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RESEARCH REPORT

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Effects of auricular vagus nerve stimulation and electrical earlobe stimulation on motor-evoked potential changes induced by paired associative stimulation

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Abstract

Paired associative stimulation (PAS) is a combination of transcranial magnetic stimulation (TMS) and peripheral nerve stimulation (PNS). PAS can induce long-term potentiation (LTP)-like plasticity in humans, manifested as motor-evoked potential (MEP) enhancement. We have developed a variant of PAS ("high-PAS"), which consists of high-frequency PNS and high-intensity TMS and targets spinal plasticity and promotes rehabilitation after spinal cord injury (SCI). Vagus nerve stimulation (VNS) promotes LTP-like plasticity and enhances recovery in SCI and stroke in humans and animals when combined with repetitive motor training. We combined high-PAS with simultaneous noninvasive transcutaneous auricular VNS (aVNS) to determine if aVNS enhances the extent of PAS-induced MEP amplitude increase. Sixteen healthy participants were stimulated for 20 min in four different sessions (PAS, PAS + aVNS, PAS + shamVNS, and aVNS) in a randomized single-blind setup. MEPs were measured before, immediately after, and at 30, 60, and 90 min post-stimulation. Stimulation protocols with PAS significantly potentiated MEPs ($p = 0.005$) when compared with aVNS ($p = 0.642$). Although not significant, MEP enhancement observed after PAS (43.5%) is further increased by aVNS (49.7%) and electrical earlobe stimulation (63.9%). Our aVNS setup failed to significantly enhance the effect of PAS, but sham VNS revealed a trend towards enhanced plasticity. Optimization of auricular VNS stimulation setup is required for possible tests of patients with SCI.

Abbreviations: ABVN, auricular branch vagus nerve; ANS, autonomic nervous system; aVNS, auricular transcutaneous VNS; ECG, electrocardiogram; HF, high frequency; HR, heart rate; HRV, heart rate variability; ISI, interstimulus interval; LTP, long-term potentiation; LF, low frequency; MEP, motor-evoked potential; MSO, maximum stimulator output; nTMS, navigated TMS; PAS, paired associative stimulation; PNS, peripheral nerve stimulation; pNN50, normal to normal intervals (NN), number of consecutive NN interval pairs differing by more than 50 ms (NN50) divided by the total number of all NN intervals, demonstrating beat-to-beat variation; RR, beat to beat RR interval; RMSSD, root mean square of successive differences between RR intervals; RMT, resting motor threshold; SCI, spinal cord injury; SO, stimulator output; TMS, transcranial magnetic stimulation; VNS, vagus nerve stimulation.

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motor-evoked potential, peripheral nerve stimulation, spinal plasticity, transcranial magnetic stimulation

1 | INTRODUCTION

Paired associative stimulation (PAS) was first introduced by Stefan et al. (2000) and has been used to induce spinal (Cortes et al., 2011) and cortical plasticity (Stefan et al., 2000). PAS is based on Hebbian plasticity (Hebb, 1949), where synchronous activation of presynaptic and postsynaptic neurons enhances synaptic strength. PAS combines a low-frequency transcranial magnetic stimulation (TMS) with peripheral electrical nerve stimulation (PNS). TMS induces neuronal activity in motor cortical circuits and the pyramidal tract, whereas PNS induces activation in somatosensory afferents. In protocols aimed at spinal cord level plasticity, PNS is designed to also activate the efferent motor neurons (Suppa et al., 2017). Using specific interstimulus intervals (ISI), the antidromic volley induced by peripheral stimulation and the orthodromic volley induced by TMS are timed to reach the dendrites and presynaptic terminals producing neuronal activations at either the cortical (Stefan et al., 2000) or spinal level (Bunday & Perez, 2012; Taylor & Martin, 2009).

The timing, frequency, and intensity of the presynaptic and postsynaptic signals are key factors for long-term potentiation (LTP)-induced plasticity (Dan & Poo, 2004). We have developed a modified version of PAS ("high-PAS"), which uses high-intensity navigated TMS (100% the maximum stimulator output, MSO) and high-frequency (100 Hz) PNS designed to activate motor neurons (Tolmacheva, Mäkelä, & Shulga, 2019). In high-PAS, the frequency and timing of cortical and peripheral stimulations are selected such that the induced multiple simultaneous orthodromic and antidromic neuronal activations coincide at the spinal level (Shulga et al., 2021). The high-PAS protocol induces a stable amplitude increase of motor-evoked potentials (MEPs, the responses to TMS measured from target muscles) and successfully enhances motor performance in patients with incomplete spinal cord injury (SCI) (Shulga et al., 2015, 2016, 2021). We observed improved motor capabilities in upper and lower limbs of patients with incomplete SCI after 20–24 weeks (Shulga et al., 2016), 4 weeks (Tolmacheva et al., 2017), 6 weeks (Tolmacheva, Savolainen, et al., 2019), 47 weeks (Rodionov et al., 2019), 8 weeks (Rodionov et al., 2020), and 6 months (Shulga et al., 2020) of high-PAS.

Invasive vagus nerve stimulation (VNS) also promotes plasticity in the context of central nervous system lesions, including an animal model of incomplete SCI. When paired with movement, VNS induces movement-specific plasticity in the motor cortex and improves upper limb function after stroke (Hays, 2016). In SCI, a similar plasticity enhancement with VNS can occur in the spinal cord neuronal synapses (Ganzer et al., 2018). VNS triggers the release of norepinephrine, acetylcholine, serotonin, brain-derived neurotrophic factor, and fibroblast growth factors, which in turn lead to expression of proteins that support spike time-dependent potentials (Hays, 2016; Hulsey et al., 2016, 2017).

VNS enhances plasticity when triggered during successful movements (Meyers et al., 2019), and high-PAS activates preserved motor pathways as a successful movement (Shulga et al., 2021). We hypothesized that VNS may enhance the action of high-PAS, which may be beneficial in the treatment of incomplete SCI. VNS is usually delivered by an invasively implanted device (Kilgard et al., 2018; Kimberley et al., 2018). Activating the vagus nerve noninvasively through auricular cutaneous vagal afferents (Butt et al., 2020) of the external ear is also feasible. This approach avoids possible adverse events associated with surgical procedures for stimulator implantation (Ben-Menachem et al., 2015). We combined

a noninvasive high-PAS with noninvasive VNS and studied their combined effect in healthy volunteers.

Cadaver studies have revealed that most substantial auricular branch vagus nerve (ABVN) innervation is in the cymba conchae (Yakunina et al., 2017) and tragus (Badran, Brown, et al., 2018). These areas may be the best locations for noninvasive VNS (Peuker & Filler, 2002). Auricular transcutaneous VNS (aVNS) has been delivered in various ways. The variable anatomical location of the electrodes, for example, tragus (Badran et al., 2022) or cymba concha (Yokota et al., 2024), intensity at sensory level (Clancy et al., 2014), or >1 mA (Redgrave et al., 2018) have been described. Short stimulation during the movement (Dawson et al., 2016; Redgrave et al., 2018) or 15-min stimulations (Bretherton et al., 2019) have been applied. aVNS has been tested in the treatment of depression (Tu et al., 2018), drug-resistant epilepsy (Yang et al., 2023), and for motor recovery after stroke (Redgrave et al., 2018). Nevertheless, aVNS effects and the most optimal use for rehabilitation are not known. A recent consensus article describes minimum standards for reporting aVNS experiments (Farmer et al., 2021); we have applied them in this study (supporting material). The earlobe, which is innervated by the great auricular nerve (Peuker & Filler, 2002), has no vagus nerve fibres and is a common site for sham stimulation in studies using noninvasive VNS (Badran, Dowdle, et al., 2018; Yakunina et al., 2017).

Heart rate variability (HRV) is a noninvasive method that measures intervals between QRS complex from electrocardiogram (ECG) (Task Force of the European Society of Cardiology the North American Society of Pacing Electrophysiology, 1996). Variability between the intervals has been used to describe neurocardiac function and the state of the autonomic nervous system (Shaffer & Ginsberg, 2017). VNS has been reported to reduce sympathetic activity (Clancy et al., 2014) or to have small nonsignificant changes on HRV (Gauthey et al., 2020). However, the results were not conclusive due to variable study designs and stimulation protocols (Soltani et al., 2023). HRV is not an accurate biomarker that can reliably indicate aVNS-induced vagal activation (Wolf et al., 2021). We have previously reported increased parasympathetic activation during PAS in healthy individuals (Haakana et al., 2023), and we recorded HRV in this study to exclude adverse effects or stress related effects to our stimulation protocols.

We have optimized the high-PAS induced MEP increase by modifications of stimulation parameters (Shulga et al., 2016, 2021; Tolmacheva, Mäkelä, & Shulga, 2019). The purpose of this single-blind, sham-controlled study was to investigate whether simultaneous aVNS can further enhance the effects of high-PAS on TMS-induced MEPs.

2 | MATERIALS & METHODS

The study was approved by the Medical Ethics Committee of the Helsinki University Central Hospital and was registered at clinicaltrials.gov (NCT04938284 first date of registration 24/06/2021). Sixteen healthy subjects (10 females, mean age 32 ± 9.5 years) participated in the study (Table 1). Exclusion criteria were brain pathology, implanted devices, regular medication, neurological diseases, skin problems in the ear area, ear infections, cardiac diseases, psychiatric diseases, drug abuse, and pregnancy. All participants provided written informed consent prior to the study, and all experiments were performed in accordance with the Helsinki declaration. Handedness was self-reported; two participants were left-handed.

All participants had four stimulation sessions (PAS, PAS + aVNS [cymba concha and cavum concha], PAS + shamVNS [earlobe], and aVNS alone). The random order of sessions was created by a randomization tool (www.randomization.com). Both aVNS and sham electrodes were attached to the cymba concha, cavum concha, and earlobe during all sessions (Figure 1) to avoid any arousal effects from skin contact. The sessions were separated by at least 1 week to avoid possible carryover effects. The total session duration was approximately 150 min, including 20 min of active stimulation. The sessions were scheduled at the same time of day. One participant with shift work had the sessions scheduled to match the participant's sleeping rhythm.

PAS was performed as described previously (Shulga et al., 2015, 2021). For PNS, six 1-ms biphasic square pulses at 100 Hz were delivered to the tibial nerve behind the left medial malleolus using a Dantec Keypoint® electroneuromyography device (Natus Medical Incorporated, California, USA). Lower limb stimulation enabled comparison with our previous data on healthy subjects. Baseline MEP values are also less variable in lower than upper limb muscles, as subjects can fully relax the lower limb muscles more easily in seated position. The stimulation intensity was defined individually by measuring the lowest intensity eliciting detectable F-responses as described previously (Shulga et al., 2015). Participants were offered local anaesthesia (5% lidocaine/prilocaine [EMLA]) ointment to reduce the pricking skin sensation associated with the stimulation (Gajraj et al., 1994). Fifteen participants chose to use this.

Navigated TMS (nTMS) was delivered by an eXimia magnetic stimulator (Nexstim Ltd, Helsinki, Finland) with a figure-of eight coil (outer diameter 70 mm). Single biphasic pulses at 100% (MSO) were used in PAS. For MEP measurements, TMS was delivered at 120% of the individual resting motor threshold (RMT). The optimal

TABLE 1 Subject characteristics and individual parameters for the high-PAS protocol.

ID	Sex	Age (years)	Handedness	F latency (ms)	F intensity (mA)	RMT persistence/10	15MEP latency (ms)	120% RMT intensity	ISI (ms)
1	F	40	R	54.8	20.0	5	42.2	78.0	12.6
2	M	50	L	50.0	18.0	8	40.8	73.0	9.2
3	F	28	R	50.8	12.5	9	38.2	91.0	12.6
4	M	31	R	48.8	28.5	8	36.7	72.0	12.1
5	M	28	R	51.3	5.0	8	45.0	70.0	6.3
6	F	36	R	48.8	7.5	8	39.4	91.0	9.4
7	F	50	R	57.3	8.0	6	42.8	92.0	14.5
8	M	38	R	60.5	10.0	3	46.9	54.0	13.6
9	F	28	L	48.7	8.5	6	40.4	59.0	7.4
10	F	22	R	48.8	5.0	10	42.4	76.0	6.4
11	F	32	R	51.3	6.5	3	42.5	56.0	8.9
12	F	24	R	48.8	6.0	5	38.9	56.0	9.9
13	M	27	R	49.8	6.0		42.1	72.0	7.7
14	M	43	R	54.8	11	1	44.2	100.0	10.6
15	F	19	R	48.1	5.2	2	38.9	96.0	9.2
16	F	20	R	48.3	5.0	2	36.9	65.0	11.4

Note: ISI was calculated based on F and MEP latencies as described previously (Shulga et al., 2015). High RMT values were needed to activate the lower limb target muscles.

Abbreviations: ISI, interstimulus interval; MEP, motor-evoked potential; PNS, peripheral nerve stimulation; RMT, resting motor threshold; SO, stimulator output.

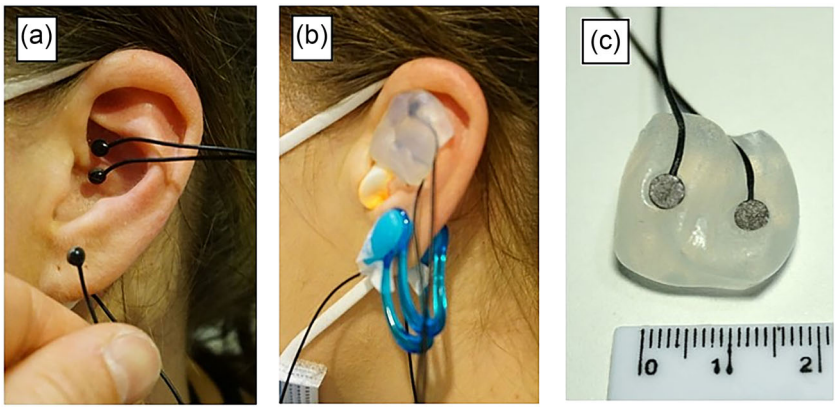

FIGURE 1 Electrode placements. (a) Cymba conchae and cavum conchae for aVNS, (b) earlobe for sham electrode attachment to the ear with silicone (aVNS), and (c) clip (sham) interelectrode distance for aVNS electrodes.

TMS target was determined using individual MRIs from the motor cortex area representing the left abductor hallucis muscle. An average amplitude of 30 MEPs was assessed before (PRE), immediately after (POST), and at 30 min (POST30), 60 min (POST60), and 90 min (POST90) after the stimulation sessions. Thirty MEPs were averaged per time point. MEPs associated with visually detected preactivation in muscle electromyography 200 ms before MEPs were excluded and subsequent MEPs (equal number to removed responses) were added.

aVNS was delivered using a Dantec Keypoint® electroneuromyography device (Natus Medical Incorporated, California, USA). Ag/AgCl electrodes with a 4-mm diameter (Biomed Products Inc., California, USA) were used. Active aVNS was delivered to the left cymba conchae (cathode) and cavum conchae (anode). The electrodes were attached with a silicone mold. The interelectrode distance was on average 0.8 cm (range 0.6–1 cm). Sham stimulation was given to the left earlobe. The electrodes were attached with a clip on both sides of the earlobe (Figure 1).

Perceptual threshold was determined at the beginning of each session for both active and sham stimulation (Table 2). The threshold level was set individually by

TABLE 2 Individual perceptual thresholds for aVNS and sham stimulation and stimulation intensities used in each session (in bold).

Session	PAS		PAS + aVNS			PAS + shamVNS			aVNS		
ID	Concha perceptual threshold mA	Earlobe perceptual threshold mA	Concha perceptual threshold mA	Earlobe perceptual threshold mA	aVNS stimulation intensity mA	Concha perceptual threshold mA	Earlobe perceptual threshold mA	shamVNS stimulation intensity mA	Concha perceptual threshold mA	Earlobe perceptual threshold mA	aVNS stimulation intensity mA
1	0.4	0.4	0.4	0.4	**0.2**	0.4	0.5	**0.3**	0.3	0.4	**0.1**
2	0.6	0.9	0.9	0.6	**0.7**	0.7	0.5	**0.3**	0.5	0.4	**0.3**
3	0.4	0.3	0.3	0.3	**0.1**	0.3	0.4	**0.2**	0.6	0.5	**0.4**
4	0.6	0.7	0.3	0.7	**0.1**	0.7	0.9	**0.7**	0.4	0.7	**0.2**
5	0.4	0.6	0.5	0.8	**0.3**	0.5	0.6	**0.4**	0.4	0.6	**0.2**
6	0.7	0.3	0.2	0.8	**0.1**	0.5	0.5	**0.3**	0.6	0.6	**0.4**
7	0.3	0.8	0.5	0.7	**0.3**	0.5	0.9	**0.7**	0.3	0.5	**0.1**
8	0.4	0.4	0.3	0.3	**0.1**	0.3	0.3	**0.1**	0.2	0.1	**0.1**
9	0.2	0.4	0.1	0.4	**0.1**	0.2	0.3	**0.1**	0.4	0.3	**0.2**
10	0.3	0.3	0.2	0.3	**0.1**	0.4	0.3	**0.1**	0.2	0.2	**0.1**
11	0.5	0.8	0.4	1.2	**0.2**	0.7	0.8	**0.6**	0.2	1.0	**0.1**
12	0.7	0.3	0.4	0.5	**0.2**	0.4	0.4	**0.2**	0.1	0.4	**0.1**
13	0.7	0.6	0.9	1.2	**0.7**	0.5	0.6	**0.4**	0.4	1.1	**0.2**
14	1.2	1.4	0.6	0.7	**0.4**	1.0	1.4	**1.2**	0.5	0.8	**0.3**
15	1.2	0.6	1.1	0.9	**0.9**	0.3	0.5	**0.3**	1.2	0.9	**1.0**
16	0.2	0.3	1.2	0.6	**1.0**	0.9	0.5	**0.3**	0.9	0.6	**0.7**

Note: The stimulation intensity was defined as cymba (aVNS) or earlobe (shamVNS) perceptual threshold minus 0.2 mA. See main text for details.

ramping the intensity up in steps of 0.1 mA and down in steps of 0.3 mA if stimulation caused sensation, as described in Llanos et al. (2020). Five-hundred-millisecond trains of 200-μs biphasic square pulses at 30 Hz, 0.2 mA below perceptual threshold, were delivered to the auricular electrodes for aVNS and to the earlobe for sham VNS. In two participants with a low perceptual threshold, the intensity was 0.1 mA below the threshold; in one participant, the intensity matched the threshold. Stimulation intensities were 0.34 ± 0.30 mA for PAS + aVNS, 0.39 ± 0.28 mA for PAS + shamVNS, and 0.28 ± 0.24 mA for aVNS session.

Electrode paste (Ten20, Weaver and company, CO, USA) was used to improve skin contact, which was continuously measured during stimulation. The device sounded an alarm if the impedance of the contact was lost. This occurred in two out of 64 sessions. In these cases, electrodes were reattached and stimulation continued according to the protocol.

TMS, PNS, and aVNS were triggered by Presentation® software (Neurobehavioral Systems Inc., Berkeley, CA, USA). PAS (TMS + PNS with an individual ISI) was delivered once every 5 s. aVNS was synchronized with every second PAS (Figure 2). Altogether, 240 PAS and 120 aVNS sequences were delivered in each session. None of the participants had received aVNS prior to this study. Four participants had previous experience with TMS.

Participants were blinded to the stimulation sequence. The sensory thresholds for aVNS and shamVNS stimulation were determined at the beginning of each session in the same order. The stimulation device was behind the participants, and they could not see the details of the stimulation. Additionally, as the stimulation intensity was below the perceptual threshold, the participants could not feel which stimulation was delivered.

HRV and blood pressure (M6 AC, Omron, Kyoto, Japan) were measured during each session. HRV was recorded with Bittium Faros 180 (Bittium, Oulu, Finland) with three ECG electrodes (Blue sensor, Ambu A/S, Ballenrup, Denmark) at sampling rate of 1000 Hz. The ECG electrodes and recording unit were attached in the beginning of the session; participants then rested for 15 min. HRV was recorded at the end of the rest period (PRE), during stimulation (STIM), immediately after stimulation (POST), and before POST30 MEPs (POST30), POST60 MEPs (POST60), and POST90 MEPs (POST90). Blood pressure was recorded twice at the same time-points. Strong body movement during TMS induced artefacts in some subjects, and these values were excluded. The values of the second measurement are reported.

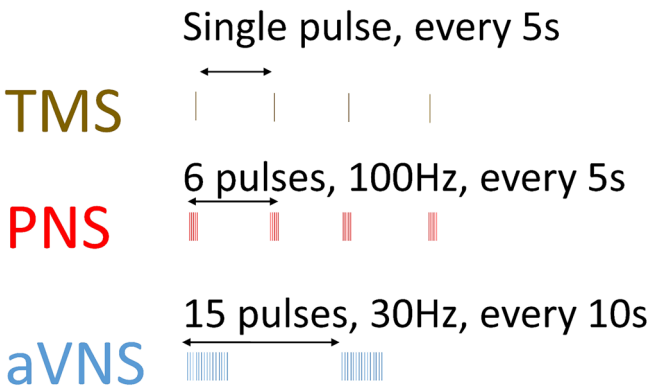

FIGURE 2 Sequence of pulses from TMS, PNS, and aVNS.

A representative 5-min segment from the area of interest without any major noise from movement or MEP stimulation was selected for the HRV analysis with Kubios HRV Scientific software (Kubios, Kuopio, Finland). HRV analysis parameters included mean heart rate (Mean HR), mean beat-to-beat RR interval (Mean RR), root mean square of successive differences between RR intervals (RMSSD), percentage of successive RR intervals differing over 50 ms (pNN50), low frequency (LF) and high frequency (HF) powers of HRV spectral density, LF/HF power ratio, ratio of Poincaré plot standard deviations (SD2/SD1), and ECG-derived respiration rate (see Table 3).

One participant was excluded due to extrasystoles. For four participants, the upper boundary of the LF band had to be decreased to 0.14 Hz due to low breathing frequency. HRV was not analysed during stimulation due to low breathing frequency (0.09, 0.08, and 0.12 Hz) for one participant and due to a stimulation artefact from TMS stimulator for another participant. Two other recording time points were removed for individual sessions for two participants, POST90 at aVNS session for ID 4 and POST30 at aVNS session for ID 14 due to low breathing frequency (0.09 and 0.13 Hz, respectively).

Before each session, participants were asked about the experienced stress and pain levels, the quality of sleep during the previous night, the type of any exercise during the previous 24 h, medications on the day of the session, and if the day of the session was an ordinary day in general. Participants were asked not to drink beverages containing caffeine before the sessions and to avoid vigorous exercise the day before. One participant drank an energy drink before all sessions and three participants drank a cup of coffee or tea once per their daily habit. Two participants had an unpleasant sensation and cold sweat during PAS sessions but felt that the effects were not strong enough to require stopping the experiments. No other adverse events were observed.

Statistical analyses were performed with IBM SPSS 27 software. The data were not normally distributed as assessed with the Shapiro Wilk test. Nonparametric tests

TABLE 3 Description of heart rate and HRV parameters in time-domain, frequency domain, and nonlinear HRV parameters with their main association to autonomic nervous system function (ANS).

HRV parameter	Units	Description	Association with ANS function	
Heart rate parameters			**Parasympathetic**	**Sympathetic**
Mean RR	(ms)	Mean of the selected beat-to-beat RR interval series, inversely proportional to mean heart rate	↑	↓
Mean HR	(bpm)	Mean heart rate, inversely proportional to mean RR	↓	↑
Time-domain HRV parameters				
RMSSD	(ms)	Root mean square of successive differences between RR intervals, demonstrating beat-to-beat variation	↑	
pNN50	(%)	NN50 divided by the total number of all NN intervals, demonstrating beat-to-beat variation	↑	
Frequency-domain HRV parameters				
LF power	(ms^2)	Low-frequency power (frequency range 0.04–0.15 Hz) extracted from RR interval time series power spectrum		↕
HF power	(ms^2)	High-frequency power (frequency range 0.15–0.4 Hz) (synchronous with respiration); estimates parasympathetic/vagal activation	↑	
LF/HF		LF/HF power ratio	Sympathetic vs. parasympathetic	
Resp	(Hz)	Respiratory rate estimated from the ECG and HRV data		
Nonlinear HRV parameters				
SD2/SD1		SD2/SD1 ratio (in Poincaré plot, the standard deviation of RR intervals perpendicular to (SD1, demonstrating beat-to-beat variability) and along (SD2, demonstrating overall variability) the line of identity)	Sympathetic vs. parasympathetic	

Note: pNN50 indicates normal to normal intervals (NN, see list of abbreviations). ↑ indicates ANS activation tends to increase the HRV or heart rate parameter, ↓ indicates ANS activation tends to decrease the HRV and heart rate parameter. Modified from Haakana et al. (2023).

were used to compare conditions and time points. MEP amplitudes between different conditions at every timepoint were compared using a global Friedman's test followed by pairwise comparisons with Wilcoxon signed-rank test with Bonferroni's correction (p values presented as corrected). The same approach was used to assess the change from PRE to POST values within sessions. We used percentage values from the PRE to compare different stimulation conditions at different time points. Absolute values were used in comparing sessions POST, POST30, POST60, and POST90 to PRE values and in comparing PRE values between different stimulation conditions. Absolute values were used for HRV analysis. Pearson correlation was used to calculate the correlation between MEP amplitudes and stimulation intensity.

3 | RESULTS

Amplitudes of averaged nTMS-induced prestimulation MEPs did not significantly differ between the treatment groups (Table 4). There was a significant post-stimulation difference between the stimulation conditions ($p < 0.001$, Kendall's $W = 0.330$). PAS ($p = 0.04$), PAS + shamVNS ($p = 0.028$), and PAS + aVNS ($p = 0.004$) increased MEP amplitude at POST compared with aVNS. The global test showed no differences between PAS and PAS + aVNS or PAS + shamVNS. No significant differences occurred between the stimulation conditions at time points POST30, POST60, and POST90.

All sessions with PAS increased MEP amplitudes significantly from PRE to immediately POST stimulation ($p = 0.005$). PAS alone increased MEP amplitudes on average by 34.7% across all timepoints and 43.5% at POST. PAS + aVNS increased MEP values by 29.1% across all timepoints and 49.7% at POST. PAS + shamVNS increased MEP amplitudes by 45.4% across all timepoints and 63.9% at POST. aVNS alone increased MEP amplitudes by 12.5% across all time points and 3.9% at POST. Changes in MEP amplitude over time in stimulation sessions are presented in Figure 3. Additional details on individual changes in MEP amplitude are shown in the Supporting Information. Within each session, PAS alone significantly increased MEP amplitudes from PRE to POST ($p = 0.005$), POST60 ($p = 0.035$), and POST90 ($p = 0.025$). PAS + aVNS increased MEPs at POST ($p = 0.005$) and PAS + shamVNS at POST,

TABLE 4 Average (± SD) MEP values in absolute values and as % from PRE value for each measurement time point per session.

	PRE		*p* value	POST	*p* value	POST30	*p* value	POST60	*p* value	POST90	*p* value
PAS (μV ± SD)	586.6 ± 371.1	PAS + aVNS	0.82	808.4 ± 535.0	1	776.9 ± 499.9	1	749.0 ± 466.0	1	766.7 ± 496.4	1
		PAS + shamVNS	1		0.716		1		1		1
		aVNS	1		0.032*		0.252		1		1
% from pre				143.5 ± 33.8	0.005¥	131.0 ± 43.5	0.075	131.5 ± 32.4	0.035¥	132.9 ± 33.7	0.025¥
PAS + aVNS (μV ± SD)	769.2 ± 686.1	PAS	0.82	959.8 ± 716.0	1	838.5 ± 612.9	1	853.9 ± 657.0	1	871.5 ± 708.7	1
		PAS + shamVNS	0.484		0.936		0.316		0.484		0.484
		aVNS	0.536		0.004**		0.652		1		1
% from pre				149.7 ± 53.8	0.005¥	118.3 ± 36.7	0.315	123.0 ± 29.0	0.315	125.6 ± 42.0	0.44
PAS + shamVNS (μV ± SD)	637.6 ± 577.7	PAS	1	863.6 ± 686.7	0.716	808.0 ± 654.5	1	782.4 ± 658.4	1	800.9 ± 601.9	1
		PAS + aVNS	0.484		0.936		0.316		0.484		0.484
		aVNS	1		0.028*		0.068		0.484		0.592
% from pre				163.9 ± 62.6	0.005¥	142.0 ± 39.8	0.005¥	134.8 ± 31.1	0.005¥	141.1 ± 40.5	0.02¥
aVNS (μV ± SD)	671.0 ± 561.8	PAS	1	648.2 ± 499.8	0.032	668.3 ± 548.3	0.252	713.9 ± 572.2	1	720.4 ± 554.3	1
		PAS + aVNS	0.536		0.004**		0.652		1		1
		PAS + shamVNS	1		0.028*		0.068		0.484		0.592
% from pre				103.9 ± 25.0	1	104.4 ± 25.4	1	119.2 ± 50.9	1	122.4 ± 49.8	1

Note: Significance levels set to *$p < 0.05$, **$p < 0.005$ differences between sessions, and ¥$p < 0.05$ significantly different from PRE value. Bonferroni's correction was applied (large corrected values truncated at $p = 1$).

POST30, and POST90 ($p = 0.005$). aVNS-only induced no significant MEP amplitude increase at any timepoint ($p = 0.535$).

At the group level, no significant correlation was observed between individual stimulation intensity and MEP amplitude for the conditions of PAS + aVNS, PAS + shamVNS, or aVNS (Figure 4). However, there was variation between individuals in the stimulation intensity used in different sessions (Table 2) and in MEP response amplitudes (see Supporting Information).

Blood pressure did not differ significantly between or within sessions (Figure 5). The analysed HRV variables did not differ significantly between the stimulation conditions at PRE test or at any other time points. In the PAS session, mean RR significantly increased from PRE to STIM (5.0%, $p = 0.048$), POST60 (8.0%, $p = 0.036$), and to POST90 (5.9%, $p = 0.012$). In the PAS + aVNS session, RR increased significantly from PRE to STIM (3.5%, $p = 0.006$). For PAS + shamVNS, RR POST90 significantly increased from PRE (5.8%, $p = 0.030$). For aVNS, RR at POST60 (6.0%) and POST90 (5.2%) were significantly increased from PRE ($p = 0.012$). Changes over time per sessions are presented in Figure 6. Results of the other variables are provided in the Supporting Information.

Mean HR did not differ significantly between any measured timepoints for different sessions. Within session PAS, HR significantly decreased from PRE to STIM (−4.5%, $p = 0.048$), POST30 (−5.2%, $p = 0.036$), and to POST60 (−7.0%, $p = 0.012$). PAS + aVNS decreased HR significantly from PRE to POST30 (−4.1%, $p = 0.024$), POST60 (−4.7%, $p = 0.012$), and POST90 (−4.7%, $p = 0.018$), as did PAS + shamVNS from PRE to POST60 (−5.1%, $p = 0.024$) and to POST90 (−5.0%, $p = 0.048$).

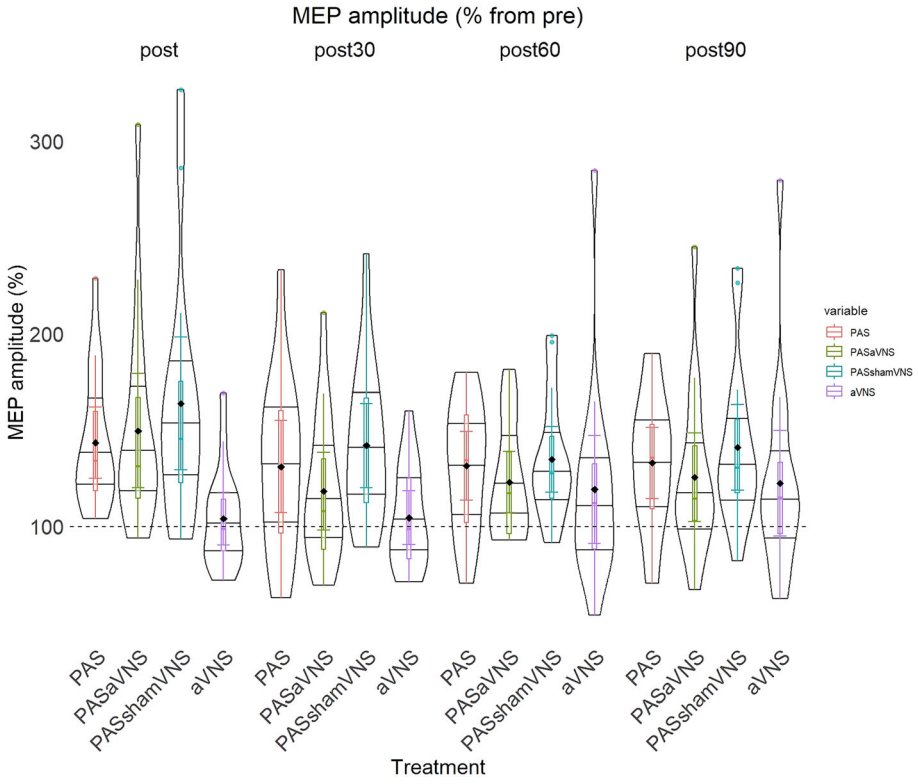

FIGURE 3 MEP values as % from PRE session presented as violin plots overlaid with boxplots. Mean value with confidence intervals and quartiles are presented for PAS, PAS + aVNS, PAS + shamVNS, and aVNS sessions.

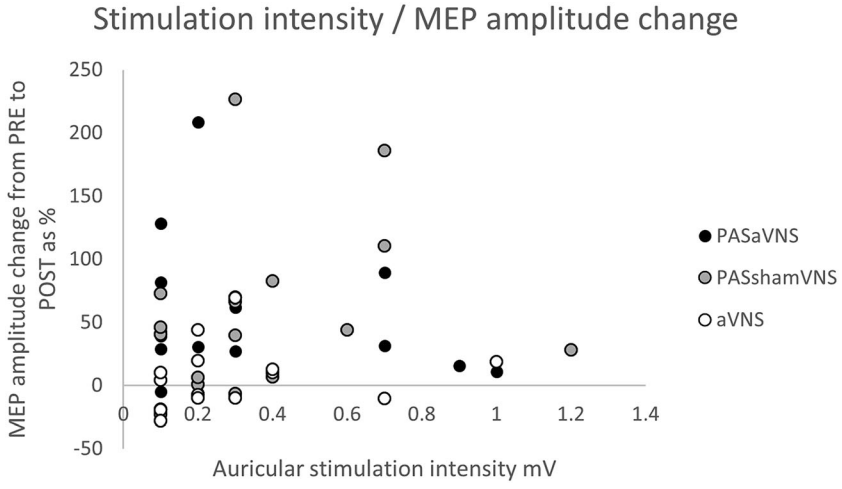

FIGURE 4 MEP amplitude change from PRE to POST as % plotted against stimulation intensity for aVNS ($p = 0.386$), PAS + aVNS ($p = 0.427$), and PAS + shamVNS ($p = 0.562$).

aVNS decreased HR significantly from PRE to STIM (−5.0%, $p = 0.006$). In the PAS session, RMSSD increased significantly from PRE to POST60 min (25.4%, $p = 0.03$).

pNN50 did not differ significantly between sessions at any timepoints or within sessions. LF power showed a significant decrease from PRE to POST90 at PAS + shamVNS session (−27.5%, $p = 0.012$). HF power increased significantly (95.9%, $p = 0.012$) from PRE to STIM and from PRE to POST30 (63%, $p = 0.018$) in PAS session. No other differences were observed between sessions at any time points or within sessions. LF/HF ratio was significantly lower at STIM in PAS + aVNS than aVNS (−35.6% vs − 5.8% from PRE, $p = 0.048$). The decrease in LF/HF ratio from PRE to STIM was significant within PAS and PAS + aVNS sessions (−56.8%, $p = 0.048$ and −35.6% $p = 0.036$, respectively). Respiration frequency decreased significantly at aVNS session from PRE to STIM (−9.7%, $p = 0.042$), and at PASaVNS session from PRE to POST90 (−9.5%, $p = 0.012$). No other differences were observed between sessions at any timepoints or within sessions. SD2/SD1 ratio did not differ between the sessions at any timepoints. During PAS and PAS + aVNS, SD2/SD1 ratio decreased significantly from PRE to STIM ($p = 0.012$, and $p = 0.036$, respectively).

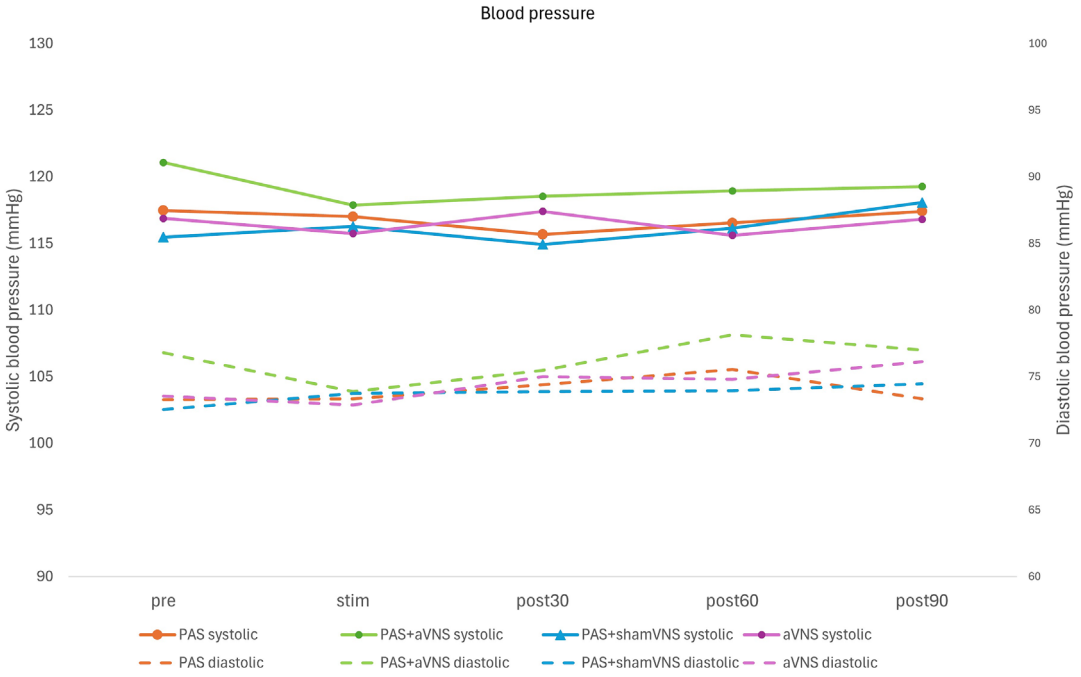

FIGURE 5 Blood pressure at different timepoints in PAS, PAS + aVNS, PAS + shamVNS, and aVNS sessions.

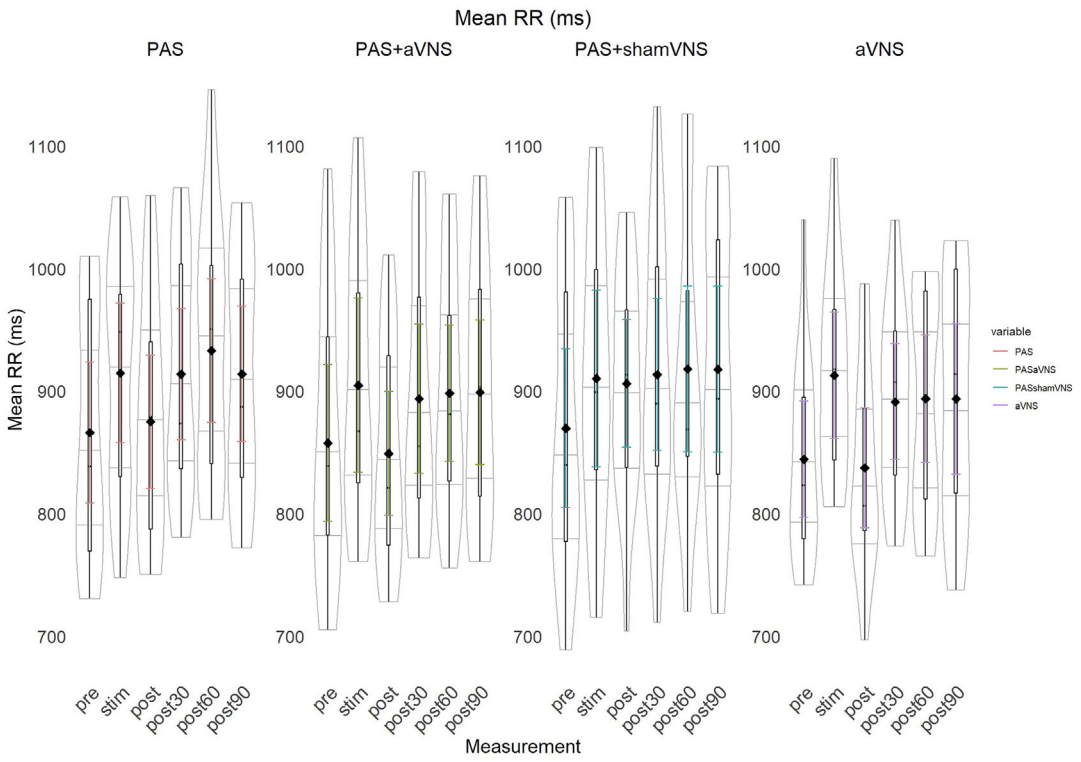

FIGURE 6 Mean RR change over time at PAS, PAS + aVNS, PAS + shamVNS, and aVNS sessions. Presented as violin plots overlaid with boxplots, highlighting mean value with confidence intervals and quartiles.

4 | DISCUSSION

We expected stronger MEP potentiation when PAS was combined with aVNS than with sham VNS or PAS alone. All sessions with PAS increased MEP amplitude significantly after stimulation. These results are consistent with our previous findings of PAS-induced increase in MEP amplitude (Mezes et al., 2020). We also observed a trend

for additional MEP increase after PAS was combined with shamVNS. aVNS did not significantly enhance the PAS effect. Thus, our auricular VNS setup did not enhance plasticity induced by high-PAS.

aVNS likely differs from implanted VNS or cervical transcutaneous VNS, as the fibre pathways of the auricular branch and cervical vagus nerve are different. Moreover, ABVN sparsely innervates the auricular area and thus may require more stimulation than the cervical vagus nerve in the implanted stimulation setup to produce the desired effect. Although our PAS + aVNS stimulation induced only a trend of enhancing MEPs when compared with PAS alone, this does not exclude the possible efficacy of invasive VNS when combined with PAS. Increasing the number of PAS + aVNS stimulation sessions over a longer period may have also enhanced its efficacy. The clinical effect of high-PAS alone peaks only after several sessions in patients with SCI (Shulga et al., 2016). Similarly, it may be that aVNS-induced brain plasticity requires a longer period of treatment to peak (Keute & Gharabaghi, 2021).

Methodological studies are important because the beneficial effects of VNS depend on the accurate delivery of a narrow range of stimulation intensities (Clark et al., 1998, 1999; Morrison et al., 2019, 2021; Revesz et al., 2008). Currently, there is no established clear consensus for aVNS parameters. Implanted VNS (Dawson et al., 2016; Redgrave et al., 2018) and transcutaneous VNS (Gerges et al., 2024) are safe and VNS has been used as a therapy for epilepsy (Selner et al., 2019), tinnitus (Vanneste et al., 2017; Ylikoski et al., 2017), pain (Chakravarthy et al., 2015), and depression (Lv et al., 2019; Wu et al., 2018) in humans. Identification of the optimal stimulation location, parameters, and protocol of aVNS required to enhance PAS will provide new insights into the biological conditions needed to alter brain networks, which may be advanced by using implanted VNS that enables precise control of stimulation intensity. For high-PAS, 5-s intervals for stimulation pulses are effective (Mezes et al., 2020; Shulga et al., 2015). We selected a 10-s interval for aVNS, as longer intervals produce a greater degree of plasticity (Borland et al., 2018).

The anatomy of the vagus nerve in the ear is not clearly defined (Butt et al., 2020). Stimulation of the vagus nerve induces neuronal activity sequentially in the nucleus tractus solitarius, locus coeruleus, and in higher brain regions (Kalia & Sullivan, 1982). It is not clear which brain areas are activated in aVNS (Yap et al., 2020). Additional studies are needed to fully understand the effects of great auricular nerve stimulation on auricular vagus activation or spinal potentiation and to find a suitable anatomical location for aVNS with optimal stimulation parameters.

VNS has been applied mainly in experiments that require induction of cortical plasticity. Cortical PAS may be more amenable for VNS to enhance PAS effects, taking advantage of the VNS-induced neurotransmitter release in cortical areas. The high-PAS setup is designed particularly to enhance motor transmission in the spinal cord (Shulga et al., 2021). Nevertheless, this setup also modifies sensorimotor cortical activity in patients with SCI (Vanhanen et al., 2022). Thus, the localization of the plasticity effects in the motor CNS is not clearly delineated in VNS or in high-PAS.

aVNS alone suppressed MEP amplitudes in some individuals. Half of the participants had decreased MEPs after stimulation (on average 16%, Supporting Information). In PAS session, all participants had increased MEP amplitudes at POST. For PAS + aVNS two participants had suppressed MEP amplitudes (on average 6%). In PAS + shamVNS one participant had suppression of 7% in MEP amplitudes after stimulation (POST). All stimulation sessions presented a variable amount of depression in MEP amplitudes at POST30, POST60, and at POST90 min after stimulation in certain participants.

MEP amplitudes vary between individuals (Pitcher et al., 2003), gender (Valero-Cabré et al., 2017), and trials (Rossini et al., 2015). Normalization of the MEP amplitudes to PRE values and averaging 30 MEPs diminishes this variability. We collected 30 MEPs per measurement. The navigated TMS system improves replicability of TMS stimulations (Rossini et al., 2015). The sessions were also scheduled at the same time of the day ±2 h. This should also decrease the variability of the TMS-induced MEPs.

Perceptual and pain thresholds have been used to determine individual levels for aVNS stimulation intensity (Yakunina et al., 2017). In our experiments, the perceptual threshold was easily and reliably detected. Defining the pain threshold is more subjective, and the intensity may vary considerably between participants. Moreover, effective blinding of the stimulation sequence would not have been possible with suprathreshold stimulation. To date, there is no consensus of optimal parameters for noninvasive VNS (Badran, Mithoefer, et al., 2018). However, 25-Hz (Redgrave et al., 2018) or 30-Hz (Dawson et al., 2016; Kimberley et al., 2018; Porter et al., 2012) VNS with pulse length of 100 μs have been applied in animal and human studies. Stimulation duration also varies; both continuous stimulation during movement (Kimberley et al., 2018; Redgrave et al., 2018) and 0.5-s trains have been applied in VNS experiments (Dawson et al., 2016; Porter et al., 2012). VNS intensity has been reported to present an inverted U-shaped relationship, where moderate VNS intensity appears to be more effective than low or high

intensities (Morrison et al., 2019; Pruitt et al., 2021). We selected the stimulation intensity below the perceptual threshold to blind the participants to the type of stimulation; the sham control setup would not have been possible with higher intensities. The selected intensity might have been too low for some participants to produce vagus nerve activation to induce plasticity. Without proper control for vagus activation (Burger et al., 2020), it is difficult to determine optimal stimulation intensity. VNS needs to be timed with movement when using a protocol to enhance movement (Hays et al., 2014). In this study, aVNS was paired with PAS-induced movement. This may have been an insufficient activation, the wrong type of exercise, or the wrong context (Hays et al., 2023).

The distance of the electrode from the nerve, distance between electrodes, or local skin properties can affect the stimulation efficacy (McAllen et al., 2018). The electrodes for aVNS were attached individually by fitting the silicone mold to the participant's ear, thus minimizing the effect of differences in ear anatomy. Consequently, the interelectrode distances varied somewhat. The interelectrode distance was monitored but not controlled. In the sham stimulation, the electrodes were set on both sides to the earlobe with a clip. Their interelectrode distance was not measured but was smaller in the earlobe than in aVNS stimulation, resulting in higher current density in sham VNS than in aVNS stimulation. Nevertheless, this should not affect the results as stimulation intensity was below the perceptual threshold. The experiment did not require focused attention to any of the stimuli. Both aVNS and sham VNS stimulation intensities were below perceptual threshold, so attending them systematically would be practically impossible. The stimulation at subthreshold level was selected to avoid attentional effects. Naturally, the subliminal state of the subject could have been modulated by aVNS or additional earlobe stimuli and involuntary attention to either nTMS or leg stimulation could have been modulated. Stimulation parameters, such as stimulation time, intensity, frequency, and timing for pairing may need to be revised (Buell et al., 2019). On the group level, sham stimulation was delivered at 0.08 mA higher intensity than aVNS stimulation. However, although there was variation in individual threshold levels, the difference between the intensity in active and sham stimulation was not significant (Table 2). Consequently, the role of aVNS vs. sham VNS intensity in MEP potentiation remains elusive.

MEP amplitudes were also increased after PAS + shamVNS. Earlobe sham stimulation activates the great auricular nerve in the earlobe. Its posterior branch (Cesmebasi, 2015) connects to the vagus nerve through a few nerve fibres. Nevertheless, if the effect of earlobe stimulation was mediated by the vagus nerve, the effect should have been weaker than that of cymba conchae and cavum stimulation, which are thought to activate the vagus nerve more efficiently. Earlobe stimulation activates similar brain areas as the VNS (Yakunina et al., 2017). Thus, the earlobe may not be an ideal site for sham stimulation in aVNS experiments. It is unlikely that TMS could have activated the somatosensory cortex innervating the earlobe, as its representation is far from the foot motor cortex targeted by our TMS.

Enhanced MEPs by PAS + shamVNS may also relate to changes in spinal activation and enhanced excitability through sensory nerve branches to C2 and C3 roots (Cesmebasi, 2015; Yakunina et al., 2017) innervating the earlobe. Epidural (Sayenko et al., 2014) and transcutaneous (Danner et al., 2011) spinal cord stimulation activate posterior roots of the spinal cord, creating antidromic potentials (Su et al., 1992; Taccola et al., 2018) for activation of motor pools (Angeli et al., 2014) and improving excitability of the spinal networks (Islam et al., 2021). A similar effect could be achieved also by C2 and C3 root stimulation induced by PAS + shamVNS. Earlobe electrical stimulation could produce stronger activation of reticular formation in the brainstem than activation of the auricular vagus nerve branches. Output projections from reticular formation go to spinal alpha motoneurons (Davis et al., 1982). Activation of the reticulospinal pathways (originating from the reticular formation) also contributes to MEPs, and stronger activation in these pathways may contribute to additional MEP potentiation by PAS + shamVNS. Although PAS here is targeting the lumbar level, increased excitability of cervical-level interneurons may have affected signal propagation efficacy in upper motor neurons that travel through the cervical level.

The ECG and blood pressure measurements did not reveal a systematic effect of aVNS on the autonomic nervous system. Other studies with aVNS revealed reduced sympathetic and increased parasympathetic activation (Clancy et al., 2014). Consistent with our previous findings (Haakana et al., 2023), HR decreased during PAS, but HRV did not vary significantly during any stimulation session. Similar findings were observed in all sessions, especially during PAS, indicating an increased parasympathetic activation (Haakana et al., 2023) that was not modified by aVNS. The literature is inconclusive about the effects of VNS on HRV, although the side (right or left ear) or stimulation parameters seem to play a role (Machetanz et al., 2021). It is probable that our aVNS intensity was too low to affect HRV, while studies with higher intensities for VNS below the pain threshold reported effects of aVNS to HRV (Gianlorenço

et al., 2024). The LF/HF ratio has been reported to significantly decrease with aVNS but not with sham stimulation (Clancy et al., 2014). Our results indicate a significant decrease of the LF/HF ratio during PAS and PAS + aVNS sessions but not during PAS + shamVNS or aVNS alone.

5 | LIMITATIONS OF THIS STUDY

The small sample size of 16 participants is a limitation of this study. Due to the low number of participants, the statistical power of the analysis may be insufficient and additional subjects might have provided more significant results.

Variation in MEP threshold is known to be high both interindividually and intraindividually (Wassermann, 2002). We made efforts to minimize this variability. Sessions were scheduled at a matching time of day and on a representative normal day of the participant's life to avoid factors eliciting variability (Ziemann et al., 2008). Other factors, such as the attentional state of each participant during testing, were not controlled.

6 | CONCLUSION

All sessions with PAS significantly enhanced the MEPs. When aVNS was combined with PAS, it did not further increase MEP amplitudes. Surprisingly, sham VNS showed a trend to potentiate the effect at all timepoints. This finding is probably not connected to aVNS effect and needs further investigation in the future. Additional experiments are needed to define the connection between the great auricular nerve and spinal cord excitability and to critically evaluate the use of the earlobe as a sham target for aVNS. All PAS sessions increased parasympathetic activation, but aVNS or sham-VNS did not enhance this effect. Optimization of auricular VNS stimulation setup is required for possible tests of patients with SCI.

AUTHOR CONTRIBUTIONS

Piia Haakana: Conceptualization; formal analysis; writing—original draft; writing—review and editing. **Anna Nätkynmäki**: Investigation; writing—review and editing. **Erika Kirveskari**: Conceptualization; writing—review and editing. **Jyrki P. Mäkelä**: Conceptualization; writing—review and editing. **Mika P. Tarvainen**: Writing—review and editing. **Michael P. Kilgard**: Conceptualization; writing—review and editing. **Anastasia Shulga**: Conceptualization; writing—review and editing; supervision. All authors approved the final version of manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/ejn.16539.

DATA AVAILABILITY STATEMENT

All data generated or analysed during this study are included in this published article (and its Supporting Information files). MEP and HRV data have been published in an open-access data repository https://doi.org/10.23729/09ca0bdf-2f5d-4598-9c71-b3f9a4684b7d.

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