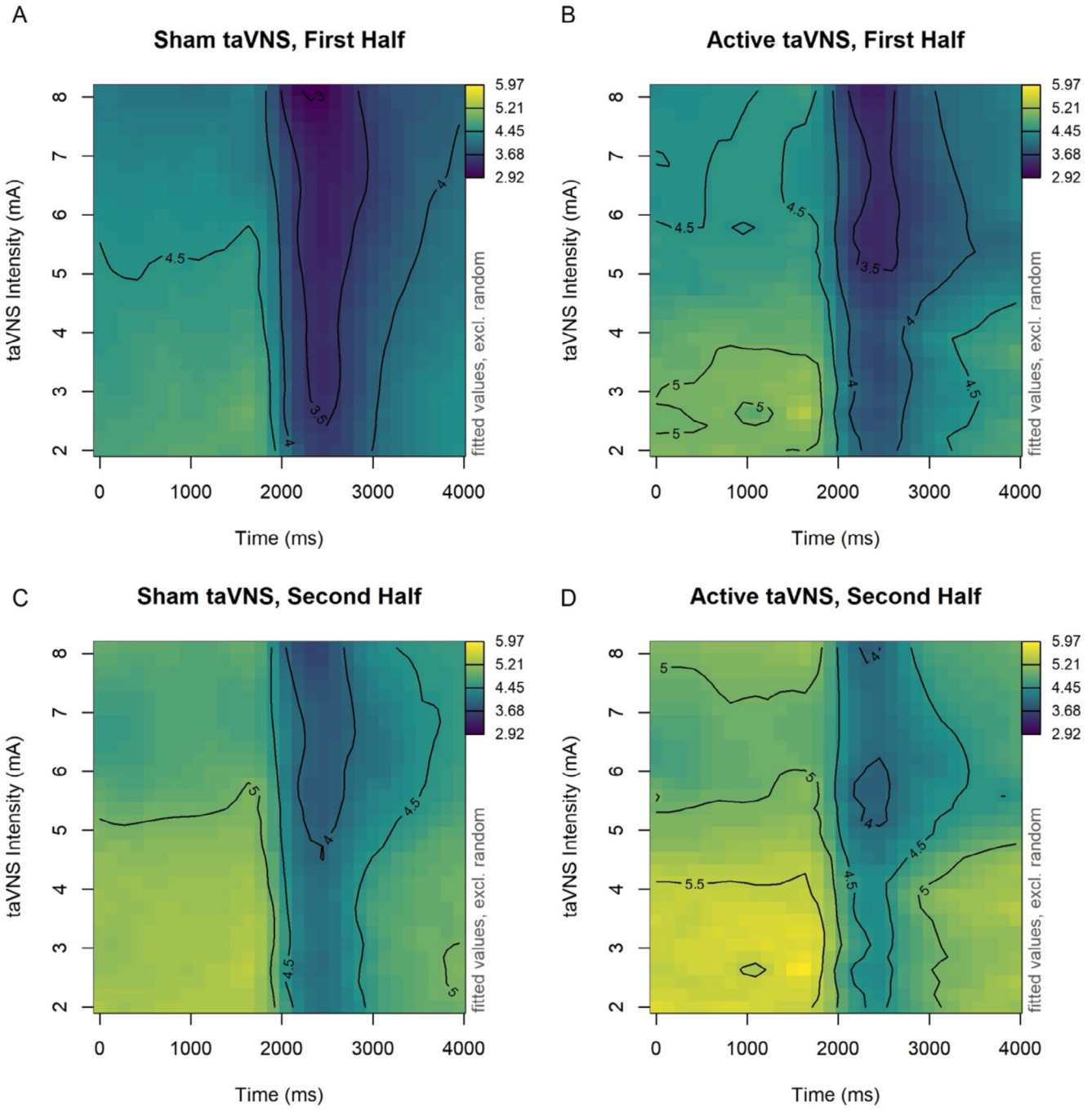

FIGURE 2 | Model-predicted pupil diameter over the course of the analysis window for each task block as a function of taVNS threshold: (A) block 1, (B) block 2, (C) block 3, and (D) block 4. Pupil diameter is represented in the z-axis with color (darker = smaller pupil diameter). The black lines indicate regions of equal pupil diameter in 0.5 mm steps. As a reference, the white disk was present on the display between 1500 and 2000 ms.

sub-perceptual taVNS intensity on resting-state pupil dilation. Pupil diameter was significantly larger during active versus sham taVNS for participants who received taVNS between 2 and ~4.8 mA, but the difference in diameter was not significant for participants who received taVNS at higher intensity levels. The model estimates indicate that taVNS at or below ~4.8 mA led to an increase in pupil size during active taVNS was sustained throughout the 3.95 s analysis window (see unshaded regions in Figure 5 heat map). However, we note that the effect of taVNS in raw measurements of pupil size varied among participants who received taVNS within this amplitude range (see Supporting Information Figure S1). We interpret this as a *transient* effect because the amplitude-dependent change in pupil size occurred while participants were receiving stimulation (active taVNS) compared to when stimulation was not being received (sham taVNS). Although the exact duration of the amplitude-dependent effect of taVNS on pupil sizes is unknown, it is transient in the sense that it appears to be short-lived.

The relationship between taVNS intensity and pupil dilation during stimulation is of particular interest because of apparent inconsistencies in the relationship between VNS intensity and changes in pupil size noted in prior studies. Importantly, the expected relationship between VNS intensity, LC activity,

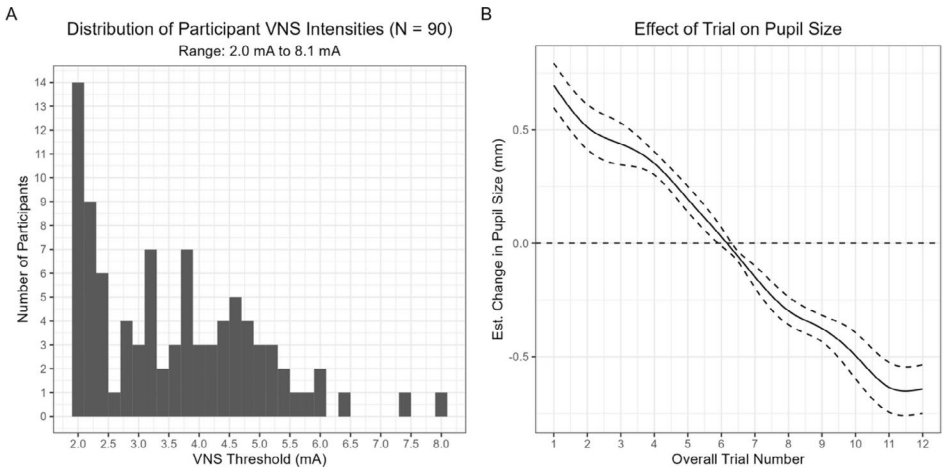

FIGURE 3 | (A) Histogram of taVNS intensity levels across participants. (B) Model-predicted change in pupil diameter from grand mean (represented by the dotted line a y = 0.0) as a function of trial number.

and pupil size may vary depending on the nature of the experimental task and the pupil analysis window. For example, pupil dilation evoked by a task stimulus, such as the appearance of a target during a target detection task, is thought to reflect LC phasic activity (Gilzenrat et al. 2010; Murphy et al. 2014). During active tasks (i.e., those that elicit behavioral responses to stimuli), the magnitude of the task-evoked pupil response (TEPR) would be expected to be maximal at moderate levels of LC activity, given the nonmonotonic (inverted-U-shaped) relationship between LC phasic and tonic activity (Aston-Jones and Cohen 2005). Phasic peak pupil dilation following VNS onset may likewise be expected to vary as a function of prestimulus baseline pupil size (Johns et al. 2024; Relaño-Iborra et al. 2022), which reflects tonic LC activity (Mridha et al. 2021). However, a *monotonic* relationship between pupil dilation and LC activity might be expected for passive tasks, given evidence from mice that shows pupil dilation to increase monotonically with LC spiking rate (Megemont, McBurney-Lin, and Yang 2022) as well as iVNS intensity, pulse width, and stimulation rate (Mridha et al. 2021).

In humans, the relationship between VNS intensity and pupil dilation has been inconsistent. Desbeaumes Jodoin et al. (2015) did not find a correlation between iVNS intensity and any pupil size metric derived from the light reflex in humans, which the authors suggested may be due to low sample size and restricted range of iVNS intensities. In contrast, Vespa et al. (2022) found a nonmonotonic, inverted-U-shaped relationship between absolute iVNS intensity and the magnitude of the late pupil dilation response (PDR; 2.5–5 s following iVNS onset) in epileptic patients. Notably, the relationship between absolute iVNS intensity and the early PDR (0–2.5 s following iVNS onset) was sigmoidal. Using taVNS, Urbin et al. (2021) found the magnitude and incidence of pupillary dilation in humans increased monotonically with increasing taVNS stimulation intensity at and above (up to two times) perceptual threshold (0.08–4.4 mA; Urbin et al. 2021); and D'Agostini et al. (2023) found a linear relationship between charge per pulse (intensity × pulse width) and pupillary dilation and its temporal derivative. However, the effects in D'Agostini et al. (2023) disappeared or were drastically reduced when the highest intensity (just below pain threshold)

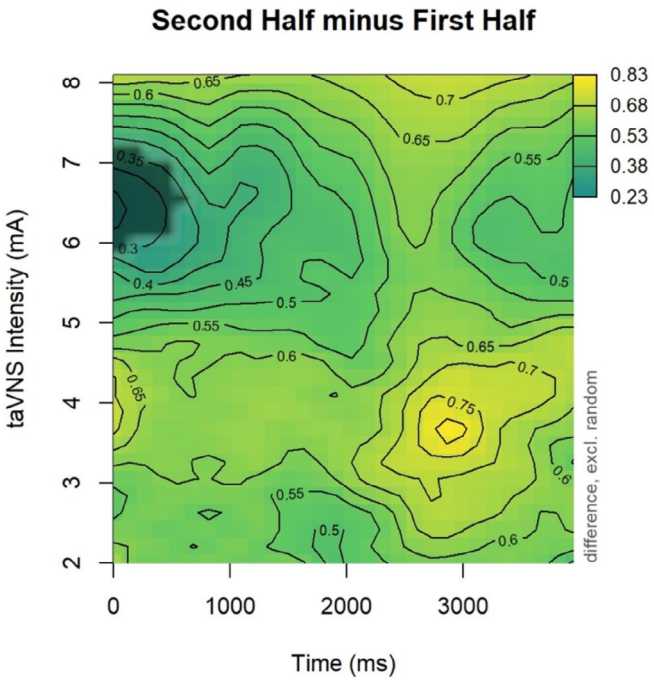

FIGURE 4 | Model-predicted differences in pupil diameter between the first and second half of the task as a function of taVNS intensity. The difference in pupil diameter between task halves is indicated along the z-axis with color. Highlighted regions indicate where differences are significant. The black lines indicate regions of equal value in 0.05 mm steps.

was excluded from the analysis. Wienke et al. (2023) found a relationship between increasing taVNS intensity across a fairly narrow range up to 2 mA and reduced pupil constriction during 300–6500 ms following the onset of the pupillary light reflex stimulus. More similar to the present study, Capone et al. (2021) found that moderate intensity (2 mA) taVNS, but not higher or lower intensities, elicited a pupil response. When comparing results, it should be noted that the above taVNS parametric studies (Capone et al. 2021; D'Agostini et al. 2023; Urbin et al. 2021) tested different taVNS intensities within the same individuals. In the present study, individually calibrated taVNS intensity was tested across individuals.

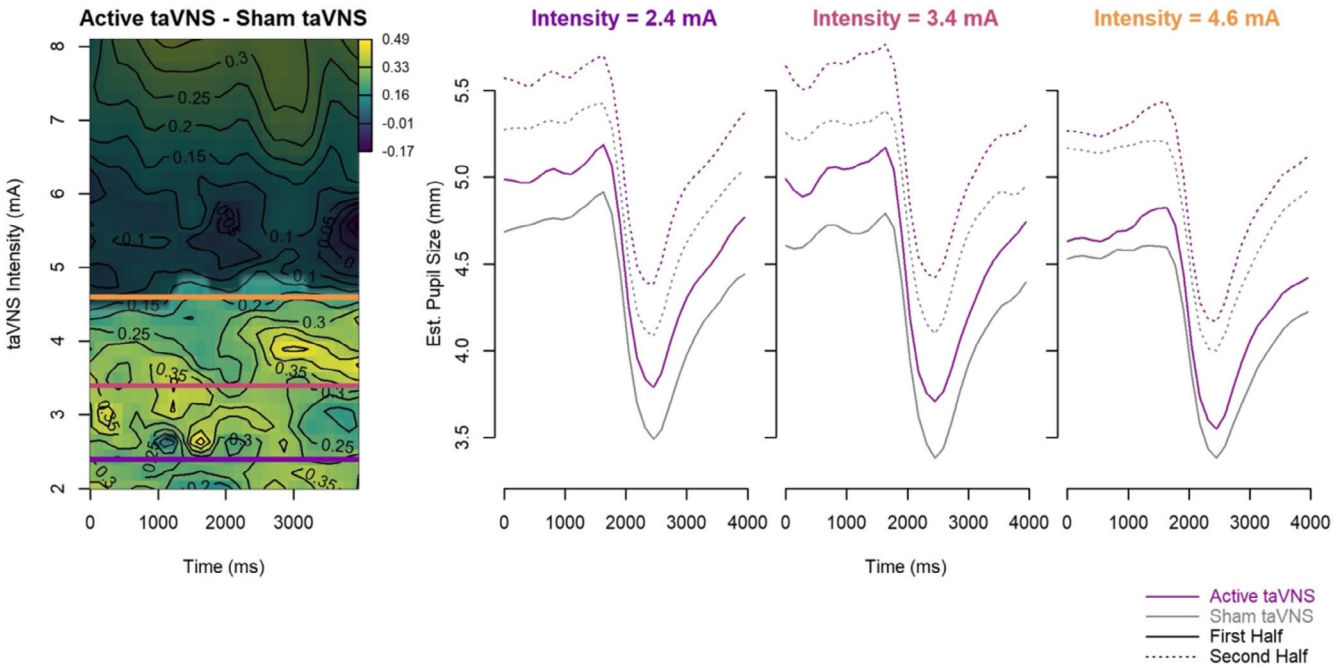

FIGURE 5 | Model-predicted differences in pupil diameter between active and sham taVNS as a function of taVNS intensity. The heatmap represents the difference between the active and sham taVNS conditions as a function of time (x-axis) and taVNS intensity (y-axis). The estimated difference in pupil diameter between the two conditions is indicated along the z-axis with color. The black lines indicate regions of equal value in 0.05 mm steps. Highlighted regions indicate a significant difference between the two conditions. Horizontal lines represent the 25th, 50th, and 75th percentile intensity values, for which fitted smooths are plotted in the three panels to the right. Separate lines are plotted for the taVNS condition and experiment half, even though this interaction was not specified in the best-fitting model because it is not possible to average over non-interacting factors when plotting GAMM-predicted values. As a reference, the white disk was present on the display between 1500 and 2000 ms.

There are several possible factors contributing to the lack of pupil dilation for active taVNS delivered above ~4.8 mA relative to sham taVNS in the present study. One possible explanation involves activation of other pathways that may increase pupil constriction or inhibit pupil dilation. For example, activation of LC-GABA neurons may inhibit activity of LC-NE neurons and, consequently, pupil dilation (Breton-Provencher and Sur 2019). Off-target activation of these neurons has been cited as a potential explanation for the lack of strong correlation between LC activity and pupil diameter in previous VNS studies (Burger, D'Agostini, et al. 2020; Capone et al. 2021).

A similar explanation involving off-target nerve activation may also help explain the monotonic relationship between VNS intensity and LC activity found in prior studies. One possibility is that increases in taVNS intensity above perceptual threshold in prior studies may potentially activate other nerves due to partial overlap in the cutaneous distribution of nerves in the auricle or via spreading or cross-communication between nerve fibers (Butt et al. 2020). This could result in a coupling of intensity and pupil size that does not depend on activation of the ABVN. For example, Mridha et al. (2021) found that the highest VNS intensity in a study delivering iVNS to mice elicited pupil dilation even after the vagus was resected. Although the anatomy of mice and humans is very different, this mechanism may be relevant to taVNS. Perhaps delivering taVNS below perceptual threshold, as in the present study, is less likely to activate off-target nerves, which could result in a functionally distinct pupil response to increasing taVNS intensity that reflects primarily, if not solely, ABVN activation.

4.2 | Persistent Effects of taVNS on Pupil Size

The second finding is that the increase in pupil size due to active taVNS in block 2 persisted into the second half of the task (blocks 3 and 4), mitigating an overall decrease in pupil size across trials. Pupil size often decreases over the course of an experimental task (McLaughlin et al. 2023; Unsworth, Robison, and Miller 2019). Our finding that pupil size generally decreased over the course of our light-reflex task is in line with this. Countering this general decrease in pupil across the 12 trials, we found that pupil size during the second half of the task was larger than expected for all but a few participants, and for those participants only during a short portion at the beginning of the trial. This effect suggests a carryover effect of taVNS across blocks.

The longevity of taVNS effects is not consistent across studies, but there is some evidence that short trains of VNS can increase NE levels for up to 80 min in rats (Hassert, Miyashita, and Williams 2004). This provides some support for the interpretation of carryover effects given the relatively short duration of this task. It is not clear why this effect was not significant during the first 500 ms of the analysis window for participants who received taVNS between ~6.0–7.0 mA. However, it is important to note that this effect was significant across the analysis window for the vast majority of participants across the range of taVNS intensities tested, including those who received taVNS above ~4.8 mA. This suggests that *all* taVNS intensities that were tested in this study led to some increase in pupil size that persisted *after* block 2 taVNS ended, although only intensities less

than ~4.8 mA significantly increased pupil size *during* taVNS. It is not obvious why taVNS intensities above ~4.8 mA would have a delayed, but not immediate, effect on pupil size. Further study is needed to replicate and explain this finding.

4.3 | Contributions of taVNS Parameters, Task, and Analysis Approach in Finding taVNS Effects

To the extent that taVNS engages the same mechanisms as iVNS, its effectiveness may similarly depend on stimulation intensity. Yet, there have been few systematic evaluations of taVNS intensity effects in humans (for exceptions see Capone et al. 2021; D'Agostini et al. 2023; Urbin et al. 2021). Studies investigating taVNS have typically tested one intensity level, which is either fixed across participants or individually titrated based on sensory thresholds, and these studies have found largely null effects on biomarkers known to reflect activity in the neuromodulatory systems taVNS is thought to engage. The use of GAMMs to fit potentially non-linear effects of taVNS intensity allowed us to find a specific range of taVNS intensities that elicited an immediate increase in pupil size without the need to limit analyses to summary measures or a priori determined windows of interest. Furthermore, GAMMs readily allow for modeling the potential influence of autocorrelation and gaze position on pupillary effects (van Rij et al. 2019). It is possible that fitting the same data with a linear model may have obscured the same effect. In addition, the ability to examine differences in height (via the parametric terms) and differences in trajectories (via the smooth terms) aids in determining whether shifts in the pupillary response are holistic (e.g., irrespective of time) or specific to certain time windows during the window of analysis.

The present analysis approach provides a template that can be used in future studies that seek to identify optimal taVNS parameters. The sections that follow highlight potential differences between the current study and previous studies, which may explain variation in the sensitivity of the pupil response to taVNS. Future studies should aim to explore these parameter spaces by testing the impact of these dimensions on the pupil response, ideally within the same participants.

4.3.1 | Active Versus Passive Tasks

The use of a passive task, only requiring the participant to fixate on a visual object during taVNS, may have also contributed to observing taVNS-related pupil dilation in this study. Seven out of nine prior studies that found taVNS effects on pupil size used a passive task that only required visual fixation (Capone et al. 2021; D'Agostini et al. 2023; Lloyd et al. 2023; Sharon, Fahoum, and Nir 2021; Skora, Marzecová, and Jocham 2024; Urbin et al. 2021; Wienke et al. 2023). In comparison, five of the eight studies that measured the TEPR elicited during active behavioral tasks did not find effects of taVNS on pupil size. These tasks required participants to identify stimuli based on discrimination of simple auditory features (D'Agostini et al. 2022; Keute et al. 2019), recognition of simple or complex visual features of target stimuli presented interspersed with (Burger, Van der Does, et al. 2020) or alongside visual distractors (Borges et al. 2021), or recognition of non-native speech sounds (McHaney et al. 2023). The three studies that found taVNS effects on pupil size measured TEPRs elicited during categorization of emotional expressions in the presence of conflicting written information (Wienke et al. 2023), discrimination of simple auditory features (Villani et al. 2022), and recognition of novel words distinguished by foreign language speech sounds. (Pandža et al. 2020). In two of these studies, active taVNS was linked to a reduced pupillary response (Pandža et al. 2020; Villani, Tsakiris, and Azevedo 2019). This finding is not unexpected given that one potential outcome of facilitating learning or improving cognitive performance with taVNS would be a reduction in the cognitive effort that is required to successfully perform the task. The facilitative effects of taVNS could reduce the cognitive effort during task performance by increasing processing efficiency (Pihlaja et al. 2020; Ridgewell et al. 2021), which would be expected to reduce TEPR amplitude (Beatty 1982). The lack of increased pupil size due to taVNS in experiments that measured TEPRs during active behavioral tasks may relate to reduction in cognitive effort during active versus sham taVNS, resulting in comparatively smaller TEPRs for active vs. sham taVNS that might cancel out effects of taVNS on LC activation that go in the opposite direction, i.e., larger pupil size for active vs. sham taVNS. Teasing these effects apart would benefit the identification of a taVNS biomarker.

4.3.2 | Variation in taVNS Intensity

Additionally, there are several differences between the taVNS parameters used in this and in prior studies that may contribute to apparent differences in the relationship between taVNS intensity and pupil dilation. Nine of the prior 16 taVNS studies that examined effects on pupil size reported testing taVNS intensities that overlapped in part or in whole with the taVNS intensity range in the present study (Capone et al. 2021; D'Agostini et al. 2022, 2023; Keute et al. 2019; Lloyd et al. 2023; Pandža et al. 2020; Sharon, Fahoum, and Nir 2021; Skora, Marzecová, and Jocham 2024; Urbin et al. 2021). Another four studies tested taVNS ranges below the minimum intensity in the present study (Burger, Van der Does, et al. 2020; D'Agostini et al. 2021; McHaney et al. 2023; Warren et al. 2019). The remaining three studies did not report taVNS ranges, but the reported taVNS intensity means and standard deviations indicate taVNS intensity was below 2.0 mA for at least some participants (Borges et al. 2021; Villani et al. 2022; Wienke et al. 2023). Ten of the twelve studies that tested overlapping taVNS intensities, but none of the four studies that tested only lower intensities, found pupil effects.

At first glance, these patterns of results might suggest that a minimum taVNS intensity level may be required to elicit a pupil response. It is not possible to evaluate this hypothesis with the present data because taVNS thresholds below 2.0 mA were not tested due to the DS8R being unable to reliably deliver lower intensities.[2] Without knowing the effects of taVNS on pupil size below 2.0 mA, it is not possible to determine whether the efficacy of the taVNS parameters tested here remains the same or changes at lower stimulation intensities. Results of prior studies also suggest that increasing taVNS intensity alone is not necessary or sufficient to elicit a pupil response. At least some participants in Urbin et al. (2021) received active taVNS below 2.0 mA, yet significant increases in pupil size over sham were

observed at both taVNS sites (concha *M(SD)* = 0.61(0.08) mA; EAM *M(SD)* = 1.04(0.1) mA; range = 0.08–4.4 mA across sites). In contrast, Keute et al. (2019) delivered 3.0 mA taVNS to all participants and found no effect on pupil size. Thus, differences in other taVNS parameters likely contribute to taVNS eliciting pupil changes.

4.3.3 | Variation in Location and Configuration of the Stimulating Electrode

Location and configuration of the stimulating electrode may also be important. All three experiments in past studies that applied taVNS to the EAM found pupil effects (Capone et al. 2021; Pandža et al. 2020; Urbin et al. 2021), while one of the two experiments that targeted the tragus found pupil effects (Villani, Tsakiris, and Azevedo 2019), and only eight of the 18 experiments in prior studies that targeted the concha found an effect on pupil size (D'Agostini et al. 2023; Lloyd et al. 2023; Sharon, Fahoum, and Nir 2021; Skora, Marzecová, and Jocham 2024; Urbin et al. 2021; Wienke et al. 2023). This suggests that stimulating the EAM is perhaps more consistently effective in increasing neuromodulatory activity. The only study to compare taVNS delivered to the EAM and the concha within participants found that perceptual thresholds were significantly greater at the EAM than the concha and that EAM stimulation generally elicited more robust pupil effects than concha stimulation (Urbin et al. 2021). Importantly, the authors noted that larger pupil dilation found for EAM versus concha stimulation were not due to differences in absolute current applied between the sites.

The anatomical evidence for the distribution of nerves in the human auricle is limited (Butt et al. 2020). The available evidence indicates that the cymba concha may be exclusively innervated by the ABVN (Peuker and Filler 2002), while the EAM, inner tragus, and cavum concha receive afferent innervation from the vagus as well as the greater auricular nerve and auriculotemporal nerve (see Butt et al. 2020 for a comprehensive review of the available evidence). Vagal innervation of the posterior aspect of the EAM noted in cadaver studies suggest the suitability of this region for applying taVNS. However, studies using functional magnetic resonance imaging (fMRI) largely suggest that applying taVNS to the cymba concha or inner tragus (Badran et al. 2018; Frangos, Ellrich, and Komisaruk 2015; Yakunina, Kim, and Nam 2017), but not the EAM, activates brain centers that are hypothesized in the taVNS mechanism of action (Yakunina, Kim, and Nam 2017). However, it should be noted that there are limitations to what can be concluded from these studies due to technical challenges associated with imaging very small brain regions targeted by taVNS and evidence that electrical stimulation of auricular sites that are not innervated by the ABVN, such as the earlobe, can elicit very similar patterns of brain activity (Butt et al. 2020).

Assuming that these imaging results reflect vagal activation, it is not clear why stimulating the EAM would more consistently elicit a pupillary response across studies. One possibility is that weak activation of NTS and LC in Yakunina, Kim, and Nam (2017) during EAM stimulation was because taVNS was applied to the inferoposterior wall of the EAM. Vagal innervation of the EAM may be localized to the posterosuperior wall (Kiyokawa et al. 2014), which is perhaps consistent with differential effects of applying taVNS to the posterior versus anterior EAM on activation of brain stem structures (Kraus et al. 2013). Pupil effects in the present and prior studies targeting the EAM may be due in part to stimulating electrodes contacting relatively large areas of the superior and posterior walls of the EAM (Pandža et al. 2020; Urbin et al. 2021) or overlapping with the inner side of the tragus (Capone et al. 2021). In addition to potentially engaging more vagal afferents with when using larger surface area electrodes (Pandža et al. 2020; Urbin et al. 2021), targeting superior and posterior aspects of the EAM may be more effective due to the larger number of A and B myelinated afferent fibers in these aspects of the EAM, which could potentially facilitate the central effects of taVNS applied to this location (Bermejo et al. 2017) as A and B nerve fiber activation is thought to drive therapeutic effects of VNS (Ruffoli et al. 2011). Although these afferents in the EAM likely do not belong exclusively to the ABVN, all may potentially drive increased activity of the NTS when stimulated with taVNS (Bermejo et al. 2017).

4.3.4 | Variation in Pulse Width and Stimulation Frequency

Another key difference is that a much shorter pulse width and higher stimulation frequency (50 μs, 300 Hz) were used in the present study compared to past taVNS studies, which almost universally tested 200–300 μs taVNS pulses delivered at 25–30 Hz. The focus on 20–30 Hz stimulation comes from iVNS work, which showed 50 Hz to be damaging to the vagus (Groves and Brown 2005). It is unclear whether the same frequency constraints apply to taVNS given the many differences from iVNS.

One study, Urbin et al. (2021), directly compared 25 to 300 Hz stimulation and found that 300 Hz taVNS elicited a shorter latency pupil response onset and peak at both the concha and EAM, but the only frequency effect on pupil response intensity was that 300 Hz elicited a greater area under the curve than 25 Hz and only when applying taVNS to the EAM. Evidence from rats and mice indicates that increasing the total charge per VNS pulse, by increasing the pulse width or stimulation intensity, increases the firing rate of LC neurons (Hulsey et al. 2017) and pupil dilation (Mridha et al. 2021), but the effect plateaus above a certain total charge (Hulsey et al. 2017). When the number of VNS pulses is held constant, increasing the frequency of pulse delivery does not alter the total amount of LC activity, but it does increase the amount of LC activity per unit time (Hulsey et al. 2017).

In principle, delivering 50 μs taVNS pulses at 300 Hz in the present study could drive higher levels of LC activity per unit time than past studies, which almost universally used 200–300 μs pulses delivered at 25–30 Hz (see Table 1). Although the pulse width in the current study is one-fifth to one-sixth the length used in prior studies, the pulse rate is ten times higher. Additional support for this idea comes from findings in rats that iVNS delivered at 300 Hz and above increased the synchrony of LC neuron firing compared to iVNS at 30 Hz and below (Farrand et al. 2023). In line with this, 100 Hz taVNS delivered to the cymba concha elicited stronger activation of the NTS and LC in humans compared to 2, 10, and 25 Hz stimulation

(Sclocco et al. 2020). One additional benefit of using a shorter pulse is that it may be possible to attain higher taVNS intensities while maintaining stimulation below perceptual threshold (Badran et al. 2019; Tyler et al. 2019, Table 1). Because pupil dilation increases with LC firing rate (Joshi et al. 2016; Megemont, McBurney-Lin, and Yang 2022; Reimer et al. 2016), perhaps some combination of higher taVNS intensity and frequency contributed to pupil dilation in the present study.

One topic that requires further investigation is the potential for different taVNS parameters to elicit qualitatively distinct physiological changes, even within the same neuromodulatory system. For example, all past studies listed in Table 1 that delivered short trains of taVNS with typical pulse width (200–400 μs) and frequency ranges (25–30 Hz) during a passive task found significant effects of taVNS on the SEPR, which is thought to reflect phasic LC activity. However, only one of the many studies that delivered longer trains of taVNS (30 or more seconds) with similar pulse width and frequency ranges found a significant effect of taVNS on resting-state pupil averaged over longer time periods, which is thought to reflect tonic LC activity (Capone et al. 2021).

4.3.5 | Variation in the Nature of the Sham Comparison

All but one prior study of taVNS effects on pupil size included an active sham taVNS comparison, whereas passive sham taVNS was used in the present study. Although the earlobe lacks vagal innervation, stimulating the earlobe, especially when above perceptual threshold, may lead to pupil dilation, thus obscuring the effects of active stimulation applied to target locations with vagus innervation (Butt et al. 2020). Indeed, active sham taVNS applied to the earlobe has been found to elicit pupil responses in some previous studies (Urbin et al. 2021).

Relatedly, although we were careful to deliver taVNS below each participant's perceptual threshold in the present study, it is possible that the observed increase in pupil size during active compared to sham taVNS blocks could be driven in part by sensation of the taVNS (Beatty and Lucero-Wagoner 2000). For example, noxious stimuli can elicit pupil dilation (e.g., electrical fingertip stimulation; Chapman et al. 1999). We cannot address this possibility directly because we did not obtain information from participants about sensations they might have perceived during the pupillary light reflex task or whether they could identify the task blocks during which they received active taVNS. However, analyses of off-target taVNS effects and blinding efficacy measures that were obtained during subsequent testing sessions when taVNS was paired with training are encouraging. In the sample of participants who completed all four training sessions, which includes all but 16 participants who are represented in the present study, those who received active taVNS did not rate feeling more pain or irritation in the ear receiving taVNS or other sensations compared to participants who received passive sham taVNS during training. Further, among participants who received active taVNS during training sessions, the vast majority (68.4%–88.2% across groups) were unable to correctly identify when they received active taVNS during the training tasks.

One further observation that supports our interpretation of the results is that only those participants who received active taVNS at or below ~4.8 mA showed an increase in pupil size during active taVNS. Participants who received taVNS at higher intensities did not show a difference in pupil size between active and sham taVNS. This is the opposite of what would be expected if taVNS-evoked sensation was responsible for the pupil size differences observed in this study since higher intensity produces stronger sensation. However, we acknowledge that using active taVNS delivered to the earlobe for the control condition would provide additional control over changes in pupil size due to unintended sensory perception of taVNS. Future studies may benefit from combining the advantages of delivering taVNS below perceptual threshold with the use of an active control condition, such as delivering sub-perceptual taVNS to the earlobe.

4.3.6 | Analysis of the Left Versus Right Eye

One final aspect of the present analysis approach may have contributed to finding taVNS effects on pupil size. The present analysis was based on pupil data averaged from the left and right eyes. A recent study found that taVNS elicited pupil dilation in the left but not right eye, ipsilateral to the stimulation ear (Capone et al. 2021). Findings in rats also indicate that LC stimulation elicits significantly larger pupil dilation in the eye ipsilateral to stimulation, and LC stimulation influences the contralateral pupil via parasympathetic pathways only, while ipsilateral pupil dilation is mediated by sympathetic and parasympathetic pathways (Bianca and Komisaruk 2007; Liu et al. 2017).

Of the nine prior studies that found a taVNS pupil effect, three analyzed pupil data from the left eye (D'Agostini et al. 2023; Urbin et al. 2021; Wienke et al. 2023), one analyzed pupil data from the right eye (Pandža et al. 2020), two analyzed data averaged for left and right eyes (Capone et al. 2021; Skora, Marzecová, and Jocham 2024), and three did not report whether analysis was based on the left or right eye (Lloyd et al. 2023; Sharon et al. 2021; Villani et al. 2022).[3] The study that the present work was based on also found left-eye pupil dilation following iVNS (Desbeaumes Jodoin et al. 2015).

Of the seven studies that did not find a pupil effect, two analyzed left-eye data (D'Agostini et al. 2021; McHaney et al. 2023), three analyzed right-eye data (Borges et al. 2021; D'Agostini et al. 2022; Keute et al. 2019), and two did not report the analysis eye (Burger, Van der Does, et al. 2020; Warren et al. 2019). Thus, while differences in the presence of pupil effects between taVNS studies examining the left versus right pupil are somewhat inconsistent, the lateralization of pupil effects noted above suggests that analysis of left-eye pupil data may be more likely to yield robust findings in future taVNS studies given that taVNS is almost always administered to the left ear to avoid potential adverse cardiac effects (Farmer et al. 2021). However, whenever possible, recording data from both eyes offers the advantage of filling in samples that are missing from one eye. Furthermore, without a strong a priori hypothesis regarding lateralization effects, analyzing the average of the two eyes (as done in the current study) may represent a more conservative approach to examining the impact of taVNS on the pupil response. Interestingly, related to the earlier discussion of stimulation rate, Liu et al. (2017) showed that the relative contribution of the ipsilateral sympathetic pathway to pupil dilation was inversely

related to stimulation rate, while there was no frequency effect for the relative contribution of the ipsilateral parasympathetic pathway.

4.4 | Limitations and Future Directions

Pupil dilation reflects activity of several neural circuits that are variously influenced by the principal neuromodulators that VNS is thought to target, including NE and ACh (Joshi and Gold 2020; Strauch et al. 2022). Given this, it is not possible to attribute taVNS-related pupil dilation in the present study specifically to LC activation (Larsen and Waters 2018). Further, because taVNS intensity was not manipulated within participants in the present study, an alternative explanation for the observed results is that individuals who perceived taVNS between 2.0 and ~4.8 mA were more responsive to taVNS, while those with higher perceptual thresholds may be taVNS non-responders. Future taVNS studies may address this possibility by analyzing whether taVNS perceptual intensity systematically differs between individuals who have been determined by some other means to be taVNS responders versus non-responders.

Measuring pupil dilation preceding and during the pupillary light reflex was used in Desbeaumes Jodoin et al. (2015) because it provides an opportunity to tease apart sympathetic and parasympathetic influences of iVNS. Pupil dilation is influenced indirectly by both inhibitory and excitatory inputs from the LC. Increased LC activity can inhibit parasympathetic activity of the Edinger-Westphal nucleus (EWN), thus inhibiting pupil constriction (Samuels and Szabadi 2008a). LC activation attenuates the pupillary light reflex via its inhibitory effects on the EWN (Samuels and Szabadi 2008b). Increased LC activity can also increase sympathetic excitatory input to the superior cervical ganglion (SCG), thus driving pupil dilation (Mridha et al. 2021). It is of interest to determine whether pupil dilation during taVNS is due to changes in one or both systems.

In principle, the use of GAMMs to analyze the data in the present study would allow differences between active and sham taVNS to appear on specific features of the pupillary light reflex and during the prestimulus resting-state period. However, our results indicated that increased pupil diameter during active versus sham taVNS was fairly consistent across the analysis window and thus do not suggest differential effects of taVNS on sympathetic and parasympathetic activity. While this is largely consistent with the findings reported in Desbeaumes Jodoin et al. (2015), it is possible that differences would be found if analyzing specific features of the pupillary light reflex, such as maximum constriction velocity or maximum constriction acceleration (selectively sensitive to parasympathetic activity), or 75% amplitude recovery time or recovery amplitude after 2.4 s, which are selectively sensitive to sympathetic activity (see Desbeaumes Jodoin et al. 2015 for details). We did not extract and analyze such features in the present study because we determined that modeling the entire pupillary response using GAMMs would provide the most informative results for the present aims. Once reliable taVNS parameters are established, it will be more practical to tease apart sympathetic and parasympathetic influences.

A further potential complication is that pupil size generally decreases over the course of a task, but the rate of constriction may level off due to physiological limits of the pupil (Unsworth, Robison, and Miller 2019). This presents a challenge for dissociating potentially cumulative or carryover effects of taVNS on resting-state pupil dilation from changes in pupil constriction related to time on task (e.g., a lessening of pupil constriction as a task progresses). This should be addressed in future work. One potential solution is to explicitly model the effect of time on task, as in the present study. This approach allows statistical models of pupil size to account for variance due to time on task, resulting in more accurate model estimates of the effect of taVNS. Experimental design may also help to dissociate pupil changes due to time on task from cumulative or carryover effects of taVNS when used in conjunction with modeling time on task. For the present study design, including a control group of participants who complete the same task but do not receive active taVNS and allowing a predictor for participant group to interact with the model terms involving trial number and task half would help strengthen the interpretation of these terms in relation to time on task versus carryover taVNS effects. In this case, the effect of task half would be expected to differ between groups while the effect of trial may not.

It is important to recognize that there is a potential dissociation between the effects of taVNS on pupil size and the desired behavioral outcomes. The link between LC-NE activation via taVNS and target behavioral outcomes, such as improved learning and memory performance, is not direct, and finding taVNS parameters that reliably dilate the pupil does not guarantee the same parameters will lead toimproved target outcomes. Animal and human studies involving iVNS have shown that iVNS is most effective at a moderate intensity (~0.4–0.8 mA), with target behavioral and physiological effects (such as cortical changes) lessening at intensities above and below this range (Borland et al. 2016; Clark et al. 1999). Thus, finding a biomarker for LC-NE activation due to taVNS is a first step. For this biomarker to be useful, further work is needed to establish its relationship to target outcomes.

5 | Conclusions

These present findings contribute to the limited evidence that has been found for the relationship between taVNS intensity and pupil dilation. The taVNS parameters used in this study, specifically stimulation of the EAM below perceptual threshold using 50 μs pulses delivered at 300 Hz, have been understudied relative to wider taVNS pulses (200–300 μs) delivered at slower rates (25–30 Hz) to other areas of the outer ear, most often the cymba conchae. The results of this study indicate that our stimulation parameters replicate and extend the effects of iVNS in Desbeaumes Jodoin et al. (2015) and the effects of taVNS in Wienke et al. (2023) on overall pupil size during stimulation, reflecting increased LC-NE activity due to taVNS. This finding adds to the evidence suggesting that 300 Hz taVNS applied to the EAM effectively engages the LC-NE system. The present study's results also suggest a non-linear relationship between taVNS intensity and resulting increases in pupil size, suggesting that pupillometry may provide a useful physiological biomarker of the effectiveness of taVNS at modulating LC-NE

activity. Establishing a reliable biomarker for LC-NE activation via taVNS is needed to titrate stimulation for different applications and individuals and is critical to advancing the use of taVNS (Yap et al. 2020).

Author Contributions

Ian Phillips: conceptualization, data curation, formal analysis, investigation, methodology, software, writing – original draft. **Michael A. Johns:** data curation, formal analysis, investigation, methodology, visualization, writing – original draft. **Nick B. Pandža:** formal analysis, investigation, methodology, writing – review and editing. **Regina C. Calloway:** investigation, methodology, writing – review and editing. **Valerie P. Karuzis:** data curation, investigation, writing – original draft. **Stefanie E. Kuchinsky:** conceptualization, funding acquisition, methodology, supervision, writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available online in the Open Science Framework (OSF; https://osf.io/8p47b/).

Endnotes

[1] Due to equipment malfunction, one participant completed the pupillary light reflex task at the end of the final post-test session of the larger study. Although they received active taVNS during 4 days of training, they had not received taVNS during the 27 days leading up to this session.

[2] A firmware update to the DS8R has since allowed intensities lower than 2.0 mA to be reliably delivered.

[3] Right-eye pupil dilation in Pandža et al. (2020) was interpreted as an index of attentional effort, rather than LC-NE activity, due to the learning nature of the task.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.