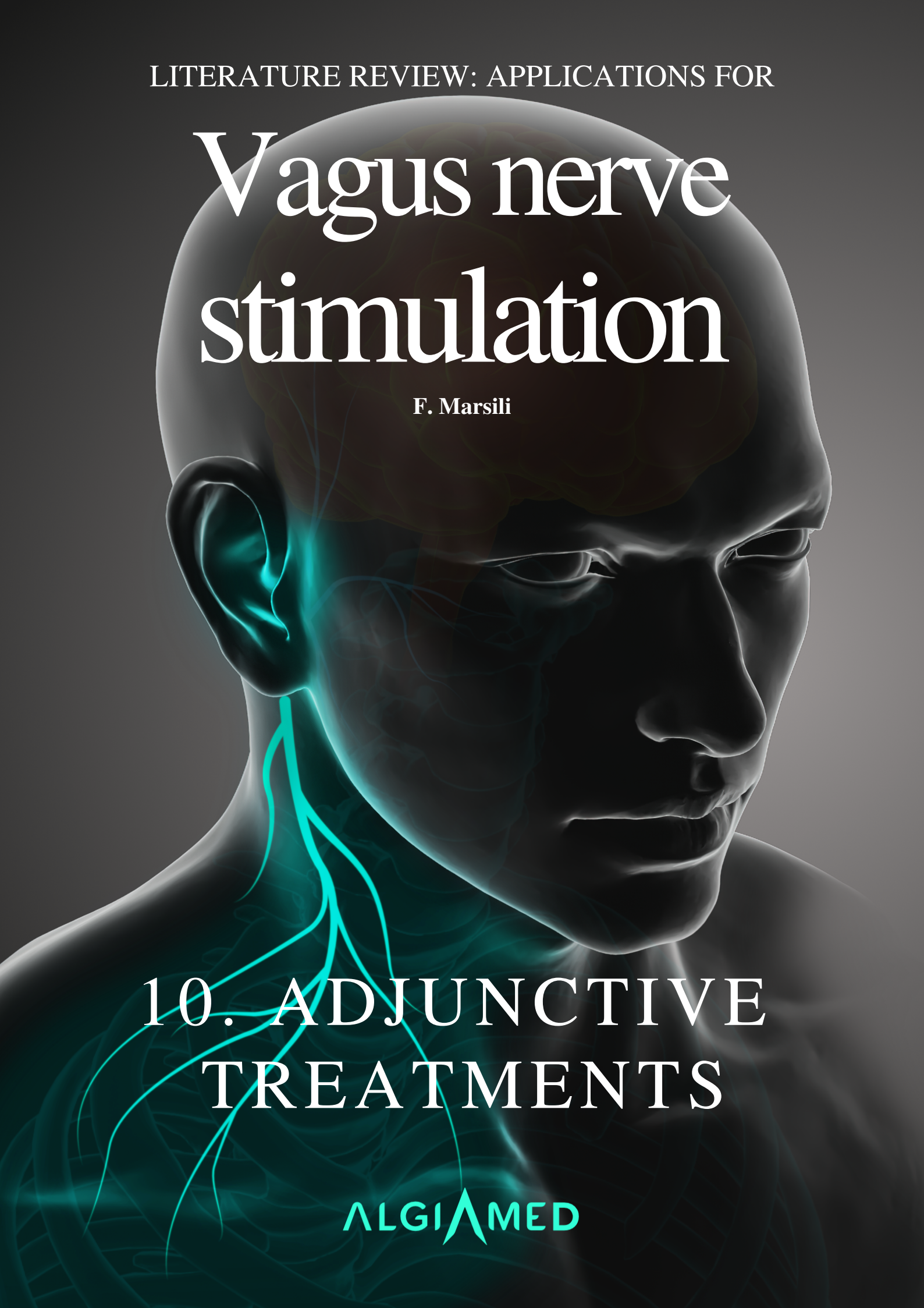
LITERATURE REVIEW: APPLICATIONS FOR

Vagus nerve stimulation

F. Marsili



10. ADJUNCTIVE TREATMENTS

ALGIAMED

Author's choice

The papers in this collection focus on the application of Vagus Nerve Stimulation (VNS) as established therapeutic solution for difficult-to-treat conditions.

The vagus nerve is the longest cranial nerve and is widely distributed throughout the body, traversing the neck, thorax and abdomen. It is composed by motor fibres and sensory fibres from sympathetic and parasympathetic branches. [1], [2]. Afferent branches of the vagus nerve innervate brain behavioural areas involved in depressive states, and it desynchronises cortical activity with anti epileptic effects [3], [4]. Efferent branches of the vagus nerve regulate gastrointestinal secretory and motor function [5]. Recent advances in the field, have unraveled an anti-inflammatory role of the efferent vagus nerve via the Cholinergic Anti-inflammatory Pathway (CAP), a known mechanism for neural inhibition of inflammation linked to the activation of the autonomic nervous system (ANS) [6], [7].

Electrical stimulation of the VN modulates the nervous system at central, peripheral, and autonomic levels without the need for pharmacological interventions. For decades, invasive techniques of VNS have demonstrated their clinical efficacy in VN-related diseases and, to these days, efforts have been made to create a more safe, effective, and non-invasive solution to VNS.

The auricular branch is the only peripheral branch of the VN on the human body, it is part of the afferent portion of the VN that directly connects to the brainstem. Thus, auricular VN has become the most favourable access point for non-invasive VNS. Neuroimaging studies on animal models and humans have confirmed the modulatory efficacy of auricular VNS (aVNS). For examples, fMRI studies show identical activation patterns in the brain between invasive and aVNS, with significant inhibitory and anti-inflammatory effects. Due to the existence of different control systems, the anti-inflammatory effects of aVNS (i.e., release of norepinephrine and noradrenaline, and neurotrophic factors) seem to occur immediately after intervention, while neuroplastic changes only occur as a consequence of sustained regenerative efforts [7].

Colleciton 1 and collection 2 are the most extensive selections, since VNS has been standard-of-care for epilepsy and depression for decades. Collection 3 explores the possibility of using VNS for the treatment of posttraumatic stress disorders. Collection 4 focuses on fibromyalgia and collection 5 on multiple sclerosis. Collection 6 and 7 corroborates the hypothesis that VNS can be used to activate the cholinergic anti-inflammatory pathway to treat inflammatory diseases, such as inflammatory bowel disease or rheumatoid arthritis. Collection 8 and 9 focus on the use of VNS for ameliorating pain sensitivity in chronic pain conditions and for rehabilitating upper limb motor fibres after ischemic strokes, respectively. In conclusion, collection 10 opens up other possibilities for clinical applications of VNS, ranging from cardiovascular diseases, through ADHD disorders, to tinnitus.

To summarise, VNS is a novel technology and its non-invasive configuration is still under investigation. Further clinical examinations are mandatory in order to understand the underlying mechanism of VNS and to open the door to new possible therapeutic applications. However, being a non-invasive, safe, and efficient therapeutic solution, VNS is an attractive tool for further implementation and new creative clinical applications.

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10. VNS and adjunctive treatments

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ORIGINAL RESEARCH
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Stress and Tinnitus; Transcutaneous Auricular Vagal Nerve Stimulation Attenuates Tinnitus-Triggered Stress Reaction

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Introduction: Tinnitus can become a strong stressor for some individuals, leading to imbalance of the autonomous nervous system with reduction of parasympathetic activity. It can manifest itself as sleep disturbances, anxiety and even depression. This condition can be reversed by bioelectrical vagal nerve stimulation (VNS). Conventional invasive VNS is an approved treatment for epilepsy and depression. Transcutaneous VNS (taVNS) stimulating the auricular branch of the vagus nerve has been shown to activate the vagal pathways similarly as an implanted VNS. Therefore, taVNS might also be a therapeutic alternative in health conditions such as tinnitus-related mental stress (TRMS). This retrospective study in 171 TRMS patients reports the clinical features, psychophysiological characteristics, and results of the heart rate variability (HRV) tests before and after test-taVNS. This study also reports the therapy outcomes of 113 TRMS patients treated with taVNS, in combination with standard tinnitus therapy.

Methods: Diagnostic tinnitus and hearing profiles were defined. To detect possible cardiac adverse effects, test-taVNS with heart rate monitoring as well as pre- and post-stimulation HRV tests were performed. Daily taVNS home therapy was prescribed thereafter. To assess therapeutic usefulness of taVNS, 1-year follow-up outcome was studied. Results of HRV tests were retrospectively analyzed and correlated to diagnostic data.

Results: The large majority of patients with TRMS suffer from associated symptoms such as sleep disturbances and anxiety. Baseline HRV data showed that more than three quarters of the 171 patients had increased sympathetic activity before test-taVNS. Test-taVNS shifted mean values of different HRV parameters toward increased parasympathetic activity in about 80% of patients. Test-taVNS did not cause any cardiac or other side effects. No significant adverse effects were reported in follow-up questionnaires.

Conclusion: TRMS is an example of a stress condition in which patients may benefit from taVNS. As revealed by HRV, test-taVNS improved parasympathetic function, most efficiently in patients with a low starting HRV level. Our tinnitus treatment program, including taVNS, effectively alleviated tinnitus stress and handicap. For wider clinical use, there is a great need for more knowledge about the optimal methodology and parameters of taVNS.

Keywords: stress, tinnitus, patients, parasympathetic, vagus, neuromodulation

INTRODUCTION

All our unconscious bodily functions are controlled by the autonomic nervous system (ANS), particularly by the CAN (Benarroch, 1993). The most common cause for the dysfunction of CAN is stress, the major cause of deteriorating health conditions and illnesses. CAN initially reacts to stressor effects with sympathetic fight/flight response that is restored back to normal by the parasympathetic nervous systemś relax/digest response (Selye, 1950). Many illnesses result from the inability of the parasympathetic activity to restore the ANS balance (for review see McEwen, 2000; McEwen and Akil, 2020). These two circuits, sympathetic and parasympathetic systems, are constantly interacting. This interaction is reflected by HRV that, hence, is a read out of ANS balance. HRV may consequently serve as a measure of stress (Akselrod et al., 1981; Thayer et al., 2012). As the vagal system with the vagus nerve in front is responsible for parasympathetic activity, neuromodulation via VNS can serve as targeted treatment in stressful conditions. VNS has been conventionally performed for more than two decades to treat severe epilepsy and depression by applying an electrode surgically implanted to the cervical trunk of the vagus nerve. More recently, it has been shown by electrophysiological and neuroimaging studies that taVNS of the ABVN activates central vagal pathways similarly as VNS with an implanted electrode (Kraus et al., 2007; Dietrich et al., 2008; Frangos et al., 2015; Yakunina et al., 2017; Badran et al., 2018a).

ANS imbalance is most often a result of the individual's exposure to concurrent stressors. Therefore, the stress-triggered clinical picture is often very heterogeneous. On the contrary, tinnitus (ringing in the ears) as a stressor usually results in a relatively regular SR in otherwise healthy individuals. Therefore, individuals with TRMS seem to be an optimal target group for investigations of the effects of taVNS on stress in patients.

Tinnitus is considered to be generated in the auditory periphery (cochlea-cochlear nerve), detected in the subcortical centers according to the lines of pattern recognition principles, and perceived and evaluated in the auditory cortex with significant participation of the limbic system and prefrontal and other cortical areas (Jastreboff, 1990). Tinnitus sound itself usually constitutes only minor symptoms. However, tinnitus connected with fear (that it is maintained or even growing worse) and threat (that it is a sign of a serious illness) leads to automatic negative thoughts, developing a SR (arousal) that may potentiate sleep problems, anxiety and depression. The end result all of this is a vicious cycle, SR worsening tinnitus and increased tinnitus worsening stress.

This was taken into consideration in the neurophysiological tinnitus model by Jastreboff (1990) and it formed the basis for the development of the TRT program (Jastreboff and Hazell, 1993). The target of TRT therapy is the stress-arousal caused by tinnitus and it leads to distress that prevents habituation. The goal of TRT is to remove the negative perception of tinnitus from patient's consciousness, thereby facilitating habituation. Furthermore, the rationale of TRT is to attenuate the conditioned stress-response (arousal) with associated sympathovagal imbalance by stimulating the parasympathetic system (Jastreboff and Hazell, 1993). TRT is a program consisting of diagnostics, instructive counseling and sound therapy, each acting in concert with the aim to stimulate the parasympathetic system.

Plans for this study were started after Engineer et al. (2011) reported that maladaptive neuronal plasticity of the central auditory system, thought to be behind tinnitus in an animal tinnitus model, can be reversed through (invasive) VNS. It was clear that conventional (invasive) VNS would not be the optimal treatment for patients with tinnitus. Therefore, we first had to develop a method and device for noninvasive VNS (taVNS). When the device development was completed, we had to start to test-use it particularly for cardiac safety.

This retrospective study reports the clinical, audiological and psychophysical diagnostic results in a historical group of 171 patients managed for TMRS. The study also reports the results of the 1-year follow-up outcome study with patients treated with taVNS. Because stress levels of patients needed to be measured and because of the novelty of taVNS and the potential cardiac complications reported by VNS, the HRV test and test-taVNS with HR monitoring were considered obligatory. We performed HRV test both before and after test-taVNS. Our data show that taVNS is safe and improves parasympathetic activity and, in conjunction with TRT, attenuates tinnitus severity based on results of symptom questionnaires.

Abbreviations: ABVN, auricular branch of the vagus nerve; AF, atrial fibrillation; ANS, autonomous nervous system; CAN, central autonomic network; CNS, central nervous system; ECG, electrocardiogram; HR, heart rate; HRV, heart rate variability; MIT, music-induced tinnitus; NIHD, noise-induced hearing disorder; NIT, noise-induced tinnitus; NTS, nucleus tractus solitarius; SR, stress reaction; taVNS, transcutaneous VNS; TCPT, tinnitus care pathway technology; THI, tinnitus handicap inventory; TRMS, tinnitus-related mental stress; TRT, tinnitus retraining therapy; VAS, visual analog scale; VNS, vagal nerve stimulation; VSEP, vagal somatosensory evoked potential.

PATIENTS AND METHODS

The present series consists of 171 consecutive patients (67 female, 104 male, mean age 49 years, range 17–84) who visited the Tinnitus Clinic of Helsinki Ear Institute between November 2014 and December 2017 due to annoying tinnitus. We have developed our own modification of TRT that is named TCPT. Its main constituents include diagnostic profiling of tinnitus and hearing, counseling, sound therapy, and a sleep module. In order to strengthen parasympathetic activation, taVNS was added to the program in 2014. In addition to other diagnostics, HRV tests and a 15–60 min test-taVNS were performed and, as adjunctive to other TCPT therapy, a taVNS device was prescribed as home-therapy if tinnitus had been defined as moderate or severe (THI, questionnaire score higher than 34/100). Some patients with lower THI scores were also instructed for taVNS treatment if they showed special interest in the device or complained of being particularly stressed. At the initial office visit, extensive otological and audiological examinations with profiling of tinnitus and hearing were done using different structured diagnostic forms and questionnaires. For clinical evaluation of the subjective severity of tinnitus annoyance and associated symptoms such as sleep disturbance and anxiety levels were quantified based on VAS questions.

Each patient's stress level was evaluated by HRV testing. The patient was then (for safety reason) test-stimulated with the Salustim taVNS device (Helsinki Ear Institute) continuously for 15–60 min after which a new HRV test was performed. We have earlier shown by magnetoencephalography that the amplitudes of auditory N1m responses in the auditory cortex are reduced by using this taVNS method (Lehtimäki et al., 2013). As there were no adverse effects related to the test-taVNS, all tested patients were then instructed to use the taVNS device at home 60–90 min per day. The long-term therapeutic outcomes of our first 113 patients were studied with structured questionnaires about 1 year after the first visit. Such follow-up data was possible to obtain during office visits scheduled according to the TCPT program or by telephone interviews in 78 patients (69%) (54 males and 59 females; age range from 18 to 84 years).

HRV Test and Heart Rate Monitoring During Test-taVNS

The main aim of HR monitoring was to detect possible cardiac side-effects during the first 15–60 min of taVNS stimulation. Other reasons were to measure the mental stress level of patients and to collect HRV data for later analyses to study whether HRV could be used for selection of presumably taVNS-responding patients. In this study, we report the results of HRV analyses and specifically correlate HRV results to clinical data in a total of 171 patients. We have previously described in detail our HRV testing procedure (Ylikoski et al., 2017). Briefly, for analyzing the dynamics of HRV signals (R-R intervals), the eMotion HRV measurement system (Mega Electronics Ltd., Kuopio, Finland) was used. Stress test with a HRV scan was performed with the patient breathing with a parasympathetic stimulating respiratory rate during which the R-R interval variability was registered by wrist electrodes with a one-lead ECG. In the eMotion HRV measurement, artifacts and interruptions were eliminated with high-end technology (and disposable surface electrodes) (Malik, 1996; Tarvainen et al., 2009). Only tests with 100% measurement quality in ECG monitoring were used for evaluation. The following HRV parameters were measured during the 1-min deep-breathing test (one-min DBT): mean HR, amplitude and ratio of HR oscillation (E-I difference, E/I ratio) (E-I = difference between the highest and the lowest HR within a breathing cycle), RMSSD (root mean square of the successive differences), SDNN (standard deviation of the R-R intervals), and Power LF (LF = low frequency; 0.04–0.15 Hz). One-min DBT was followed by 5-min short-term HRV (s-HRV) where the HRV parameters HR, SD1 (width of the Poincare plot, reflecting short-term variability), SD2 (length of the Poincare plot, reflecting short-term variability), SDNN, Stress Index, Power HF (HF = high frequency; 0.15–0.4 Hz), Power LF, Power VLF (very low frequency), and Total Power were determined as well. Parameters were compared through correlation analysis and agreement analysis by Bland-Altman plots. The results of HRV tests were evaluated on the basis of eMotion tests performed in normal individuals (Camm et al., 1996; Tarvainen et al., 2009; Weinschenk et al., 2016).

Vagal Somatosensory Evoked Potential Test

In order to biomonitor the electrical stimulation of ABVN, we measured the VSEP response (Fallgatter et al., 2003). We used EGI GTEN 100 EEG system with 8 kHz collection rate (256 electrodes) (Palva and Palva, 2018) and with different stimulation parameters. We used uni- or bipolar pulses, pulse width 100–560 microseconds, frequencies 1, 2, 4, 8, 15, 20, 25, 30 Hz, amplitude at or just below the pain threshold, and delivered 200–500 epochs at each setting.

taVNS

We used the taVNS instrument consisting of one ear clip electrode connected to a wired TENS-neurostimulating device (Salustim, Helsinki Ear Institute). The clip-electrode was placed on the tragus of the left ear. The clinical efficacy of taVNS requires activation of the thick myelinated afferent fibers of the vagus nerve. Fibers of a sensory peripheral nerve, such as the ABVN, mediate touch sensation. Consequently, the stimulus intensity of taVNS will be adjusted to a level above the individual's detection threshold and clearly below the individual's pain threshold. The taVNS device offers a stimulus intensity between 0.1 and 30 mA with a stimulation frequency of 25 Hz and pulse duration of 250 μs. After the individual adjustments the level of stimulation in patients ranged from 0.3 to 3.0 mA. The 15–60 min continuous test stimulation was performed under medical supervision in the office, with continuous HR monitoring. After the initial test stimulation, patients were instructed to use the taVNS device at home for 60–90 min daily, 5 days a week.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, United States). Normality of data was tested with D'Agostino & Pearson test. The non-parametric Wilcoxon's matched pairs signed rank test was used to estimate the *p*-values between pre/post -taVNS data, as all data did not follow a normal distribution. Comparison between age groups was also done using the non-parametric Kolmogorov–Smirnov test. Comparison between super-responders vs. non -responders was done using unpaired two-tailed Student's *t*-test as the data followed a normal distribution. Data is presented as mean ± standard deviation (SD). Statistical significance was set up at $P < 0.05$. Bonferroni adjustment were performed for multiple comparisons by dividing the initial significance level of 0.05 by the number of tests to obtain a modified significance level. Treatment effect sizes were calculated by Cohen's *d* as the difference in means, divided by the pooled standard deviation of the two means (Cohen's, 1988). The magnitude of Cohen's *d* can be expressed as small (0.2), moderate (0.5), and large (0.8).

RESULTS

Baseline Values

All the patients had clinically relevant tinnitus; chronic in two thirds, subchronic in about 20% and acute (tinnitus duration less than 3 months) in about 15%. In acute cases, most patients had visited our tinnitus clinic one to 6 weeks after the start or worsening of tinnitus. The most common cause of tinnitus was acoustic overstimulation ("NIT", 47%), usually of a result of exposure to loud music in a festival or restaurant. About one third of NIT cases was defined as music-induced tinnitus (MIT). The MIT patients were usually professional or hobby musicians. Other causes were self-reported stress (6%), otitis media (4%) and other causes such as flight travel (2%). The cause of tinnitus was unknown in 40% of cases (**Figure 1A**).

Otological examinations were normal and there were no audiometrically detectable acute hearing impairment, except in five patients that had been exposed to shooting noise. They had a mild (10–25 dB) dip-type hearing loss either at 4 or 6 kHz. Two thirds of patients showed normal or age-related hearing loss in pure tone audiometry. Tinnitus was high-pitched (8 kHz or higher) in two thirds (66%) and >4 kHz in about 85% of cases. The most common tinnitus frequency was detected between 7 and 9 kHz (27%), followed by 11–14 kHz (19%), 9–11 kHz (14%) and 5–7 kHz (13%) (**Figure 1B**).

The mean THI was 55, and it was between 34 and 100 in 81% of patients indicating moderate or severe tinnitus (**Figure 2**). Tinnitus was frequently associated with sleep disturbances (92%) and anxiety (96%) (**Figure 2**). Both sleep disturbances and anxiety were severe or very severe (above 50/100 in VAS in more than half of the patients (57% and 54%, respectively). It may be noteworthy that the patients were typically stressed because, prior to visiting us, they had visited general practitioners or ENT specialists and had received negative counseling ("nothing can be done", "you just have to learn to live with it"). One third of patients had a history or ongoing therapy of depression, ranging from mild (12%) to moderate (18%) to severe (3%).

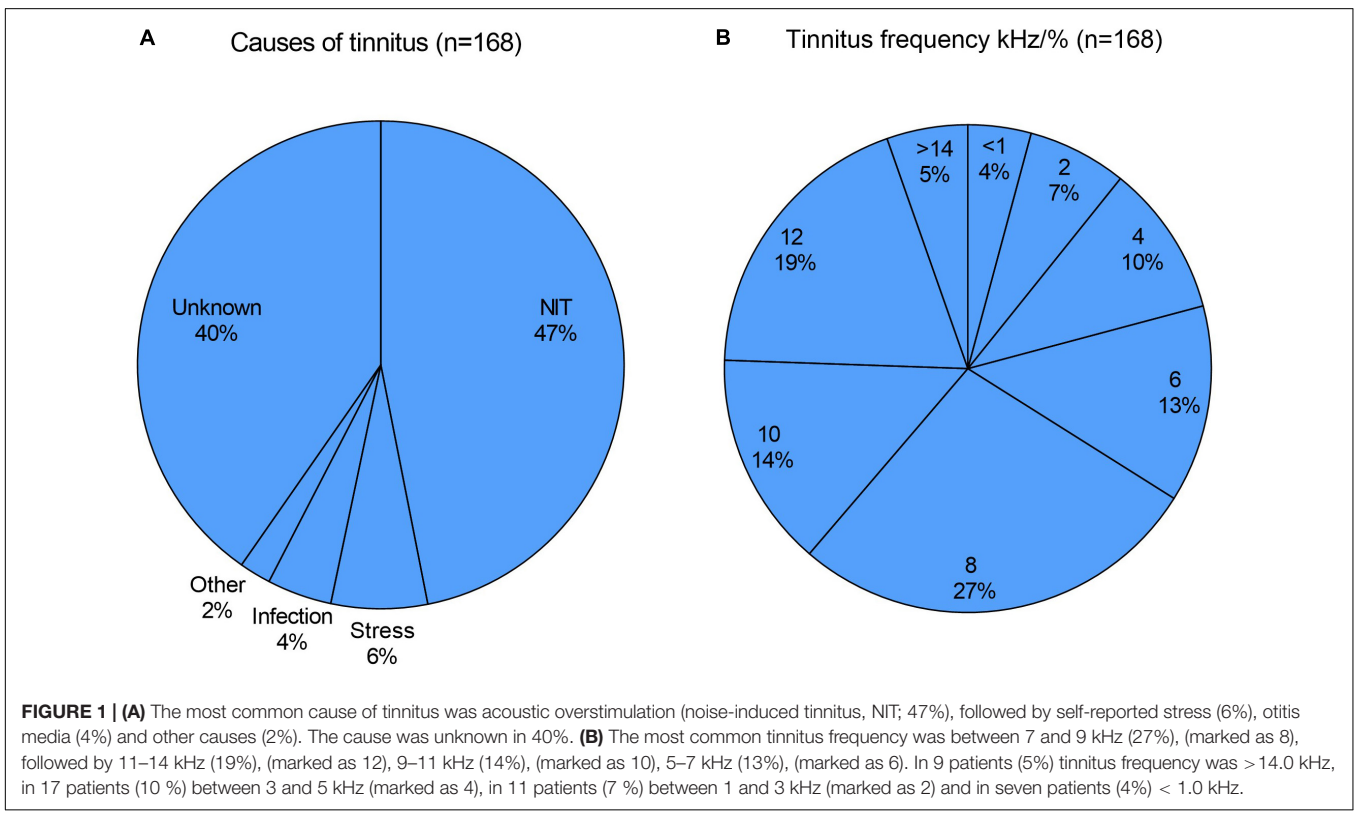

FIGURE 1 | (A) The most common cause of tinnitus was acoustic overstimulation (noise-induced tinnitus, NIT; 47%), followed by self-reported stress (6%), otitis media (4%) and other causes (2%). The cause was unknown in 40%. **(B)** The most common tinnitus frequency was between 7 and 9 kHz (27%), (marked as 8), followed by 11–14 kHz (19%), (marked as 12), 9–11 kHz (14%), (marked as 10), 5–7 kHz (13%), (marked as 6). In 9 patients (5%) tinnitus frequency was >14.0 kHz, in 17 patients (10 %) between 3 and 5 kHz (marked as 4), in 11 patients (7 %) between 1 and 3 kHz (marked as 2) and in seven patients (4%) < 1.0 kHz.

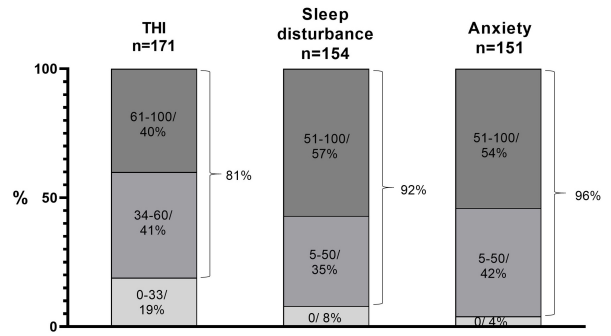

FIGURE 2 | THI was between 34 and 100 in 81% of patients. The mean THI value was 55 and the mean VAS-scaled (0–100) values of both loudness and annoyance were 55. Tinnitus was associated with sleep disturbances in 92% and with anxiety in 96%, both being severe or very severe (>50/100 in VAS scale) in 57% and 54% of patients, respectively.

HRV Tests and Results of Test-taVNS

The main aim of the initial test-taVNS was to ascertain the cardiac safety of the method. We found no cardiac adverse effects in our 171 patients. This applies actually to more than 250 taVNS patients treated so far by us: none of them reported cardiac or other serious side-effects during the first test-taVNS or during home treatment. Several of our patients have used the taVNS device regularly, practically daily for 2–3 years, some up to 5 years.

Baseline data from 1-min DBT-HRV and 5-min short-term HRV (s-HRV) showed that more than three quarters of TRMS patients had increased sympathetic activity before test-taVNS. This was deduced from the stress index (data not shown) and the HRV age, which was in this patient population approximately 16 years higher than the mean chronological age. **Table 1** shows the mean values of different HRV parameters, considered to describe the vasovagal tone and the changes of HRV parameters immediately after the 15–60 min test-taVNS. The taVNS significantly increased all HRV parameters: the mean R-R interval in 81%, Flexibility = E-I (difference between the highest and the lowest heart rate within a breathing cycle) in 63%, Dynamics = RMSSD, (root of the mean square of successive differences) in 69%, Tone = mean HR in DBT in 68% of patients, and decreased the HRV age by 9 years (**Table 1**).

From our previous experience (Ylikoski et al., 2017) and from the results of the current study, we deduced that R-R interval, HRV age and RMSSD are the most useful markers for stimulation changes in HRV (**Table 1**). RMSSD is mainly related to beat-to-beat variations reflecting parasympathetic output (Faber et al., 1996). In practice, the change in vasovagal tone is best illustrated by the HRV age that was determined by algorithms based on values of HRV parameter values, as described by Weinschenk et al. (2016).

If in cases where R-R interval was decreased (in 19%) after test-taVNS, the results of HRV age were taken into consideration, either the HRV age or R-R interval showed increased parasympathetic activity in more than 95% of cases.

To compare the changes in R-R interval, RMSSD and HRV age with the age of patients, we selected the youngest patients (age < 41 years) for one group (n = 41) and the oldest patients (age > 63 years) for another group (n = 21) (**Table 2**, **Figure 3**). Test-taVNS increased all the three HRV parameters much more often in patients of the older group: R-R interval in 86% in >63 group, 73% in < 41 group, RMSSD in 81% in >63 group, 66% in < 41 group and HRV age in 81% in >63 group, 58% in < 41 group (**Table 2**). The taVNS-induced numerical increases of the three HRV parameters were also greater in the older patients. However, only the RMSSD changes reached statistical significance (**Table 2**, **Figure 3**). This indicates that taVNS is more efficient in older patients, a result that could be explained by lower starting levels of HRV in this group due to age-related decline in parasympathetic activity. The magnitude of taVNS-induced changes has been shown to be higher in individuals with lower starting HRV level (Bretherton et al., 2019).

TABLE 1 | Mean HRV test data of 171 patients with TRMS.

	R-R-interval	HRV-age	Flexibility %	Dynamic % (RMSSD)	Tone %
	pre/post taVNS	pre/post taVNS/chronol	pre/post taVNS	pre/post taVNS	pre/post taVNS
Mean	815/868	65/56/49	32/44	32/49	38/49
SD	141/140	22/23/16	28/30	30/34	30/30
p-Value	<0,0001	<0,0001	<0,0001	<0,0001	<0,0001
Cohen's *d*	0,377	0,400	0,414	0,530	0,367
taVNS induced change	+52 (6.4%)	−9 (13.7%)	+11 (34.5%)	+18 (53.2%)	+12 (30.5%)
Increased	139 (81.3%)	12 (7.0%)	107 (62.6%)	118 (69.0%)	116 (67.8%)
Decreased	32 (18.7%)	135 (79.0%)	36 (21.1%)	16 (9.4%)	22 (12.9%)
Unhanged	–	24 (14.0%)	28 (16.4%)	37 (21.6%)	33 (19.3)

The taVNS significantly increased all HRV parameters: the mean R-R interval in 81%, Flexibility in 63%, Dynamics (RMSSD) in 69%, Tone in 68% of patients, and decreased the HRV age by 9 years. The mean pre-taVNS HRV age was 16 years higher than the chronological age. The taVNS induced change represents the mean difference between pre- and post-taVNS values of all patients. The mean percentage change is also shown. Increases of RR-interval, flexibility, RMSSD and tone and decrease of HRV-age indicate increased parasympathetic or decreased sympathetic tone. Pre = baseline data; post = post-taVNS stimulation data. Wilcoxon's matched pairs signed rank test was used to calculate p-values. The Bonferroni adjustment method for multiple testing produced a rejection p-value of 0.01. All p-values remained statistically significant. Effect sizes (Cohen's d) ranged from small to medium, the largest was observed in Dynamic % (RMSSD).

TABLE 2 | Correlations between HRV parameters and scores of sleep and anxiety between age groups.

	R-R-interval	HRV-age	RMSSD	sleep/anxiety
	pre/post taVNS	pre/post taVNS	pre/post taVNS	
All patients (*n* = 171)	+52 (6.4%)	−9 (13.7%)	+18 (53.2%)	55
<41 year, *n* = 41	+53,6 (6,9%)	−7,76 (−15,5%)	+15,2 (42,6%)	61
>63 year, *n* = 21	+85,0 (10,5%)	−11,7 (−14,7%)	+30,1 (93,3%)	52
p-Values	0,515	0,0718	0,0392	0,456
Cohen's *d*	0,395	0,315	0,574	0,276

Correlations between HRV parameters (R-R interval, HRV age and RMSSD), and scores of questionnaires for sleep and anxiety between Group A (age < 41 years) and Group B (age > 63 years). Test-taVNS increased all three HRV parameters much more often in patients of the older group: R-R interval in 86% in >63, 73% in < 41 groups, RMSSD 81% in >63, 66% in < 41 groups and HRV age 81% in >63, 58% in < 41 group. Shown is the mean taVNS induced change (mean difference between pre- and post-taVNS values) of all patients as well as of the two age groups. The taVNS-induced numerical increases of these three HRV parameters were also greater in the older group. Only the RMSSD changes reached statistical significance using the non-parametric Kolmogorov–Smirnov test (when comparing mean changes between the two groups) That significance was removed by the Bonferroni correction as the adjustment for multiple testing produced a rejection p-value of 0.0125. Effect sizes (Cohen's d) ranged from small to medium, the largest was observed in Dynamic % (RMSSD). A positive effect means that the change of the >63 years group was larger than the change of the <43 years group.

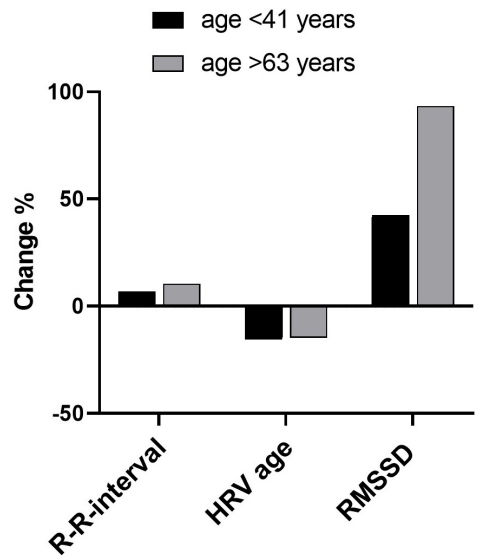

FIGURE 3 | Correlations between HRV parameters between Group A (below 41 years) and Group B (above 63 years) shown in **Table 2**. The taVNS-induced numerical increases of these three HRV parameters were greater in the older group. However, only the RMSSD changes reached statistical significance.

To compare taVNS responses to questionnaire-based clinical data, we selected two patient groups, based on the magnitude of taVNS responses. Group A (21 patients) consisted of super-responders showing a post-taVNS RMSSD increase of 400–2000%. Group B (43 patients) consisted of non-responders showing unchanged or decreased post-taVNS RMSSD. Comparison of the values of THI, sleep disturbance and anxiety showed that group A comprized of more patients with mean THI scores > 60/100 and with sleep disturbance and anxiety scores > 60/100. However, the differences were not statistically significant (**Table 3**, **Figure 4**). Super-responders had also higher average tinnitus pitch (8.4 kHz) than non-responders (6.6 kHz) (**Table 3**, **Figure 4**).

Results of VSEP Testing

There was a strong stimulation artifact (0 ms) after which oscillations were registered at about 3 ms. These have been earlier described to be of brainstem origin (VSEP) (Fallgatter et al., 2003). This response, however, was interpreted and presumed to be of local origin, arising from muscles in the ear region, not in the brainstem, in accordance to Leutzow et al. (2013). Therefore, we have not used VSEP as a biomarker for taVNS.

Results of the 1-Year Follow-Up Outcome Questionnaires

One-year follow-up therapeutic outcome data was possible to receive from 78 out of 113 patients (69%). Both the loudness and annoyance of tinnitus had decreased in about two thirds and, importantly, stress had decreased in more than 80% of patients (**Figure 5A**). About 76% of patients reported that they had benefited from the TCPT (including taVNS) treatment. When asked whether they would recommend similar treatment for a friend or near relative with similar health problems, 90% answered yes or probably yes (**Figure 5B**). When asked what constituent of the TCPT therapy regimen was most efficient in 1 to 5 scale, counseling showed an efficacy of 3.4, followed by taVNS (3.1) and sound therapy (2.8) (**Figure 5C**). Thus, it seems that taVNS is a useful addition to tinnitus treatments. We stress, however, that the most important constituent of tinnitus therapy is directive counseling. That has been our opinion for decades and it was also supported by the results of this follow-up study.

DISCUSSION

The main aim of this study is to share our experience of the usage of taVNS in the treatment of distressing tinnitus. Therefore, we discuss only matters that we feel important in clinical practice. Our study shows that taVNS is safe. Baseline HRV data of 171 patients showed that more than three quarters of TRMS patients had increased sympathetic prevalence (preponderance) before the first test-taVNS. The mean values of different HRV parameters changed toward increased parasympathetic activity by test-taVNS in about 80% of patients. These changes were more pronounced in patients showing greater tinnitus handicap, more severe associated symptoms, higher stress levels and higher age before the test stimulation. No significant adverse effects were reported in follow-up questionnaires. Our conclusion is that our tinnitus treatment program, including taVNS, alleviates stress and handicap caused by tinnitus.

TABLE 3 | Comparison between taVNS responses and questionnaire-based clinical data in super-responders and non-responders.

	THI	Sleep	Anxiety	Loudness	Annoyance	Frequency
A. Super responders (RMSDD increase 400–2000%)	61,05 (*n* = 21)	70,00 (*n* = 20)	68,95 (*n* = 19)	67.35 (*n* = 19)	74.12 (*n* = 19)	8398 Hz (*n* = 20)
B. Non-responders (decrease or no change)	56,67 (*n* = 43)	61,22 (*n* = 37)	58,11 (*n* = 37)	60,63 (*n* = 37)	70,47 (*n* = 37)	6562 Hz (*n* = 40)
p-Values	0,246	0,642	0,198	0,147	0,197	0,122
Cohen's *d*	0,301	0,128	0,358	0,429	0,386	0,414

Comparison between taVNS responses and questionnaire-based clinical data and tinnitus frequency in two patient groups. Group A (n = 21): super-responders (taVNS-induced RMSSD increase of 400–2000%). Group B (n = 43): non-responders (RMSSD decreased or unchanged). The mean values of THI, sleep disturbance, anxiety and tinnitus frequency were all greater in group A, but the differences were not statistically significant (Student's t-test). Bonferroni adjustment method for multiple testing produced a rejection p-value of 0.0083. Effect sizes (Cohen's d) ranged from small to medium. A positive effect indicates that the mean of the super-responder group was larger than the mean of the non-responder group.

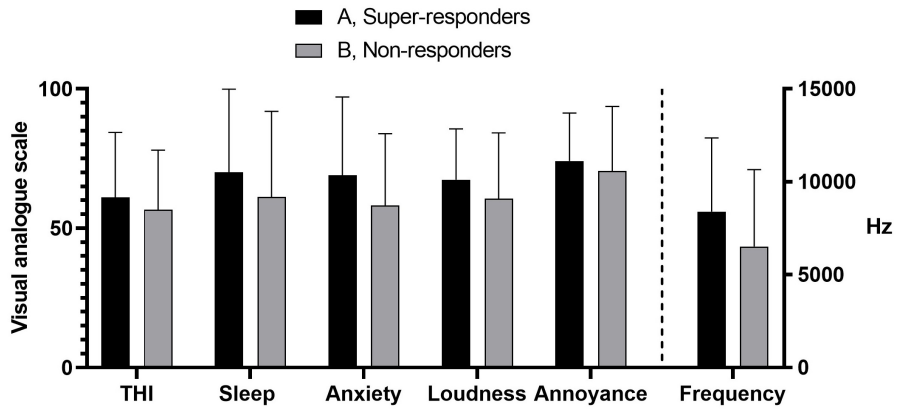

FIGURE 4 | Comparison between taVNS responses and questionnaire-based clinical data and tinnitus frequency in groups A (super-responders) and B (non-responders), shown in **Table 3**. The mean values of THI, sleep disturbance, anxiety and tinnitus frequency were all greater in group A, but the differences were not statistically significant. Error bars represent standard deviations. THI = tinnitus handicap inventory.

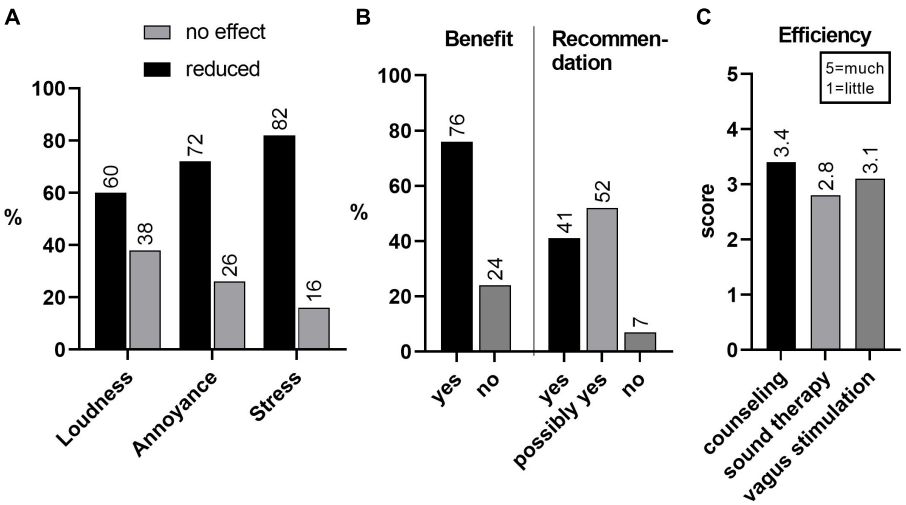

FIGURE 5 | (A) Results of 1-year-follow-up outcome of 113 patients of which 78 (69%) data was received. Tinnitus annoyance had decreased in 72% and tinnitus-triggered stress in 82%. Symptoms had increased in 2%. **(B)** 76% of patients reported that they had benefited from TCPT treatment, including taVNS; 41% would recommend and 52% possibly recommend TCPT plus taVNS t for a friend or relative if suffering from similar health problems. **(C)** Of the components of treatment, counseling was reported most helpful (score 3.4 from the range 1–5), followed by taVNS (3.1) and sound therapy (2.8).

The main problem of patients with disturbing moderate or severe tinnitus is usually TRMS (Andersson and Hesser, 2013) and associated imbalance of the ANS, leading to sympathetic prevalence and correspondingly reduced parasympathetic activity (Thayer et al., 2012; Chalmers et al., 2014). Therefore, the optimal TRMS treatment would be by trophotropic

(parasympathetic activity enhancing) means. This could be achieved by behavioral methods such as cervical vagal massage, Valsalva maneuver or respiratory VNS (Gerritsen and Band, 2018) or by general relaxation generating methods such as yoga, mindfulness, biofeedback and cognitive behavioral therapy. The present study, in agreement with our previous results (Ylikoski et al., 2017), suggests that the therapeutic trophotropic effect can be accentuated by taVNS in TRMS and, thereby, tinnitus handicap can be attenuated. This would be in accordance with recent studies reporting that (implanted) VNS paired with tones as well as taVNS constitute promising novel treatments for tinnitus (Lehtimäki et al., 2013; Tyler et al., 2017; Yakunina et al., 2018). On the other hand, another clinical study did not report improvement of tinnitus with taVNS alone, although the therapy was found to be safe (Kreuzer et al., 2014).

The vagus nerve provides a unique therapeutical entrance to the CNS. Although VNS has become an established intervention therapy for therapy resistant epilepsy and depression, the exact mechanisms remain unsolved. Preclinical studies have shown that VNS therapy results in intermittently increased release of multiple neuromodulators, including norepinephrine, acetylcholine, serotonin and brain-derived neurotrophic factor (BDNF) (Hassert et al., 2004; Dorr and Debonnel, 2006). BDNF is a key player in the CNS neuronal plasticity. Maladaptive plasticity is thought to be involved in many medical entities, particularly in illnesses such as phantom pain, dystonias and tinnitus (Flor et al., 2001; Engineer et al., 2011; Kilgard, 2012). In addition, enhancement of neuronal plasticity through VNS has been shown to improve functional recovery in animal models and patients with stroke-induced upper limb paresis (Dawson et al., 2016; Engineer et al., 2019) and in animal models of spinal cord injury (Ganzer et al., 2018). BDNF, norepinephrine and serotonin have been suggested to play a key role in this enhanced plasticity (Hulsey et al., 2017). BDNF is an activity-dependent neurotrophic factor (Barde et al., 1982). Therefore, therapeutic sessions have consisted of VNS combined with simultaneous activities such as physical therapeutic movements in upper limb paresis or pairing with tones in tinnitus (Engineer et al., 2011; Dawson et al., 2016). In depression, the activity part is thought to consist of psychotherapy.

We emphasize that taVNS should not be applied as a solo but an adjunctive therapy. In our study the treatment of patients with distressing tinnitus was always started with a 1.5 h office visit during which patient's complaints were dealt with our TRT modification, the TCPT program. This consists of careful diagnostics with hearing and tinnitus profiling, counseling and instructions for sound therapy. The taVNS has been the fourth component of TCPT, initiated at the office visit and continued, together with sound therapy (the activity component), as home therapy. Our inquiries (**Figure 5**) indicate that counseling is the most important therapy constituent for this kind of distressed patients. During counseling, which takes about 1 h, we try to demystify tinnitus by explaining what tinnitus is all about and how one should behave in order to diminish its annoyance; not to be afraid that it worsens or never disappears. It has been shown that uncertainties and fears constitute a potent stressor and can easily cause diseases (Peters et al., 2017). Specifically, fear and anxiety can be significant co-factors, possibly modulated by amygdala, in triggering TRMS (Andersson et al., 1999; Cima et al., 2012). This type of counseling should be applied also to the therapeutic regimen of anxious and depressive patients.

Mechanisms of TRMS

The prominent feature of our patients was the SR (arousal) caused by tinnitus. SR is such a personal experience that it is not possible to ascertain – or even speculate- why most of the patients had developed a severe SR. Also, the reliability of our main markers for SR, the questionnaires and HRV tests, are not accurate and often even debatable. However, there are some general clinical and test-based characteristics, which allow to suggest speculative stress pathways at least in the patients in which tinnitus was initially triggered by exposure to excessively loud sounds. These patients had contracted NIHD that is now known to be the most common consequence of noise trauma. Its most common symptoms are tinnitus and hyperacusis, the audiometrically measurable hearing impairment being a much less common feature (Kähäri et al., 2001; Szibor et al., 2018). Usually, the intensity of the exposing sound (mostly music) is relatively low in NIHD and its action on the cochlea is thought to be cellular stress/damage that does not lead to significant hair cell loss. Low-level noise exposure is known to cause synaptopathy, the damage of the synapses between inner hair cells and auditory nerve fibers (Kujawa and Liberman, 2009). It is generally accepted that tinnitus frequency and the predicted location of the lesion along the tonotopic axis of the cochlear are closely related. In the present study, tinnitus frequency tended to be very high, higher than 6 kHz in more than 85% of cases, indicating that the presumable cellular stress response was localized to the extreme basal coil of the cochlea. Most researchers agree that tinnitus can be linked to changes at one or more relays along the peripheral and central auditory pathways including auditory cortex (Jastreboff, 1990; Lockwood et al., 1998; Giraud et al., 1999; Møller, 2003; Eggermont and Roberts, 2004; Rauschecker et al., 2010). Although it is generally accepted that the dysfunction of the auditory system is necessary for tinnitus to occur, it is unclear whether this defect alone is sufficient to cause chronic tinnitus or whether additional mechanisms outside the auditory-sensory regions are involved. Clinically, there is a clear relationship between tinnitus and the emotional state (Sullivan et al., 1988; Dobie, 2003) and it has led to the suggestion that the limbic system plays a role in modulating or perpetuating tinnitus (Jastreboff, 1990; Rauschecker et al., 2010). Indeed, the lifetime incidence of clinical depression in tinnitus patients is estimated to be more than twice of the national average (∼35% vs. 15%, respectively, Folmer et al., 1999). Treatment regimens that include forms of cognitive-behavioral therapy have been shown to be effective for many tinnitus patients (Jastreboff, 2007; Robinson et al., 2008). Although the exact nature of the involvement of the limbic system in chronic tinnitus remains to be shown, there is substantial – mainly neuroimaging – evidence indicating that networks such as the corticostriatal circuit and the amygdala-anterior cingulate cortex axis are involved (Leaver et al., 2011; Chen et al., 2017; Xu et al., 2019). It has been shown that the corticostriatal circuit, which includes the nucleus

accumbens and ventromedial posterior frontal cortex, does indeed differ in the brains of individuals with tinnitus (Leaver et al., 2011). The corticostriatal circuit is part of the general "appraisal network", determining which sensations are important and ultimately affecting how (or whether) those sensations are experienced (Simmons et al., 2020). Our theory of the pathogenetic mechanism of NIHD is schematically summarized in **Figure 6**.

The Inflammatory Reflex or Neuroinflammation in the Pathogenesis of Stress and Tinnitus, and Possible Attenuation by taVNS

Although the common pathways between stress exposure and pathophysiological processes leading to tissue damage are still debatable, several results indicate that stress can activate an inflammatory response in the brain and in the periphery (Calcia et al., 2016, for review). In this damaging process, stress-induced pro-inflammatory factors including C-reactive protein, IL-6, TNFα, IL-1β and NF-κB, have an important role (Miller et al., 2008). In common, over-activated immune system, increased sympathetic nervous system activity and reduced glucocorticoid (GC) responsiveness may work tandemly in the activation of inflammatory responses during stress. GCs, catecholamines, cytokines and other mediators are thought to be the main mediators of the stress-induced pro-inflammatory effect. Correspondingly, when the auditory system in a rodent model was exposed to acoustic overstimulation causing hearing impairment and tinnitus, neuroinflammation in the central auditory system was found to be importantly involved (Wang et al., 2019).

After exposure to acoustic overstimulation from loud noise or music, the resulting tinnitus is high-pitched (Szibor et al., 2018). It can be very difficult to tolerate and habituate this tinnitus and, therefore, it may lead to sleep disturbances, anxiety and finally to SR. Tinnitus can be regarded as the consequence of multisensory interactions between the auditory and limbic systems. This is because extensive functional networks and tinnitus distress strongly correlate with enhanced effective connectivity that is directed from the amygdala to the auditory cortex (Rauschecker et al., 2010; Chen et al., 2017). When the stimulation patterns and dynamics of functional networks during VNS were examined by fMRI, the vagus nerve was found to convey signals to the brain through the polysynaptic neuronal pathways, by projecting to the brainstem nuclei (NTS, locus coeruleus), subcortical areas and lastly to the cortex (Henry, 2002; Ressler and Mayberg, 2007), thus covering the entire CAN. fMRI and a spatially independent component analysis were utilized in a recent experimental study (Cao et al., 2017). That study demonstrated that VNS activated 15 out of 20 brain networks and that the activated regions covered > 75% of the brain volume.

Very soon after the acoustic trauma, which means during ongoing inflammatory response or neuroinflammation, patients usually seek medical assistance because of uncertainty (and fear) with questions regarding possible consequences and management. If no appropriate treatment or even counseling are available and only negative counseling is offered ("nothing can be done"), a complete SR with self-perpetuating cycle develops: distress worsens tinnitus and worsening tinnitus accentuates SR. This is about the clinical picture characterizing most of the patients included in the present study. Therefore, it is not surprising that our TCPT therapeutic regimen, including taVNS as the adjunctive treatment, significantly benefited the great majority of patients. In this type of condition, taVNS may be especially effective, perhaps due to a dual action: it may attenuate the underlying neuroinflammation or inflammatory process in parallel or subsequent to SR.

Of special interest are our findings indicating that aged patients are more responsive to acute taVNS than younger ones, as revealed by HRV tests. Our results are preliminary and appropriate controls are missing, but if age-related differences in HRV responses hold true also in controlled studies, it may open new avenues for the treatment of hearing disorders, particularly the two most common disorders, presbyacusis and NIHD. There is no effective treatment available for them today. Targeting neuroinflammation with taVNS might be a novel therapeutic possibility for NIHD with tinnitus. There is strong preclinical scientific evidence of the beneficial role of VNS in the treatment of immunologic reflex-associated disorders, particularly rheumatoid arthritis [reviewed by Tracey (2018)]. As a method taVNS is safer than VNS, because ABVN has no efferent neurons.

In the pathogenesis of AF, another common medical entity, accumulating evidence indicates that the inflammatory pathways play a significant role (Aviles et al., 2003; Hu et al., 2015). In a recent clinical trial, chronic, intermittent taVNS (with the Salustim device used in this study as well) resulted in lower AF burden in about 50% of patients compared to sham control stimulation. These results support the use of taVNS to treat paroxysmal AF in selected patients (Stavrakis et al., 2020).

How to Improve the Efficacy of taVNS?

The stimulation of ABVN is an easy and non-invasive method to obtain the beneficial effects of vagal system activation. However, there are still uncertainties concerning the modes of stimulation, including the optimal stimulation site and parameters. These can be defined only after the appropriate biomonitoring tests become available. While clinical taVNS applications have been widely noted in the literature, the physiological mechanisms supporting such clinical effects are poorly understood, particularly in humans.

May be the most important proof of the usefulness of taVNS has so far been obtained from clinical studies. taVNS has been employed for patients suffering from various disorders, including epilepsy (Stefan et al., 2012), tinnitus (Lehtimäki et al., 2013; Yakunina et al., 2018), depression (Rong et al., 2012; Hein et al., 2013), pain (Napadow et al., 2012; Laqua et al., 2014; Janner et al., 2018) and migraine (Straube et al., 2015; Garcia et al., 2017). Clinical studies do not, however, directly show that the beneficial effects are due to ABVN stimulation. This is because the outer ear has an innervation not only from the cranial nerve X, but also from the cranial nerves VII and V as well as the cervical plexus.

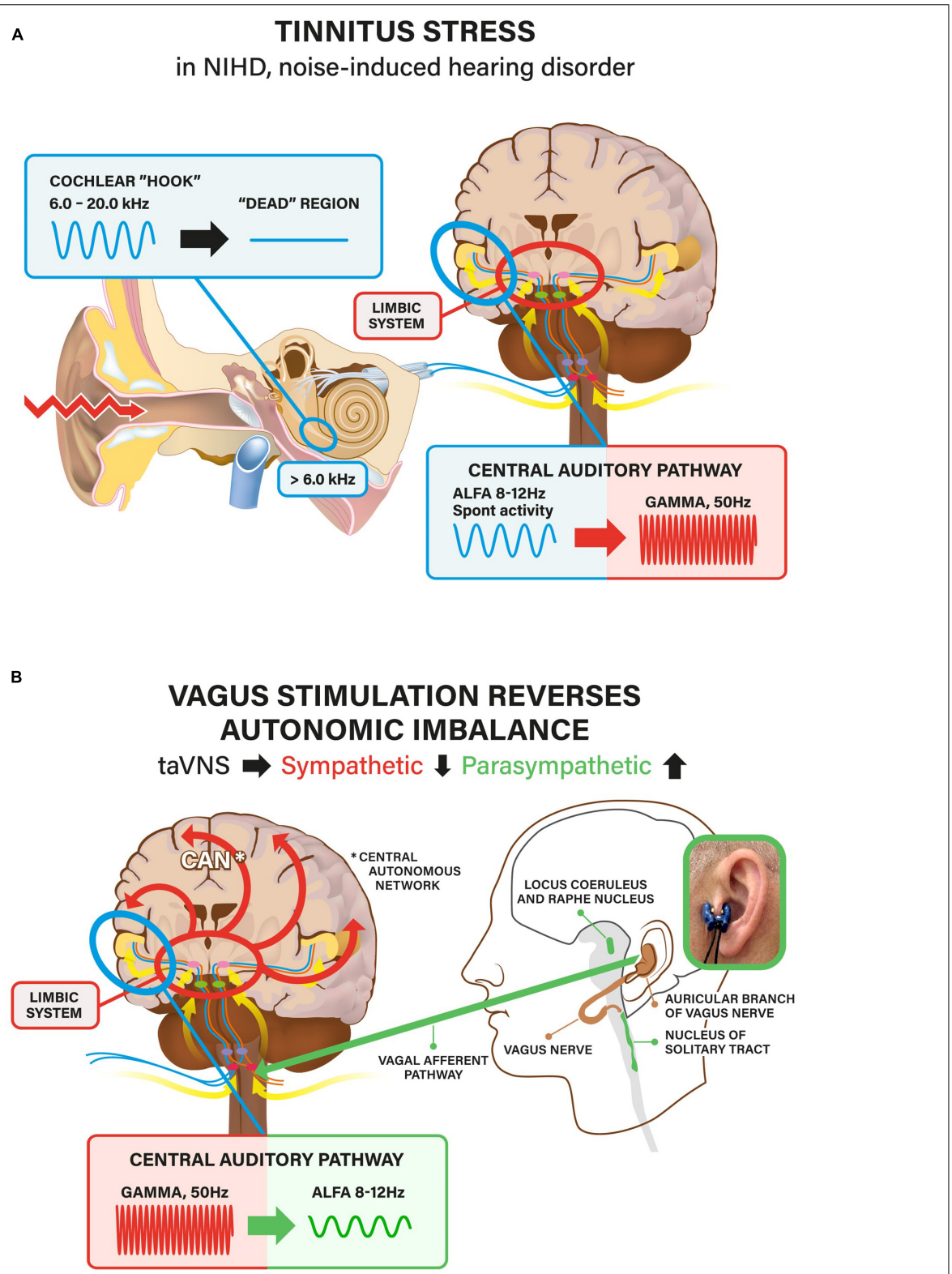

FIGURE 6 | (A) Summary of the hypothesis. Noise exposure (most commonly music) causes dysfunction/damage in the high-frequency (>6.0 kHz) region of the cochlea) in >85% of patients. This leads to high-pitched tinnitus, most commonly at about 8.0 kHz. Bioelectrical impulse flow from the damaged cochlear region toward auditory cortex diminishes or ceases ("dead" region). This leads to reduced (inhibitory) regulation with subsequent neuronal hyperactivity in the central auditory pathway, first in the auditory nuclei of brainstem, later in the auditory cortex. The normal (spontaneous) alpha activity (in EEG) changes to gamma activity.

(Continued)

FIGURE 6 | Continued
The central auditory pathway is intimately connected to the limbic system (that controls emotions). Tinnitus is experienced as an emotionally negative sensation including uncertainties and fears ("what is this all about?"; "does it ever go away?"). Thereby, the perceptive (hearing) network is connected to the distress network (stress). The stressor leads to imbalance of the central autonomous network (CAN) with hyperactivity of the sympathetic nervous system (flight or fight or freeze response) and, correspondingly reduced activity of the parasympathetic nervous system (PNS) (relax, calm down). **(B)** Vagus nerve is the main player of the PNS. Therefore, activation of the vagal system increases PNS activity. For taVNS we have used a specially designed Salustim device that uses an ear-clip electrode inserted to the tragus and electrically stimulates ABVN. The taVNS reverses sympathetic hyperactivity in the limbic system and the CAN imbalance toward parasympathetic direction. Reduction of distress also facilitates the reversal of gamma-hyperactivity back to normal alpha-activity in the auditory central pathway.

Much of our present understanding of the mechanisms and presumed efficacy of taVNS comes from fMRI studies. These studies have shown that taVNS produces significant cortical effects in the vagal afferent pathway. Thus, outer ear stimulation in the regions innervated by ABVN activates afferent vagal networks (Kraus et al., 2007; Dietrich et al., 2008; Frangos et al., 2015; Yakunina et al., 2017; Badran et al., 2018a). These studies have, however, failed to convincingly demonstrate that taVNS activates the crucial brainstem nuclei such as NTS. This has now changed when it was recently demonstrated using a ultrahigh-field (7T) fMRI that taVNS evokes activation in the ipsilateral NTS and upstream monoaminergic source nuclei of the brainstem (Sclocco et al., 2019). This finding supports the idea that the selective stimulation of ABVN is responsible for NTS activation. Corresponding selective NTS activation, comparable to tragal ABVN stimulation, may be possible by using percutaneous ABVN stimulation (Kaniusas et al., 2019). Percutaneous stimulation must, however, be considered as a mini-invasive procedure, because the skin is penetrated. It may be appropriate for the treatment of diseases in medical offices, but not for continuous home-therapy.

Importantly, NTS activity is known to be modulated by respiration, both through the bottom-up afferent pathway from pulmonary stretch receptors and aortic baroreceptors and through the top-down effects from respiratory nuclei in the medulla (Sclocco et al., 2019). Specifically, NTS receives inhibitory influence during inhalation and facilitatory influence during exhalation (Miyazaki et al., 1999; Baekey et al., 2010). Therefore, it has been proposed that NTS targeted by taVNS can be enhanced by gating stimulation to the exhalation phase of the respiratory cycle via respiration-gated auricular vagal afferent nerve stimulation (RAVANS) (Napadow et al., 2012; Garcia et al., 2017). Our taVNS treatment protocol includes instructions for slow breathing ("Respiratory VNS") (Gerritsen and Band, 2018), but electrical stimulation in our device was not synchronized to give electrical stimuli specifically during exhalation.

HRV

Because HRV is a biomonitor for ANS function, we also analyzed HRV changes before and immediately after acute test-taVNS. HRV as well as HR have been found to be useful in monitoring the effects of taVNS (Clancy et al., 2014; Antonino et al., 2017; De Couck et al., 2017; Ylikoski et al., 2017; Badran et al., 2018b; Bretherton et al., 2019). Clancy et al. (2014) demonstrated that 15 min of taVNS administered to the tragus significantly increased HRV, at least partly through reduction of sympathetic nerve activity. In addition, the acute taVNS has been demonstrated to improve spontaneous baroreflex sensitivity (BRS) that may be the most sensitive measure of ANS function and thereby the parasympathetic activity (Antonino et al., 2017; Bretherton et al., 2019). Normal aging is associated with increase in sympathetic prevalence and/or decreases in the vagal tone and overall variability, which is reflected in HRV (Stein et al., 1997; Kuo et al., 1999). There is a general consensus that we all have our own dominant parasympathetic and sympathetic regulation that gradually decreases with advancing age due to a significant reduction of nocturnal parasympathetic activity. Hence, the preservation of parasympathetic function may serve as a biomarker related to the healthy longevity and vitality in late life span (Zulfiqar et al., 2010). In addition to normal aging, a shift toward sympathetic prevalence may contribute to age-related conditions, such as hypertension, heart failure and AF. Evidence suggests that taVNS could play a role in ameliorating these conditions. Bretherton et al. (2019) have suggested that age-related autonomic dysfunction (decrease of HRV and BRS), QoL, mood and sleep changes improve with taVNS administered daily for 2 weeks. This is in line with our previous study (Ylikoski et al., 2017) and also with the observations in the present study showing that the HRV improvement after acute test-taVNS was greater in elderly individuals (with TRMS) than in younger ones. The findings of Bretherton et al. (2019) also point to the influence of initial values in determining the magnitude and direction of change following taVNS: high initial sympathetic prevalence, tension, anger, depression as well as low energy and sleep quality were associated with greater improvements of HRV and BRS. This is also in line with our findings of HRV changes after acute test-taVNS: when the HRV-RMSSD values were correlated to clinical data, patients with high scores in THI, tinnitus annoyance, sleep disturbances and anxiety showed largest changes in RMSSD. Overall, our findings support the idea of Bretherton et al. (2019), when they state: "considering the ease of application and affordability of taVNS, there is significant potential in attenuating symptoms associated with age-related conditions and prolonging the period of healthy ageing."

VSEP

Different physiological and neurophysiological tests have been used to biomonitor the effects of taVNS. According to the literature, VSEP is the most useful online biomonitor. Therefore, we investigated whether VSEP could be used for our biomonitoring purposes. In order to reveal the anatomic site where VSEP arises (hypothetically NTS of the brainstem), we registered VSEP responses using EGI GTEN 100 EEG system with 256 electrodes (Palva and Palva, 2018) and with multiple

stimulation parameters. We found a strong stimulation artifact (0 ms) and thereafter oscillations at about 3 ms, previously described to originate from the brainstem. We interpreted that this response has a local origin, presumably arising from muscles in the ear region, not in the brainstem, in accordance to Leutzow et al. (2013). Therefore, we had to abandon VSEP as a biomarker for taVNS. VSEP seemed to be unrelevant also because of the low numbers (50–100) of epochs reported in prior VSEP studies (Fallgatter et al., 2003; Polak et al., 2007). It is well known that in the most commonly employed brainstem response test, auditory brainstem response (ABR), the minimal number of epochs needed for reliable results vary between 500 and 1000. We are currently investigating whether other features of EEG could be used as online biomonitoring methods for taVNS.

Limitations

The results of the present study should be interpreted with caution because they only represent a retrospective clinical cohort study. However, all our clinical data are based on structured diagnostic forms and questionnaires that were used in the management of the patient population. Furthermore, the consistent improvement of HRV -a seemingly useful marker for mental stress- in 80–90% of our patients suggests that taVNS is a useful (adjunctive) therapeutic means in severe tinnitus. This study encourages future controlled clinical studies on the usefulness of taVNS in tinnitus. The major defect in our retrospective study is the lack of appropriate controls and sham procedures, which are the crucial components of prospective randomized controlled trials (RCTs). Our patient population consists of nonselective series in contrast to that of RTCs in which participants are recruited through various procedures enhancing the selection factor. This aspect is particularly important when the target of investigation is such a common symptom as mental stress.

CONCLUSION

TRMS is an example of a tinnitus-triggered stress condition in which patients may benefit from taVNS. Our clinical data and HRV results before and after test-taVNS suggest that patients with TRMS have ANS imbalance with increased sympathetic activity and, correspondingly, reduced parasympathetic function. Acute test-taVNS increased parasympathetic activity, more in elderly and patients with more severe stress symptoms. Although our follow-up outcome study primarily aimed to study the TCPT therapeutic efficacy in patients with TRMS, showing that this therapeutic program alleviated tinnitus severity, the results can also be interpreted such that the majority of stressed tinnitus patients get additional benefit from taVNS as an adjunct therapy. HRV seems to serve as an easy and rapid method for assessment of SR and thereby ANS balance. Combining clinical data to HRV results may be useful in selecting patients for taVNS. We have now clinical experience on the long-term use of taVNS by several of our patients. They have used taVNS daily for more than 4 years without any adverse effects. They continue to use the device because of subjective benefits. Currently, at the same setting where the present study was performed, we offer taVNS treatment for all our patients who show THI scores of 34 or over. We regard this as an alternative to a possible need for e.g., tranquilizers. However, taVNS should not be used as a solo therapy but as an adjunct to a treatment program in which all the constituents are aimed to restore the sympathovagal imbalance through parasympathetic activation. Generally, this study offers additional support to the idea that taVNS might offer a new, targeted therapeutic tool for patients in whom sympathovagal imbalance is involved. Furthermore, taVNS is patient-friendly and of low-cost. However, as there are not (yet) appropriate online biomarkers available for taVNS, there is still a great need for additional research to find optimal therapeutic regimen as well as better stimulating devices.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

JY contributed to the design of the study, interpretation of data, and writing the manuscript. JY, JL, and MY conducted the clinical work. MM conducted the data analysis and illustrations. UP, ZJ, SS, and AM revised the manuscript. All authors contributed to the article and approved the submitted version.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Article

Vagus Nerve Stimulation with Mild Stimulation Intensity Exerts Anti-Inflammatory and Neuroprotective Effects in Parkinson's Disease Model Rats

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Abstract: Background: The major surgical treatment for Parkinson's disease (PD) is deep brain stimulation (DBS), but a less invasive treatment is desired. Vagus nerve stimulation (VNS) is a relatively safe treatment without cerebral invasiveness. In this study, we developed a wireless controllable electrical stimulator to examine the efficacy of VNS on PD model rats. Methods: Adult female Sprague-Dawley rats underwent placement of a cuff-type electrode and stimulator on the vagus nerve. Following which, 6-hydroxydopamine (6-OHDA) was administered into the left striatum to prepare a PD model. VNS was started immediately after 6-OHDA administration and continued for 14 days. We evaluated the therapeutic effects of VNS with behavioral and immunohistochemical outcome assays under different stimulation intensity (0.1, 0.25, 0.5 and 1 mA). Results: VNS with 0.25–0.5 mA intensity remarkably improved behavioral impairment, preserved dopamine neurons, reduced inflammatory glial cells, and increased noradrenergic neurons. On the other hand, VNS with 0.1 mA and 1 mA intensity did not display significant therapeutic efficacy. Conclusions: VNS with 0.25–0.5 mA intensity has anti-inflammatory and neuroprotective effects on PD model rats induced by 6-OHDA administration. In addition, we were able to confirm the practicality and effectiveness of the new experimental device.

Keywords: anti-inflammation; less invasive therapy; new experimental device; Parkinson's disease; vagus nerve stimulation

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1. Introduction

The main pathology of Parkinson's disease (PD) is characterized by a loss of dopamine (DA) neurons in the substantia nigra pars compacta (SNc) associated with prolonged neuroinflammation [1]. Glial cell activation of microglia and astrocyte in the SNc stands as common features of both human PD patients and animal models of PD, playing vital roles in DA neuronal degeneration [1–4]. In postmortem analysis of PD patients, activated microglia, HLA-positive microglia [2], and increased density of astrocytes populated the SNc [3,5]. These proliferated and activated glial cells induce inflammatory mediators

including tumor necrosis factor α (TNFα), interleukin 1β (IL-1β), IL-6, IFN-γ, and subsequently cause oxidative damage, altogether contributing to the acceleration of DA neuronal degeneration in the SNc [1,4,6,7].

PD onset and progression are caused not only by loss of DA neurons in the SNc but also by significant degeneration of noradrenalin (NA) neurons in the locus coeruleus (LC). Postmortem brains of PD patients showed remarkable degeneration of NA neurons in the LC as well as the loss of DA neurons in the SNc [8–10]. The LC serves as the largest source of NA production in the central nervous system with axonal projections to multiple sites in the whole brain. NA neurons in the LC also send direct projections to the striatum and SNc, protecting DA fibers and neurons and subsequently functionally regulating DA neurons in the SNc [11]. Consequently, the degeneration and loss of NA in the LC significantly contribute to DA neuronal degeneration in the SNc and to the progression of PD pathology [12,13]. In advanced PD patients treated with long-term administration of anti-PD drugs, deep brain stimulation (DBS) exhibits therapeutic effects. Despite the effectiveness of DBS, it has the crucial problem of cerebral invasiveness and this disadvantage might limit the application of DBS for PD. There is an underlying necessity for complementary and alternative surgical therapies to treat advanced PD patients with less invasiveness. Vagus nerve stimulation (VNS) is conducted without cerebral invasiveness. In the laboratory, VNS produces robust amelioration of chronic inflammatory and autoimmune disorders, primarily through its anti-inflammatory properties [14,15].

In this study, we assessed the neuroinflammation in PD by examining the anti-inflammatory effects of VNS. Recognizing the need for a clinically feasible approach in providing continuous electrical stimulation, we utilized a wireless controllable stimulation device. We evaluated the therapeutic effects of VNS with behavioral and immunohistochemical outcome assays, with emphasis on detailing the status of glial cells and NA neurons under different stimulation conditions.

2. Materials and Methods

2.1. Electrical Stimulation System for Animal Studies

We developed a wireless controllable electrical stimulation device named SAS-200 for animals with technical assistance from Unique Medical Co., Ltd. (Tokyo, Japan). SAS-200 was designed exceedingly miniaturized and light-weight. The size of the device is as follows: 40 × 20 × 20 mm (width × depth × height). The weight is 26 g including a lithium-ion battery (3.7 V, 250 mAh) in an aluminum chassis (Figure 1a–c). SAS-200 allows outputting biphasic pattern of square pulses, and to set 10 patterns of stimulation intensity ranging 0–2.0 mA, 11 patterns of duration ranging 0–300 Hz, 3 patterns of pulse width, and 5 patterns of stimulation cycle. Thus, SAS-200 consists of over 1500 patterns of stimulation conditions. SAS-200 receives a parameter command from control software for a Windows PC via Bluetooth dongle which is designed for this system. We can detect the battery residual amount and the signals for changing the stimulation parameter by the glimmering of the LED light located inside the device through the transparent screw (Figure 1d). The stimulation system consisted of SAS-200 as a stimulator, a standard Windows PC as a command post, and Bluetooth dongle for SAS-200 (Figure 1e). In the experiment, SAS-200 was fixed to the back of a rat with a suture through holes in the base. The battery was charged by a power supply from PC via USB cable and easily exchanged by loosening two screws at the lateral side of SAS-200 (Figure 1f). The greatest advantage of this system is that all rats received continuous electrical stimulation without anesthesia and moved freely during the experiment.

2.2. Electrode

We used cuff-type electrodes purchased from Unique Medical Co., Ltd. (Tokyo, Japan) (Figure 2a). Electrodes were composed of two curved silver wires (0.08 mm diameter) covered with a 4 mm section of polyethylene tubing (outer/inner diameter: 1.0 mm/0.5 mm). The silver wires were aligned 1.5 mm apart in parallel inside the cuff. A cut was made

lengthwise along the tubing to allow the cuff to be wrapped around the nerve and then closed by a suture (Figure 2b). The insulation was removed to provide conductivity, allowing bipolar stimulation limited in surrounding the nerve. These electrodes were designed based on a similar method used by several previous studies [16–18].

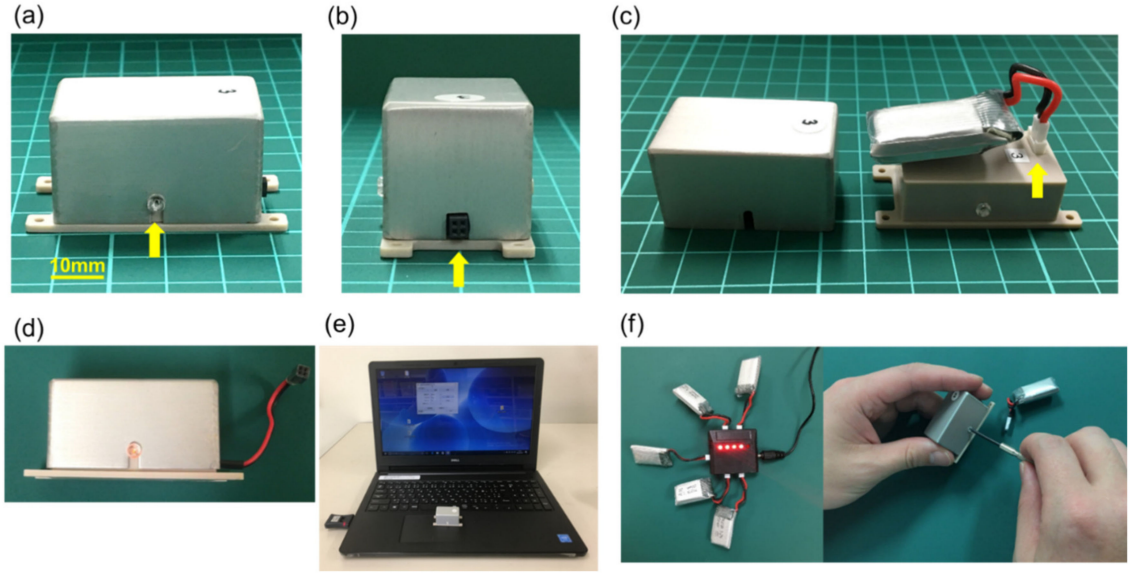


Figure 1. Newly developed stimulating system. (**a**) The lateral side of the SAS-200. A yellow arrow shows the transparent screw in which the LED light is attached to the electronic circuit board to confirm the energization. (**b**) The frontal side of the SAS-200. A yellow arrow is showing the external terminal of the SAS-200 where the stimulation electrode is connected. The external terminal can be extended with a lead type connection. (**c**) The lithium-ion battery in the aluminum chassis. The lithium-ion battery is connected to the control board inside the plastic chassis (arrow). (**d**) Flickering of the LED light located inside of SAS-200 through the transparent screw can indicate the stimulation command input and battery residual. The extensional lead can be attached to SAS-200. (**e**) SAS-200, Windows PC, and Bluetooth dongle for SAS-200. The recent Windows PC adapted with SAS-200 and its dongle. (**f**) Charging lithium-ion battery via USB cable. The battery can be exchanged by loosening two screws on the lateral side of SAS-200.

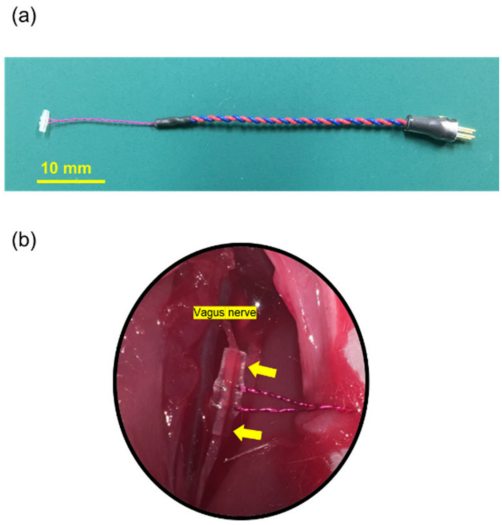


Figure 2. The cuff type electrode for vagus nerve stimulation. (**a**) The cuff type electrode used in this study. The electrode is 60 mm long, composed of 20 mm of silver wire and 40 mm of flexible lead. (**b**) The cuff type electrode surrounding the left vagus nerve. A yellow arrow shows the point to be fixed by 5–0 silk.

2.3. Ethics Statement and Animals

All experimental procedures were conducted in accordance with Okayama University guidelines for animal experiments and were approved by the University's committee on animal experimentation (examination protocol, #OKU-2017449 approved on 23 October 2017). Adult female Sprague-Dawley rats (SHIMIZU Laboratory Supplies Co., Ltd., Tokyo, Japan) weighing 200–250 g at the beginning of the study were used for all experiments. They were singly housed per cage in a temperature- and humidity-controlled room that was maintained on a 12 h light/dark cycle with free access to food and water. A total of 50 rats were used in this study. Ten rats were used for 5 groups, respectively. The data obtained from a total of 46 animals were used in the analysis, with the exclusion of 2 rats with more than 20% weight loss and of 2 dead rats.

2.4. Surgical Procedure

2.4.1. VNS Surgery

All rats were anesthetized with a combination of three anesthetics intraperitoneally (i.p.) injected (0.3 mg/kg of medetomidine, 4 mg/kg of midazolam, and 5 mg/kg of butorphanol). Rats were then fixed in supine position on the heating pad at 37 °C. A 20 mm skin incision was made on the left ventral side of the neck. The sternohyoid and sternomastoid muscles were separated longitudinally and then retracted laterally until the carotid artery within the carotid sheath was exposed. The left vagus nerve was then carefully separated from the surrounding connective tissue. The left vagus nerve was surrounded by a cuff-type silver electrode, and both ends were closed with sutures. Thereafter, a small incision was made on the dorsal side of the neck, then a subcutaneous tunnel was made with the passer to allow the electrode terminal to be passed through. The electrode terminal was fixed at the dorsal neck with a suture, followed by connection to the SAS-200 external terminal. A rat and SAS-200 were covered with handmade jacket to keep the stimulator in a stable position during the experiment [19]. A control group of animals was subjected to the same surgery with a dummy pulse generator.

2.4.2. 6-OHDA Lesioning

After the operation for VNS, rats were placed in a stereotaxic instrument (Narishige, Japan). Furthermore, 20 μg of 6-OHDA (4 μL of 5 mg/mL dissolved in saline containing 0.2 mg/mL ascorbic acid; Sigma-Aldrich Co. LLC, St. Louis, MO, USA) was injected into the left striatum with a 28 G Hamilton syringe. The lesion coordinates were as follows: 1 mm anterior to the bregma, 3 mm lateral to the sagittal suture, and 5 mm ventral to the surface of the brain with the tooth-bar set at −1.0 mm. The injection rate was 1 μL/min. After the injection, the syringe was left in place for additional 5 min, followed by being retracted slowly (1 mm/min).

2.5. VNS Stimulation Parameters and Experimental Protocol

Fifteen minutes after the 6-OHDA injection, VNS electrical stimulation commenced. We applied the conventional parameters for VNS used in clinical settings: cycle of 30 s on 5 min off, pulse frequency of 30 Hz, pulse width of 500 μs, for 14 consecutive days. Except for the stimulation intensity, these fixed parameters were identical for every rat in this study. Rats were randomly assigned into 5 groups, namely, control group and 0.1 mA, 0.25 mA, 0.5 mA, and 1 mA VNS groups. To evaluate the behavioral symptoms of PD, cylinder test and methamphetamine-induced rotation test were performed by blinded investigators on day 7 and day 14 after 6-OHDA lesioning. On day 7, the SAS-200 was removed from the body at the time of behavioral tests, and then re-fixed under anesthesia after the behavioral tests. Following behavioral tests on day14, animals were euthanized for immunohistochemical investigations. These experimental designs are shown in Figure 3.

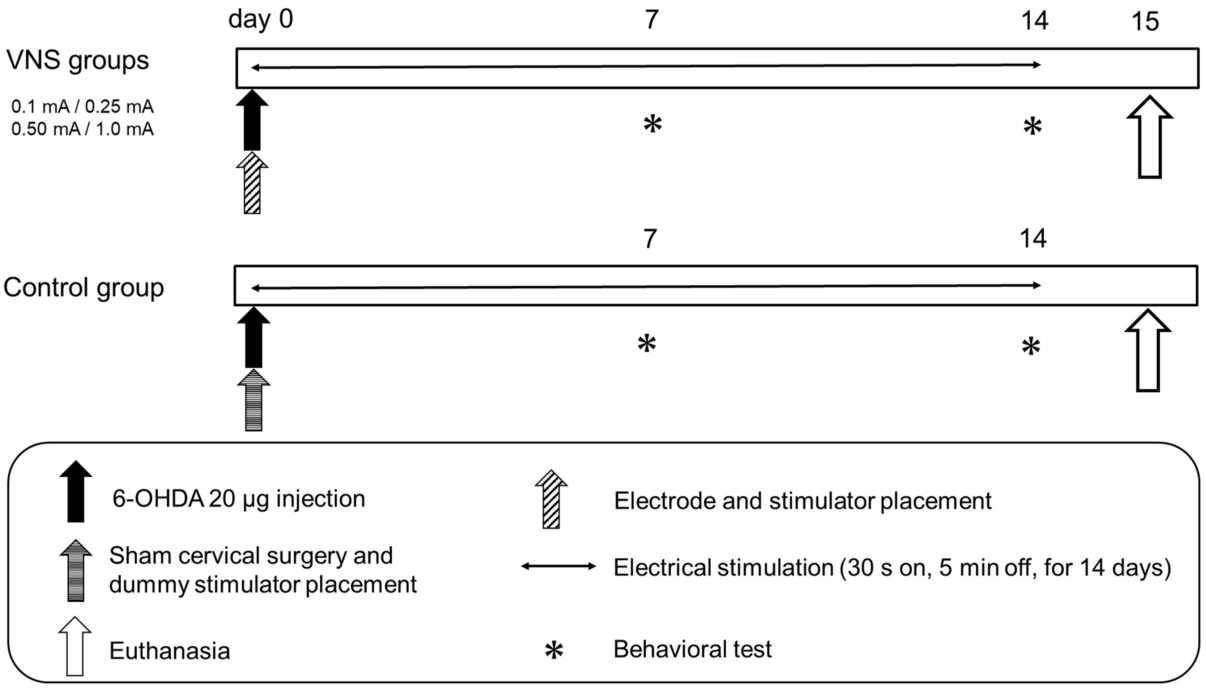


Figure 3. Experimental protocol. Fifty rats were randomly divided into five groups (n = 10 in each). On day 0, the left vagus nerve was exposed, and the cuff type silver electrode was placed. The electrode lead was connected to the SAS-200 and fixed to the back of the rats. Thereafter, 20 μg of 6-hydroxydopamine (6-OHDA) was stereotactically administered into the left striatum to prepare a Parkinson's disease (PD) model. Vagus nerve stimulation (VNS) was started immediately after 6-OHDA administration and was continued for 14 days. On days 7 and 14, behavioral evaluation was performed with cylinder test and methamphetamine-induced rotation test. All rats were then euthanized on day 15.

2.6. Behavioral Tests

2.6.1. Cylinder Test

We performed the cylinder test to assess the degree of forepaw asymmetry, on day 7 and day 14 after 6-OHDA injection. Rats were placed in a transparent cylinder (diameter: 20 cm, height: 30 cm) for 3 min. and the number of forepaw contacts to the cylinder wall was counted [20]. The score of the cylinder test was calculated as a contralateral bias: ((the number of contacts with the contralateral limb) − (the number of contacts with the ipsilateral limb)/(the number of total contacts) × 100)) [21–23]. In the cylinder test, asymmetry in forelimb use is evaluated to reveal spontaneous locomotor activity of unilateral dopamine-depleted rats [20].

2.6.2. Methamphetamine-Induced Rotation Test

All rats were tested with intraperitoneal administration of methamphetamine (3 mg/kg, Dainippon Sumitomo Pharma, Osaka, Japan) on day 7 and day 14 after the cylinder test. Fifteen minutes after injection, the rotational behaviors were assessed for 90 min. with a video camera. Full 360° turns ipsilateral to the lesion were counted [22,23].

Methamphetamine-induced rotation test is used to evaluate the degree of preservation of functional DA neurons, imbalances in dopamine, and motor impairment caused by 6-OHDA lesion [24].

2.7. Fixation and Sectioning

On day 15, all rats were deeply anesthetized by injection of overdosed sodium pentobarbital (100 mg/kg). Rats were perfused with 150 mL of 4 °C phosphate-buffered saline (PBS) transcardially, followed by 150 mL of 4% paraformaldehyde (PFA) in PBS. Brains were carefully removed and incubated in 30% sucrose at 4 °C for 3 days.

For the preparation of cryostat sections, brains were frozen and stored at −70 °C. Six series of 40 µm-thick coronal brain sections including striatum and SNc were obtained with a cryostat, respectively. Brain sections were stored at −20 °C and used for immunohistochemical investigations [22,23].

2.8. Immunohistochemical Investigations

2.8.1. Tyrosine Hydroxylase (TH) Immunohistochemical Investigations

Free-floating sections were blocked with 3% hydrogen peroxide in 70% methanol for 10 min. Sections were washed 3 times for 5 min. each time in PBS, followed by incubation overnight at 4 °C with rabbit anti-TH antibody (1:500; Chemicon, Temecula, CA, USA) with 3% normal horse serum. After several rinses in PBS, sections were incubated for 1 h in biotinylated donkey anti-rabbit IgG (1:500; Jackson Immuno Research Lab, West Grove, PA, USA), then for 30 min in avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA, USA). Subsequently, the sections were treated with 3,4-diaminobenzidine (DAB, Vector) and hydrogen peroxide, then mounted on albumin-coated slides and embedded with cover glass [22,23].

2.8.2. Fluorescent Immunostaining of Microglias, Astrocyte and Noradrenaline Neurons

To explore the distribution of immunoreactive glial cells in neurons, astrocytes, and microglias, double immunofluorescence staining of glial fibrillary acidic protein (GFAP) and ionized calcium binding adaptor molecule 1 (Iba1) were performed. For analyzing the viability of NA neurons in the LC, double immunofluorescence staining of dopamine β hydroxylase (DβH) was performed. Sections of 40-µm-thickness of the striatum, SNc, and LC were used. The slices were washed 3 times with PBS, followed by incubation with 10% normal horse serum and primary antibodies; rabbit anti-GFAP antibody (1:1000; Novus Biologicals, Littleton, CO, USA) and rabbit anti-Iba1 antibody (1:250; Wako Pure Chemical Industries, Osaka, Japan) and rabbit-anti DβH antibody (1:500; Sigma-Aldrich Co. LLC, St. Louis, MO, USA) for 24 h at 4 °C, respectively. After rinsing in PBS, sections were incubated for 1 h in FITC-conjugated affinity-purified donkey anti-rabbit IgG (H + L) and 4,6-diamidino-2-phenylindole (DAPI; 2 drops/mL, R37606; Thermo Fisher, Waltham, MA, USA) in a dark chamber. The sections were then extensively washed with PBS and coverslipped. Both TH and fluorescent immunoreactivities were visualized using an inverted fluorescence phase-contrast microscope BZ-X710 (Keyence, Osaka, Japan).

2.9. Morphological Analyses

2.9.1. TH Immunostaining

The optical density of TH-positive fibers in the striatum was determined and analyzed with a computerized analysis system as previously described [25]. Three sections at 0.5 ± 1.0 mm anterior to the bregma were randomly selected for quantitative analyses. The two areas adjacent to the needle tract of lesioned side and the symmetrical areas in the intact side were analyzed, respectively. The percentages of lesion to the intact side were evaluated in each section and the averages were used for statistical analyses. The images were computer-processed into binary images using an appropriate threshold (Image J; National Institutes of Health, Bethesda, MD, USA). Using Image J, we first defined the threshold of the TH-positive fibers on the lesion side, and then applied the same threshold to the intact side. Each area was then calculated for statistical analyses. The number of TH-positive neurons was counted of three sections at 4.8, 5.3 and 5.8 mm posterior to the bregma in the SNc, respectively. The number of TH-positive neurons was measured by manually counting the cell bodies. The percentage of the number of TH-positive neurons in the lesioned SNc to the intact side was analyzed and the average was used for the statistical analyses. For all 3 areas, both the lesion side and intact side of the striatum and SNc in each rat was calculated and analyzed [22,23].

2.9.2. Activation of Microglia and Astrocytes in Striatum and SNc

The number of Iba1-positive cells and GFAP-positive cells in the lesion side of the striatum and SNc was counted in the two fixed areas (each 500 × 500 μm square) to evaluate glial reaction. Three different sections were randomly selected which were the same level corresponding to TH-immunostaining. In total, 6 representative areas for both the striatum and SNc were counted in each rat. Cell number averages were used for statistical analyses [23].

2.9.3. Preservation of Noradrenergic Neurons in the LC

The density of NA neurons in the LC was analyzed. Three sections at 10 ± 1.0 mm posterior to the bregma were randomly selected for analyses. The percentage of the number of DβH-positive neurons in the lesioned LC to the intact side was analyzed and the average was used for the statistical analyses to evaluate preservation of the NA neurons. For all 3 areas, both the lesion side and intact side of the LC (each 700 × 500 μm) in each rat was analyzed and the average was used for the statistical analyses.

2.10. Statistical Analyses

All data were analyzed using SPSS ver. 20.0 software (SPSS, Chicago, IL, USA). To investigate statistical significance between multiple groups, one-way analysis of variance with subsequent Tukey's tests were used. Statistical significance was preset at $p < 0.05$. The variance homogeneity was confirmed with Levene's test. Mean values are presented with standard error (SE).

3. Results

3.1. Body Weight

All rats showed a decrease of body weight on day 7 and recovery on day 14 without significant differences (Figure 4). In the 0.5 mA and 1 mA VNS groups, one rat from each group lost more than 20% of its body weight during the experiment (sacrificed on day 7). In the 1 mA VNS group, two rats died on day 10 and day 12 with decreased body weight, respectively. In the control group, 0.1 mA, and 0.25 mA VNS groups, body weight was maintained within normal limits and no rat died during the experiment. In total, 4 rats were excluded from this study.

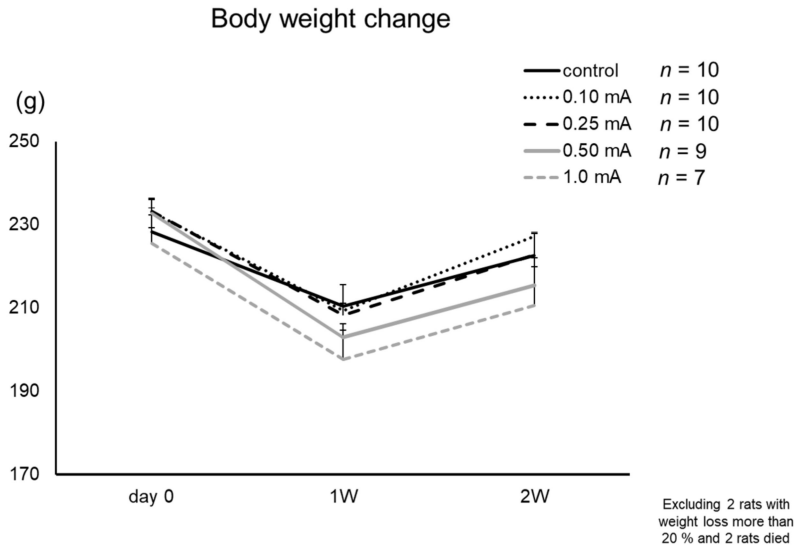


Figure 4. Body weight changes. Body weight decreased slightly with moderate intensity (0.1–0.25 mA) stimulation. In the 0.5 mA VNS group, one rat lost over 20% weight during the experiment (euthanized at day 7). In the 1 mA VNS group, one rat lost over 20% weight (euthanized at day 7) and two rats died before 2 weeks. Four rats were excluded from this study.

3.2. Behavioral Tests

3.2.1. Cylinder Test

Rats in 0.25 mA and 0.5 mA VNS groups showed significant improvement on day 14 (contralateral bias: 21.4 ± 8.4 (0.25 mA) and 17.8 ± 8.6% (0.5 mA)), compared to control group (78.4 ± 5.3%: one-way ANOVA, $F_{4,41} = 7.92$ both $p < 0.001$) and 0.1 mA VNS group (65.2 ± 10.2%, both $p = 0.013$) (Figure 5A). Slight improvement was shown in 1 mA VNS group (44.4 ± 14.9%), but not in 0.1 mA VNS group (65.2 ± 10.2%) compared to control group.

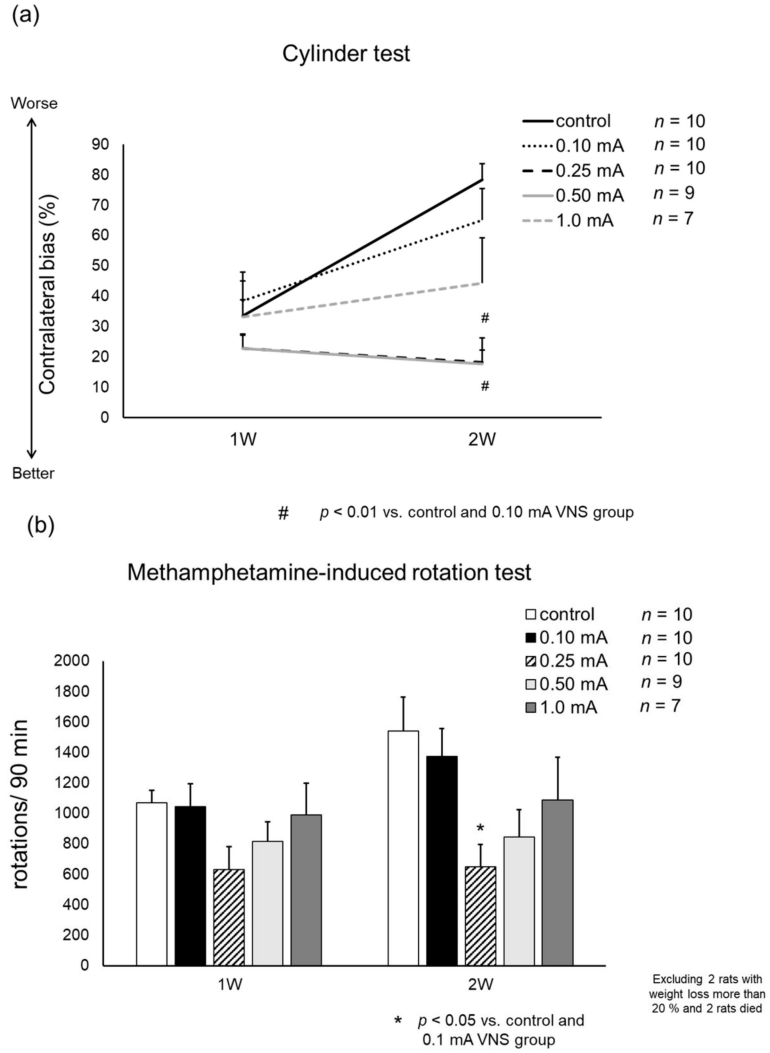


Figure 5. Results of behavioral tests. (**a**) The results of contralateral bias in the cylinder test. In the 0.25 mA and 0.5 mA VNS groups, the improvement of contralateral bias was observed at days 7 and 14 ($^{\#}p < 0.01$ at day 14 vs. control and 0.1 mA VNS groups). (**b**) The number of methamphetamine-induced rotations per 90 min. The number of methamphetamine-induced rotations decreased in 0.25 mA and 0.5 mA VNS groups compared to the control group on days 7 and 14. The 0.25 mA VNS group significantly decreased rotation number at day 14 (* $p < 0.05$ vs. control and 0.1 mA VNS group). The data are presented as means ± SE and analyzed by one-way ANOVA and Turkey's post hoc tests. n = 7–10 rats/group.

3.2.2. Methamphetamine-Induced Rotation Test

Rats in the 0.25 mA VNS group showed significant reduction in methamphetamine-induced rotations on day 14 (648 ± 149 turns/90 min) compared to control group (1541 ± 221 turns/90 min: one-way ANOVA, $F_{4,40} = 3.91$, $p = 0.011$) and 0.1 mA VNS

group (1373 ± 182 turns/90 min, $p = 0.049$) (Figure 5B). The number of rotations on day 14 in the 0.5 mA (849 ± 179 turns/90 min) and 1 mA VNS groups (1089 ± 279 turns/90 min) slightly decreased, compared to the control group.

3.3. Immunohistochemical Investigations

3.3.1. TH

Rats in 0.25 mA (29.5 ± 2.2%) and 0.5 mA (27.1 ± 2.9%) VNS groups showed significantly preserved density of TH-positive fibers in the striatum, compared to that in the control group (18.5 ± 2.2%; one-way ANO VA, $F_{4,41} = 4.73$, $p = 0.0077$ and $p = 0.0462$) (Figure 6a). Rats in the 0.1 mA (20.3 ± 1.5%) and 1 mA (21.2 ± 3.4%) VNS groups showed limited therapeutic effects on TH-positive fibers in the striatum.

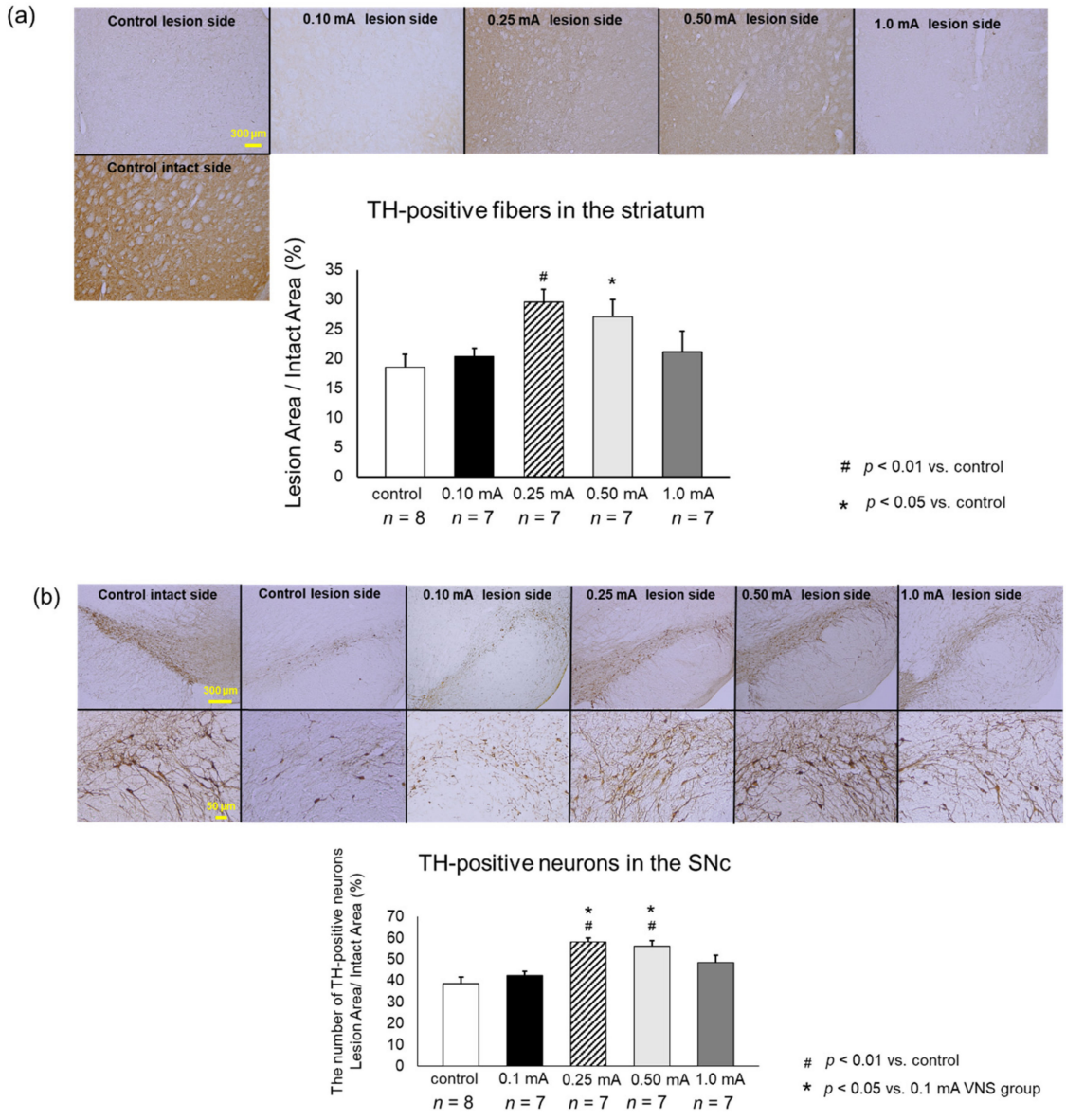


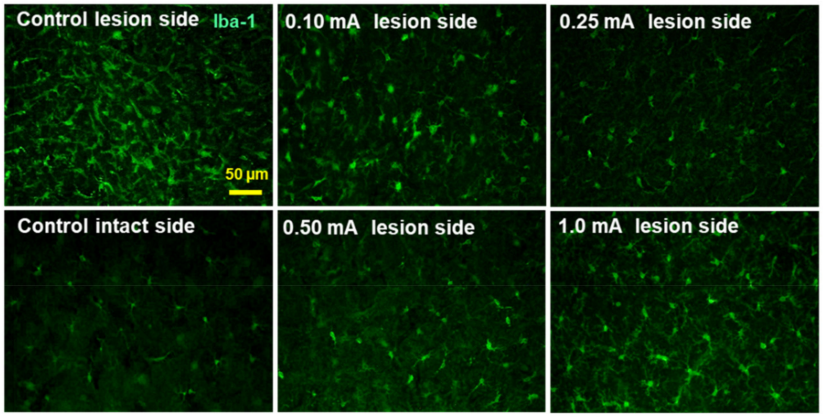
Figure 6. VNS with mild to moderate stimulation intensity preserved tyrosine hydroxylase (TH)-positive fibers in the striatum and TH-positive neurons in the SNc. (**a**) TH-positive fibers in the striatum. The ratio of TH-positive fibers in the lesioned striatum to the intact side was significantly preserved in the 0.25 mA and 0.5 mA VNS groups compared to that in the control group ($^{\#}$ $p < 0.01$ vs. control, * $p < 0.05$). (**b**) TH-positive neurons in the SNc.TH-positive neurons in the SNc in 0.25 mA and 0.5 mA VNS groups were significantly preserved compared to those in the control group and 0.1 mA VNS group ($^{\#}$ $p < 0.01$ vs. control, * $p < 0.05$ vs. control). The data are presented as means ± SE and analyzed by one-way ANOVA and Turkey's post hoc tests. n = 7–8 rats/group.

TH-positive neurons in the SNc of rats in the 0.25 mA (58.2 ± 1.8%) and 0.5 mA (56.1 ± 2.7%) VNS groups was significantly preserved, compared to those in the control group (38.9 ± 3.0%; one-way ANOVA, $F_{4,40} = 7.39$, $p < 0.001$ and $p = 0.0044$) and in the 0.1 mA VNS group (42.3 ± 2.0%, $p = 0.011$ and $p = 0.045$) (Figure 6b). Rats in the0.1 mA and 1 mA (48.5 ± 3.6%) VNS groups showed non-significant effects on TH-positive neurons in the SNc.

3.3.2. Iba1

In the 0.25 mA and 0.5 mA VNS groups, the number of Iba1-positive microglia significantly reduced both in the striatum (0.25 mA: 33.3 ± 1.8 cells/field, one-way ANOVA, $F_{4,20} = 10.67$, $p < 0.001$; 0.5 mA: 33.7 ± 1.1 cells/field, $p < 0.001$) and SNc (0.25 mA: 26.3 ± 0.9 cells/field, one-way ANOVA, $F_{4,20} = 35.18$, $p < 0.001$; 0.5 mA: 31.9 ± 1.8, $p < 0.001$), compared to the control group (striatum: 68 ± 7.4 cells/field; SNc: 50 ± 1.7) (Figure 7a,b). The suppressive effects against migrating microglia by 1 mA VNS group were significant in the SNc (43 ± 2.1 cells/field, $p = 0.043$) but not significant in the striatum (50 ± 5.8 cells/field, $p = 0.08$), compared to the control group. Moreover, the suppressive effects against migrating microglia by the 1 mA VNS group were significantly inferior to those in the 0.25 mA and 0.5 mA VNS groups (0.25 mA: $p < 0.001$, 0.5 mA: $p = 0.004$). Compared with the 0.1 mA VNS group in the SNc (48 ± 2.0 cells/field), the 0.25 mA and 0.5 mA VNS groups significantly suppressed migrating microglia (both: $p < 0.001$).

(a)



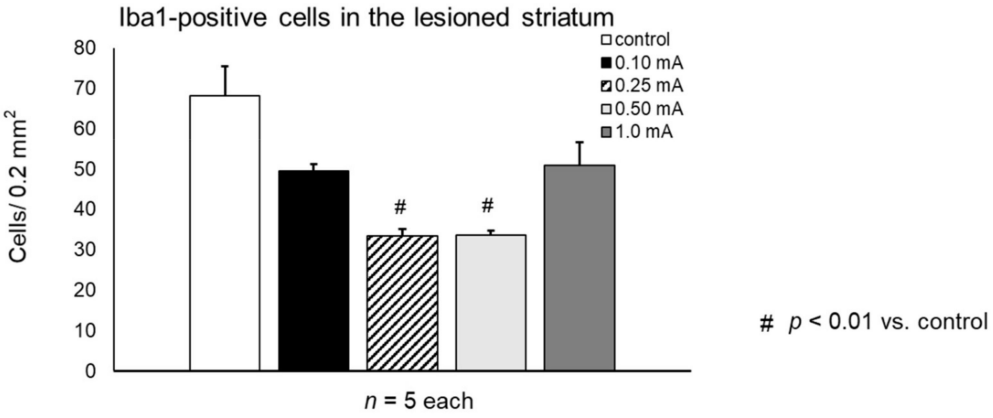


Figure 7. *Cont.*

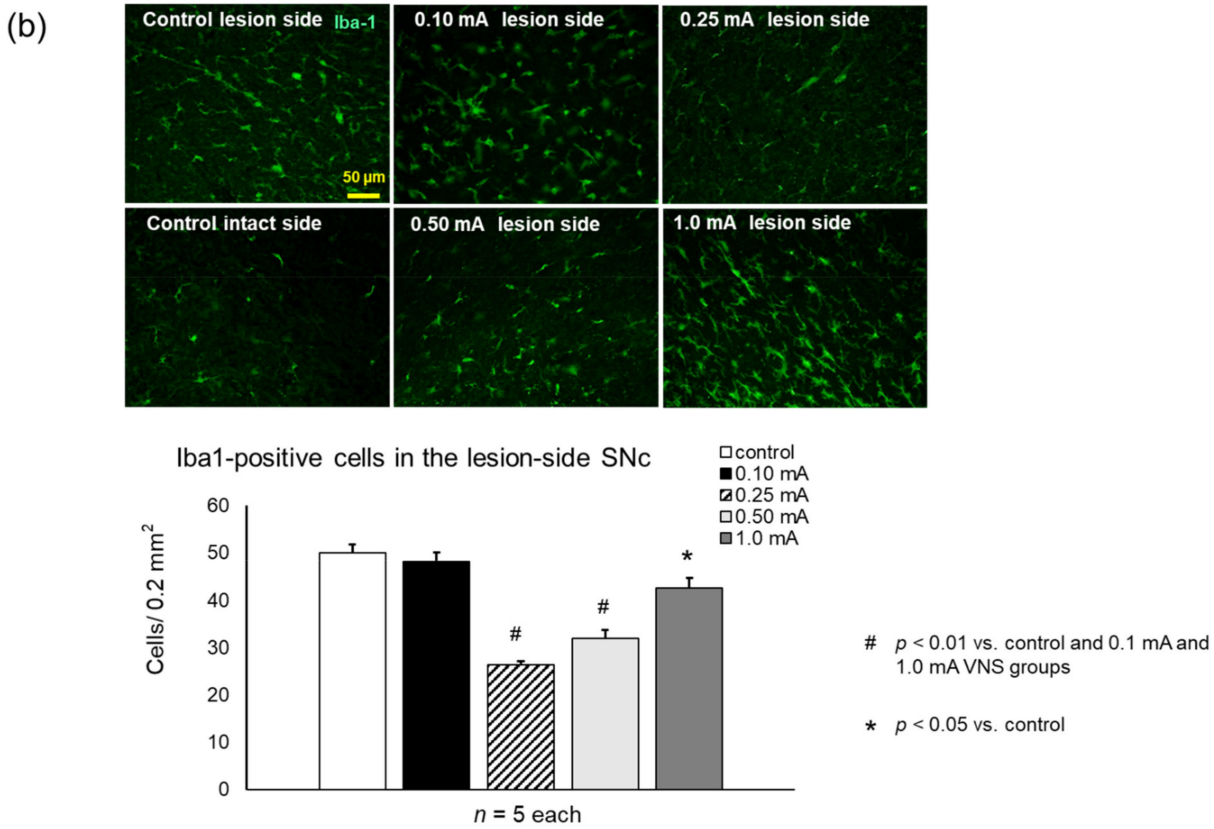


Figure 7. VNS with mild to moderate stimulation intensity inhibited proliferation of microglia both in the striatum and SNc. (**a**) Ionized calcium binding adaptor molecule 1 (Iba1)-positive microglia in the striatum of the lesion side. The number of Iba1-positive microglia in the lesioned SNc significantly decreased in the 0.25 mA and 0.5 mA VNS groups compared to the control group ($^{\#}$ $p < 0.01$). (**b**) Iba1-positive microglia in the SNc of the lesion side. The number of Iba1-positive microglia in the lesioned SNc significantly decreased in the 0.25 mA and 0.5 mA VNS groups compared to the control and 0.1 mA VNS groups ($^{\#}$ $p < 0.01$). Iba1-positive neurons in the SNc in the 1 mA VNS group also significantly decreased compared to the control group (* $p < 0.05$), although the effect was less than that of the 0.25 mA and 0.5 mA VNS groups. The data are presented as means ± SE and analyzed by one-way ANOVA and Turkey's post hoc tests. $n = 5$ rats/group.

3.3.3. GFAP

Rats in the 0.25 mA, 0.5 mA, and 1 mA VNS groups showed significant suppression of GFAP-positive cells both in the striatum (0.25 mA:17 ± 0.9 cells/field, one-way ANOVA, $F_{4,20} = 48.62$ $p < 0.001$; 0.5 mA:18 ± 0.6, $p < 0.001$, 1 mA:26 ± 1.1, $p < 0.001$) (Figure 8a) and SNc (0.25 mA:17 ± 1.2 cells/field, one-way ANOVA, $F_{4,20} = 25.59$, $p < 0.001$; 0.5 mA: 18 ± 0.9, $p < 0.001$, 1 mA:26 ± 1.2, $p = 0.018$), compared to the control (striatum: 33 ± 1.5 cells/field, SNc: 34 ± 2.4) (Figure 8b). Rats in the 0.25 mA and 0.5 mA VNS groups showed significant suppression of GFAP-positive cells both in the striatum and SNc, compared to the 1 mA VNS group (striatum: 0.25 mA and 0.5 mA, $p < 0.001$; SNc: 0.25 mA and 0.5 mA, $p = 0.006$ and $p = 0.03$). Compared to the 0.1 mA VNS group, the 0.25 mA and 0.5 mA VNS groups displayed significant suppression of GFAP-positive cells both in the striatum and SNc (all: $p < 0.001$).

3.3.4. DβH

The density of NA neurons in LC was significantly increased in the 0.25 mA (29.5 ± 2.2%, one-way ANOVA, $F_{4,20} = 19.6$, $p < 0.001$), 0.5 mA (27.1 ± 2.9%, $p < 0.001$), and 1 mA (21.2 ± 3.4%, $p = 0.038$) VNS groups compared to the control group (18.5 ± 2.2%) and 0.1 mA VNS group (20.3 ± 1.5%) (Figure 9). The increased density of NA neurons in the 0.25 mA VNS group were significantly higher than those of the 1 mA VNS group ($p = 0.038$).

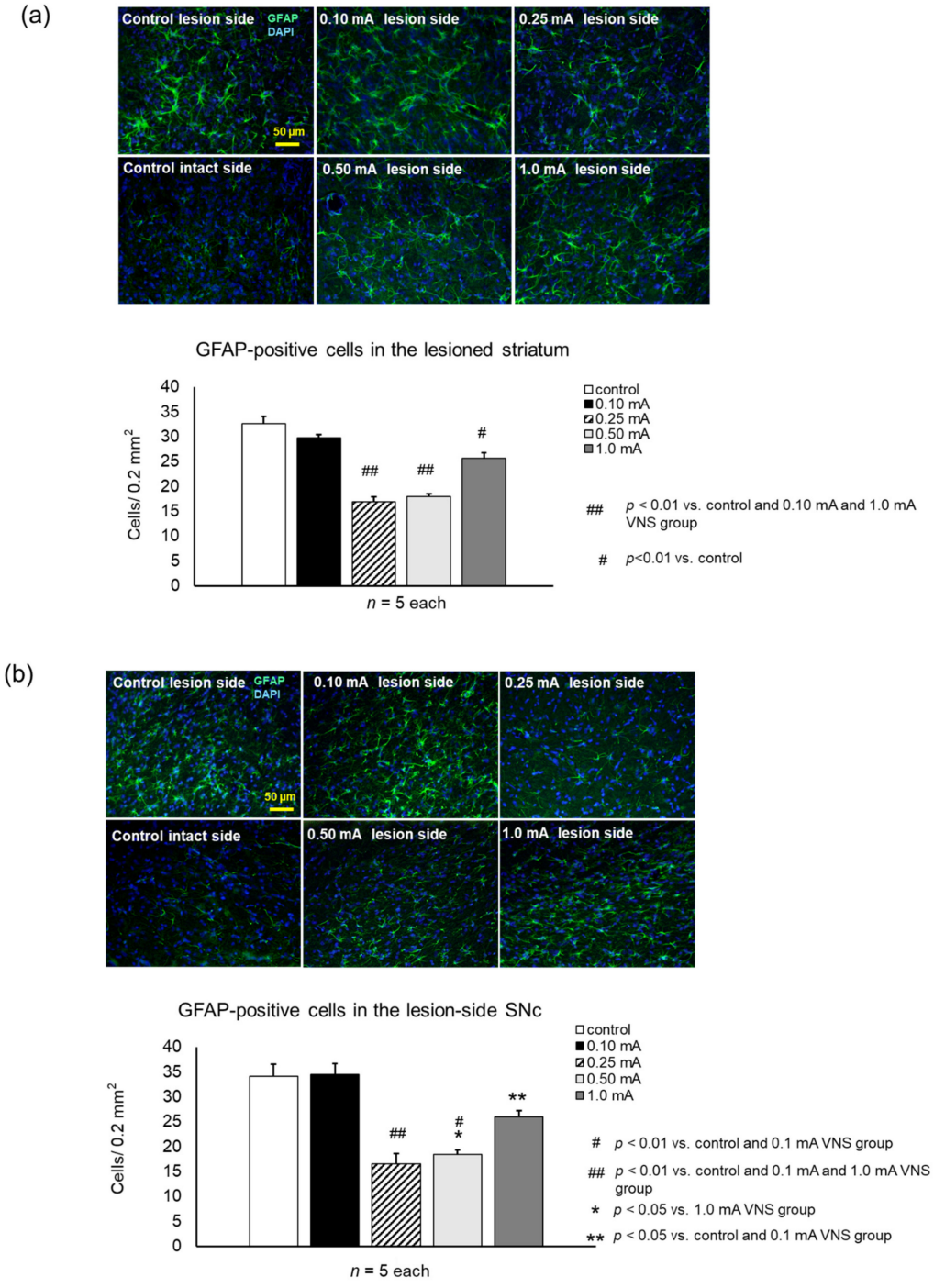


Figure 8. VNS with mild to moderate stimulation intensity inhibited astrocytic proliferation both in the striatum and SNc. (**a**) Glial fibrillary acidic protein (GFAP)-positive astrocytes in the striatum of the lesion side. The number of GFAP-positive astrocytes in the lesioned striatum significantly decreased in the 0.25 mA and 0.5 mA VNS groups to all other groups ($^{\#\#}$ $p < 0.01$). GFAP-positive astrocytes also significantly decreased in the 1 mA VNS group, compared to the control group ($^{\#}$ $p < 0.01$), although the effect was less than that of the 0.25 mA and 0.5 mA VNS groups. (**b**) GFAP-positive astrocytes in the SNc of the lesion side. The number of GFAP-positive astrocytes in the lesioned SNc significantly decreased in the 0.25 mA, 0.5 mA, and 1 mA VNS groups. Especially, 0.25 mA and 0.5 mA VNS groups displayed remarkable effects. Furthermore, the 0.25 mA and 0.5 mA VNS groups reduced astrocytes compared to all the other groups ($^{\#\#}$ $p < 0.01$ in 0.25 mA VNS group, $^{\#}$ $p < 0.01$ and * $p < 0.05$ in the 0.5 mA VNS group). The 1 mA VNS group also reduced astrocytes compared to the control and 0.1 mA VNS groups (** $p < 0.05$). The data are presented as means ± SE and analyzed by one-way ANOVA and Turkey's post hoc tests. $n = 5$ rats/group.

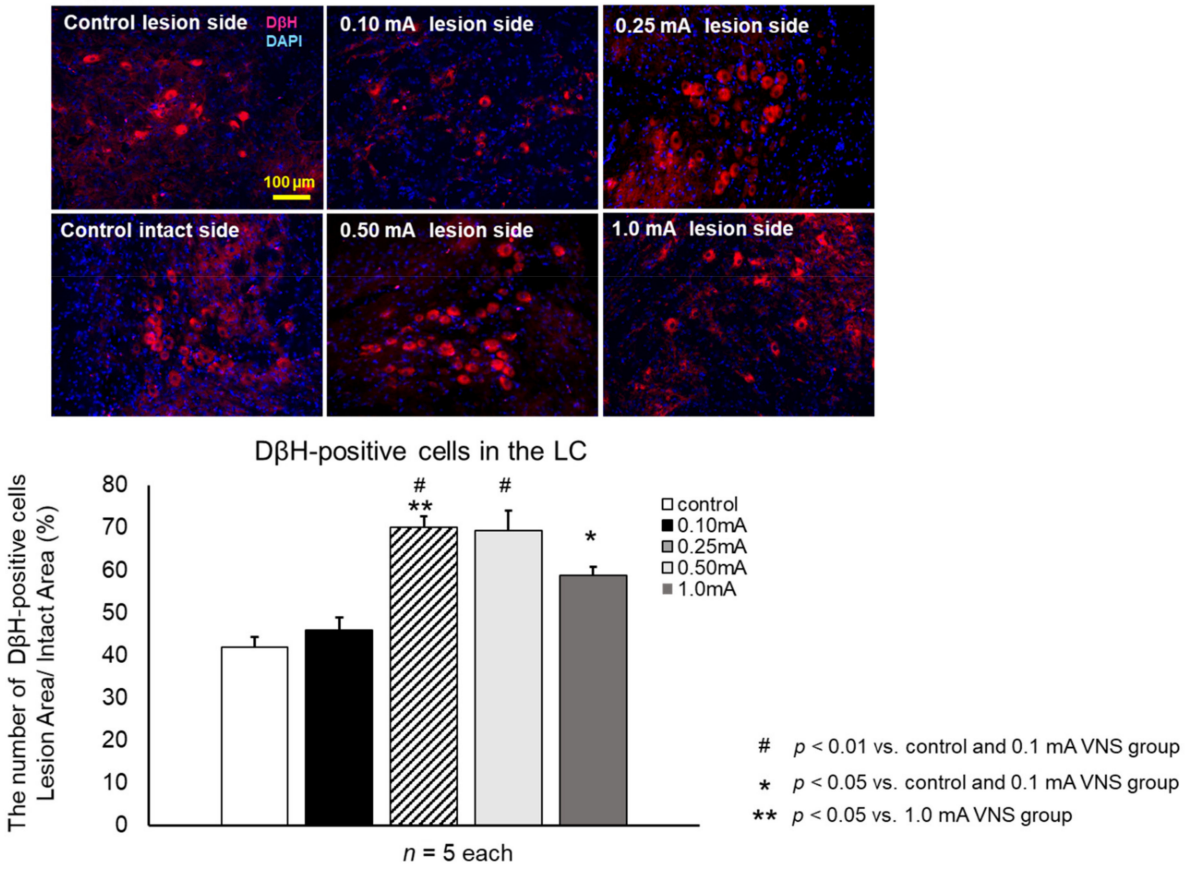


Figure 9. VNS with mild to moderate stimulation intensity preserved noradrenergic neuron in the LC. The ratio of dopamine β hydroxylase (DβH)-positive noradrenergic neurons in the lesioned LC to the intact side was significantly increased in the 0.25 mA, 0.5 mA, and 1 mA VNS groups compared to the control and 0.1 mA VNS groups ($^{\#\#}$ $p < 0.01$ in 0.25 mA VNS group, $^{\#}$ $p < 0.01$ in 0.5 mA VNS group, ** $p < 0.05$ in 1 mA VNS group). In addition, the preservation effect of the 0.25 mA VNS group was significantly superior to that of the 1 mA VNS group (* $p < 0.05$). The data are presented as means ± SE and analyzed by one-way ANOVA and Turkey's post hoc tests. $n = 5$ rats/group.

4. Discussion

In this study, we demonstrated the therapeutic effects of VNS (0.25 mA and 0.5 mA) on PD rats using a wireless controllable electrical stimulation device with several intensities to simulate clinical settings. The behavioral improvement and DA neuronal preservation in these animals may be due to anti-inflammatory effects and potentiation of increased DA/NA neuronal preservation induced by VNS.

4.1. VNS in Clinical Settings

VNS is an established treatment for refractory epilepsy, depression, and cluster headache [15,26,27]. Several basic and clinical studies demonstrated the therapeutic efficacy of VNS in ischemic stroke [28–31], cerebral hemorrhage [32], traumatic brain injury [16,33–35], migraine [36,37], and Alzheimer's disease [38]. Beyond the central nervous system, VNS was also reported to provide therapeutic effects in systematic or local inflammatory disorder such as septic shock [39–41], acute myocardial infarction [42,43], acute lung injury [44], ileus [45], obesity [46,47], and rheumatoid arthritis [48]. For PD, DBS is usually performed safely, but intracranial intervention still has potential risks of intracranial hemorrhage and infection [49,50] with subsequent critical complications. VNS is relatively safe without intracranial manipulation and surgically performed with ease. Thus, the therapeutic potential of VNS for PD may become more apparent because of its enhanced safety and feasibility.

The vagus nerve plays multiple roles in homeostatic regulation of visceral functions. The vagus nerve is composed of 80% afferent sensory fibers that project upward from the viscera into the medulla and 20% efferent motor fiber that regulates visceral organs [14,15]. The vagus nerve is composed of three types of fibers: A fiber (large and myelinated), B fiber (mid-size and myelinated), and C fiber (small and unmyelinated) [51,52]. The right vagus nerve contains fibers to the sinus node that is a risk for bradycardia, so the left VNS generally tends to be favored as surgical targets. Although the exact mechanisms of anti-inflammatory effects of VNS are not completely understood, both afferent and efferent pathways via NA and acetylcholine may be involved [14,53]. Vagal afferents project to the nucleus of the solitary tract (NTS) which widely feeds to brainstem, forebrain structures, and both directly and indirectly linked to the LC leading to synergistic subsequent regulation of NA secretion [14,15]. VNS protects NA fibers in the LC via its afferent fibers with subsequent protection of DA neurons in the SN [54]. VNS modifies the electrical activity of LC while depletion of NA in the LC eliminated the effects of VNS [55]. In this study, we demonstrated that the therapeutic potential of VNS was accompanied by NA and DA neuronal preservation. In concert with a previous study, VNS increased locomotor activity in 6-OHDA-induced PD model rats, maintained striatal TH-positive fibers and nigral TH-positive cells, suppressed glial cell expression, and retained NA neurons in LC [54]. Our results paralleled these reported findings, but the stimulation system and conditions were different. We directly applied clinical VNS parameters used for epilepsy treatment and varied stimulation intensity to the PD model in this study. This is because, in addition to mimicking common parameters for actual clinical application, it has been reported to elevate NA in brainstem including LC [16,56]. Our results demonstrated that 0.25 mA and 0.5 mA conventional VNS for 14 consecutive days was effective. The antiepileptic effects of VNS are thought to be mediated by the elevation of NA in LC [57]. The direct application of this stimulation parameter possibly increased NA neurons in LC and DA neurons in SN, which may have direct therapeutic application in PD patients. In addition, long-term intermittent VNS will be more effective for PD patients, considering the neurodegenerative nature of the disease.

4.2. Anti-Inflammatory Effects of VNS

In addition, mild to moderate stimulation intensity of 0.25 mA and 0.5 mA remarkably suppressed the activation of microglia and astrocytes induced by 6-OHDA, but the very weak and strong stimulation intensity of 0.1 mA and 1 mA were less effective. Microglia and astrocyte have a major role to play in immune defense in the central nervous system. Activated glial cells release anti-inflammatory substances with neuroprotective effects. However, prolonged overactivation of glial cells produces inflammation mediator leading to DA neuronal degeneration in the SN [1,4,6,7]. Reduction in NA following LC degeneration leads to immune-overactivity of glial cells with consequent neurodegeneration [58–60]. In our study, the 0.25 mA and 0.5 mA VNS groups showed strong anti-inflammatory effects with suppression of the morphological change of glial cells. The anti-inflammatory effects of 0.25 mA and 0.5 mA VNS with subsequent therapeutic effects on DA/NA neuronal preservation were stronger than those of 0.1 mA and 1 mA VNS. Indeed, the VNS experiment examining the plasticity of motor cortex showed that moderate stimulation intensity was effective, while very weak and strong stimulation were less effective [61,62]. This phenomenon is called the inverted-u effect. It is hypothesized that the activation of A and B fibers, which have low current thresholds, is important for the anti-inflammatory effect of VNS [51,55,63]. C fibers are small fibers that compose most of the vagus nerve, but the activation of A and B fibers, which are lesser components of the vagus nerve, is especially important for the therapeutic effect of VNS [51,55,63]. We speculated that the inverted-u effect is due to the increased activity of A and B fibers caused by the mild-moderate intensity. As a possibility, a mild-moderate stimulation intensity of VNS (0.25 mA and 0.5 mA) demonstrated remarkable therapeutic efficacy via the inverted-u effect for LC, SN, and striatum as well. It was recently reported that VNS with high-intensity stimulation inhibited the

onset of the treatment effect for a longer time and inhibited neuroplasticity [64]. Although the exact pathway may differ, our experiment also confirmed that high-intensity stimulation is unlikely to respond to treatment for PD. In addition, the non-intensity dependent therapeutic response may be due to the damage induced by higher intensity, imbalanced stimulation between afferent/efferent nerves, among other unknown mechanisms that require further investigation.

Although not statistically, the body weight decreased as the stimulation intensity increased. VNS reduced fat and contributed to body weight loss [46,47]. However, in this experiment, some rats died from the strong stimulation intensity of 1 mA despite the body weight loss of 20% or less. In this regard, the strong stimulation of VNS appears not to be suitable for PD treatment. The overall results might indicate that 0.25 mA and 0.5 mA VNS have increased NA neurons in LC, nurturing NA to protect DA neurons in SN by suppressing glial cell activation with consequent attenuation of behavioral PD symptoms and of DA neuronal degeneration.

4.3. PD Pathogenesis and Inflammation

The pathological process of PD may originate from inflammation of peripheral nerve system in visceral organs, and progresses through the vagus nerve to the brainstem and SNc like a prion mechanism [65]. This inflammation-based hypothesis has become one of the mainstreams of the cause and progression of PD. The association between abnormalities in the intestinal flora and PD onset implicates key intestinal infections as the cause of PD. In tandem, abnormal intestinal flora may lead to amplified inflammatory flora and decreased anti-inflammatory flora, causing chronic constipation and ultimately a risk of developing PD [66]. Abnormal α-synuclein produced by glial cells in the intestinal tract propagate through the vagus nerve, reach the NTS to LC, and propagate to the SN leading to the development of PD [67]. Indeed, a recent large cohort study found that PD was suppressed in patients who had previously undergone abdominal vagotomy for gastric ulcer [52] or appendectomy [68]. The abnormal signals from the intestine via the vagus nerve may be suppressed. Although the exact mechanisms are still unclear, we recognized that the inflammatory pathogenesis of PD acts as a key target to treat PD. To this end, VNS may sequester this inflammation-plagued neurodegeneration in PD via its anti-inflammatory effects. If PD onset involves the spread of inflammation and neurodegeneration from the periphery to LC and SN through the vagus nerve pathway, cervical VNS may directly alter this pathway in providing relief for PD. In our study, VNS was started immediately after 6-OHDA administration. An early VNS initiation may suppress the glial cell activation and maintain the potency of LC-NA and SN-DA signaling, thereby preventing the progression of PD symptoms in its initial stages.

4.4. Study Limitations

We have several limitations in this experiment. We used a two-week-stimulation with a single condition without stepwise adjustment which is usually employed in the clinic. During the long-term treatment, the gradual increase of the stimulation intensity may enhance more neuroprotective effects. In addition, considering the pathophysiology of PD, it would be more ideal to perform VNS at 2–4 weeks after 6-OHDA administration. Further studies of late intervention against the PD model are needed to simulate clinical settings. The therapeutic mechanisms of VNS may require the participation of either afferent, efferent, in combination, stimulation, which will be explored in subsequent experiments including research for cholinergic anti-inflammatory pathways via efferent nerve and dynamics of inflammatory cytokines in the tissue. In addition, recent reports have suggested that the right vagus nerve has a greater number of TH positive nerves [69], and the right VNS is important in influencing DA regulation in the SN [70]. It is necessary to try the right VNS in PD research as well, instead of being limited to the traditional left VNS. In addition, although we performed PD treatment by varying the stimulation intensity in four steps, we need to conduct further searches by changing the stimulation intensity more

closely, and further experiments by changing the intervals and frequency of stimulation. For example, as 1% of the stimulation intensity used for treating epilepsy in humans has neural plasticity on cortical lesion in rats [18,61,62]. Moreover, low frequency stimulation should be tried, as it predominantly activates the efferent fibers [14]. Additionally, most recently, the efficacy of burst VNS with high-frequency stimulation was reported to be effective for PD treatment in rats [71]. Further research is needed to determine what stimulation patterns are most effective in which areas of the brain. Since SAS-200 can create more than 1500 different stimulation parameters, additional experiments using these parameters may support us to discover appropriate stimulation parameters for PD treatment.

5. Conclusions

VNS with mild to moderate intensity exerted anti-inflammatory and neuroprotective effects on the PD rat model induced by 6-OHDA administration. These effects accompanied the preservation of DA neurons in nigrostriatal systems, increased NA neurons in LC, and robust behavioral improvements. The clinical entry of VNS emphasizes the crucial role of regulating the inflammation in the pathogenesis of PD. However, further studies of late intervention against the PD model are needed to simulate clinical settings. In addition, the usability and effectiveness of the new experimental device is hopeful for subsequent researches with electrical stimulation.

Author Contributions: I.K., conceptualization, data curation, investigation, methodology, draft writing; T.S., conceptualization, format analysis, project administration, review; T.Y., data curation, resources, review/editing; M.K., validation, review; T.A., conceptualization; M.O., investigation, methodology; K.H., methodology, investigation; Y.O., methodology, investigation; S.Y., methodology, visualization; S.K., investigation, methodology; K.K. (Ken Kuwahara), investigation; J.M., validation; K.K. (Kyohei Kin), data curation, review; M.U., methodology; Y.T., methodology; N.T., review/editing; C.V.B., review/editing; I.D., supervision. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with Okayama University guidelines for animal experiments and were approved by the University's committee on animal experimentation (examination protocol, #OKU-2017449 approved on 23 October 2017).

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Transcutaneous Auricular Vagus Nerve Stimulation Improves Spatial Working Memory in Healthy Young Adults

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Working memory (WM) is one of the core components of higher cognitive functions. There exists debate regarding the extent to which current techniques can enhance human WM capacity. Here, we examined the WM modulation effects of a previously less studied technique, transcutaneous auricular vagus nerve stimulation (taVNS). In experiment 1, a within-subject study, we aimed to investigate whether and which stimulation protocols of taVNS can modulate spatial WM performance in healthy adults. Forty-eight participants performed baseline spatial n-back tasks (1, 3-back) and then received online taVNS, offline taVNS, or sham stimulation before or during (online group) the posttest of spatial n-back tasks in random order. Results showed that offline taVNS could significantly increase hits in spatial 3-back task, whereas no effect was found in online taVNS or sham group. No significant taVNS effects were found on correct rejections or reaction time of accurate trials (aRT) in both online and offline protocols. To replicate the results found in experiment 1 and further investigate the generalization effect of offline taVNS, we carried out experiment 2. Sixty participants were recruited and received offline taVNS or offline earlobe stimulation in random order between baseline and posttests of behavioral tests (spatial/digit 3-back tasks). Results replicated the findings; offline taVNS could improve hits but not correct rejections or aRT in spatial WM performance, which were found in experiment 1. However, there were no significant stimulation effects on digit 3-back task. Overall, the findings suggest that offline taVNS has potential on modulating WM performance.

Keywords: taVNS, working memory, n-back task, cognitive enhancement, non-invasive neuromodulation

INTRODUCTION

Working memory (WM), a core component of higher cognitive functions, is a system that combines attentional control with temporary storage and information manipulation (Chiesa et al., 2011). The field of cognitive psychology has underlined the importance of the ability to maintain and manipulate information over a period of seconds in WM and the vital role of WM for complex mental abilities, including problem solving, reasoning, and learning (Baddeley and Hitch, 1974). Specifically, one of the central limitations of human cognition is the restricted amount

of information that can be kept in WM (Cowan, 2001), and the differences in WM capacity among individuals are associated with variation in several important abilities such as academic performance (Gathercole et al., 2003), non-verbal reasoning ability (Kyllonen and Christal, 1990), and control of attention (Kane et al., 2007). Many clinical populations, including individuals with schizophrenia, attention-deficit/hyperactivity disorder (ADHD), stroke, and traumatic brain injury, also exhibit a lower WM capacity. Moreover, deficits in WM play a crucial role in normal neurocognitive aging and the rapid cognitive deterioration associated with dementias, such as Alzheimer's disease (Park and Reuter-Lorenz, 2009; Grady, 2012). Fortunately, researches at the beginning of the 2000s showed that the WM was an ability that could be increased by training or psychosocial inventions rather than an immutable individual characteristic (for review, see Constantinidis and Klingberg, 2016). Therefore, the available ways to improve the capacity of WM are urgently needed.

At present, non-invasive brain stimulation techniques, such as transcranial direct current stimulation (tDCS; Andrews et al., 2011; Arkan, 2019; Živanović et al., 2021), transcranial alternating current stimulation (tACS; Ermolova et al., 2019; Benussi et al., 2021), and transcranial magnetic stimulation (TMS; Chen et al., 2015; Hulst et al., 2017), have become one of the mainstream clinical treatment approaches to moderate the WM because of their potential, convenience, and safety. Although some of previous studies have demonstrated the availability of transcranial current stimulation on modulating WM by altering the activity of neurons through changing the resting membrane potential of neurons (Bindman et al., 1962; Nitsche and Paulus, 2000), some recent studies found limited positive effects of tDCS on WM accuracy with a minor reaction time enhancement in healthy cohorts (e.g., Koshy et al., 2020; Shires et al., 2020; for meta-analysis, see Hill et al., 2016; Medina and Cason, 2017). Indeed, the effect of tDCS on WM heavily relies on the stimulation form (online/offline), stimulation duration, current density, and stimulation area (right or left dorsolateral/ventral lateral prefrontal cortex, posterior parietal cortex, or premotor cortex, etc.), which have been further studied (e.g., Nikolin et al., 2018; Živanović et al., 2021). The same problems also appeared in tACS and TMS studies (Chung et al., 2018; Pavlov et al., 2020). At the same time, transcutaneous auricular vagus nerve stimulation (taVNS), as an emerging cranial nerve stimulation method, represents a promising alternative (van Leusden et al., 2015).

The cranial nerves are a specialized part of the peripheral nervous system that emerges directly from the brain rather than through the spine. For each cranial nerve, there is a relatively accessible portion, and each of them is intimately linked to perception and regulation of central nervous system, with "bottom-up" functions in cognition and clinical disorders, which makes them a special target for neuromodulation. The vagus nerve, which is made up of approximately 80% sensory afferent fibers, is the longest cranial nerve (Agostoni et al., 1957). It projects to the nucleus tractus solitarii (NTS) in the medulla, before it is relayed further to other brainstem nuclei and higher-order structures, including the thalamus, hippocampus, amygdala, and insula (Goehler et al., 2000; Saper, 2002). Since the end of the last century, multiple studies in clinical populations have found the special enhancement of vagus nerve stimulation (VNS) on cognition and memory (e.g., Clark et al., 1999; Schachter, 2004; Ghacibeh et al., 2006; Merrill et al., 2006). Recently, some brain imaging studies found that, taVNS, a non-invasive neurostimulation technique that targets the auricular branch of the vagus nerve, produced increased blood oxygen level–dependent signal in the contralateral postcentral gyrus, bilateral insula, frontal cortex, right operculum, and left cerebellum (Badran et al., 2017). Yakunina et al. (2017) suggested that taVNS could modulate the activities in the locus coeruleus (LC) and the areas innervated by this region, including the insula, hippocampus, amygdala, and thalamus. As frontal cortex, hippocampus, and the neurotransmitters, such as norepinephrine (NE), which is released by LC, are known to be important for many cognitive functions, including WM (Gu, 2002; Duffau, 2006; Funahashi, 2017), taVNS gains ever-increasing scientific interest in cognition modulation. In healthy volunteers, several clinical studies have demonstrated that taVNS could modulate a series of cognitive processes, such as emotion recognition (Colzato et al., 2017), divergent thinking (Colzato et al., 2018), inhibitory control processes (Beste et al., 2016), response selection functions (Steenbergen et al., 2015), conflict adaptation (Fischer et al., 2018), attentional processes (Ventura-Bort et al., 2018), and post-error slowing (Sellaro et al., 2015), and so on. In addition, after the first study to explore the effect of taVNS on memory performance (Jacobs et al., 2015), studies have investigated the enhancement of taVNS on verbal memory (Mertens et al., 2020), high-confidence recognition memory (Giraudier et al., 2020), memory reinforcement (Hansen, 2019), long-term emotional episodic memory (Ventura-Bort et al., 2021), and associative memory (Jacobs et al., 2015).

Nevertheless, most of these studies did not refer to WM performance; thus, the effect of taVNS on WM is still unknown. Although direct evidence has been scant, the findings from invasive VNS have shown that the VNS over the left cervical vagus nerve improved immediate WM of epilepsy patients (Sun et al., 2017). Therefore, it is valuable to explore the immediate regulatory effect of taVNS on WM. In the current study, we aimed to investigate the effects of taVNS on WM in healthy volunteers by using n-back tasks. There were two specific questions: first, is there any difference between online and offline taVNS protocols in modulation effect on WM? Up to now, both online (e.g., Jacobs et al., 2015; Colzato et al., 2018; Giraudier et al., 2020) and offline (e.g., Alicart et al., 2020; Warren et al., 2020) taVNS could be seen in researches, and to the best of our knowledge, none of the studies have compared their efficiency. Meta-analyses of tDCS studies have suggested that for healthy population the significant effect could be found only in offline stimulation (see Hill et al., 2016), which might be caused by different neurobiological processes; namely, the online effects might result from resting membrane potential alterations, whereas the offline effects appear to result from modulation of synaptic plasticity (Stagg and Nitsche, 2011; Medeiros et al., 2012; Hill et al., 2016). In addition, the neurotransmitter release needs time to take effect, which might lead to a stronger effect of offline protocol

than online stimulation. However, several studies, such as that of Neuser et al. (2020), reported a significant online taVNS effect on motivation. Thus, we compared the effects of online and offline taVNS in the first experiment. Second, are there some generalization effects of taVNS on modulating WM performance, namely, does taVNS have effects on more than one modality of WM tasks? As taVNS has extensive activation on cerebral cortex (Yakunina et al., 2017) and the neurotransmitters released by taVNS might affect a series of cognition, it might have a comprehensive effect on WM performances. However, according to previous studies, there were different neural bases for verbal (like digit) and non-verbal (like spatial) WM tasks (Owen et al., 2005). Thus, the specific effects of taVNS on different modalities and WM tasks are valuable to investigate. To testify this question, we used spatial WM tasks in the first experiment because it has been heavily investigated, and numerous studies have found that it could be improved by the increased activity of prefrontal neurons and dopaminergic transmission (for review, see Constantinidis and Klingberg, 2016) and then tested the corresponding taVNS effects on digit WM tasks in the second experiment. To sum up, in this study, we aimed at (1) investigating the taVNS effects on spatial WM performance and choosing the optimal stimulation protocol between online and offline stimulation and (2) replicating the taVNS effects on spatial WM performance and further investigating its influence on digit WM tasks.

MATERIALS AND METHODS

Experiment 1

In this experiment, we aimed to investigate the enhancement of taVNS on spatial WM by using online taVNS, offline taVNS, and sham groups with two n-back tasks, namely 1-back and 3-back tasks. The behavioral changes between baseline and posttest per condition (online taVNS, offline taVNS and sham) were calculated to evaluate the effects of taVNS on WM.

Participant

Forty-eight healthy students at Xidian University were included in this experiment. Each of them had to participate in three sessions, including online taVNS, offline taVNS, and sham. All participants were right-handed, with no smoking, neurological disease, or brain damage history. No participants reported ear injuries, drinking, or taking drugs 48 h before the experiment. Before the experiment, participants were provided with information about the stimulation procedure and experimental protocols and written informed consent. Participants were instructed that they could withdraw from the experiment at any time if they did not wish to continue, and all of them could receive corresponding remuneration. All research procedures were conducted in accordance with the Declaration of Helsinki and approved by the institutional research ethics committee of Xijing Hospital of the Air Force Medical University (KY20192008-X-1). Finally, 46 participants completed the experiment successfully (25 female, average age = 20.39 $\pm$ 1.96 years, range = 18–25 years), whereas two subjects were excluded from the data analysis because of withdrawing.

Design

The experiment was a within-subjects design, with each participant completing three separate sessions, which were different in stimulation conditions [i.e., online taVNS, offline taVNS, and sham (stimulation equipment placebo); **Figure 1A**]. In the online taVNS condition, participants first tested the baseline of behavioral tasks and then had a 25-min rest. Then, they received a taVNS stimulation at the beginning of the posttest of behavioral task until the end, which lasted approximately 15 min. In the offline taVNS section, participants completed 15-min baseline test of the behavioral tasks, a 25-min taVNS stimulation, and a 15-min posttest of behavioral tasks in turn. The process of sham condition was similar with offline taVNS, except that the 25-min stimulation was instead by a 30-s stimulation at the beginning and end time. The three sessions were separated by a period of at least 2 days (M_{days} = 3.46 $\pm$ 1.50), and the stimulation orders were counterbalanced between participants. One or 2 days before the formal experiment sessions, participants needed to come to the laboratory to familiarize themselves with the experimental procedure, practice the behavioral tasks (completed whole tasks until reached an accuracy rate of 60%), and test the acceptability of taVNS.

taVNS Stimulation Equipment and Parameters

The electrical stimulation equipment used in this study was made by our joint laboratory (XD-Kerfun BS-VNS-001), an upgrade version of the one that has been successfully used in previous researches (e.g., Shen et al., 2021; Sun et al., 2021). The taVNS channel was connected to two silver chloride electrodes (outer diameter 7 mm). The anode and cathode of taVNS were both placed in the left cymba conchae with the cathode inside and 0.5 cm apart from the anode. The electrical stimulation waveform was a single-phase rectangular pulse with a pulse width of 500 μs and frequency of 25 Hz. The current was delivered with a cycle of 30 s on and 30 s off to avoid habituation.

As perceived and tolerated stimulation intensity varies across participants, the current intensity was determined by each participant by using the threshold method to match the subjective experience of the stimulation. Before formal test of each session, there was a threshold test. In the threshold test section, participants were asked to give direct feedback on their feeling of each stimulation intensity on a 10-point scale ranging from (1) no perception to (3) light tingling to (6) strong tingling to (10) intense pain. The stimulation started with an intensity of 0.1 mA and increased stepwise in 0.1-mA increments until the subject reported a slight feeling of pain (corresponding to $\geq$7 on the subjective sensation scale) and then decreased in 0.1-mA increments until 0.1 mA below the light tingling threshold (corresponding to $\leq$3 on the subjective sensation scale). The protocol was repeated twice, and the average of the intensities rated as 5 (mild tingling) was used as the stimulation threshold (Neuser et al., 2020). The individual stimulation intensities varied from 0.1 to 1.3 mA for the online taVNS group (M_{online} taVNS = 0.7 $\pm$ 0.36 mA) and from 0.2 to 1.6 mA for the offline taVNS group (M_{offline} taVNS = 0.69 $\pm$ 0.38 mA). For the sham group, all the participants tested only the threshold that varied from 0.3 to 1.5 mA and received an intensity at

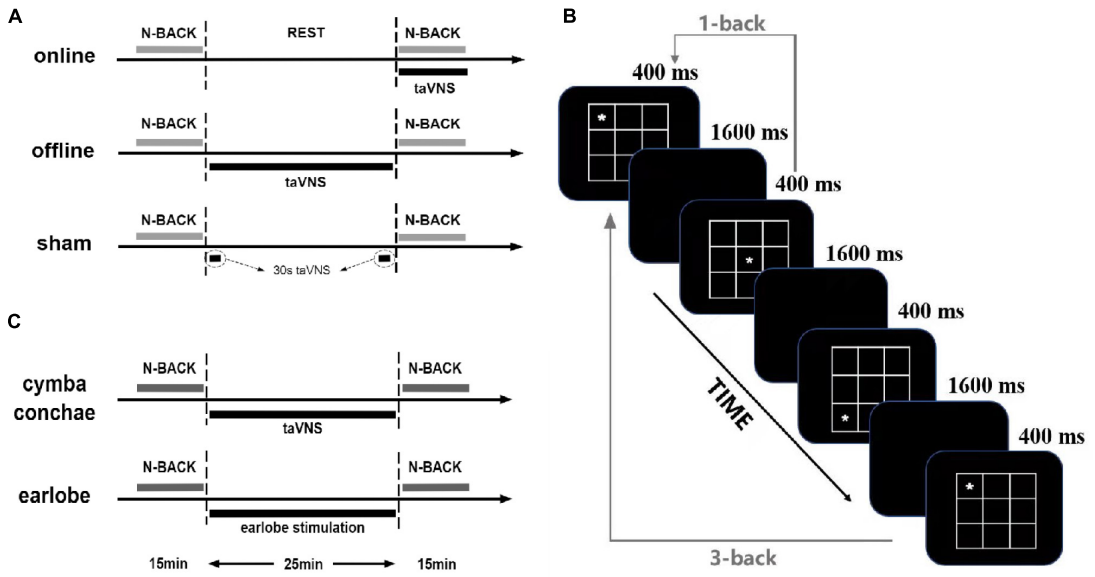

FIGURE 1 | Overview of the study and n-back tasks: **(A)** the protocol of experiment 1; **(B)** the paradigm of spatial n-back tasks, changing the spatial stimuli by digits is the digit 3-back task; **(C)** the protocol of experiment 2.

the beginning and the end of the stimulation section for 30 s (M_{sham} = 0.73 ± 0.27 mA).

Working Memory Tasks

The n-back task is one of the most frequently used paradigms in the assessment of WM capacity, which needs continuous updating of the transient memory storage with novel stimuli in order to compare the new stimuli with previously presented ones (Jarrold and Towse, 2006). In this experiment, we used 1- and 3-back tasks with spatial stimuli. There were four blocks (1-back, 3-back, 1-back, 3-back) with 72 experiment trials in each block. Each block was separated by a 30-s rest period. Before these four blocks, there was a training block with 16 trials for 1-back and 3-back tasks, respectively. Participants were instructed to press "F" when the site of the symbol ("*") was the same as in one or three trials earlier (namely, "matching" trial), but otherwise pressed "J" (namely, "mismatching" trial, **Figure 1B** for detailed parameters). Each trial was inserted as picture format with 257 × 257 pixels of width and height. One-third of the trials were matching. Training trials were before experiment trials, and participants could not start the formal experiment unless their training accuracy rate reached more than 75% and the average reaction time was less than 1,000 ms. Psychology experiment computer program E-Prime version 3.0 was used to administer the tasks and record response accuracy and reaction time of all the participants.

Experiment 2

Based on the design in experiment 1, in experiment 2 we used an active sham group (stimulation placebo), that is, earlobe stimulation group, which was used widely in taVNS modulation studies (e.g., Giraudier et al., 2020; Mertens et al., 2020; Neuser et al., 2020) to further replicate the results that were found in experiment 1. Besides, we added a 3-back task of digit to explore the generalization effect of taVNS on different WM tasks.

Participants

Sixty healthy students at Xidian University were included. The inclusion criteria were the same as in experiment 1, mainly including the right handedness, no smoking, no neurological disease, and no brain damage history. No participants reported ear injuries, drinking, or taking drugs 48 h before the experiment. One subject was excluded because of confusing matching and mismatching response, and another subject was excluded because of low baseline accuracy (<30%). Finally, there were 58 students in data analysis (24 female students; average age = 19.90 ± 1.49 years, range = 18–23 years). Each participant was provided written informed consent, and all research procedures were conducted in accordance with the Declaration of Helsinki and approved by the institutional research ethics committee of Xijing Hospital of the Air Force Medical University (KY20192008-X-1).

Design

The experiment was a within-subjects design, too, with each participant completing two separate sessions, which were different in stimulation conditions [i.e., offline taVNS and offline earlobe stimulation (offline ES); **Figure 1C**]. Despite the stimulation site, all the other conditions were the same in the two groups. There was at least a 2-day period (M_{days} = 2.93 ± 0.49) between two sessions.

taVNS Stimulation Equipment and Parameters

All the information of taVNS and stimulation intensity threshold was the same as in experiment 1, except that the anode and

cathode of taVNS were both placed in the left earlobe for the active sham group with anode front side and cathode back side. The stimulation intensity threshold was tested in the same way as in experiment 1, with stimulation intensities varying from 0.3 to 2.7 mA for the taVNS group (M_{off}line taVNS = 0.74 mA ± 0.37) and from 0.3 to 2.4 mA for the earlobe group ($M_{earlobe-sham}$ = 0.84 mA ± 0.39). Both offline taVNS and offline ES groups would receive a 25-min stimulation between baseline test and posttest.

Working Memory Tasks

Both spatial and digit 3-back tasks were used in this experiment with two blocks of each form. In total, there were four blocks (spatial, digit, spatial, and digit) with 72 trials in each block. The spatial 3-back paradigm was the same as in experiment 1. The procedure of digit 3-back task was the same as in spatial 3-back task, but the stimuli were changed from the site of "*" to nine Arabic numbers (1, 2, 3, 4, 5, 6, 7, 8, 9). The font of each number was Times New Roman, and the font size was 72. Participants were instructed to press "F" when the number was the same as in three trials earlier, but otherwise pressed "J." One-third of the trials were matching, and there was a 30-s period between each block. The paradigms and requirement of training blocks were the same as in experiment 1.

Data Analysis

There are four indicators that are often used, that is, hits (the accuracy of matching trials), correct rejections (the accuracy of mismatching trials) or false alarms (one minus correct rejections), *d* prime (*d*', hits minus false alarms), and reaction time (e.g., Jongkees et al., 2017). *d*' was first introduced based on signal detection theory to avoid distorted hits by false alarms (Haatveit et al., 2010). However, it is more of a receptivity indicator than a WM memory ability indicator, which mainly focused on participants' reaction tendency (Macmillan and Creelman, 1991). In a task with unbalanced matching and mismatching trials, there might be different change tendencies of hits and false alarms, whereas *d*' might weaken or even conceal these changes (e.g., Haatveit et al., 2010). As the present study was implemented in a healthy cohort whose improvement potential of WM is small, we used both hits and correct rejections (has similar power with false alarms), rather than *d*', as indicators to avoid missing any changes. Besides, we used the mean reaction time for accurate trials (aRT). Thus, three indicators were calculated for both experiments in the baseline (T0) and during stimulation/poststimulation (T1) of each condition for each participant. For each experiment, the trial if participant missed to response was regarded as a response error. One-way repeated-measures analysis of variance (ANOVA) and paired *t* test were used to check whether the indicators in T0 matched across conditions. The statistical analyses were performed in SPSSv26 (IBM Corp., Armonk, NY, United States) and MATLAB2019b (The MathWorks, Natick, MA, United States).

In experiment 1, the effect of taVNS on indicators were first assessed by using 2 × 3 repeated-measures ANOVA, with both time (T0, T1) and condition (online taVNS, offline taVNS, and sham) as within-subjects factors. *Post hoc* effect analysis was used in significant interaction effects via the paired *t* test for time (T0 vs. T1). In addition, one-way repeated-measures ANOVAs were used to directly compare the change scores from baseline (Δ score = T1 - T0) between conditions. Bonferroni correction was used to explore any significant effects.

In experiment 2, the effects of taVNS on indicators were first assessed using 2 × 2 repeated-measures ANOVAs, with both time (T0, T1) and condition (offline taVNS, and offline ES) as within-subjects factors. *Post hoc* effect analysis was used in significant interaction effects via the paired *t* test for time (T0 vs. T1). In addition, the paired *t* test was used to directly compare the change scores from baseline (Δ score = T1 - T0) between conditions. Bonferroni correction was used to explore any significant effects.

RESULTS

Experiment 1

There was no significant difference in baseline performance among the three conditions. There were no obvious feeling difference and adverse reactions of both online and offline taVNS, compared with the sham group (see **Supplementary Material** for detailed information).

Effects of taVNS on Spatial 1-Back Task

There was no significant main effect of conditions for hits [$F(2,90) = 0.18$, $p = 1.00$, $\eta_p^2 = 0.004$], correct rejections [$F(2,90) = 0.94$, $p = 1.00$, $\eta_p^2 = 0.02$] and aRT [$F(2,90) = 0.03$, $p = 1.00$, $\eta_p^2 = 0.001$]. The main effect of time was not significant in hits [$F(1,45) = 0.31$, $p = 1.00$, $\eta_p^2 = 0.01$] and correct rejections [$F(1,45) = 2.98$, $p = 0.55$, $\eta_p^2 = 0.06$], but was significant in aRT [$F(1,45) = 9.93$, $p = 0.02$, $\eta_p^2 = 0.18$]. The aRT at posttest was significantly shorter than that at baseline. The two-way interaction between time and groups was not significant in hits [$F(2,90) = 0.93$, $p = 1.00$, $\eta_p^2 = 0.02$], correct rejections [$F(2,90) = 1.04$, $p = 1.00$, $\eta_p^2 = 0.02$], and aRT [$F(2,90) = 0.01$, $p = 1.00$, $\eta_p^2 < 0.001$]. It suggested that both online and offline taVNS had no significant modulation on 1-back spatial WM (**Table 1** and **Figure 2**).

Effects of taVNS on Spatial 3-Back Task

There was no significant main effect of conditions for hits [$F(2,90) = 1.08$, $p = 1.00$, $\eta_p^2 = 0.02$], correct rejections [$F(2,90) = 3.09$, $p = 0.30$, $\eta_p^2 = 0.06$], and aRT [$F(2,90) = 0.11$, $p = 1.00$, $\eta_p^2 = 0.002$]. The main effect of time was not significant in hits [$F(1,45) = 5.39$, $p = 0.15$, $\eta_p^2 = 0.11$] and correct rejections [$F(1,45) = 0.72$, $p = 1.00$, $\eta_p^2 = 0.02$], but was significant in aRT [$F(1,45) = 32.46$, $p < 0.001$, $\eta_p^2 = 0.42$]. The reaction at posttest was much faster than that at baseline. The two-way interaction between time and groups was not significant in correct rejections [$F(2,90) = 1.20$, $p = 1.00$, $\eta_p^2 = 0.03$] and aRT [$F(2,90) = 0.54$, $p = 1.00$, $\eta_p^2 = 0.01$], but was significant in hits [$F(2,90) = 5.58$, $p = 0.03$, $\eta_p^2 = 0.11$]. The detailed information is presented in **Table 1**. *Post hoc* analysis showed that there were no differences between baseline and posttests in online taVNS [$t(45) = 0.07$, $p = 1.00$] and sham [$t(45) = -0.45$, $p = 1.00$] groups, whereas there was a significant improvement in the offline aVNS group

TABLE 1 | Accuracy and reaction time for n-back tasks per condition of experiment 1.

	Online group				Offline group				Sham group				Statistical test
	Baseline		Post		Baseline		Post		Baseline		Post		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	*F*(2,90)
1-Back													
Hits	0.870	0.012	0.869	0.013	0.866	0.013	0.871	0.013	0.872	0.011	0.855	0.018	0.93
Correct rejections	0.970	0.003	0.969	0.007	0.967	0.005	0.973	0.004	0.971	0.003	0.977	0.003	1.04
Accurate RT (ms)	540.86	17.91	511.39	17.31	538.32	17.76	510.96	14.35	537.88	15.93	507.78	13.39	0.008
3-Back													
Hits	0.757	0.021	0.766	0.021	0.751	0.023	0.808	0.018	0.758	0.021	0.751	0.026	5.58*
Correct rejections	0.918	0.021	0.940	0.008	0.930	0.021	0.958	0.007	0.930	0.023	0.945	0.008	1.25
Accurate RT (ms)	665.93	26.03	590.60	19.01	663.13	27.27	605.81	20.05	660.51	22.69	612.58	24.21	0.54

Hits means accuracy in matching trials; correct rejections means accuracy in mismatching trials; accurate RT means specifically referring to reaction time in all correct trials. F values referred to the two-way interaction. One asterisk indicates a corrected p < 0.05.

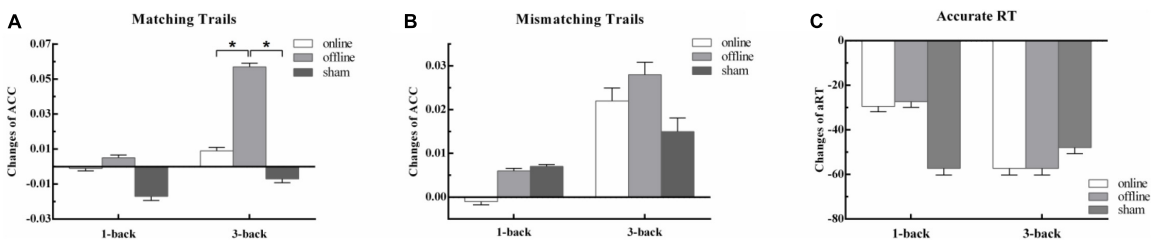

FIGURE 2 | The behavior outcomes of experiment 1. Task outcome changes (T1 - T0): **(A)** the change of accuracy rate for per condition in matching trials; **(B)** the change of accuracy rate for per condition in mismatching trials; **(C)** the change of mean reaction time of correct trials for per condition, and the quantities' units were ms. One asterisk indicates a corrected *p* value less than 0.05.

[$t(45) = 4.04$, $p = 0.001$]. One-way repeated-measures ANOVA of the Δ score (T1 - T0) among the online taVNS, offline taVNS, and sham groups found a significant difference [$F(2,90) = 5.59$, $p = 0.005$], which showed that the Δ score of the offline taVNS group was significantly higher than that of the online group ($d = 0.05$, $p = 0.02$) and sham group ($d = 0.06$, $p = 0.01$). The results are shown in **Figure 2**.

Experiment 2

There was no significant difference in baseline performance between the two conditions. There were no obvious feeling difference and adverse reactions of both offline taVNS and offline ES (see **Supplementary Material** for detailed information).

Effects of taVNS on Spatial 3-Back Task

The main effect of stimulus site for hits [$F(1,57) = 3.78$, $p = 0.34$, $\eta_p^2 = 0.06$], correct rejections [$F(1,57) = 0.81$, $p = 1.00$, $\eta_p^2 = 0.01$], and aRT [$F(1,57) = 0.42$, $p = 1.00$, $\eta_p^2 = 0.01$] was not significant. The main effect of time was significant in aRT [$F(1,57) = 15.87$, $p = 0.001$, $\eta_p^2 = 0.22$] and was marginal significant in hits [$F(1,57) = 7.36$, $p = 0.05$, $\eta_p^2 = 0.11$] and correct rejections [$F(1,57) = 6.73$, $p = 0.07$, $\eta_p^2 = 0.11$]. The aRT of posttest was shorter than that at baseline, and the hits at posttest were higher than those at baseline, whereas the hits and correct rejections at posttest were higher than those at baseline. The two-way interaction between time and groups was not significant in aRT [$F(1,57) = 0.07$, $p = 1.00$, $\eta_p^2 = 0.001$], but was significant in hits [$F(1,57) = 11.32$, $p = 0.006$, $\eta_p^2 = 0.17$] and correct rejections [$F(1,57) = 9.36$, $p = 0.02$, $\eta_p^2 = 0.14$]. The detailed information was presented in **Table 2**. *Post hoc* analysis of hits showed that there were no differences between baseline and posttests in earlobe group [$t(57) = 0.41$, $p = 1.00$], whereas there was a significant improvement in the taVNS group [$t(57) = 4.25$, $p < 0.001$]. The paired *t* test of the Δ score of hits between the two groups showed a significant difference [$t(57) = 3.36$, $p = 0.001$], which suggested that the Δ score of the taVNS group was significantly higher than that of the earlobe group (**Figure 3**). The paired *t* test of the Δ score of correct rejection between the two groups showed a significant difference [$t(57) = -2.22$, $p = 0.03$], which suggested that the Δ score of the taVNS group was significantly lower than that of the earlobe group (**Figure 3**).

Effects of taVNS on Digit 3-Back Task

There was no significant main effect of conditions for hits [$F(1,57) = 0.25$, $p = 1.00$, $\eta_p^2 = 0.004$], correct rejections [$F(1,57) = 0.50$, $p = 1.00$, $\eta_p^2 = 0.01$], and aRT [$F(1,57) = 0.63$, $p = 1.00$, $\eta_p^2 = 0.01$]. The main effect of time was not significant in hits [$F(1,57) = 1.76$, $p = 1.00$, $\eta_p^2 = 0.03$] and correct rejections [$F(1,57) = 0.55$, $p = 1.00$, $\eta_p^2 = 0.01$], but was significant in aRT [$F(1,57) = 7.99$, $p = 0.04$, $\eta_p^2 = 0.12$]. The aRT at posttest was significantly shorter than that at baseline. The two-way interaction between time and groups was not significant in hits [$F(1,57) = 0.04$, $p = 1.00$, $\eta_p^2 = 0.001$], correct rejections [$F(1,57) = 0.32$, $p = 1.00$, $\eta_p^2 = 0.01$], and aRT [$F(1,57) = 0.01$,

TABLE 2 | Accuracy and reaction time for spatial and digit 3-back tasks per condition of experiment 2.

	taVNS group				Earlobe group				Statistical test
	Baseline		Post		Baseline		Post		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	*F*(1,57)
Spatial 3-back									
Hits	0.825	0.018	0.870	0.014	0.828	0.021	0.832	0.017	11.32**
Correct rejections	0.965	0.005	0.967	0.005	0.955	0.006	0.970	0.004	9.36*
Accurate RT (ms)	612.22	21.92	555.42	19.81	618.12	25.61	567.06	22.07	0.07
Digit 3-back									
Hits	0.871	0.015	0.885	0.013	0.868	0.020	0.878	0.016	0.04
Correct rejections	0.972	0.004	0.975	0.005	0.976	0.003	0.976	0.003	0.32
Accurate RT (ms)	525.66	19.42	496.91	17.81	533.56	19.98	506.53	17.09	0.01

Hits, correct rejections, accurate RT, and F values have the same meaning with ***Table 1****. One asterisk indicates a corrected p < 0.05, and two asterisks indicate a corrected p < 0.01.*

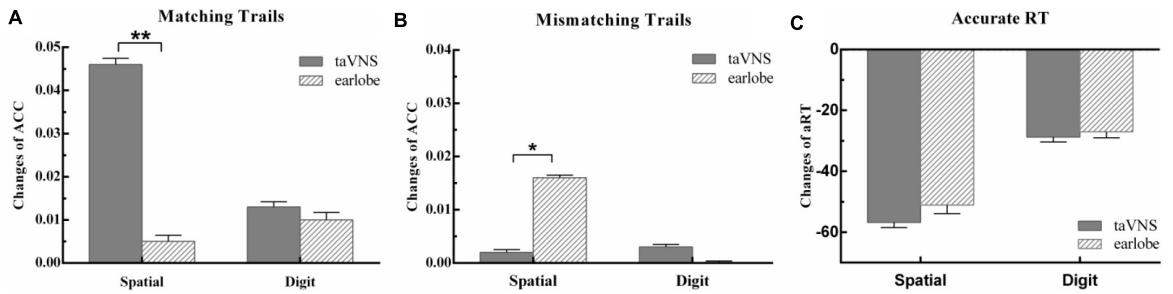

FIGURE 3 | The behavior outcomes of experiment 2. Task outcome changes (T1 - T0): **(A)** the change of accuracy rate for per condition in matching trials; **(B)** the change of accuracy rate for per condition in mismatching trials; **(C)** the change of mean reaction time of correct trials for per condition, and the quantities' units were ms. One asterisk indicates a corrected *p* value less than 0.05, and two asterisks indicate a corrected *p* value less than 0.01.

$p = 1.00$, $\eta_p^2 < 0.001$]. It suggested that both taVNS and earlobe groups had no significant modulation on 3-back digit WM (**Table 2** and **Figure 3**).

DISCUSSION

The current study assessed the effects of taVNS on WM performance under varying conditions: online and offline protocols, stimulation equipment sham and active sham (earlobe stimulation), 1-back and 3-back spatial WM tasks, and spatial and digit modalities of WM tasks. Overall, the experiments yielded relatively robust findings about the improvement of taVNS on offline spatial WM performance, no matter compared with online protocol, equipment sham, or active sham group. However, the enhancement of taVNS specifically appeared in offline 3-back spatial WM tasks, but not in online 1-back spatial or 3-back digit tasks.

To the best of our knowledge, this is the first study to investigate the immediate enhancement of WM by taVNS in healthy adults. In the first experiment, we discovered the improvement of offline taVNS on spatial WM capacity by comparing with online taVNS and sham groups, whereas in the second experiment, we replicated the results in experiment 1 with an active sham (offline ES) group. With the exploration and replication samples, we put relatively robust results about the improvement of offline taVNS on WM. There might be three reasons for the improvement. First, as we know, the vagus nerves project to the NTS in the medulla, before being relayed further to other brainstem nuclei and higher-order structures, including the thalamus, hippocampus, amygdala, and insula (Goehler et al., 2000; Saper, 2002). When the vagus nerve projects to the NTS and activates the noradrenergic neurons in the LC and cholinergic neurons in the nucleus basalis, NE and acetylcholine consequently release in wide areas of the cortex (Gu, 2002; Hassert et al., 2004; Roosevelt et al., 2006; Nichols et al., 2011). Subsequently, α1-adrenergic receptors in the dorsal raphe nucleus are activated by NE and release serotonin (Manta et al., 2009). These neurotransmitters can enhance behavioral and cognitive processes, including WM capacity by facilitating neural plasticity (Gu, 2002; Duffau, 2006). Second, long-term potentiation (LTP) as a process involving persistent strengthening of synapses that leads to a long-lasting increase in signal transmission between neurons is widely recognized as a cellular mechanism of memory formation (Bliss and Collingridge, 1993; Bear and Malenka, 1994). As NE is known to facilitate this early LTP through activating β-noradrenergic receptors, the VNS-induced LC-NE release system has been proposed as another possible mechanism of modulating memory performance (Harley, 2007; Mueller et al., 2008). Third, attentional mechanisms might contribute to the improvement of taVNS on WM performance. WM and attention

are interacting constructs and tightly intertwined, as attention provides the basis for selecting what information will be encoded in WM (Awh et al., 2006). Previous studies found that VNS could increase early visual N1 amplitude, which is similar to what is seen with increased level of attention (Mangun and Hillyard, 1991; Luck and Ford, 1998). Sun et al. (2017) further discovered that VNS could increase the WM capacity of epilepsy patients by attentional mechanisms.

However, the improved effect of taVNS on WM was absent in the online stimulation group. One would argue that the absence of enhancement in online condition can be attributed to the shorter stimulation time (approximately 15 min) compared with the offline group (25 min). This is possible, but not highly plausible, as no studies have found compelling evidence that increasing stimulation time led to stronger effects on cognitive performance. In fact, the stimulation time of online taVNS in previous studies was highly dependent on the length of behavioral tasks, which varied from 13 to 75 min (e.g., Giraudier et al., 2020; Neuser et al., 2020; Tona et al., 2020), whereas the positive results did not increase with the increase in stimulation time. Besides, if the timing was crucial, there is probably some systemic difference between the 1- and 3-back tasks, which are completed in turn, but this was not the case. Further researches are needed to investigate the specific effects of stimulation time. However, the most likely explanation in the current studies lies in different mechanism behind online and offline taVNS effects, which need more researches. However, as discussed previously, the effect of taVNS on WM mainly depends on the LC-NE release system, which need time to take effect, and this might be the first possible explanation. Furthermore, researches from tDCS showed that there was a trend toward improvement for offline WM performance but was not on online performance in the healthy subjects, whereas the neuropsychiatric cohort exhibited an opposite pattern (e.g., Živanović et al., 2021; for review, see Hill et al., 2016). As online tDCS alters neuronal firing by changing membrane potential, whereas the aftereffects of tDCS stem from changes in synaptic strength, these authors attributed their findings to the optimal cortical excitation/inhibition balance and insufficient neuronal excitability changes in online stimulation in healthy adults. The same pattern appeared here, as Sun et al. (2017) found an online VNS effect in epilepsy patients, whereas this study showed an offline taVNS effect in healthy participants. Thus, we consider that there might be a similar reason that the insufficient vagus nerve excitability changes during online taVNS in healthy adults restrict behavioral changes. Finally, the distracting effect of online stimulation might cover up the modulation effect of online taVNS, and this should be taken into account in further studies.

Beyond the stimulation protocols, the modulation of taVNS might also depend on the properties of the output measure used. Namely, although n-back is a typical paradigm for WM assessment, the numbers of steps back, for example, 1-back (Sandrini et al., 2012), 2-back (Keshvari et al., 2013; Hill et al., 2019), and 3-back (Hill et al., 2019; Živanović et al., 2021), as well as the stimuli of the task, for example, spatial (Živanović et al., 2021), letters (Hill et al., 2019), digits (Nozari and Thompson-Schill, 2013), and objects (Keshvari et al., 2013), are highly variable in the literature. The current study specifically found the taVNS effect on 3-back spatial WM task but not on spatial 1-back or digit 3-back tasks. A meta-analysis suggested that the verbal n-back, like the digit n-back task, was associated with enhanced activation in the left ventrolateral prefrontal cortex, whereas the non-verbal location n-back task was associated with enhanced activation in a set of regions that have been described as a spatial attention network, including right dorsolateral prefrontal, lateral premotor, and posterior parietal cortex (Owen et al., 2005). Given the discussion above, we know that the attentional mechanisms might be one of the reasons for the taVNS effect on WM performance. The improved selective attention induced by VNS (Sun et al., 2017) might have a larger effect on the spatial attention network and contributed to the difference in the improvement of taVNS on spatial and digit WM performance. Besides, the researches in tDCS found that the modulation of electric field on WM depends on the baseline performance (e.g., Assecondi et al., 2021). The individuals or tasks with lower baseline outcome were more likely to have a higher improvement. For 1-back spatial task, the high baseline performance might restrict the modulation of taVNS. It should be noted that the baseline performance of the digit 3-back task was similar to that of the spatial 1-back task, and it might be another probable reason for the uselessness of taVNS. Unfortunately, the present study did not use a digit WM with higher difficulty, and further researches are needed.

Finally, except the difference between the effective stimulation protocol by Sun et al. (2017; online stimulation) and the current study (offline stimulation), these two studies found that for both epilepsy patients and healthy adults, the increase in WM outcome appeared only in hit reactions but not in reaction time, missing response, or correct rejections. These results show consistency between the clinical study and laboratory investigation, which make these results more convincing. According to Sun et al. (2017), the improvement of selective attention increased accuracy in target trials, that is, hits, whereas the unchanged general level of attention was attributed to the unchanged aRT and correct rejection rate. Besides, the high baseline of correct rejections might restrict the increased potential of posttest, and the improved familiarity of the tasks leads to a comprehensive main effect of aRT in all groups. However, as shown in the meta-analysis, tDCS could improve the reaction time in healthy adults (Hill et al., 2016); the synergistic effects of tDCS and taVNS might lead to a more comprehensive improvement in WM, which has been proven in neuroimaging study (Sun et al., 2021), whereas the effects on behavior need to be investigated in the future. Another interesting finding that should be noted is that the offline ES increased participants' correct rejection rate. It might be caused by the special effect of nervus auricularis magnus, which was activated by earlobe stimulation. The further effect and mechanism of earlobe stimulation need more studies.

Nevertheless, there are still some limitations to the current study. First, the optimal condition offline taVNS, especially the stimulation time, was not clear in the present study. Although the stimulation time in this study has led to a strong effect, we know little about the effects in longer or shorter stimulation conditions. As the stimulation time influences the convenience

and safety and is important for standard protocol, it needs more investigation. Besides, the modulation effect of online taVNS might also depend on stimulation time more or less. The optimal stimulation time might lead to a stronger and more efficient online taVNS effect on behavior performance, which needs more researches in the future. Second, if the taVNS modulation effects exist only in the offline protocol, it is valuable to investigate the potential mechanism difference between online and offline taVNS, such as the excitability changes of vagus nerve, which might put new perspective about the effects of taVNS and need further researches. Third, as mentioned previously, although we failed to identify the generalization effects of taVNS from spatial WM to digit WM task, the absent improvement of taVNS on digit WM performance is not clear in detailed reasons, namely, whether it is caused by the specificity of taVNS or the high baseline performance. Thus, further studies with more difficult digit/verbal WM tasks or some subjects with lower WM capacity, such as aging people or patients, are needed. Fourth, beyond n-back task, there are many other tasks that require WM capacity, like Sternberg task. Therefore, studies with other tasks are needed to further verify the generalization effects of taVNS on WM ability. Lastly, although the immediate improvement of WM was strong after offline taVNS, it is unknown to date whether acute improvements can predict the sustained therapeutic effects of potential taVNS-based treatment. Translation to clinical settings remains as a vital question and urgently needs more researches.

CONCLUSION

To summarize, although the vagus nerve is known to play a vital role in the regulation of cognition, the immediate modulatory effects of vagal afferent signals on WM in healthy cohort are largely elusive to date. Here, using taVNS, we demonstrate that stimulation of the vagus nerve increases performance of offline spatial WM tasks in healthy populations, whereas the evidence of improvement of taVNS on digit WM tasks was absent and needs further researches. In general, our results shed light on the role of peripheral physiological signals in regulating WM and highlight the potential for non-invasive cranial nerve stimulation techniques to improve a person's cognition and behavior.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Research Ethics Committee of the Xijing Hospital of the Air Force Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

J-BS, Q-QT, and WQ: conception and study design. J-BS, Q-QT, X-YG, Y-PC, and CW: data collection or acquisition. J-BS, CC, and Q-QT: statistical analysis. J-BS, CC, HY, X-JY, and HD: interpretation of results. J-BS and CC: drafting the manuscript work or revising it critically for important intellectual content. All authors approval of final version to be published and agreement to be accountable for the integrity and accuracy of all aspects of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.790793/full#supplementary-material

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REVIEW
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"The Wandering Nerve Linking Heart and Mind" – The Complementary Role of Transcutaneous Vagus Nerve Stimulation in Modulating Neuro-Cardiovascular and Cognitive Performance

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The vagus nerve is the longest nerve in the human body, providing afferent information about visceral sensation, integrity and somatic sensations to the CNS *via* brainstem nuclei to subcortical and cortical structures. Its efferent arm influences GI motility and secretion, cardiac ionotropy, chonotropy and heart rate variability, blood pressure responses, bronchoconstriction and modulates gag and cough responses *via* palatine and pharyngeal innervation. Vagus nerve stimulation has been utilized as a successful treatment for intractable epilepsy and treatment-resistant depression, and new non-invasive transcutaneous (t-VNS) devices offer equivalent therapeutic potential as invasive devices without the surgical risks. t-VNS offers exciting potential as a therapeutic intervention in cognitive decline and aging populations, classically affected by reduced cerebral perfusion by modulating both limbic and frontal cortical structures, regulating cerebral perfusion and improving parasympathetic modulation of the cardiovascular system. In this narrative review we summarize the research to date investigating the cognitive effects of VNS therapy, and its effects on neurocardiovascular stability.

Keywords: vagus nerve stimulation, cognition, neurocardiovascular control, cerebral blood flow, LC-NE system, inhibitory control, executive function

INTRODUCTION

Vagus nerve stimulation (VNS) as a neurostimulation technique and has received renewed attention in recent years. Traditionally invasive VNS (iVNS) devices were sutured under the skin of the chest with a lateral left neck dissection undertaken to expose the left cervical vagus nerve and wrap a stimulating electrode around it. Each iVNS device is costly and up to 30% of patients have side effects post implantation (Morris and Mueller, 1999). Since the development in the early 2000s of peripheral stimulating devices that harness the vagus nerve's innervation of the skin of the

external ear demonstrating efficacy in treating epilepsy, depression and headaches, interest in wider therapeutic potentials of this treatment have grown (Yap et al., 2020).

Declining cognition associated with aging is a burgeoning global health crisis, with at least 152.8 million persons projected to have dementia worldwide by the year 2050 (Nichols et al., 2022). There are few effective treatments for cognitive decline and dementia, with no current cure (Cummings et al., 2021) and although the first disease modifying anti-amyloid agent has been licensed by the FDA (Steinbrook, 2021), more therapies are urgently needed to help alleviate the personal, societal and economic cost of increasing dementia diagnoses (Xu et al., 2017). Impaired cognition is associated with impaired autonomic function, specifically impaired parasympathetic measures of heart rate variability (HRV) (Forte et al., 2019; Cheng et al., 2022; Liu et al., 2022) likely reflective of the complex interplay between cognition and cardiac modulation, *via* the central autonomic network.

Studies of patients with intractable epilepsy and treatment -resistant depression treated with iVNS devices showed signals indicating increased alertness and potentially cognitive improvements (Ghacibeh et al., 2006a; McGlone et al., 2008; Schevernels et al., 2016; Sun et al., 2017; van Bochove et al., 2018) and a small pilot study investigated iVNS devices in patients with Alzheimer's Disease with overall positive results (Sjögren et al., 2002; Merrill et al., 2006). Recent meta-analysis of t-VNS in young healthy adults has found an overall moderate effect especially for improved cognitive performance especially executive function (Ridgewell et al., 2021). However the neuroanatomical substrates of persons with treatment-resistant depression or epilepsy are likely both widely variable, and grossly different to both a young cognitively healthy adult and a person with mild cognitive impairment (MCI) or dementia and dedicated larger studies are required to investigate if t-VNS has therapeutic potential in this population.

The purpose of this narrative review will be to outline the research to date investigating both cognitive outcomes of VNS in healthy and clinical populations, and the effect VNS has on HRV as a measure of autonomic tone. The mechanisms of action of VNS including neurotransmitter release, local increased cerebral blood flow and modulation of peripheral hemodynamics are discussed and future research recommendations outlined.

ANATOMY AND PHYSIOLOGY OF THE VAGUS NERVE

The longest nerve in the body, the vagus nerve derives its name from the Latin for 'straying' or 'wandering.' Aptly named, the nerve has an extensive course, traveling from the medulla to the gut. The vagus nerve's function is to transmit information to and from the central nervous system (CNS) regarding control of the gastrointestinal, cardiovascular, and respiratory systems. It is comprised of approximately 80% afferent and 20% efferent fibers (Foley and Dubois, 1937; Agostini et al., 1957) including A, B and C fibers classified by conduction velocity (Erlanger and Gasser, 1937). Vagus neurons may involve visceral (cardiac, bronchopulmonary, gastrointestinal) or somatic (soft tissues, muscles of palate, pharynx) modulation. Afferent fibers are further sub classified as general visceral afferent, general somatic afferent, or special visceral afferent. Two efferent fiber types are recognized, namely special visceral efferent and general visceral efferent (see **Table 1**). Fibers connect centrally to four vagal nuclei; the nucleus of the solitary tract (NTS) and spinal trigeminal nucleus which contain vagal afferent fibers and the nucleus ambiguous and dorsal motor nucleus of the vagus (DMN) from where vagal efferent fibers leave (Rutecki, 1990; Berthoud and Neuhuber, 2000).

Afferent vagus fibers enter the medulla at the level of the olive, and terminate primarily in the NTS (Beckstead and Norgren, 1979; Kalia and Sullivan, 1982). Each vagus nerve (VN) synapses bilaterally in the NTS; so vagal afferent information is processed bilaterally in the CNS (Henry, 2002). Second order afferent fibers from the NTS project most densely to the parabrachial nucleus of the pons (PBN) with the NTS also projecting to noradrenergic (locus coeruleus) and serotonergic (raphe nuclei) neuromodulatory systems (Rutecki, 1990; Saper, 2000). From here vagal information is relayed to a number of mostly subcortical structures, including the hypothalamus, the central nucleus of the amygdala, the bed nucleus of the stria terminalis, and the intralaminar thalamic nucleus. Vagal afferent information is also sent to the anterior insular cortex which communicates with more rostral regions of the cortex (orbital and ventrolateral prefrontal cortex) and also indirectly with the medial prefrontal cortex (Öngür and Price, 2000; Saleem et al., 2008).

These central structures are part of the central autonomic network (CAN) which is thought to be the origin of autonomic, behavioral, cognitive, and endocrine responses, capable of modulating the functioning of the autonomic nervous system (ANS) *via* descending pathways projecting onto sympathetic pre-ganglionic neurons in the spinal cord and onto the DMN at the origin of vagal efferents (Benarroch, 1993). The central connections of the DMN are considerable, with afferent projections arising from sites including the NST, magnocellular paraventricular nuclei and several medullary nuclei (Roges et al., 1980; Hansen, 2019). Whilst a minority of efferent fibers connect centrally, most DMN fibers project to GI organs *via* parasympathetic ganglia located close to or in the walls of viscera. Further efferent fibers originate from the nucleus ambiguous (NA), a motor nucleus located in the reticular formation of the medulla which gives rise to preganglionic neurons innervating the heart and lungs (Llewellyn-Smith and Verberne, 2011) which exert a cardio-inhibitory effect mediated *via* the sinoatrial and atrioventricular ganglia (Massari et al., 1995; Gatti et al., 1996). The right vagus nerve mostly innervates the sinoatrial node (involved in the pacemaker function of the heart) whereas the left vagus is mostly thought to innervate the atrioventricular node (regulating the force of contraction of the cardiac myocytes with less influence over heart rate) however comprehensive human studies confirming this precise delineation are needed (Coote, 2013). The dorsal branchiomotor division of the NA is the site of origin of efferent fibers innervating striated muscle of the palate, pharynx, larynx and upper esophagus.

TABLE 1 | The constituent fibers of the vagus nerve.

	FIBER				
	Aα	**Aβ**	**Aδ**	**B**	**C**
Fiber diameter	13–20 mm	6–12 mm	1–5 mm	1–5 mm	0.4–2 mm
Gross anatomical structure	Large	Large	Large	Small	Small
Main function afferent	Somatic touch pain temperature	Somatic touch	Visceral: pain stretch chemical, temperature	Visceral	Visceral: pain stretch chemical, temperature
Main function efferent	Muscle tone	Muscle preganglionic	preganglionic	preganglionic	preganglionic
Myelin	+	+	+	+	−
Threshold mA	0.02–0.2 mA	0.02–0.2 mA	0.02–0.2 mA	0.04–0.6 mA	0.3–6 mA
Conduction velocity ms	8–120 ms	35–75 ms	3–30 ms	3–15 ms	0.5–2 ms
Purported effect of VNS on EEG	Synchronization	Synchronization	Synchronization	Synchronization	Desynchronization

Adapted from Groves et al. (2005).

See **Figure 1** for a schematic representation of the VN fibers and central projections.

HISTORY OF VAGUS NERVE STIMULATION

Vagus nerve stimulation was initially proposed as a therapeutic intervention in 1871 (Neftel, 1871) and a device was designed to stimulate bilateral vagus nerves in the late 19th century (Lanska, 2002). Preclinical studies in the 1930–1950s demonstrated *via* EEG signaling that VNS had cortical stimulating activity (Bailey and Bremer, 1938; Zanchetti et al., 1952), and could terminate canine seizures (Zabara, 1985, 1992).

Invasive VNS (iVNS) received United States regulatory approval for the adjunctive treatment of refractory seizures in 1997 and for use in treatment resistant depression in 2005 (O'Reardon et al., 2006). However, given the invasive nature of iVNS (requiring general anesthesia, thoracic implantation of a battery generator, and neck dissection to attach stimulating electrodes to the left cervical vagus nerve), the concept of non-invasive VNS was proposed in 2000 whereby, drawing on evidence from studies of auricular acupuncture, it was postulated that transcutaneous vagal stimulation could represent a valuable tool in epilepsy treatment (Ventureyra, 2000). Non-invasive VNS involves using stimulating electrodes on the skin to excite afferent vagal fibers and can be performed *via* the ear (transcutaneous auricular VNS: t-VNS) or the neck (transcutaneous cervical VNS: tcVNS). For the purposes of this narrative review non-invasive VNS will refer to auricular t-VNS.

The technique of t-VNS exploits the peripheral anatomy of the vagus nerve, activating vagal afferent projections through stimulation of the auricular branch of vagus nerve (ABVN) at the ear (Peuker and Filler, 2002; Mercante et al., 2018) see **Figure 2** for a schematic representation of the anatomy of the ABVN and central structures it modulates. Anti-seizure efficacy equivalent to iVNS was demonstrated in preclinical studies before the feasibility and therapeutic significance of this technique in humans were demonstrated (Stefan et al., 2012) and evidence from multiple functional brain imaging studies confirms significant activation of central vagal projections *via* this non-invasive method (Kraus et al., 2013; Frangos et al., 2015; Yakunina et al., 2017; Badran et al., 2018a).

Transcutaneous auricular vagus nerve stimulation waveforms can be delivered at a variety of different parameter settings which vary frequency (Hz), amplitude (mA), pulse width (μs-msec) and duration of stimulation. It is currently being investigated as a therapeutic intervention for a variety of medical disorders including epilepsy, migraine and cluster headaches, tinnitus, atrial fibrillation, Parkinson's disease, schizophrenia, impaired glucose tolerance, obesity, and pain (Goadsby et al., 2014; Huang et al., 2014; Laqua et al., 2014; Hasan et al., 2015; Hyvarinen et al., 2015; Nesbitt et al., 2015; Stavrakis et al., 2015; Cakmak et al., 2017; Obst et al., 2020). There is particular interest in the evolving literature reporting the use of t-VNS in cognitive disorders (Broncel et al., 2020; Lam et al., 2021). Potential mechanisms of action include modulation of HRV, impacts on cerebral perfusion, and noradrenergic neuromodulation. The complementary role of vagus nerve stimulation in modulating neuro-cardiovascular and cognitive performance is explored in detail below.

See **Figure 2** for a schematic diagram of the area of innervation of the ABVN and its central projections.

COGNITIVE PERFORMANCE AND VAGUS NERVE STIMULATION

Brain imaging during t-VNS demonstrates strong activation of vagal projections to subcortical nuclei and frontal brain regions, i.e., superior frontal gyrus and medial frontal gyrus during stimulation (Kraus et al., 2013) (See below in "Mechanisms of Action" for further detailed discussion regarding the neuroanatomical structures modulated during VNS). Cognitive effects of both iVNS and t-VNS in both clinical populations and healthy volunteers will be examined under the following themes: Cognitive control, i.e., the non-automatic regulation of behavior to achieve a goal (Gonthier, 2014) a primarily

executive function that involves suppression of goal-irrelevant stimuli *via* response and attention-inhibition (Tiego et al., 2018) and it primarily involves the lateral prefrontal cortex (Dixon, 2015); Language, both assessing categorical fluency a semantic memory language task involving the temporal lobe, and word recognition and retrieval which mostly involves episodic working memory, involving prefrontal cortex and medial temporal structures (Squire and Zola, 1998; Camina and Güell, 2017); Associative memory, a subcategory of declarative episodic memory and involves the ability to link disparate novel stimuli (Naveh-Benjamin, 2000); Emotion recognition as a subtype of cognition involves areas of the brain involved in perceiving social information including the medial prefrontal cortex and the orbitofrontal cortex (Bachmann et al., 2018) and regions implicated in emotional processing, including the cortical orbitofrontal cortex and the anterior cingulate cortex but also subcortical structures including the amygdala, hypothalamus, basal ganglia and the periaqueductal gray matter (van den Stock et al., 2011).

Interest in the potential role of VNS as a cognitive enhancer started following a preclinical rodent study of an inhibitory-avoidance task. Subjects received a single exposure to a foot shock followed immediately by VNS or sham. Those undergoing true VNS stimulation had longer step times demonstrating enhanced avoidance and this effect was modulated by the intensity of the stimulus, with 0.4 mA being an effective level of stimulation and 0.2 and 0.8 mA having no significant effect (Clark et al., 1995). Subsequent in-human trials tested word recognition in patients with intractable epilepsy who had iVNS devices implanted 2–24 weeks prior to testing. The stimulation parameters were 30Hz, 0.5 mA at 0.5 ms pulse width compared to an amplitude of 0.75–1 mA, and improved word recognition was only found in the group stimulated at the lower amplitude (Clark et al., 1999). These results paved the way for further investigation in this area as detailed below.

VAGUS NERVE STIMULATION AND COGNITIVE CONTROL, i.e., EXECUTIVE FUNCTION IN HEALTHY VOLUNTEERS

Inhibitory control is commonly measured using performance on tasks such as the Stroop, Eriksen Flanker (Flanker), and Simon tasks, i.e., forced-choice reaction time tasks that require participants to selectively attend and respond to target stimuli whilst ignoring goal-irrelevant distracting stimuli (Kornblum et al., 1990; MacLeod, 1991; Eriksen, 1995).

Enhanced response times, as reflected by participants' ability to stop a process and change to another response simultaneously and sequentially, and increased post error slowing were demonstrated during t-VNS (Sellaro et al., 2015; Steenbergen et al., 2015). Post error slowing refers to appropriate slowing after negative feedback or unforeseen errors and is linked to the activity of the locus coeruleus–norepinephrine (LC–NE) system and therefore postulated to be enhanced by VNS. As with the above trials, there were fewer false alarms during a more challenging paradigm with t-VNS when working memory processes were simultaneously engaged (Beste et al., 2016) and improved response selection and control performance was demonstrated with t-VNS in a serial reaction time test in young volunteers (Jongkees et al., 2018). In a sequence learning paradigm, the presentation of so-called reversal trials is associated with longer response latencies as compared to non-reversal trials, a result attributable to the 'inhibition of return' type phenomenon. Inhibition of return refers to an inhibitory after-effect of attention whereby, following exogenous orientation of attention to a stimulus, processing of stimuli at this location is first facilitated and then inhibited (Wang et al., 2018). Jongkees et al. (2018) demonstrated that active t-VNS, as compared to sham stimulation in the context of a serial reaction time test, reduced reaction time for reversal trials, eliminating the inhibition of return like effect described above.

In a similar experimental set up, increased attention, globally enhanced accuracy and reduced performance costs were demonstrated in a Stop-Change paradigm with t-VNS (Keute et al., 2020).

Results in this area have not been uniformly positive. In a testing paradigm in healthy volunteers using higher than average amplitude settings (see **Table 2**) there were no improvements in a Stroop test, Modified Flanker test or a number/letter working memory task with t-VNS. Improved accuracy in a dimensional change card sorting task was however noted (Borges et al., 2020). Similar previous studies failed to show improved behavioral performance with t-VNS (Fischer et al., 2018; Ventura-Bort et al., 2018) however non-performance parameters, namely a frontal EEG signal (P3 amplitude) thought to change with response inhibition and higher salivary amylase levels, were noted in the intervention group (Ventura-Bort et al., 2018). Further studies investigating EEG amplitudes affected by t-VNS and cognitive control paradigms included one involving an acoustic rather than visual oddball paradigm. In this context, t-VNS augmented the P3 amplitude, and with random noise stimulation with t-VNS reaction times were reduced (Rufener et al., 2018). There are myriad potential reasons for replication challenges in this newly expanding area of research and may include stimulation parameter differences including lack of pre-testing active stimulation.

The most recent studies in this area have involved a spatial stimulation and response inhibition multitask, with notable improved results in accuracy with 25 min pre-assessment t-VNS stimulation (Sun et al., 2021) and improved objective attention, arousal and multitasking ability in sleep deprived military personnel (McIntire et al., 2021).

VAGUS NERVE STIMULATION AND LANGUAGE IN HEALTHY VOLUNTEERS

Fluency scores in healthy volunteers during a convergent and divergent thinking task were significantly higher during active t-VNS at the left conchae, and categorical flexibility (i.e., participants' ability to think of more and varied categories of nouns) was also significantly improved (Colzato et al., 2018). However, an experimental design investigating the difference in

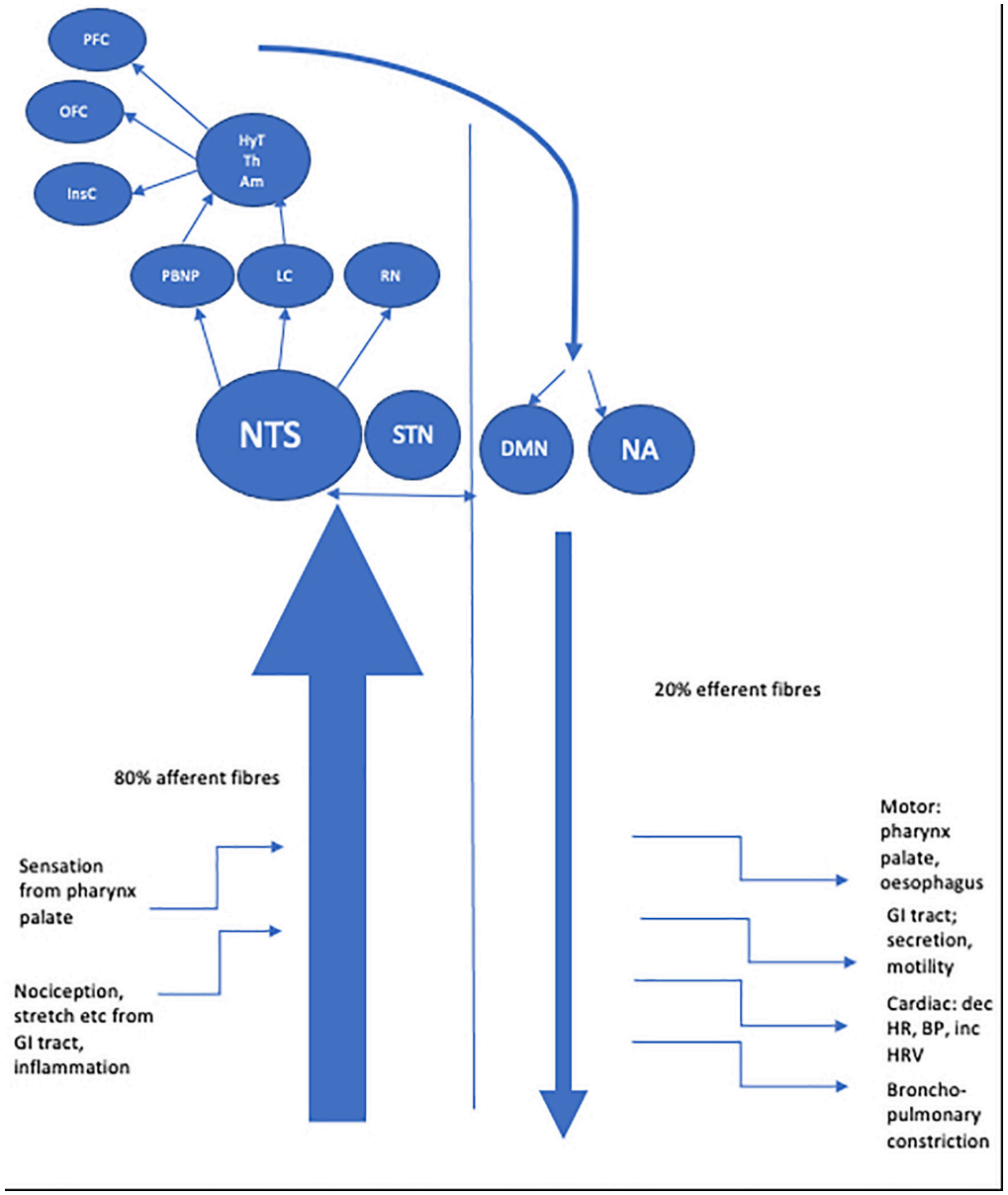

FIGURE 1 | Schematic representation of afferent and efferent fibers of the vagus nerve and central projections.

effect of t-VNS on word recognition memory in young compared to older volunteers (average age 22.2 and 55.1) whereby t-VNS was delivered for 30 s during the consolidation phase of a word recognition memory task showed no improvement in accuracy scores for immediate recall or delayed recognition in both age groups (Mertens et al., 2020). Possible reasons for this may be that 30 s of t-VNS may be insufficient for a non-invasive device to effectively stimulate the vagal afferent pathway, that longer and more repetitive stimulation of the vagus nerve might be required to effectively modulate hippocampal processes *via* synaptic plasticity. A recent investigation of word retention, stimulating the left tragus with t-VNS at again similar parameters but wider amplitude found improved accuracy in word retention but only in items that rhymed, i.e., were phonologically similar (Kaan et al., 2021).

VAGUS NERVE STIMULATION AND ASSOCIATIVE MEMORY IN HEALTHY VOLUNTEERS

Transcutaneous auricular vagus nerve stimulation has been tested in a group of healthy older adults to determine the technique's impact on performance in a face-name association task (Jacobs et al., 2015). VNS was employed in the encoding and consolidation phases of the task with active and sham stimulation

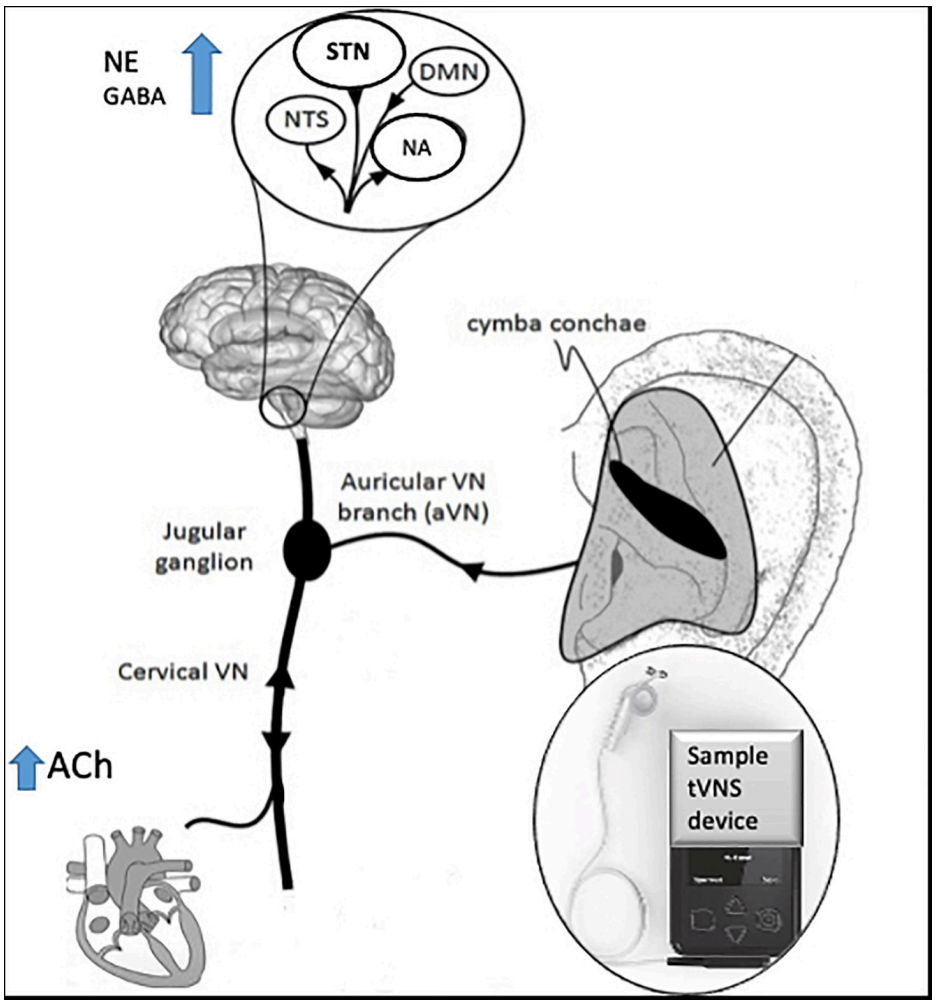

FIGURE 2 | Schematic diagram of innervation of ABVN and central projections, adapted with permission from Kaniusas et al. (2019).

compared in a randomized crossover design. Active t-VNS was demonstrated to increase the number of 'hits' on the memory task. Stimulation parameters employed differed somewhat from those seen in the broader literature concerning the impact of VNS on cognitive function. A stimulation intensity of 5.0 mA, a pulse width of 0.2 ms, and a frequency of 8Hz were utilized, citing previous functional and electrophysiological studies (Kraus et al., 2007; Polak et al., 2009). A stimulation lead in time of 17 min was also utilized, which has been theorized to be beneficial for targeted neuronal plasticity (Hays et al., 2013).

VAGUS NERVE STIMULATION AND EMOTION RECOGNITION IN HEALTHY VOLUNTEERS

This ability to recognize different emotions in others was investigated and found to be enhanced by t-VNS at the left outer auditory canal in young healthy adults but only for objectively easy, not challenging, items *via* the Reading the Mind in the Eyes test (Colzato et al., 2017). Subsequent investigations of fear conditioning and extinction in young volunteers, after previous positive studies, found that t-VNS at the left cymba conchae did not infer any difference in physiological or declarative indices of fear or improve fear extinction (Burger et al., 2019). Further studies are needed in this area to elucidate if t-VNS has a specific beneficial effect, given its ability to modulate both cortical and subcortical structures.

See **Table 2** for parameters settings and outcomes in trials of VNS in healthy volunteers.

VAGUS NERVE STIMULATION AND COGNITION IN CLINICAL POPULATIONS

In this section we highlight the studies to date investigating the cognitive effects of VNS on clinical populations, mostly with treatment-resistant depression or epilepsy. Many studies investigating the role of VNS in clinical populations has involved

TABLE 2 | Cognition and VNS in healthy volunteer populations.

COGNITION AND VNS: Healthy volunteers

		Stimulation Parameters						
Study	**iVNS/tVNS**	**Hz**	**mA**	**Pulse width**	**Time**	**Population**	**Task**	**Outcome**
Steenbergen et al., 2015	tVNS	25Hz	0.5mA	200–300 μs	30 s blocks	Healthy young adult volunteers $n = 30$	Stop change paradigm	Enhanced response selection and faster response times when two actions executed in succession
Sellaro et al., 2015	tVNS left outer auditory canal	25Hz	0.5 mA	200–300 μs	30 s blocks	Healthy young adult volunteers $n = 40$	Modified Flanker test	Increased post error slowing during active tVNS
Jacobs et al., 2015	tVNS left external acoustic meatus	8Hz	5.0 mA	200 μs	17 min	Healthy older adults avg age 60.5 $n = 30$	-Face name recognition task −15 word learning test -Digit span forward/backward -Verbal fluency test -Concept shifting task -Letter digit subtraction -Stroop color word test	Higher number of accurate "hits" during tVNS for face name recognition
Beste et al., 2016	tVNS left inner ear	25Hz	0.5 mA	200–300 μs	30 s on/30 s off	Healthy young volunteers $n = 51$	Inhibitory control (go-no-go task)	Fewer false alarms in the more challenging paradigm, i.e., when working memory processes also engaged
Colzato et al., 2017	tVNS Left outer auditory canal	25Hz	0.5 mA	200–300 μs	30 s on/30 s off	Healthy young volunteers $n = 38$	Emotion recognition Reading the mind in the Eyes test	Enhanced emotion recognition for easy (not challenging) items suggesting it promoted the ability to decode salient social cues
Fischer et al., 2018	tVNS	25Hz	Avg 1.3 mA (0.4–3.3)	200–300 μs	Continuous	Healthy adult volunteers $n = 21$	-Adapted response conflict Simon task -Novelty oddball task	No behavioral change noted Down-regulated N2 potential EEG reading
Ventura-Bort et al., 2018	tVNS Left cymba conchae	25 Hz	−1.3 mA (0.4–3.3) active −1.49 mA (0.6–4.8) sham	200–300 μs	28 min task 1 7 min task 2	Healthy young volunteers $n = 21$	-Novelty oddball task -number version of the Simon task	-No difference with tVNS with difficult targets or novel stimuli -Difference between tVNS and sham stimulation (P3 amplitude) in EEG parameters for easy targets associated with larger increase in sAA levels after tVNS
Rufener et al., 2018	tVNS Left cymba conchae	25Hz	0.5 mA	250 μs	30 s on/30 s off Started 90 min prior to task	Healthy young volunteers $n = 20$ avg age 24.8	-Acoustic oddball paradigm (respond as quickly as possible whenever a target tone was detected)	- tVNS increased EEG parameter P3 amplitude - Random noise stimulation reduced the reaction time
Maharjan et al., 2018	tVNS at left ear both anterior (cymba conchae) and posterior of ear	−80Hz −10Hz -No stim	10–15 mA	180 μs in square waveform	25–35 min lead in time	Healthy adult males $n = 18$	Two olfactory tests (odor threshold test (OTT) and supra-threshold test (STT)	High frequency (80Hz) VNS positively modulated olfactory performance in healthy participants and showed significant increase in NIRS recordings of the right hemispheric orbitofrontal cortex
Colzato et al., 2018	tVNS left concha $n = 40$ sham left earlobe $n = 40$	25Hz	0.5 mA	200–300 μs	15 min lead in time	Healthy young volunteers $n = 80$ (50 females, 30 males, mean age 20.96)	Convergent and divergent thinking tasks	-Fluency scores were significantly higher in the active tVNS group (able to generate more answers) -tVNS affected cognitive flexibility, i.e., participants could think of more different categories than sham
Jongkees et al., 2018	tVNS left medial acoustic meatus	25Hz	0.5 mA	200–300 μs	30 s blocks 15 min lead in time	Healthy young adult volunteers $n = 40$	Serial reaction time test	Enhanced response selection process and action control performance

(Continued)

TABLE 2 | (Continued)

COGNITION AND VNS: Healthy volunteers

		Stimulation Parameters						
Study	**iVNS/tVNS**	**Hz**	**mA**	**Pulse width**	**Time**	**Population**	**Task**	**Outcome**
Burger et al., 2019	tVNS Left cymba conchae	25Hz	0.5 mA	250 μs	30 s on/30 s off 10 min lead in time	Healthy young volunteers $n = 61$	Computerized fear conditioning, fear generalization, and fear extinction paradigm	No difference in physiological and declarative indices of fear between tVNS and sham conditions
Mertens et al., 2020	tVNS cymba conchae	25Hz	0.5 mA for 16 0.54–0.57 mA for rest	250 μs	30 s during consolidation	Healthy volunteers $-n = 41$ age avg 22.2 $-n = 24$ age avg 55.1	Word recognition task	No effect on verbal word memory
Giraudier et al., 2020	tVNS left cymba conchae (active) or left earlobe (sham)	25Hz	Active 1.48 mA ± 0.59 sham 1.31 mA ± 0.5	200–300 μs	30 s on/30 s off Stimulated for 23 min 5 min before 13 min during and 5 in after lexical decision task	Healthy volunteers $-n = 60$ $-46 =$ female -avg age 23.45	Lexical decision task and recognition memory task of selected German words (either emotionally charged or neutral) Also – BP, HR and sAA	Overall no effect of tVNS on task performance or word recognition memory – however higher recollection based memory performance was observed during tVNS than sham
Borges et al., 2020	tVNS	25Hz	2.19 mA (±0.93)	200–300 μs	30 s on/30 s off 4 min lead in time	Healthy adult volunteers $n = 35$	-Modified Flanker test -Spatial Stroop task -Number/Letter task -Dimensional change card sorting task	Only the DCCS shows improvement with tVNS
Keute et al., 2020	tVNS left cymba conchae	25Hz	2.37 mA (±0.16)	200 μs	30 s on/30 s off 30 min lead in time	Healthy adult volunteers $n = 22$	Stop Change paradigm (go-no-go task)	Globally enhanced accuracy across conditions -Reduced the performance costs of go/change response conflicts -increased attention
Sun et al., 2021 Study 1	tVNS Left cymba conchae Sham: no stimulation	25Hz	Online 0.7 ± 0.36 mA Offline 0.69 ± 0.38 mA Sham 0.73 ± 0.27 mA	500 μs	30 s on and 30 s off 25 min pre task (offline) or 15 min during task (online)	Healthy young volunteer $n = 46$ (25 female, average age 20.39 ± 1.96)	Spatial stimuli task Four blocks with 72 experiment trials in each block	Offline (pre-task stim for 25 min) tVNS significantly increased hits in spatial 3-back task but not rejections or reaction times
Sun et al., 2021 Study 2	tVNS left cymba conchae sham; active stimulation of earlobe	25 Hz	Active: 0.74 mA ± 0.37 Sham: 0.84 mA ± 0.39	500 μs	30 s on and 30 s off Both 25 min stimulation	Healthy young volunteers $n = 58$ (24 female, average age 19.9 ± 1.49)	Spatial stimuli task Four blocks with 72 experiment trials in each block	Offline (pre- task stimulation for 25 min) tVNS improved hits but not correct rejections or reaction time of accurate trials in spatial WM performance
Kaan et al., 2021	tVNS Left tragus	25Hz	1–6 mA	250 μs		Healthy young volunteers $n = 33$ control $n = 29$ experiment	-Word retention: – non-rhyming, easily separable words -rhyming words	tVNS was associated with higher accuracy but only when the items are phonologically similar
McIntire et al., 2021	Cervical VNS *via* gammaCore device cVNS vs. sham ($n = 20$ both groups)	25Hz	Not available	Not available	2 min cycles	Healthy young military recruits $n = 40$ (M:F 33:7) avg age 28 ± 6 years	34 h of continuous sleep deprivation Air Force–Multi-Attribute Task Battery (AF-MATB); simultaneously monitor and respond to four separate cognitive process tasks: a visual system alert monitoring task, a visual–motor tracking task, an auditory communication monitoring task and a management task	cVNS significantly improved objective arousal and multitasking for as long as 24-h post-stimulation Subjective ratings of fatigue also improved

invasive VNS (iVNS). A further potential confounder is the impact some of these underlying pathologies have on cognition, the altered medial temporal anatomy especially in cases of epilepsy and the medications used to manage these conditions can also have deleterious effects on cognition.

VAGUS NERVE STIMULATION AND COGNITIVE CONTROL, i.e., EXECUTIVE FUNCTION IN CLINICAL POPULATIONS

Vagus nerve stimulation has been shown experimentally to have mixed results when examining the subdomain of decision making, specifically on the Iowa Gambling Task (IGT). In one paradigm eleven patients with refractory epilepsy and iVNS devices completed a gambling task involving control and experimental trials with active VNS synchronized to stimulate in the latter. Whilst improved performance was demonstrated in the earlier part of the task, this trend was reversed later in the experimental trial with active stimulation trending toward being detrimental to performance (Martin et al., 2004). Technical failure and a cumulative stimulation-dose effect were amongst the potential explanations proposed by the authors to explain this phenomenon. Decision-making may depend on intact working memory (Bechara and Martin, 2004) and several studies have demonstrated working memory involvement in the IGT (Bagneux et al., 2013) which may have affected results in this study.

Working memory refers to a cognitive process that provides temporary storage and manipulation of the information necessary for complex cognitive tasks (Baddeley, 2010). Literature concerning the impact of acutely administered VNS on working memory is promising but limited to a small number of studies. In one experimental paradigm, twenty participants with poorly controlled epilepsy were required to perform a computer-based Executive-Reaction Time (Executive RT) Test, wherein ability to memorize and store the orientation of a triangle and indicate its position in response to a go signal were assessed whilst VNS was delivered in a cyclic fashion. Active iVNS stimulation was associated with fewer errors in the subtask relying on working memory (Sun et al., 2017).

The effect of active iVNS on response inhibition was also assessed by employing a classic stop-signal task in participants with refractory epilepsy (Schevernels et al., 2016). Quicker response inhibition has been demonstrated during active stimulation in patients who had previously shown a larger therapeutic effect of VNS. The beneficial effects of VNS on cognitive control may be maximally demonstrated in so-called 'VNS responders' (for the primary clinical indication) as demonstrated by patients with iVNS devices who undertook the Eriksen Flanker task during both VNS 'on' and 'off' stimulation. Only those deemed VNS responders (i.e., those whose seizure frequency had decreased by >50% post-device implantation) had demonstrable improved reaction times and reduced distractor interference during active stimulation (van Bochove et al., 2018). There is a subcategory of patients with refractory epilepsy who do not respond to iVNS therapy, i.e., do not have seizure reduction of 50%, and deemed "non-responders." It is notable that a current output of 2.28 mA was utilized in the VNS "responder" group and it's possible that, in keeping with previous studies examining optimal amplitude for stimulation, that the higher amplitudes employed exceeded that at which cognitive control is optimized for the iVNS "non-responders." Further research is needed in this area in particular regarding stimulation parameters and iVNS responders.

VAGUS NERVE STIMULATION AND LANGUAGE IN CLINICAL POPULATIONS

In the first study of its kind, building on previous preclinical research, the impact of iVNS on word retrieval memory was assessed *via* an experimental protocol whereby participants with iVNS devices inserted for epilepsy control, were required to read a series of paragraphs, and subsequently identify words that were highlighted in the text. The study population comprised two groups of patients who were administered active (0.5–1.5 mA) or sham VNS, delivered 2-min after learning in the memory consolidation phase. An inverted U-shaped relationship was demonstrated regarding stimulus intensity and modulation of cognitive performance, with memory enhancing effects demonstrated only at moderate intensities, namely 0.5 mA (Clark et al., 1999). These results were in part corroborated by a subsequent study which employed higher stimulation intensities (>1.0 mA) and failed to demonstrate enhancement of verbal recognition memory, in fact demonstrating a reversible deterioration in figural memory (Helmstaedter et al., 2001). However, study design may have impacted cognitive outcomes here as delivery of stimulation was not restricted to the consolidation period. The propensity for iVNS to positively impact word retrieval memory in a population of patients being treated with iVNS for intractable epilepsy was highlighted again in 2006 whereby the impact of iVNS on performance in the Hopkins Verbal Learning Test was assessed, demonstrating a significant improvement in word retention when active (amplitude 0.5 mA) as opposed to sham stimulation was applied during memory consolidation (Ghacibeh et al., 2006b).

VAGUS NERVE STIMULATION AND EMOTIONAL RECOGNITION IN CLINICAL POPULATIONS

The effect of t-VNS on participants' ability to recognize facial emotions in three experimental paradigms (graded presentation, static images and in a go-no-go task) was assessed in a group of adolescents diagnosed with major depressive disorder (MDD). In non-depressed controls t-VNS delivered at 1Hz, 0.5 mA 30 s block with 15 min lead in time, demonstrated enhanced recognition of emotions but notably led to a significant decrease in the ability of those with MDD to recognize sad emotions (Koenig et al., 2021).

TABLE 3 | VNS and cognition in clinical populations.

COGNITION AND VNS: Clinical Populations

		Stimulation Parameters						
Study	**iVNS/tVNS**	**Hz**	**mA**	**Pulse width**	**Time**	**Population**	**Task**	**Outcome**
Clark et al., 1999	iVNS 2–24/52 post implantation	30Hz	−0.5 mA −0.75–1.5 mA	0.5 ms	30 s	Intractable epilepsy $n = 10$	Word recognition task	Improved word recognition memory only when 0.5 mA delivered post reading
Sjögren et al., 2002	iVNS Assessed at 3 and 6 months	20Hz	0.25 mA, increased 0.25 mA increments over 2 weeks then fixed	500 μs	30 s followed by 5 min pause	Probable Alzheimer's $n = 10$ age 67 ± 7.6 8 women 2 men	Median change in ADAS-cog Median change in MMSE after 3 and 6/12 Depression, behavior and QOL variables	After 6/12 8 of 10 patients showed improvement from 3/12 ADAS-cog scores After 6/12 7 of 10 patients improved MMSE score by average 2.5 points No change in other variables
Martin et al., 2004	iVNS	30Hz	0.5 mA	500 μs	60 s	Intractable epilepsy $n = 11$	Iowa Gambling Task	Conflicting results, deleterious at higher doses
Merrill et al., 2006	iVNS At least 1 year of VNS treatment	20Hz	0.25 mA, increased in 0.25 mA increments over 2 weeks then fixed	500 μs	30 s followed by 5 min pause	Probable Alzheimer's $n = 17$ (age 63 range 57–81) 11 women 6 men	Median change in ADAS-cog Median change in MMSE after 1 year Depression, behavior and QOL variables	At 1 year, 41% had improvement or no decline from baseline on ADAS-cog 70% had improvement or no decline on MMSE No change in other variables
Helmstaedter et al., 2001	iVNS 5–7/12 post implantation	30Hz	Mean 1.75 mA (range 1–2.5)	500 μs	30 s–4.5 min	Intractable Epilepsy $n = 11$	Word recognition task Design recognition task	Deterioration in figural recognition memory
Dodrill and Morris, 2001	iVNS 12–16/52 after implantation	30 Hz in high stim group 1 Hz in low stim group	Avg 1.3 mA in high simulation group Avg 1.2 mA in low stimulation group	500 μs 130 μs	30 s on every 5 min 30 s on every 3 h	Intractable Epilepsy $n = 160$	Wonderlic personell test, Stroop test, Digit cancelation, Symbol Digit Modalities	No significant changes were noted in the cognitive tests in low or high stimulation
Ghacibeh et al., 2006b	iVNS >3/12 post implantation	X	0.5 mA	x	30 s	Intractable Epilepsy $n = 10$	Hopkins verbal learning test	Improved retention index
McGlone et al., 2008	iVNS 12/12 post implantation	30 Hz	0.5–3 mA avg 1.72 ± 0.53	500 μs	30 s every 5 min	Intractable epilepsy $n = 16$	Memory Observation Questionnaire	Improved subjective and objective memory scores compared to baseline, but similar to medical management
Schevernels et al., 2016	iVNS >18/12 post implantation	Avg 25 (20–30)	Avg 2.3 mA (0.75–3.0)	Avg 431 μs (130–500 μs)	7 s on/ 18 s off	Intractable epilepsy $n = 20$	Stop signal task	VNS responders demonstrated quicker response inhibition
Sun et al., 2017	iVNS 2–130 months post implantation	30Hz	1.5–1.75 mA	250 μs	30 s on/48 s off	Intractable epilepsy $n = 20$	Executive reaction time test (go-no-go task)	Improved working memory (only when 3 participants with cognitive impairment removed)
van Bochove et al., 2018	iVNS	20 or 30 Hz	Avg 2.28 mA (0.75–3.0)	250 μs or 500 μs	7 s on/ 18 s off	Intractable epilepsy $n = 17$	Eriksen Flanker task	VNS responders demonstrated improved reaction times and decreased distraction interference
Koenig et al., 2021	tVNS Left conchae	1Hz	0.5 mA	250 μs	30 s on/30 s off 15 min lead in time	-Adolescents with major depressive disorder $n = 33$ control group: adolescents with headache $n = 30$	Facial emotional recognition in three tests 1. As a graded presentation 2. As static images 3. in a go – no -go task	-In non-depressed controls tVNS enhances the general ability to recognize emotions -tVNS specifically led to a decrease in the recognition of sad emotions in patients with MDD

TABLE 4 | VNS and neurocardiovascular assessment.

Neurocardiovascular assessment AND VNS

		VNS Stimulation Parameters						
Study	**iVNS/tVNS/site specific**	**Hz**	**mA**	**Pulse width**	**Time**	**Analysis parameters**	**Population**	**Result**
Kamath et al., 1992	iVNS for refractory epilepsy (left cervical vagus)	2 Hz 30 Hz	0.1 mA 1 mA	130 ms 500 ms	Not specified	Baseline 45 min ECG readings pre implantation and at 2/52 post implant	Refractory epilepsy $n = 8$ High stimulation and low stimulation groups avg age 34 ± 7.8 range 21–47	HiStim group: LF:HF ratio decreased from 2.5 ± 1.5 preimplant to 1.5 ± 0.49 ($P < 0.02$) with iVNS Significantly higher HF power in the HiStim compared to LoStim group
Setty et al., 1998	iVNS for refractory epilepsy (left cervical vagus) implanted for minimum 1/12	30 Hz	Max tolerated threshold	750 μs	30 s on 5 min off	Pre and post stimulation ECG (7 min baseline, 2.5 min of stimulation and a 7 min post-stimulation)	Refractory epilepsy $n = 10$ (avg age 28 range 14–46) 8 men	No significant effect noted on HRV variables
Handforth et al., 1998	iVNS for refractory epilepsy (left cervical vagus)	30Hz in high stimulation group I Hz in low stimulation group	Avg 1.3 mA in high simulation group Avg 1.2 mA in low stimulation group	500 μs 130 μs	30 s on every 5 min 30 s on every 3 h	Study mainly aimed at seizure reduction in two groups (high vs. low stimulation) in refractory epilepsy	Refractory epilepsy High stimulation group $n = 95$ age 32.1 ± 10.8 Low stimulation $n = 103$ age 34.2 ± 10.1	"Autonomic function assessments revealed no significant changes in Holter function measures; mean heart rate, mean lowest or highest heart rate, heart rate variability, occurrences of bradycardia"
Galli et al., 2003	iVNS for refractory epilepsy (left cervical vagus)	30 Hz	0.25 mA adjusted	500 μs	30 s on every 5 min	24-h analysis of RR variability at baseline (t0), 1 month (t1, short-term VNS) and 36 months after VNS initiation (t2, long-term VNS).	Refractory epilepsy $n = 7$ (4 men) age 47 ± 11.2 range 34–63 f	No significant changes in HRV variables, trend to increased HF at night-time
Ronkainen et al., 2006	iVNS for refractory epilepsy (left cervical vagus)	30 Hz	2.9 mA avg	500 ms	30 s on 5 min off	Pre and 1 year post implantation 24 h Holter HRV variables	Refractory epilepsy $n = 14$ (eight male and six female age 34.3 ± 9.3; 20–52) compared to matched controls	VNS had no significant effects on any HRV indices despite a significant reduction in seizure frequency
Barone et al., 2007	iVNS for refractory epilepsy (left cervical vagus)	30 Hz	0.75–1.75 mA	500 μs	30 s on, 5 s off	24 h ECG holter at baseline and after 3/12 implantation	Refractory epilepsy 8 patients (age 32 range 9–65 2 men)	No significant change in HRV parameters after 3/12 iVNS
Sperling et al., 2010	iVNS (left cervical vagus) for treatment resistant depression (post implantation 6–40 months)	15–30Hz	0.25–2.5 mA	500 μs	30 s on 5 min off	ECG testing at baseline, switched on and switched off conditions	Patients with major depressive disorder (ICD-10) $n = 9$ (51.6 years, 5 women, 4 men) Compared to age and sex matched controls	RMSSD increased significantly in switched on conditions during stimulation (30 s) in six patients compared to stimulation-free intervals and baseline
Clancy et al., 2014	tVNS on inner and outer surface of the tragus of the ear Sham – on tragus but disconnected Either active or sham tVNS	30Hz	10–50 mA	200 μs	Continuous 15 min stimulation	HRV frequency and spectral analysis Muscle sympathetic nerve activity (MSNA) recordings	Healthy volunteers $n = 48$ age 20–62 years old (M:F 1:1)	Significant decrease in LF/HF ratio during active tVNS Greater response to tVNS in those who had higher sympathetic predominance at baseline (higher LF/HF ratio)

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TABLE 4 | (Continued)

Neurocardiovascular assessment AND VNS

		VNS Stimulation Parameters						
Study	**iVNS/tVNS/site specific**	**Hz**	**mA**	**Pulse width**	**Time**	**Analysis parameters**	**Population**	**Result**
de Couck et al., 2017 Study 1	tVNS cymba conchae left or right ear vs. sham (earlobe)	25Hz	0.7 mA average	250 μs	30 s on/30 s off 10 min	HRV frequency and spectral analysis	Healthy older volunteer $n = 30$ age 23–58	Right stimulation alone significantly increased SDNN compared to baseline
de Couck et al., 2017 Study 2	tVNS cymba conchae right ear	25Hz	1 mA average	250 μs	30 s on/30 s off 1 h	HRV frequency and spectral analysis	Healthy older volunteer $n = 30$ age range 30–65	SDNN significantly increased after 35 min and after 1 h specifically in female participants LF and LF/HF significantly increased after 35 min of stimulation
Antonino et al., 2017	tVNS active – tragus- inner and outer surface sham ear lobe Electrodes placed bilaterally (1) active tVNS (2) sham- olunteer placed on tragus –no current (3) olunteer placed on the earlobe current applied	30 Hz	45 ± 1 mA	200 μs	Continuous 15 min	HRV, BP variability, cBRS	Healthy young male olunteer $n = 13$ age = 23 ± 1	Active tVNS acutely improved spontaneous cBRS, olunteer LF/HF ratio and evoked slight decrease in HR Nil change with two sham conditions
Lamb et al., 2017	tVNS left tragus/auditory meatus or sham (no current)	20Hz	5.6 mA range 3–11.3 mA	100 μs	unavailable	Postural HRV *via* Tilt Table Test Startle Blink Paradigm	Military veterans with PTSD and mild TBI $n = 12$ or healthy control $n = 10$ age 30 ± 7	Significantly increased RSA (HF HRV) in tilt during tVNS Trend toward reduced reactivity (*via* electrodermal response monitoring) to startle
Badran et al., 2018b Study 1	tVNS to the inner side of the left tragus (anode in the ear canal, cathode on the surface of the tragus) of the left ear for 9 different stimulation rounds sham = left earlobe crossover design	1Hz 10 Hz 25 Hz	At 100 μs: tragus 9.28 ± 2.56 mA earlobe 6.5 ± 1.83 mA At 200 μs tragus 5.32 ± 1.60 mA earlobe 3.64 ± 1.26 mA At 500 μs tragus 3.0 ± 0.93 mA earlobe 1.97 ± 0.70 mA	100 μs 200 μs 500 μs	Stimulation period (60s) recovery period (180s)	Heart rate analysis	Healthy young adult olunteer $n = 15$ (M:F 1:1) age 26.5 ± 4.9	Active stimulation olunteer HR more than control stimulation on with these parameters: 500 μs at 25 Hz 500 μs at 10 Hz
Badran et al., 2018b Study 2	tVNS to the inner side of the left tragus (anode in the ear canal, cathode on the surface of the tragus) of the left ear for 10 stimulation rounds sham = left earlobe crossover design	10 Hz 25 Hz	tragus- 2.09 ± 0.97 mA earlobe 2.04 ± 0.82 mA	500 μs	Stimulation period (60s) recovery period (90s)	Heart rate analysis	Healthy young adult olunteer $n = 20$ (M:F 1:1)	The parameters 500 ms at 10 Hz alone induced a significant decrease in HR
Bretherton et al., 2019 Study 1	tVNS left tragus 1 week later sham (electrodes on tragus but no current)	30Hz	2–4 mA	200 μs	15 min	Baroreceptor sensitivity §	Healthy participants aged ≥55 years $n = 14$ Age 69.11 ± 1.52	Baseline LF/HF ratio power significantly predicted response to tVNS where higher resting LF/HF ratio was associated with greater olunteer during tVNS

(Continued)

TABLE 4 | (Continued)

Neurocardiovascular assessment AND VNS

		VNS Stimulation Parameters						
Study	**iVNS/tVNS/site specific**	**Hz**	**mA**	**Pulse width**	**Time**	**Analysis parameters**	**Population**	**Result**
Bretherton et al., 2019 Study 2	tVNS left tragus no sham	30 Hz	2–4 mA	200 μs	15 min	Baroreceptor sensitivity, HRV frequency and spectral analysis	Healthy participants aged ≥55 years $n = 51$ Age 65.20 ± 0.79	Total power, mean RR interval, Δ RR, SDRR were significantly affected during tVNS A higher LF/HF ratio predicted a greater decrease to tVNS
Bretherton et al., 2019 Study 3	tVNS left tragus daily at home for 15 min for 2 weeks	30Hz	2–4 mA	200 μs	15 min daily for 14 days	HRV frequency and spectral analysis	Healthy participants aged ≥55 years $n = 29$ Age 64.14 ± 0.89	RMSSD, pRR50, SD1 and nSD1, were significantly higher after 2 weeks tVNS
Tobaldini et al., 2019	tVNS left cymba conchae Cross-over design 2-day protocol, 1 day with tVNS and a control day, at least 24 h difference	25Hz	1–6 mA adjusted to sensory threshold	200 μs	10 min supine stimulator on (rest tVNS on), 15 min orthostatic position with tVNS on (tilt tVNS on)	(1) ECG (2) Respiration (3) Non-invasive beat-to-beat arterial blood pressure at rest and during a 75° tilt test	Healthy young olunteer $n = 13$ (5 males, 8 females) age 27 ± 4 years	Clinostasis: tVNS reduced HR, systolic BP variability and cardiac and peripheral sympathetic modulation Responsivity of HR and BP to orthostatic stress during tVNS was significantly higher when compared to control
Borges et al., 2019 Study 1	tVNS to left cymba conchae	25Hz	0.5, 1, and 1.5 mA	200–300 μs	30 s on/off cycling 10 min stimulation	RMSSD	Healthy young olunteer $n = 61$ (16 female) avg age 23.32	Increase in RMSSD during stimulation compared to the resting phases for all mA settings
Borges et al., 2019 Study 2	tVNS to left cymba conchae	25Hz	1 mA Compared to 1.78 mA ± 1.13	200–300 μs	30 s on/off cycling 10 min stimulation	RMSSD	Healthy young olunteer $n = 62$ (26 females avg age 24.77)	RMSSD values showed a significant overall increase during the stimulation phase none of the different stimulation conditions significantly differed from each other regarding RMSSD values
Borges et al., 2019 Study 3	tVNS to left cymba conchae vs. sham (earlobe)	25 Hz	Active 2.5 mA ± 0.93) Sham 2.76 mA ± 1.01	200–300 μs	30 s on/off cycling 10 min stimulation each	RMSSD	Healthy young volunteers $n = 60$ (31 females, age avg 23.62)	No difference between active and sham stimulation
Sclocco et al., 2019	tVNS (1) to cymba conchae no current (2) to cymba conchae active during exhalation (3) to cymba conchae active during inhalation (4) sham to earlobe	25 Hz	(1) 1.6 mA ± 2.3 (2) 1.7 mA ± 2.4 (3) 1.4 mA ± 1.1	450 ms pulse width duration of 1 s	32 min	-Instantaneous HF-HRV index -four 8-min duration fMRI scans (1) passive control (2) active stimulation exhalation (3) active stimulation inhalation (4) active control	Healthy adult participants $n = 16$ (9 female, age 27.0 ± 6.6)	Exhalation tVNS but not inhalation enhanced cardiovagal modulation, i.e., increased instantaneous HF hRV index Exhalation found significantly signal at MRI site of LC/NTS
Gauthey et al., 2020	tVNS Cymba Crossover design	5Hz 20Hz active 5Hz sham	1.5 ± 0. mA 1.2 ± 0. mA 5.5 ± 1. mA	0.2 ms	10 min stimulation 10 min washout	Muscle sympathetic nerve activity (MSNA) recorded by microneurography at rest, during apnoea and tVNS HRV power and spectral analysis	Healthy, young male volunteers $n = 28$ (age 27 ± 4)	Acute right cymba tVNS did not induce any effects on HRV nor MSNA variables when compared to active control

(Continued)

TABLE 4 | (Continued)

Neurocardiovascular assessment AND VNS								
		VNS Stimulation Parameters						
Study	**iVNS/tVNS/site specific**	**Hz**	**mA**	**Pulse width**	**Time**	**Analysis parameters**	**Population**	**Result**
Machetanz et al., 2021	tVNS right $n = 7$ left $n = 6$ -cymba conchae -cavum conchae -outer tragus -inner tragus -crus helicis -fossa triangularis	25 Hz at a periodicity of 1 Hz	0.2–2 mA 0.096–0.769 mA 0.05–0.4 mA	100 μs 260 μs 260 μs	90 s (i.e., 3 s × 30 s) at each stimulation site 144 parameter combinations	HRV power and spectral analysis	Healthy adults $n = 13$ (age 24 ± 3, 8 female)	Significant differences between right- and left-sided stimulation for the SDNN and RMSSD analysis only (increasing with right ear stimulation) HRV increases were highest at cymba conchae and fossa triangularis, to a lesser extent to stimulation at the inner tragus
Sinkovec et al., 2021	tVNS to right tragus during rest (60 min) and autonomic nervous system testing (15 min) (Valsalva, wet cold face, etc.) sham = no stimulation, preceded stimulation	20Hz	Adjusted individually to barely perceptible <150 μA	1 ms rectangular pulse width	1 h resting tVNS vs. sham 15 min ANST vs. sham	Continuous cardiac measurements with impedance cardiography Non-invasive arterial BP monitor ECG for HRV analysis	Healthy male volunteers $n = 15$ (age 23 range 20–25)	Indices of LV contractility, LV output, and LV work significantly decreased SBP and TPR significantly increased No difference HRV or ANST parameters

See **Table 3** for parameters settings and outcomes in trials of VNS in clinical populations and please see below "VNS, cognition and HRV" for a discussion of VNS in Alzheimer's disease.

LINKING BRAIN AND HEART: POTENTIAL MECHANISMS OF ACTION OF VAGUS NERVE STIMULATION-MEDIATED COGNITIVE ENHANCEMENT

There are many potential mechanisms through which VNS may exert its cognitive enhancing effects, including direct neurotransmitter release, increased cerebral perfusion to discreet neuroanatomical structures, reduced neuro-inflammation and *via* modulation of peripheral hemodynamics. For the purposes of this narrative review, we will analyze the link between cerebral blood flow, cerebral autoregulation and cardiac modulation. Beyond the scope of this review is how t-VNS may therapeutically affect the inflammatory cascade *via* activating the cholinergic anti-inflammatory pathway and the beneficial effects this may have in aging populations.

MECHANISM OF ACTION: VAGUS NERVE STIMULATION AND LOCAL NEUROTRANSMITTER RELEASE

The main neurotransmitters centrally released *via* the afferent projections of the vagus nerve are thought to be GABA and Norepinephrine (NE). For a comprehensive review of the preclinical and clinical studies detailing the evidence supporting the modulation of these neurotransmitters during iVNS and t-VNS see (Colzato and Beste, 2020).

As the primary inhibitory neurotransmitter in the brain, higher levels of GABA decrease cortical excitability, and is the accepted proposed method for VNS' anti-seizure efficacy. It has been suggested that increased cortical inhibition due to high GABA levels can sharpen task-relevant representations in the cortex and inhibit competing responses, thereby facilitating response selection and inhibition processes (Munakata et al., 2011; de la Vega et al., 2014).

Norepinephrine is a crucial neurotransmitter modulating arousal and attention, and is primarily released *via* the locus coeruleus (LC). There are two distinct modes of LC firing that are associated with equally distinct modes of attentional strategy. Connections with the orbitofrontal cortex and anterior cingulate cortex are thought to drive the LC-NE system into one of these two stable states of activity, a high tonic (sustained) mode or a phasic (bursting) mode accompanied by moderate tonic activity (Aston-Jones and Cohen, 2005). This switching of attentional state *via* tonic LC activity is thought to result in a flexible attentional system that allows cycling between behaviors to find and meet task demands in one's environment, i.e., the adaptive gain theory (Aston-Jones and Cohen, 2005).

Interestingly, and similar to the effects noted with iVNS stimulation levels and responses by Clark et al. (1995), moderate levels of NE augment prefrontal cortex function, whereas high and low concentrations of NE impair function, i.e., NE exhibits an inverted-U relationship between LC-NE activity and optimal performance on attention tasks (Berridge and Waterhouse, 2003). However, in general as NE levels rise executive function improves, likely *via* enhanced activation of the prefrontal cortex and frontoparietal control network (Xing et al., 2016; Unsworth and Robison, 2017). Inhibitory control for action cancelation is specifically enhanced with noradrenergic modulation, likely *via* this prefrontal cortical network (Chambers et al., 2009; Duann et al., 2009).

Older adults with more dense LC innervation (i.e., higher neuromelanin MRI contrast) had overall better performance on a reversal memory tasks (Hämmerer et al., 2018) and had improved cognitive reserve (Clewett et al., 2016). Similarly in a post-mortem study of patients with Alzheimer's disease, lower LC cell integrity and greater cortical tangle density was associated with greater tau burden beyond the medial temporal lobes and worsening memory decline, identifying LC integrity as a promising indicator of initial AD-related processes (Jacobs et al., 2021).

Studies have also demonstrated a decline in GABA concentration in frontal and parietal regions in aging populations, areas crucial for cognitive control (Gao et al., 2013; Porges et al., 2017). NE and GABA may in fact work synergistically to facilitate executive functioning; GABA by encouraging response inhibition of task irrelevant stimuli and NE *via* the LC-NE system increasing frontal NE release and thus executive functioning (Ridgewell et al., 2021).

MECHANISM OF ACTION: VAGUS NERVE STIMULATION INCREASES CEREBRAL PERFUSION

Cerebral autoregulation is the phenomenon by which the brain receives the same cerebral blood flow (CBF) despite variations in perfusion pressure. The aim of autoregulation is to protect the brain against hypoxia and edema as a result of decreased or critically high arterial blood pressures respectively. Multiple factors physiologically modify autoregulation including blood CO2 levels, hypoxia etc. While still controversial, the ANS may play a prominent role in cerebral autoregulation in response to such stimuli, inducing vasodilation or constriction, and parasympathetic and sympathetic nerves are anatomically located in the same perineural sheath innervating cerebral arteries (Tamayo and Siepmann, 2021). The means by which VNS exerts its cognitive enhancing effect is probably multimodal, however modulating CBF is likely a crucial factor.

Multiple modalities have been utilized to assess for CBF changes due to vagus nerve stimulation, including position emission tomography (PET), functional magnetic resonance imaging (fMRI) and single photon emission computed tomography (SPECT) studies and trials of patients with iVNS treatment for epilepsy and depression have demonstrated a variety of CBF modulatory effects at specific cortical and subcortical areas. Increased CBF at the orbitofrontal cortex (Henry et al., 1998; Bohning et al., 2001; Lomarev et al., 2002; Mu et al., 2004; Vonck et al., 2008), temporal lobe (Ko et al., 1996; Lomarev et al., 2002; Liu et al., 2003; Vonck et al., 2008; Conway et al., 2012), insular cortex (Liu and Hu, 1988; Henry et al., 1998, 2004), bilateral frontal lobes (Sucholeiki et al., 2002), left dorsolateral prefrontal cortex (Kosel et al., 2011) and subcortical structures including thalamus, hypothalamus, basal ganglia and other nuclei (Narayanan et al., 2002; Sucholeiki et al., 2002; Conway et al., 2012) has been observed. For a comprehensive review see Chae et al. (2003).

Notably analysis undertaken during acute iVNS has noted bilateral decreased hippocampal CBF (Henry et al., 1998; Mu et al., 2004; Vonck et al., 2008). This has been replicated in t-VNS functional imaging studies which have confirmed stimulation and increased CBF at vagally innervated brain regions during auricular t-VNS and notably decreased perfusion at hippocampal regions (Kraus et al., 2007, 2013; Frangos and Komisaruk, 2017). T-VNS has also demonstrated efficacy in increasing arousal in comatose patients who respond to auditory signaling and again the brain regions noted on fMRI to be activated were similar to previous iVNS studies, including left superior temporal gyrus, left prefrontal cortex, left insular cortex, left middle frontal gyrus among other cortical and subcortical structures (Yu et al., 2021).

It is worth considering that intermittently stimulating neurons at different frequencies produces drastically different changes in neuronal behavior with low frequency stimulation inducing long term depression (LTD) and less connectivity while intermittent high frequency stimulation produces long term potentiation (LTP) and increased signaling (Lomarev et al., 2002; Kealy and Commins, 2010). Therefore acute VNS stimulates brain regions mostly involved in alertness and frontal processing, whereas chronic stimulation may improve LTP in classic memory-associated regions, including the hippocampus. Evidence for this can be seen in preclinical studies (Zuo et al., 2007) but also significant increases in hippocampal gray matter volume over time has been observed in patients with iVNS devices inserted for treatment-resistant depression (Perini et al., 2017). More recently, Near Infrared Spectroscopy (NIRS) has been utilized to monitor cerebral blood flow and increased frontal perfusion in patients with epilepsy was noted during iVNS when paired with a cognitive task (Kunii et al., 2021).

Both dementia and even its prodromal stage, MCI, are characterized by a reduction in cerebral blood flow (Mazza et al., 2011; Sierra-Marcos, 2017). A meta-analysis of twenty-six studies investigating CBF in MCI found overall reduced tissue oxygenation, CBF and velocity in MCI compared to healthy controls (Beishon et al., 2017) and studies are underway investigating the CBF changes that may occur with cognitive stimulation in MCI and dementia (Beishon et al., 2019). Similar findings have been noted in patients with Alzheimer's disease, with reduced CBF in many cortical regions including temporal (Sandson et al., 1996; Alsop et al., 2000; Asllani et al., 2008; Yoshiura et al., 2009; Ding et al., 2014) parietal (Alsop et al., 2000; Johnson et al., 2005) and other regions including precuneus,

frontal and posterior cingulate cortex (Alsop et al., 2008; Yoshiura et al., 2009).

MECHANISM OF ACTION: VAGUS NERVE STIMULATION MODULATES PERIPHERAL HEMODYNAMICS

As well as modulating central neurotransmitter release and cerebral blood flow, VNS has been shown to have positive peripheral modulatory effects in pathological states characterized by impaired autonomic regulation including postural orthostatic tachycardia syndrome (POTS) (Petelin Gadze et al., 2018) specifically patients with POTS and impaired vagal cardiac control, as defined by reduced HRV (Jacob et al., 2019). T-VNS has also shown benefits in modulating blood pressure in induced orthostatic hypotension (Tobaldini et al., 2019). These studies suggest VNS may have a role in positively manipulating the peripheral baroreceptor-reflex and thus cerebral autoregulation, and potentially may improve cortical perfusion *via* this route, however further dedicated studies are required to precisely delineate this relationship.

VAGUS NERVE STIMULATION AND HEART RATE VARIABILITY

Heart rate variability analysis can be performed *via* a variety of approaches and is based on the extrapolation of time intervals between each R wave peak (Shaffer and Ginsberg, 2017), discounting any ectopic beats or arrhythmias, e.g., atrial fibrillation. The most commonly applied methods to determine HRV are time-domain analysis and frequency/spectral analysis. Indices deriving from the time domain analysis quantify the amount of variance in the selected inter-beat interval employing statistical measures, such as the standard deviation of the normal beat intervals (SDNN) and the root mean square of successive differences between normal beats (RMSSD) (Shaffer et al., 2014). The spectral analysis of HRV identifies oscillatory rhythms that occur in specific frequency ranges. Three main components of the spectrums can be identified as: the very low frequency band (VLF), below 0.04 Hz, likely influenced by thermoregulatory mechanisms and circadian rhythms; the low-frequency band (LF) between 0.04 and 0.15 Hz in humans, a marker influenced by baroreflex (Furlan et al., 2019) sympathetic and parasympathetic modulation; the high-frequency band (HF) in the range from 0.15 to 0.4 Hz, a marker of vagal modulation that is influenced by respiratory activity (Montano et al., 2009; Shaffer et al., 2014). One of the limitations of HRV analysis is high within and between individual variability, which may be reduced by longer measurement intervals, i.e., 24 h but which is resultantly harder to process. For a comprehensive review on the various indices please see Merrick et al. (2017).

The ANS influences cardiac beat-to-beat interval length in response to several factors. The sympathetic and parasympathetic systems are the principal rapidly reacting systems that control heart rate. The two systems have different latency periods with sympathetic effects on heart rate slower than parasympathetic (Warner and Cox, 1962; Pickering and Davies, 1973; Koizumi et al., 1983) i.e., the parasympathetic system has the ability to alter heart rate within 1–2 beats, while sympathetic effects take up to 10 s to take effect.

Low HRV has been associated with poorer prognosis in cardiovascular diseases, cancer, Metabolic Syndrome and Alzheimer's disease and it has been postulated that related pathophysiological mechanisms often contribute to their occurrence and progression, namely inflammatory responses, sympathetic overactivity, and oxidative stress (Entschladen et al., 2004; Thayer and Lane, 2007; de Couck et al., 2012). Lower vagal nerve activity has been found to be significantly correlated with oxidative stress (Tsutsumi et al., 2008), with inflammatory markers in healthy individuals as well as in those with cardiovascular diseases (Haensel et al., 2008) and anxiety disorders have also been characterized by low HRV (Chalmers et al., 2014). Experimental studies have long demonstrated the success of behavioral (Stein and Kleiger, 2003) and pharmacological (Sandrone et al., 1994) interventions in manipulating HRV. Increases in HRV seen with physical fitness training are associated with improvements in executive function (Hansen et al., 2004). The links between executive function and cardiac autonomic regulation were further highlighted by a recent study examining the impact of cognitive and motor training on HRV indices. Physical training alone failed to impact HRV in older adults whereas dual cognitive and motor training significantly improved global and parasympathetic autonomic nervous system activity (Eggenberger et al., 2020). These studies point toward a duality; the vagal communications between heart and mind can be bidirectionally manipulated to improve both parasympathetic control of HRV and, synergistically, executive cognitive function.

Preclinical research has noted that VNS, particularly to the right vagus nerve, increases vagally mediated (vm-) HRV measures (Huang et al., 2010; Sun et al., 2013). In a canine study, VNS treatment enhanced HRV at 4 and 8 weeks and reduced heart failure development (Zhang et al., 2009) and a Japanese study in rabbits founds that intermittent VNS, but not constant VNS, increased the HF (vagal) component of HRV (Iwao et al., 2000). Discrepancies in this preclinical work may be due to different species, devices and parameters but indicate that manipulating the vagus nerve electrically can have positive impacts on cardiac function and HRV.

VAGUS NERVE STIMULATION AND HEART RATE VARIABILITY IN HEALTHY VOLUNTEERS

Transcutaneous auricular vagus nerve stimulation devices and their stimulation effect on HRV have been examined in several experimental paradigms involving multiple auricular positions, left vs. right ear stimulation, and different stimulation settings. There is a trend toward positive findings, i.e., improved HRV indices, with t-VNS in healthy volunteer populations when the right auricular branch of the vagus is stimulated

(de Couck et al., 2017; Machetanz et al., 2021). It is notable that greater responses to t-VNS (i.e., improved vagally medicated HRV signals) have been demonstrated in those with higher sympathetic balance at baseline in both younger and older volunteers, both acutely and with 2 weeks t-VNS at home for 15 min daily (Clancy et al., 2014; Bretherton et al., 2019). An experimental design comparing left and right t-VNS at multiple stimulation targets found that SDNN and RMSSD both were most significantly improved when the right cymba conchae and fossa triangularis were stimulated (Machetanz et al., 2021).

When specific parameters of stimulation at the left tragus were sequentially analyzed, the settings that had the most significant impact on heart rate analysis in young volunteers were 500 μs at 10 Hz (Badran et al., 2018b). Studies investigating the effect of t-VNS and 70-degree tilt table testing on HRV at the left tragus found that the RSA measure of HRV (HF domain) was also significantly increased during an orthostatic maneuver (Lamb et al., 2017) and similarly stimulation at the left cymba conchae during 75-degree tilt found that responsivity, i.e., degree of change of heart rate and systolic blood pressure during t-VNS were significantly higher during orthostasis compared to control (Tobaldini et al., 2019).

Research in this area has not been consistent. Some initial findings indicated improved HRV measures with t-VNS to the left cymba conchae but ultimately no difference compared to sham and at multiple intensities (Borges et al., 2019). In an experimental crossover design employing a variety of amplitudes at the right cymba, there was no positive signal in affecting HRV measures (Gauthey et al., 2020) and similarly t-VNS to the right tragus during rest and autonomic nervous system testing, with appreciably different stimulation parameters to what was previously cited in the literature, also did not have any effect on HRV (Sinkovec et al., 2021). Inconsistent results are likely due to the use of different anatomical sites and stimulation parameters being utilized, some with "lead in" times and some without, and reporting on this area has been of variable quality, and recent international consensus has called for standardized reporting of this research (Farmer et al., 2021).

VAGUS NERVE STIMULATION AND HEART RATE VARIABILITY IN CLINICAL POPULATIONS

Initial studies in clinical populations involved patients with iVNS devices inserted for control of refractory epilepsy. The earliest study demonstrated a reduction in LF:HF ratio and significantly higher HF power was noted in the higher stimulation group than lower stimulation (see **Table 4**; Kamath et al., 1992). These results were not however replicated in further studies of similar populations with comparable stimulation settings at timeframes ranging from minutes to 1 year of stimulation (Handforth et al., 1998; Setty et al., 1998; Galli et al., 2003; Ronkainen et al., 2006; Barone et al., 2007). A small study analyzing HRV in patients with iVNS devices implanted for management of treatment-resistant depression noted an increase in the RMSSD (increased vagal predominance) during stimulation compared to baseline and healthy controls (Sperling et al., 2010). It is notable that iVNS devices are for the most part inserted to activate the vagus *via* its left cervical branch, thereby appropriately reducing adverse cardiac effects but also not demonstrably influencing HRV measures in these populations.

Please see **Table 4** for further analysis of the specific neurocardiovascular assessments, specific t-VNS parameters and outcomes measures in discreet populations in this area.

VAGUS NERVE STIMULATION, COGNITION AND HEART RATE VARIABILITY

Heart rate variability can be conceptualized as a biomarker of parasympathetic modulation, and it is associated with a network of brain regions involved in autonomic nervous system regulation, known as the central autonomic network (Benarroch, 1993; Thayer et al., 2009). This network, which comprises prefrontal cortical (anterior cingulate, insula, orbitofrontal, and ventromedial cortices), limbic (central nucleus of the amygdala, hypothalamus), and brainstem regions, areas of the brain intimately involved in emotional regulation and executive functioning, leading to the proposal that vagally mediated HRV may index these aspects of prefrontal cortical function (Thayer and Lane, 2007; Thayer et al., 2009). Higher HRV has been linked to better cognitive function in healthy adults including healthy older individuals (Frewen et al., 2013; Grässler et al., 2020) and a meta-analysis found a positive overall correlation ($r = 0.09$) between vagally mediated HRV indices and emotional regulation processes (including executive functioning, emotion regulation, and effortful or self-control) in mostly healthy participants across a number of age groups (Holzman and Bridgett, 2017).

Autonomic system dysfunction is common in patients with MCI, with studies suggesting MCI participants are 5.6 times more likely than controls to have autonomic dysfunction, specifically on assessment of HRV and cardiac reflexes (Collins et al., 2012). A meta-analysis of MCI with dementia also found autonomic dysfunction, as defined by reduced HRV, was significantly associated with cognitive impairment (da Silva et al., 2017). Reduced HRV is associated with worse performance on tests of global cognitive function, more than cardiovascular risk factors (Zeki Al Hazzouri et al., 2014).

Recent meta-analyses of HRV in patients with neurodegenerative conditions including MCI, Alzheimer's disease, Lewy Body dementia (DLB), vascular dementia, Parkinson's disease and multiple sclerosis found a significant, moderate effect ($r = 0.25$) indicating that higher HRV was related to better cognitive and behavioral scores, which was not influenced by mean age or cognitive status (Liu et al., 2022). These results were mirrored in a similar recent meta-analysis of patients with dementia compared to healthy controls, which found significantly lower resting HRV for parasympathetic function and total variability in those with dementia. On subgroup analysis then most striking differences, i.e., worse HRV analysis was found in those with MCI or DLB (Cheng et al., 2022).

Heart rate variability and CBF are linked *via* vagal afferents, and a meta-analysis revealed that HRV was significantly associated with regional cerebral blood flow in the ventromedial prefrontal cortex (including anterior cingulate regions) and the amygdala (Thayer et al., 2012). In both younger and older adults scanned while at rest, higher HRV is associated with higher medial prefrontal cortex and amygdala functional connectivity (Sakaki et al., 2016). The Neurovisceral Integration Model holds that HRV, executive cognitive function, and prefrontal neural function are integrally associated (Thayer et al., 2009).

In an interesting Swedish clinical trial in 2002, iVNS devices were implanted in a small group of patients with likely Alzheimer's Dementia (AD) as defined by the criteria of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA), with a view to assessing its impact on cognition *via* memory test scores. In the primary trial, 10 patients with average Mini Mental State Exam (MMSE) scores of 21 (range 16–24) had iVNS devices implanted and the median change in MMSE and Alzheimer's Disease Assessment Scale-cognitive subscale (ADAS-cog) scores among a battery of tests was assessed at 3 and 6 months, with improvements in both assessments noted in the majority (6 out of 10) of cases (Sjögren et al., 2002). The follow up trial by the same research group involved 17 patients with likely AD, who had iVNS devices implanted and had outcomes measured and available at 1 year post implantation. At 1 year, 7 of 17 (41%) had improvement or no decline from baseline in ADAS-cog scores and 12 of 17 (70%) had improvement or no decline in MMSE scores. There was no change in noted in other outcomes including depressive symptoms (Merrill et al., 2006). There are a small number of trials registered investigating the therapeutic potential of t-VNS in older populations, both healthy and with cognitive impairment (for a recent review see (Vargas-Caballero et al., 2022)) however there are no known published studies to date investigating t-VNS in populations with dementia or MCI, and the associated effect on HRV.

SUMMARY

There is mounting evidence of the potential benefits of VNS in myriad disease states, with notable promise in the area of cognition. VNS shows promise as a neuromodulatory technique in cognitive decline and this may be *via* its ability to regulate both cardiac autonomic function and increase cerebral perfusion. Dementia is a multifactorial process and together with reduced cerebral perfusion is associated with neuroinflammation and altered synaptic plasticity, both of which may also be favorably modulated by VNS. It has been noted that perfusion to cortical and subcortical areas increases with VNS, specifically to areas that modulate executive function and attention, i.e., insular, orbitofrontal and prefrontal cortex. These areas are hypothesized by the neurovisceral integration model to be crucial areas in modulating the ANS (Thayer et al., 2009). Given that the LC-NE system is intimately involved in the therapeutic effects of VNS, and likely improves cognition *via* norepinephrine release and improved executive performance, it is notable that the earliest stages of pathological tau accumulation in Alzheimer's disease are seen in the LC. Whether this small midbrain nucleus will prove to be pivotal in our understanding of how to modulate the vagus nerve and harness its benefits cognitively remains to be elucidated. VNS can now be delivered safely and non-invasively *via* t-VNS devices with equivalent neuromodulatory effects on brain imaging as invasive devices, which broadens its therapeutic applicability considerably, especially to an older population with cognitive complaints for whom device implantation may not be feasible. Globally, the need for effective therapies to both treat the cause and symptoms of cognitive decline are needed urgently as rates of dementia increase due to population expansion. Dedicated studies into the potential therapeutic effects of t-VNS in early cognitive decline and dementia are needed. Research to date has been limited by myriad issues, including studies on cognition in clinical populations with altered neuroanatomy, lack of standardization in device usage, parameter settings, frequency of use, duration of stimulation. Minimum reporting standards have recently been published to help ameliorate some of these issues. Further rigorous studies of the therapeutic benefit of VNS are required, especially in populations with autonomic instability and cognitive decline.

AUTHOR CONTRIBUTIONS

HD did most of the research, writing, and editing of the article. Significant contributions were made by each author, specifically TD with manuscript reading, editing, and direction, SC with direction RE psychological assessments and plasticity, CF with neurocardiovascular assessments, ANS testing. PM and SK assisted significantly with overall editorial support and guidance. All authors contributed to the article and approved the submitted version.

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Self-Administration of Right Vagus Nerve Stimulation Activates Midbrain Dopaminergic Nuclei

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Background: Left cervical vagus nerve stimulation (l-VNS) is an FDA-approved treatment for neurological disorders including epilepsy, major depressive disorder, and stroke, and l-VNS is increasingly under investigation for a range of other neurological indications. Traditional l-VNS is thought to induce therapeutic neuroplasticity in part through the coordinated activation of multiple broadly projecting neuromodulatory systems in the brain. Recently, it has been reported that striking lateralization exists in the anatomical and functional connectivity between the vagus nerves and the dopaminergic midbrain. These emerging findings suggest that VNS-driven activation of this important plasticity-promoting neuromodulatory system may be preferentially driven by targeting the right, rather than the left, cervical nerve.

Objective: To compare the effects of right cervical VNS (r-VNS) vs. traditional l-VNS on self-administration behavior and midbrain dopaminergic activation in rats.

Methods: Rats were implanted with a stimulating cuff electrode targeting either the right or left cervical vagus nerve. After surgical recovery, rats underwent a VNS self-administration assay in which lever pressing was paired with r-VNS or l-VNS delivery. Self-administration was followed by extinction, cue-only reinstatement, and stimulation reinstatement sessions. Rats were sacrificed 90 min after completion of behavioral training, and brains were removed for immunohistochemical analysis of c-Fos expression in the dopaminergic ventral tegmental area (VTA) and substantia nigra pars compacta (SNc), as well as in the noradrenergic locus coeruleus (LC).

Results: Rats in the r-VNS cohort performed significantly more lever presses throughout self-administration and reinstatement sessions than did rats in the l-VNS cohort. Moreover, this appetitive behavioral responding was associated with significantly greater c-Fos expression among neuronal populations within the VTA, SNc, and LC. Differential c-Fos expression following r-VNS vs. l-VNS was particularly prominent within dopaminergic midbrain neurons.

Conclusion: Our results support the existence of strong lateralization within vagal-mesencephalic signaling pathways, and suggest that VNS targeted to the right, rather

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than left, cervical nerve preferentially activates the midbrain dopaminergic system. These findings raise the possibility that r-VNS could provide a promising strategy for enhancing dopamine-dependent neuroplasticity, opening broad avenues for future research into the efficacy and safety of r-VNS in the treatment of neurological disease.

Keywords: VNS (vagus nerve stimulation), lateralization, dopamine, self-administration, ventral tegmental area, substantia nigra, neural stimulation, c-fos

INTRODUCTION

Stimulation of the left cervical vagus nerve (l-VNS) is an FDA-approved therapeutic approach for a wide range of neurological diseases, including epilepsy, major depressive disorder, migraine, and stroke. Moreover, recent research is rapidly expanding the clinical indications for which stimulation of the "wandering" vagus nerve may provide therapeutic benefit. Cervical VNS is currently under investigation for multiple other neurological and neurodegenerative disorders, including Alzheimer's disease (Chang et al., 2018; Slater and Wang, 2021), trauma and anxiety disorders (Marin et al., 2014; Noble et al., 2019), and autism (Engineer et al., 2017; van Hoorn et al., 2019), among others (Hays, 2016; Wang et al., 2021). Though the mechanisms of l-VNS efficacy are incompletely understood, it has been shown that VNS exerts wide-ranging neurological effects in part through activation of the broadly projecting nucleus of the solitary tract and several downstream neuromodulatory nuclei, which include the noradrenergic locus coeruleus (LC), the serotonergic raphe nuclei, and the cholinergic medial forebrain (Detari et al., 1983; Krahl et al., 1998; Dorr and Debonnel, 2006; Osharina et al., 2006; Cunningham et al., 2008; Manta et al., 2009; Ruffoli et al., 2011; Collins et al., 2021). Coordinated activation of these neuromodulatory systems is thought to promote therapeutic neuroplasticity, resulting in improved clinical outcomes (Hays et al., 2013; Conway and Xiong, 2018; Wang et al., 2021).

Midbrain dopaminergic signaling is widely recognized to play a key role in promoting reward-related neuroplasticity throughout the brain (Schultz, 1998; Baik, 2013; Volkow et al., 2017; Speranza et al., 2021), though the role of dopamine in VNS efficacy is less well-studied. Recently, Han et al. (2018) reported a remarkable lateralization in the anatomical and functional connectivity between the vagus nerves and midbrain dopaminergic nuclei. Specifically, these authors demonstrated that optogenetic stimulation of selectively targeted, gut-innervating vagal neurons located in the right, but not left, nodose ganglion (NG) resulted in strong activation of the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc). Moreover, right, but not left, NG stimulation was sufficient to induce striatal dopamine release and appetitive behavioral responses. The clinical implications of these recent findings for the further development of therapeutic VNS remain unclear. VNS-mediated manipulation of the dopamine system could offer powerful additional neuroplasticity-promoting mechanisms by which to achieve therapeutic effects. However, it is unknown whether stimulation of the right vagus nerve (r-VNS) using traditional non-selective electrical stimulation of the cervical fibers would be sufficient to activate the midbrain dopamine system. Nor is it clear whether traditional cervical VNS produces differential dopaminergic activation when applied to the right vs. the left cervical nerves.

In the current study, we compare the appetitive behavioral effects of r-VNS and l-VNS in rats, and ask whether lateralized stimulation produces differential activation of neurons within midbrain dopaminergic nuclei. We first tested the appetitive effects of r-VNS and l-VNS using a VNS self-administration assay. After completion of the behavioral task, animals were sacrificed and c-Fos expression within the expression within the VTA and SNc was quantified to examine neuronal activation in these regions following r-VNS vs. l-VNS. Our behavioral and histological results are consistent with a striking lateralization of vagal-mesencephalic signaling, and suggest that standard electrical stimulation of the right cervical vagus nerve is capable of producing strong activation of the midbrain dopaminergic system.

MATERIALS AND METHODS

All procedures were approved by the University of Texas at Dallas Institutional Animal Care and Use Committee and are in accordance with the National Institutes of Health guide for the care and use of laboratory animals.

Animal Subjects

Fourteen adult female Long-Evans rats, aged 8–14 weeks at study start, were used in these experiments. Rats were housed in a 12:12 h reverse light cycle room with *ad libitum* access to water (lights on: 6:00 pm) and all handling and training occurred during their active cycle. Prior to cuff implantation surgery, rats were handled for at least 3 daily 15-min habituation sessions.

Vagus Nerve Cuff Electrode Implantation

At study start, rats were randomly assigned to l-VNS (n = 7) or r-VNS (n = 7) treatment groups. Vagus nerve cuff electrodes consisted of platinum-iridium leads (Sigmund Cohn, #10IR9/4T) ensheathed in MicroRenathane tubing (Braintree Scientific, #MRE080), and were assembled in-house according to published methods (Sanchez et al., 2020). The stimulating cuff electrode was implanted around the targeted cervical vagus nerve as previously described (Porter et al., 2012; Tseng et al., 2020). Briefly, an incision was made 1 cm from the midline on either the right or left side, and the targeted cervical vagus nerve was bluntly dissected from the carotid artery and placed inside the cuff. A second incision was then made on the midline of the skull at

the occipital and parietal bones, and cuff electrode leads were tunneled subcutaneously, exited through this second incision, and attached to a headcap/connector (Omnetics, #A24002-004). Cuff function was validated during surgery, for both left- and right-side implants, by delivering a single 10-s train of electrical stimulation (amplitude = 0.8 mA, pulse frequency = 30 Hz, pulse width = 100 μs biphasic) using an isolated pulse stimulator (A-M Systems, Model 2100) to evoke a brief cessation of breathing consistent with the Hering-Breuer reflex (Bucksot et al., 2020). Following cuff validation, the neck incision was sutured. Fascia was cleared from the skull, the headcap was secured with bone screws and dental cement, and the cranial incision closed with sutures. Rats were given a 1-week surgical recovery period prior to the start of behavioral training. For 3 days post-surgery, rats were administered Baytril (enrofloxacin, 0.5 mg/5 g) and Rimadyl (carprofen, 2 mg/5 g) tablets (Bio-Serv, Flemington, NJ, United States).

Vagus Nerve Stimulation Self-Administration

After recovery from surgery, rats underwent the VNS self-administration assay, which included Acclimation, VNS Self-Administration (VNS-SA), Extinction (EXT), and Reinstatement (R) stages. Throughout the duration of the training protocol, beginning 24 h prior to the start of Acclimation, rats were lightly food restricted. Subjects received 5 pellets of rat chow (ca. 14–18 gm; Labdiet Prolab RMH 1800) each day, delivered in the homecage immediately following the training session. Weights were monitored daily prior to feeding to ensure animals maintained at least 90% of their free-feeding weight throughout the study.

During Acclimation, rats were placed in a MotoTrak training booth (30 cm × 13 cm × 25 cm booth; Vulintus, Inc., Louisville, CO) overnight (8–12 h) and trained to press a lever (>1.5 degree deflection from horizontal) extending 1 cm inside the booth to receive a 45 mg food pellet (Bio-Serv, Flemington, NJ; #F0021). A 2-s time-out period followed each pellet delivery before a subsequent trial could be initiated. Rats were required to perform at least 100 rewarded presses within a single overnight Acclimation session before beginning VNS-SA. If a subject failed to perform at least 100 presses, they were given a 24-h rest period before receiving an additional acclimation session. All rats completed the Acclimation stage in 1–4 sessions (mean = 2.4).

Following Acclimation, rats underwent five 2-h VNS-SA sessions (1/day) in which food pellets were removed and pressing behavior was instead paired with VNS delivery and the onset of a visual cue (**Figure 1A**). VNS stimulation parameters were identical to those shown in our previous studies of l-VNS to induce neuroplasticity within the motor cortex (Tseng et al., 2020; Brougher et al., 2021). Immediately upon detection of a lever press, a single 0.5 s train of 16 pulses (amplitude = 0.8 mA, pulse frequency = 30 Hz, pulse width = 100 μs biphasic) was delivered through the implanted cuff electrode. The same stimulation parameters were used for both l-VNS and r-VNS groups. The visual cue consisted of a green (488 nm) LED located outside of the booth directly above the lever. Visual cue onset was simultaneous with and for the same duration as the VNS train (0.5 s).

Following VNS-SA, rats received five 2-h EXT sessions (1/day). During EXT, the VNS stimulator and LED remained off, and lever presses no longer resulted in stimulation or visual cue delivery.

After EXT, animals underwent a single 2-h session of visual cue-only reinstatement (R1). During R1, successful lever presses resulted in the presentation of the visual cue only, but no VNS was delivered.

Following R1, subjects underwent a second 2-h cue + VNS reinstatement session (R2). During R2, r-VNS rats received delivery of both the visual cue and VNS immediately upon detection of each lever press, as they did during VNS-SA sessions. To ensure both r-VNS and l-VNS treatment groups received equal amounts of stimulation during this final R2 session, stimulation of each rat in the l-VNS group was yoked to that of an r-VNS subject. Yoked r-VNS and l-VNS rats were run simultaneously and both received cue and VNS delivery contingent on the lever pressing behavior of the r-VNS rat. Ninety minutes after the completion of R2, rats were sacrificed for histological analyses.

c-Fos Immunohistochemistry

Ninety minutes after the final reinstatement session, rats were deeply anesthetized with sodium pentobarbital/phenytoin (150/50 mg/kg, i.p.) and transcardially perfused with ice-cold phosphate buffered saline (PBS), followed by 4% paraformaldehyde in PBS. Brains were removed and stored in 4% paraformaldehyde overnight for fixation. The following day, brains were transferred to a 30% sucrose solution for cryoprotection.

Three subjects in each group were randomly chosen for inclusion in the histological analyses. A cryostat was used to make brain slices through the VTA/SNc (AP: −5.2 to −5.3 mm from Bregma) and the LC (−9.6– to −9.7 mm from Bregma) at 20 μm thickness. Slices were washed (3X in PBS), followed by 30 min permeabilization with 0.5% Triton-X in PBS. Slices were again washed and blocked for 1 h in 2.0% BSA in PBS. Slices were then washed and incubated overnight at 4°C in a primary antibody cocktail to label tyrosine hydroxylase (TH) and c-Fos (chicken anti-TH, 1:1,000 dilution, Abcam #ab76442; mouse anti-c-fos, 1:1,000, Abcam #ab208942). The following day, slices were washed and incubated at room temperature for 1 h in secondary antibody solution (anti-chicken IgY conjugated to Alexa Fluor 555, 1:1,000 dilution, Abcam #ab150170; anti-mouse IgG conjugated to Alexa Fluor 488, 1:1,000 dilution, ThermoFisher #A28175). Finally, slices were washed and mounted on slides in a DAPI containing mounting medium (DAPI Fluoromount-G, SouthernBiotech #0100-20).

For each subject examined, three alternating slices were imaged per nucleus of interest. VTA, SNc, and LC in both left and right hemispheres were imaged for subsequent Mean Gray Value (MGV) and cell counting analyses. Images were made using an Olympus BX51 fluorescent microscope. Images for MGV were taken at 10x magnification; images for cell counting were taken at 20x magnification.

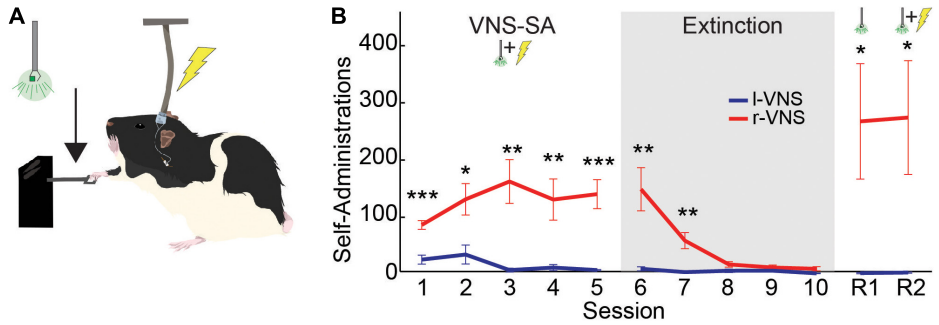

FIGURE 1 | Rats self-administer r-VNS but not l-VNS. **(A)** During VNS-SA sessions, r-VNS or l-VNS was delivered along with a visual cue (488 nm LED) immediately upon detection of a lever deflection. **(B)** Lever-press performance of r-VNS (red; $n = 7$) and l-VNS (blue; $n = 7$) cohorts throughout the self-administration assay. Training stages included VNS self-administration (VNS-SA), Extinction, cue-only reinstatement (R1), and yoked-stimulation reinstatement (R2) sessions. Student's *t*-tests were used to test for between-group differences in behavioral performance within each training session and corrected for multiple comparisons using false discovery rate. For sessions in which FDR-corrected comparisons indicated the presence of a statistically significant between-group difference, uncorrected *p*-values are denoted: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

MGV quantification was performed bilaterally in each analyzed slice imaged at 10x. For each hemisphere and nucleus of interest, regions of interest (ROIs) were drawn by hand in ImageJ using TH+ fluorescence as an indicator for nucleus boundaries based on prior literature (Boekhoudt et al., 2017; Farrand et al., 2020). For each image, ROIs were accepted for analysis if detected TH fluorescence (raw integrated density) within the ROI made up at least 95% of the total TH fluorescence within the image. In images containing multiple TH+ nuclei (i.e., VTA and SNc), sections of the adjacent, non-target nuclei were cropped prior to ROI validation. Midline was determined in VTA images by the presence of the periaqueductal gray dorsal to the VTA. For each ROI, overall c-Fos expression was quantified in ImageJ as the MGV of c-Fos immunofluorescence within the ROI. MGVs were then averaged across the 3 slices per nucleus, to obtain a measure of nucleus- and hemisphere-specific c-Fos expression for each rat.

Specific cell counts were also obtained for each nucleus of interest using additional images taken at 20x magnification. Images were centered on the densest population of TH+ cells within each nucleus. As for MGV, ROIs were drawn in ImageJ using the boundaries of TH expression to define nucleus boundaries, and ROIs were accepted for analysis if 95% of the TH+ signal in the image was contained within the ROI. Images were manually quantified to obtain specific cell counts for DAPI+, TH+, and/or c-Fos+ cells. Images were pseudocolored in ImageJ for quantification (TH = red, c-Fos = green, DAPI = blue). Each ROI was quantified by 2 graders, both blinded to the subject's treatment condition and the other grader's counts. Only DAPI+ cells within the plane of focus were counted; cells were classified into 1 of 4 categories: (1) DAPI+ only, (2) TH+ and DAPI+, (3) c-Fos+ and DAPI+, or (4) c-Fos+ and TH+ and DAPI+. For each grader and nucleus of interest, percentages of (1) TH+ cells, (2) c-Fos+ cells within the TH+ population, and (3) c-Fos+ cells within the TH− population were calculated and averaged across relevant ROIs in all three slices per nucleus, and then averaged across the 2 hemispheres to obtain average count values for each cell type in each nucleus. Percentages were then averaged across the two graders to obtain cell-type-specific quantification of c-Fos expression within each nucleus for each rat.

Data Analysis

Behavioral data (lever presses per session) were analyzed in R 4.0.3 (R Core Team, 2021) using a two-way mixed ANOVA, with treatment group as a between-subject factor and session number as a within-subject factor. As Mauchly's test indicated a lack of sphericity ($p < 0.0001$), Greenhouse-Geisser corrected within factor results are reported. *Post hoc t*-tests were then used to compare lever pressing between l-VNS and r-VNS treatment groups within each session, and corrected for multiple comparisons using false discovery rate (FDR). For all behavioral analyses, statistical significance is reported for FDR-adjusted $p < 0.05$. All summary statistics are reported as mean ± SEM.

For histological MGV analysis, 2-way ANOVA was used to test for differences in c-Fos expression across brain hemispheres and VNS treatment groups. For each nucleus of interest, two-way ANOVAs were followed by Tukey *post hoc* comparisons of c-Fos expression across all four (brain hemisphere × stimulation side) contingencies. Significant differences are reported for $p < 0.05$.

For histological cell counts, the percentages of TH+, c-Fos+/TH+, and c-Fos+/TH− cells within each nucleus of interest were compared between l-VNS and r-VNS treated rats using unpaired Student's *t*-tests, which were corrected for multiple comparisons using false discovery rate. Significant differences are reported for FDR-adjusted $p < 0.05$.

RESULTS

Fourteen female rats were implanted with stimulating cuff electrodes around the right (r-VNS: $n = 7$) or left (l-VNS: $n = 7$) cervical vagus nerve. After surgical recovery, all rats were habituated to the lever press task using food rewards during 1–4 overnight sessions prior to the start of VNS-SA (see section "Materials and Methods"). Rats in r-VNS and l-VNS groups performed similarly during habituation (Lever

presses during final habituation session: r-VNS: 144 ± 15.66, l-VNS = 137 ± 13.63; $p = 0.742$, unpaired t-test). During daily 2-h VNS-SA sessions, each lever press was paired with simultaneous onset of a visual stimulus and delivery of a brief train of VNS (**Figure 1A**). VNS stimulation parameters were matched in r-VNS and l-VNS treatment groups, and identical to those used in prior studies (Tseng et al., 2020; Brougher et al., 2021).

Rats Self-Administer Right Cervical Vagus Nerve Stimulation but Not Left Cervical Vagus Nerve Stimulation

During VNS-SA, rats in the r-VNS treatment group quickly began to lever press at high rates to self-administer vagal stimulation, while l-VNS failed to drive similar levels of lever responding [Greenhouse-Geisser corrected 2-way mixed ANOVA, group effect: $F_{(1,\ 12)} = 21.528$, $p = 5.7e\text{-}4$; session effect: $F_{(1.56,\ 18.7)} = 4.487$, $p = 0.033$; interaction: $F_{(1.56,\ 18.7)} = 4.602$, $p = 0.031$]. Lever press performance in the r-VNS and l-VNS treatment groups began to significantly diverge during the first VNS-SA session (SA1), ca. 65 min into the 2-h session (**Supplementary Figure 1**). In each of the 5 VNS-SA sessions, rats that received r-VNS performed significantly more presses per session than rats that received traditional l-VNS (**Figure 1B** and **Table 1**). Rats in the r-VNS group increased their rates of lever pressing throughout VNS-SA sessions (r-VNS group, SA1 vs. SA5: $p = 0.038$, paired t-test), and performed over 120 presses on average in SA sessions 2 through 5 (**Table 1**). By contrast, rats that received l-VNS at matched stimulation parameters performed fewer than 40 presses per session (**Table 1**), and decreased their response rate across VNS-SA sessions (l-VNS group, SA1 vs. SA5: $p = 0.047$, paired t-test). Taken together, these results suggest that r-VNS, but not l-VNS, was highly behaviorally reinforcing.

Following VNS-SA sessions, rats underwent 5 days of extinction training in which the visual cue and VNS were no longer delivered upon detection of a lever press. During extinction, r-VNS treated rats significantly decreased their lever pressing (r-VNS group, SA5 vs. EXT5: $p = 0.002$), and lever responding in the l-VNS treatment group further declined (l-VNS group, SA5 vs. EXT5: $p = 0.0003$, paired t-test). Rats in the r-VNS group continued to press significantly more than those in the l-VNS group during the first two extinction sessions, but response rates were similarly low in both groups during the final three sessions of extinction (**Figure 1B** and **Table 1**).

Following extinction, rats underwent two sessions of reinstatement. During the first reinstatement session (R1), the visual cue alone was presented upon lever pressing, but no VNS was delivered. Rats in the l-VNS group continued to press the lever at very low rates during cue-only reinstatement. By contrast, rats in the r-VNS group resumed high levels of lever responding during R1 (**Figure 1B** and **Table 1**), suggesting that the visual stimulus itself had acquired strong appetitive value during r-VNS and was sufficient to reinforce lever responding.

During the second reinstatement session on the following day (R2), both visual cue and VNS were delivered. To ensure that l-VNS and r-VNS treatment groups received equal amounts of stimulation during R2, stimulation of each l-VNS rat was yoked to the performance of a rat in the r-VNS treatment group. In R2, as in R1, r-VNS treated rats continued to press the lever at high rates, whereas l-VNS rats continued to exhibit low levels of lever engagement (**Figure 1B** and **Table 1**).

TABLE 1 | Comparison of lever pressing performance for l-VNS vs. r-VNS treated rats throughout self-administration, extinction, and reinstatement sessions.

	l-VNS	r-VNS	*t*-test
Session	**Mean (SEM)**	**Mean (SEM)**	***p*-value (FDR *q*-value)**
VNS self-administration			
SA1	25.86 (7.91)	86.71 (7.70)	**0.0001** (0.001)
SA2	34.71 (16.66)	131.71 (27.62)	**0.0109** (0.019)
SA3	7.43 (3.43)	163.00 (38.38)	**0.0016** (0.007)
SA4	11.86 (5.35)	131.29 (36.16)	**0.0067** (0.014)
SA5	7.43 (1.02)	140.86 (25.40)	**0.0002** (0.001)
Extinction			
EXT1	9.71 (3.62)	149.43 (37.85)	**0.0032** (0.008)
EXT2	3.86 (1.63)	59.14 (14.12)	**0.0022** (0.007)
EXT3	6.29 (2.96)	17.43 (4.72)	0.0684 (0.075)
EXT4	6.43 (1.90)	12.00 (4.33)	0.2617 (0.262)
EXT5	1.71 (0.47)	9.71 (3.68)	0.0518 (0.062)
Reinstatement			
R1	2.14 (1.18)	268.57 (101.26)	**0.0219** (0.029)
R2	3.14 (1.10)	275.29 (99.83)	**0.0184** (0.028)

Student's t-tests were used to compare lever pressing between treatment groups during each training session and corrected for multiple comparisons using false discovery rate (FDR). Bold denotes a statistically significant difference in behavioral performance between groups for FDR-adjusted q < 0.05.

Taken together, our results demonstrate that rats will readily self-administer brief bursts of 30 Hz r-VNS, but that l-VNS delivered at equivalent stimulation parameters does not produce similar appetitive behavioral responses. Extensive literature details the importance of dopaminergic signaling in the reinforcement of self-administration behaviors, including during acquisition, extinction, and reinstatement (Olds and Milner, 1954; German and Bowden, 1974; Volkow et al., 2017; Namba et al., 2018; Pitchers et al., 2018; Salinas-Hernández et al., 2018; Wise and Robble, 2020). Our behavioral findings reveal a striking laterality in the reinforcing effects of cervical vagus nerve stimulation, and are consistent with strong activation of the midbrain dopaminergic reward nuclei by r-VNS, but not l-VNS.

Right Cervical Vagus Nerve Stimulation Self-Administration Enhances c-Fos Expression in Dopaminergic and Noradrenergic Nuclei

To specifically test whether r-VNS self-administration engages midbrain dopaminergic nuclei, we examined c-Fos expression in the VTA and SNc of our rats following the completion of the self-administration assay. As l-VNS efficacy has been previously shown to depend on noradrenergic signaling (Krahl et al., 1998; Furmaga et al., 2011; Grimonprez et al., 2015; Hulsey et al., 2019), we additionally asked whether r-VNS and l-VNS produced similar levels of LC activation.

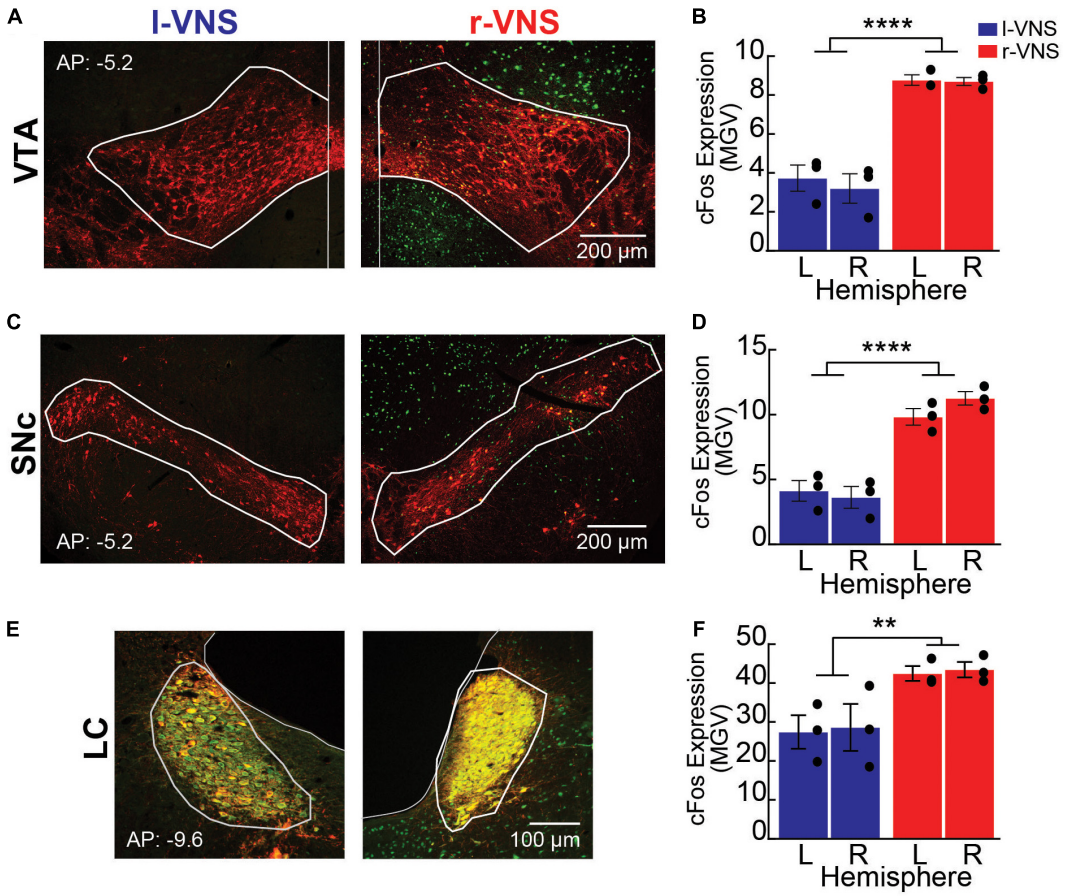

FIGURE 2 | Compared to l-VNS, r-VNS self-administration increases c-Fos expression within catecholaminergic nuclei. **(A,C,E)** Representative 10x images and ROI boundaries used to quantify c-Fos expression within the VTA **(A)**, SNc **(C)**, and LC **(E)** following either l-VNS (left) or r-VNS (right). Sections were stained for tyrosine hydroxylase (red) to label catecholaminergic neurons, c-Fos (green) as a marker of neuronal activation, and the nuclear marker DAPI (omitted for clarity). **(B,D,F)** Mean gray value (MGV) of c-Fos fluorescence was significantly greater following r-VNS self-administration (red) than in l-VNS treated rats (blue) within both left (L) and right (R) brain hemispheres of the VTA **(B)**, SNc **(D)**, and LC **(F)**. $^{**}p < 0.01$; $^{****}p < 0.0001$; 2-way ANOVA between-group comparisons. Within each treatment group, no significant difference in c-Fos expression was observed between left and right brain hemispheres; full statistical results are presented in **Tables 2**, **3**.

TABLE 2 | Comparison of c-Fos labeling intensity (mean gray value) in left (LH) vs. right (RH) brain hemispheres (hemi) following l-VNS vs. r-VNS treatment (vns_side), for ventral tegmental area (VTA), substantia nigra pars compacta (SNc), and locus coeruleus (LC).

	l-VNS		r-VNS		2-way ANOVA		
	LH	RH	LH	RH			
Nucleus	**Mean (SEM)**				p_{vns_side} [F_{vns_side}]	p_{hemi} [F_{hemi}]	p_{int} [F_{int}]
VTA	3.73 (0.7)	3.20 (0.8)	8.77 (0.3)	8.70 (0.2)	**0.000 [98.46]**	0.575 [0.34]	0.658 [0.21]
SNc	4.13 (0.8)	3.63 (0.8)	9.83 (0.6)	11.27 (0.52)	**0.000 [86.7]**	0.540 [0.41]	0.214 [1.82]
LC	27.43 (4.3)	27.3 (4.9)	42.47 (1.9)	43.47 (2.0)	**0.002 [19.6]**	0.894 [0.02]	0.875 [0.03]

Bold denotes a statistically significant effect for $p < 0.05$.

For these analyses, rats were sacrificed 90 min after the conclusion of the R2 reinstatement session, and c-Fos expression was examined in the VTA, SNc, and LC bilaterally. Sections were co-stained for TH to identify the boundaries of each nucleus of interest (**Figures 2A,C,E**). We first compared total c-Fos expression between hemispheres and between l-VNS and r-VNS treated subjects by computing the mean gray value (MGV) within regions of interest (ROIs) defining the VTA, SNc, and LC (**Figures 2B,D,F**). For all 3 catecholaminergic nuclei, 2-way ANOVAs revealed a significant main effect of stimulation side on c-Fos expression, but no effect of brain hemisphere or interaction effects (**Table 2**). Tukey *post hoc* comparisons confirmed that r-VNS self-administration resulted in significantly greater c-Fos expression than l-VNS, and this effect was seen in both the VTA

TABLE 3 | Tukey *post hoc* comparisons of c-Fos labeling intensity (mean gray value) between left (LH) vs. right (RH) brain hemispheres and r-VNS vs. l-VNS treatments for VTA, SNc, and LC.

	RH	LH	LH vs. RH comparisons		r-VNS | RH vs. l-VNS | LH	r-VNS | LH vs. l-VNS | RH
	r- vs. l-VNS comparisons		r-VNS	l-VNS		
VTA	**0.0022**	**0.0003**	0.5289	0.9564	**0.0013**	**0.0005**
SNc	**0.0007**	**0.0004**	0.9997	0.8790	**0.0003**	**0.0008**
LC	*0.0651*	**0.0471**	0.9964	1.000	*0.0635*	0.1483

Bold denotes statistically significant differences in c-Fos intensity for $p < 0.05$; italics denotes trend toward statistical significance for $p < 0.1$.

and SNc dopaminergic nuclei, as well as in the noradrenergic LC (**Table 3**). Combined, these results suggest that, compared to l-VNS administration, activation of midbrain dopaminergic "reward" circuits is strongly enhanced following r-VNS self-administration.

Right Cervical Vagus Nerve Stimulation Enhances c-Fos Expression in Dopaminergic and Non-dopaminergic Midbrain Neurons

We next examined whether enhanced neuronal activity within the VTA, SNc, and LC occurred within the catecholaminergic or non-catecholaminergic cell populations in each nucleus. The nuclear marker DAPI was used to label cells in VTA, SNc, and LC and DAPI-labeled cells were classified as TH+ or TH−, as well as c-Fos+ or c-Fos- (**Figures 3A,C,E**). Within the VTA and SNc, we observed similar percentages of TH+ cells in r-VNS and l-VNS treated subjects (**Figures 3B,D**, **Table 4**, and **Supplementary Table 1**). However, compared to l-VNS treated rats, r-VNS treated subjects exhibited significantly greater c-Fos expression within both TH+ and TH− cell populations in these regions (**Figures 3B,D**, **Table 4**, and **Supplementary Table 1**). In VTA, r-VNS treated rats had approximately 4 times more c-Fos+ non-dopaminergic cells, and approximately 8 times more c-Fos+ dopaminergic neurons, than l-VNS treated rats. In SNc, r-VNS treated rats had ca. 2 times more c-Fos+ non-dopaminergic cells, and ca. 13 times more c-Fos+ dopaminergic neurons, than l-VNS treated rats. These findings indicate that r-VNS self-administration drives stronger midbrain neuronal activation than l-VNS, in both dopaminergic and non-dopaminergic populations within the VTA and SNc.

In the noradrenergic LC, r-VNS self-administration was associated with a significant increase in TH+ staining compared to l-VNS treatment (**Figure 3F**, **Table 4**, and **Supplementary Table 1**). The overall percentage of c-Fos+ cells did not differ, however, between r-VNS and l-VNS treatment groups, for either TH+ or TH− cell populations in the LC. Within the TH+ noradrenergic population, the majority of neurons in both l-VNS and r-VNS treated rats were found to be c-Fos+, consistent with prior reports that VNS drives neural firing in the LC (Groves and Brown, 2005; Dorr and Debonnel, 2006; Hulsey et al., 2017) and enhances noradrenaline release throughout the brain (Dorr and Debonnel, 2006; Roosevelt et al., 2006; Follesa et al., 2007; Manta et al., 2009; Raedt et al., 2011). Our results further suggest that, compared to l-VNS, r-VNS self-administration results in greater overall activation of LC noradrenergic neurons, which may be accompanied by enhanced catecholamine synthesis within the LC.

DISCUSSION

In the current study, we tested whether VNS induces differential activation of midbrain dopaminergic nuclei when delivered to the right vs. left cervical vagus nerves. Our findings provide the first evidence, to our knowledge, that standard electrical stimulation of the right cervical vagus nerve is sufficient to reinforce learned behaviors, and to drive strong activation of midbrain dopaminergic neurons within the VTA and SNc. Notably, these effects were not observed with traditional l-VNS delivered at equivalent stimulation parameters. These results suggest that, compared to l-VNS, r-VNS can engage additional neuroplasticity-promoting signaling pathways, opening broad possibilities for further research into the therapeutic potential of r-VNS for the treatment of neurological disorders.

Our finding that r-VNS promotes appetitive behavioral responses while activating midbrain dopaminergic nuclei is consistent with recent literature detailing similar lateralization in the anatomical and functional connectivity between the upper gut and the midbrain DA system (Han et al., 2018). Using optogenetics to selectively target stomach and duodenum-innervating vagal cell bodies located in the left vs. right nodose ganglia (NG), Han et al. (2018) showed that activation of gut-innervating right, but not left, NG neurons drives striatal dopamine release and induces both place preference and increased nose poke behaviors. These functional effects were consistent with differential anatomical connectivity between left and right gut-innervating NG cells and brainstem and midbrain nuclei. In the current study, lateralization of VNS-driven reward-related signaling may be hypothesized to arise from activation of these lateralized gut-innervating vagal fibers characterized by Han et al. (2018) However, electrical stimulation should non-selectively activate vagal fibers innervating the upper gut in addition to those targeting the intestines, liver, pancreas and other organs capable of conveying reward-related nutritive or metabolic information to the brain (Yuan and Silberstein, 2016; Browning et al., 2017; Shechter and Schwartz, 2018; Berthoud and Neuhuber, 2019; de Araujo et al., 2020). Indeed, other authors have recently reported an increase in VTA activation following optogenetic stimulation of left nodose ganglion neurons (Fernandes et al., 2020). Together, these

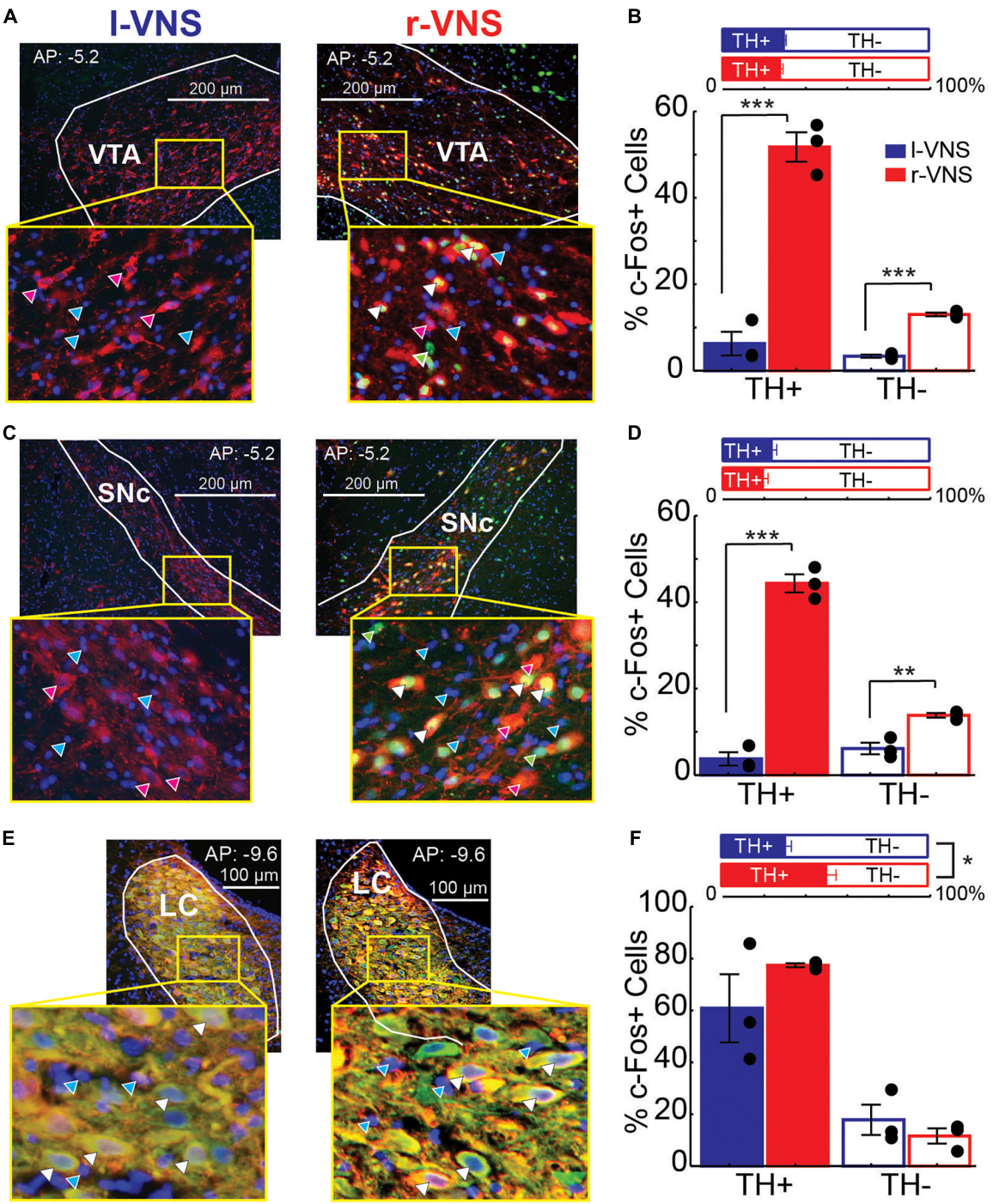

FIGURE 3 | Compared to I-VNS, r-VNS self-administration significantly increases c-Fos expression in both TH+ and TH− cells within catecholaminergic nuclei. **(A,C,E)** Representative 20x images and ROI boundaries used to quantify single-cell c-Fos expression within the VTA **(A)**, SNc **(C)**, and LC **(E)** following either I-VNS (left) or r-VNS (right). Sections were co-stained for tyrosine hydroxylase (red), c-Fos (green), and DAPI (blue). Arrow heads in enlarged insets show example cells classified as exclusively DAPI+ (cyan arrows); DAPI+, c-Fos+, and TH− (green arrows); DAPI+, c- Fos-, and TH+ (magenta arrows); or DAPI+, c-Fos+, and TH+ (white arrows). **(B,D,F)** In both VTA **(B)** and SNc **(D)**, the percentage of TH+ neurons did not differ between r-VNS and I-VNS treatment groups (top). However, the percentage of c-Fos+ cells (bottom) was significantly greater in the r-VNS group, in both TH+ and TH− cell populations. **(F)** In the LC, r-VNS self-administration resulted in a higher percentage of TH+ cells than I-VNS (top), but the percentage of TH+ and TH− cells that were found to be c-Fos+ did not differ between groups (bottom). In **(B,D,F)**, Student's *t*-tests were used to test for between-group differences in TH+ population size, as well as in c-Fos expression within TH+ and TH− populations; multiple comparisons were corrected using false discovery rate. For FDR-corrected comparisons in which statistically significant differences were observed, uncorrected *p*-values are indicated: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

TABLE 4 | Between-group comparisons of the percentage of TH+ cells, as well as percentage of c-Fos+ cells within the separate TH+ vs. TH− populations within VTA, SNc, and LC.

	% TH+ cells			% c-Fos+ of TH+ population			% c-Fos+ of TH− population		
	r-VNS	l-VNS	*t*-test	r-VNS	l-VNS	*t*-test	r-VNS	l-VNS	*t*-test
	Mean (SEM)		*p*-value (FDR *q*-value)	Mean (SEM)		*p*-value (FDR *q*-value)	Mean (SEM)		*p*-value (FDR *q*-value)
VTA	28.38 (0.98)	30.25 (0.63)	0.1856 (0.278)	51.74 (3.40)	6.26 (2.74)	**0.0005** (0.001)	13.00 (0.42)	3.35 (0.34)	**0.0001** (0.000)
SNc	20.52 (1.97)	24.74 (1.98)	0.2059 (0.265)	44.34 (2.10)	3.44 (1.54)	**0.0001** (0.000)	13.81 (0.52)	6.12 (1.33)	**0.0058** (0.013)
LC	51.32 (4.32)	31.39 (2.60)	**0.0167** (0.030)	77.45 (0.75)	60.86 (13.12)	0.2756 (0.310)	11.61 (2.96)	17.85 (5.85)	0.3952 (0.395)

For each region and cell population, percentages were compared between r-VNS and l-VNS treatments using Student's t-tests, corrected for multiple comparisons using false discovery rate. Bold denotes statistical significance for FDR-adjusted q-values < 0.05.

findings suggest that additional studies are needed to fully specify the peripheral origins of lateralized VNS effects.

The acclimation protocol used here presents an important limitation of our study. In the current experiments, we find evidence of strong lateralization in VNS-driven reward-related signaling in rats that had been previously trained to lever press for food reward. A recent study by Fernandes et al. (2020) provides some evidence that this food-reinforced training period may impact appetitive vagal-mesencephalic connectivity. These authors found that intragastric infusions of a sucrose solution were strongly reinforcing in mice previously trained to lever-press for oral sucrose delivery, but that the same infusions were not rewarding in naïve mice. Their findings are consistent with prior reports that significant plasticity occurs within reward-related gut-brain signaling pathways following orogastric reward consumption (Berthoud, 2008; Uematsu et al., 2009; Myers et al., 2013; Schier and Spector, 2016; Shechter and Schwartz, 2018; Bai et al., 2019; de Araujo et al., 2020). Further research is necessary to clarify the impact of such plasticity on r-VNS driven dopamine signaling.

Traditional l-VNS has been shown to enhance several learning and memory processes. Preclinically, l-VNS is seen, for example, to induce significant neuroplasticity within the motor system (Porter et al., 2012; Morrison et al., 2019; Tseng et al., 2020), and to improve functional recovery following stroke and other neural injuries (Hays et al., 2014; Pruitt et al., 2016; Meyers et al., 2018, 2019). Left VNS has also been found to speed extinction and prevent reinstatement of drug seeking (Childs et al., 2017, 2019) and of conditioned fear (Peña et al., 2013, 2014; Childs et al., 2015; Burger et al., 2016; Noble et al., 2017, 2019; Szeska et al., 2020; Souza et al., 2021). These effects of l-VNS are thought to depend on the coordinated activation of multiple neuromodulatory systems, including broadly projecting cholinergic, noradrenergic, and serotonergic systems (Detari et al., 1983; Krahl et al., 1998; Dorr and Debonnel, 2006; Osharina et al., 2006; Cunningham et al., 2008; Manta et al., 2009; Ruffoli et al., 2011; Hays, 2016; Hulsey et al., 2016, 2019; Meyers et al., 2019; Collins et al., 2021). Consistent with the results of the current study, we recently demonstrated that cortical dopamine is not required for l-VNS induced neuroplasticity to occur (Brougher et al., 2021), nor is l-VNS typically found to be inherently rewarding (Noble et al., 2019; Hickman et al., 2021; Müller et al., 2021). Taken together with this prior literature, the results of the current study thus suggest that, unlike l-VNS, r-VNS strongly engages the midbrain dopaminergic system. Given the strong dependence on multiple neuromodulatory signaling pathways, it is perhaps unsurprising that l-VNS efficacy exhibits an inverted U-shaped curve, with maximum efficacy in rats occurring at the parameters similar to those used in the current study, and reduced effectiveness occurring at lower and higher intensities of stimulation (Borland et al., 2016; Buell et al., 2018; Morrison et al., 2019; Pruitt et al., 2020; Souza et al., 2021). Dopaminergic signaling is known to exert similar inverted U-shaped effects on working memory, attention, and impulsivity (Williams and Dayan, 2005; Gjedde et al., 2010; Cools and D'Esposito, 2011). The parametric responses of midbrain dopaminergic activity to l-VNS and r-VNS have not been well characterized, but understanding this relationship will be critical for optimizing the therapeutic potential of targeted vagal-mesencephalic stimulation.

Importantly, l-VNS is specifically approved for clinical use due to concerns that stimulation of the right nerve may be more likely to induce adverse cardiac effects. This concern largely arises from the anatomical observation that the sinoatrial node is preferentially innervated by right vagus fibers, while the cardiac ventricles receive innervation from both right and left nerves (Krahl, 2012; Coote, 2013). However, evidence that more severe cardiac effects are produced by r-VNS is mixed and varies according to the model species used (Ardell and Randall, 1986; Lockard et al., 1990; Lewis et al., 2001; Krahl et al., 2003). Due to its hypothesized cardiac effects, right cervical VNS is now under investigation for the treatment of heart failure (Zannad et al., 2015; Gold et al., 2016; Anand et al., 2020; Hadaya and Ardell, 2020). While r-VNS has been safe and well-tolerated in these trials, it was found to be ineffective (Zannad et al., 2015; Gold et al., 2016), or no more effective than l-VNS (Premchand et al., 2014, 2016; Nearing et al., 2021), at improving cardiac function. Moreover, in several clinical case reports (McGregor et al., 2005; Spuck et al., 2008), as well as in preclinical studies (Krahl et al., 2003; Sun et al., 2012), r-VNS was seen to improve neurological symptoms, without inducing severe adverse effects. While significantly more data are needed, existing evidence indicates that r-VNS may be safe and well-tolerated for neurological indications. Our current findings suggest that targeting the right cervical nerve rather than the left could potentially enhance the therapeutic efficacy of VNS for indications in which dopamine signaling is known to be

disrupted, including, for example, Parkinson's disease, major depressive disorder, or obesity.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Texas at Dallas.

AUTHOR CONTRIBUTIONS

JB and CT conceived the experiments, analyzed the data, and wrote the manuscript. JB, UA, NA, MC, AJ, IS, and SS performed the experiments and histological quantification. All authors contributed to the manuscript and approved of the final submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.782786/full#supplementary-material

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REVIEW
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Closed-Loop Vagus Nerve Stimulation for the Treatment of Cardiovascular Diseases: State of the Art and Future Directions

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The autonomic nervous system exerts a fine beat-to-beat regulation of cardiovascular functions and is consequently involved in the onset and progression of many cardiovascular diseases (CVDs). Selective neuromodulation of the brain-heart axis with advanced neurotechnologies is an emerging approach to corroborate CVDs treatment when classical pharmacological agents show limited effectiveness. The vagus nerve is a major component of the cardiac neuroaxis, and vagus nerve stimulation (VNS) is a promising application to restore autonomic function under various pathological conditions. VNS has led to encouraging results in animal models of CVDs, but its translation to clinical practice has not been equally successful, calling for more investigation to optimize this technique. Herein we reviewed the state of the art of VNS for CVDs and discuss avenues for therapeutic optimization. Firstly, we provided a succinct description of cardiac vagal innervation anatomy and physiology and principles of VNS. Then, we examined the main clinical applications of VNS in CVDs and the related open challenges. Finally, we presented preclinical studies that aim at overcoming VNS limitations through optimization of anatomical targets, development of novel neural interface technologies, and design of efficient VNS closed-loop protocols.

Keywords: autonomic nervous system, vagus nerve stimulation, cardiovascular diseases, neural decoding, closed-loop

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INTRODUCTION

Cardiovascular diseases (CVDs) still represent a major disease burden worldwide, despite advances in pharmacological treatments (1). Therefore, new therapeutical strategies are currently being investigated as an alternative to classical schemes. Among those, the solutions offered by Bioelectronic Medicine (BM) (2–5)—a new, highly interdisciplinary field incorporating neuroscience, engineering, and molecular medicine (4, 6)—are emerging as appealing candidates. The development of BM was inspired by the growing comprehension of the autonomic nervous system (ANS), which plays a key role in the control of whole-body homeostasis. Dysfunctions

of the ANS are consequently implicated in the development and progression of many diseases, including those affecting the cardiovascular system (4, 7–13). BM utilizes this body of knowledge as a reference for the design of implantable devices (5) that modulate signals of the peripheral nervous system to visceral organs for therapeutic purposes (14–16).

The neural control of cardiovascular functions involves multiple interactions among central and peripheral components of the so-called "cardiac neuraxis" (17), which comprises the intrinsic cardiac nervous system, vagus nerves (VNs), intrathoracic sympathetic ganglia, spinal cord, brain stem, and multiple central regions up to the insular cortex. Acting together, these hierarchically organized functional units coordinate and regulate cardiac activity to preserve an adequate match between cardiac output and blood flow demand (18). Autonomic dysregulation at various levels of the cardiac neuroaxis, from central nuclei to peripheral effectors, is now recognized as a fundamental contributor to the progression of CVDs (1, 19). For instance, altered neural signals, such as the pathological activation of cardiac afferent neurons by acute myocardial ischemia and reperfusion, induce maladaptive responses such as sympathetic overdrive and parasympathetic withdrawal (autonomic imbalance) that in turn contribute to the development of systemic cardiovascular alterations (1, 19, 20). Therefore, the selective modulation of the cardiac neuraxis to achieve targeted control of cardiovascular functions has been proposed as a potentially impactful application of BM (21, 22). Peripheral nerves and ganglia of the ANS are attractive targets for BM for their favorable location for surgical interventions compared with deep and anatomically less characterized ANS centers of the CNS like the periaqueductal gray matter of the midbrain or hypothalamic nuclei (11, 23). In this perspective, the most widely studied intervention is represented by the electrical stimulation of the VN (VNS) (24, 25).

The VN (or X cranial nerve) is a paired asymmetric and the most extensively distributed nerve in the body, as well as a major component of the cardiac neuraxis (26). Sensory signaling through the VN plays a critical role in maintaining homeostasis of feeding, digestion, respiration, and cardiovascular functions (27). For this reason, VN neuromodulation is being tested as a potential therapeutical strategy for many pathological conditions, including CVDs (28). However, despite promising results achieved in preclinical studies (29), VNS is accompanied by side effects and still needs to be optimized for better exploitation of its full potential in the clinical setting. Herein we will review the state of the art of VNS for CVDs and discuss the current perspectives of VNS optimization. First, we will summarize the anatomy and physiology of the cardiac vagal system; then, we will describe the principles of VNS for CVDs and its main clinical applications, with related open challenges. Finally, we will review preclinical studies aimed at overcoming VNS limitations through optimization of anatomical targets, development of novel neural interface technologies, and design of efficient VNS closed-loop protocols. In the latter case, we will particularly focus on neural decoding strategies that aim at the identification of timely- and spatially selective feedback signals to drive VNS for CVDs properly (8, 30–32).

ANATOMY AND PHYSIOLOGY OF VAGUS NERVE IN THE CARDIOVASCULAR SYSTEM

Gross Anatomy

The VN originates bilaterally in the medulla as multiple filaments that extend toward the jugular foramen and then converge to form a single trunk. Within or just caudal to the jugular foramen are located the superior (jugular) and inferior (nodose) ganglia of the VN (**Figure 1A**) (26). At its emergence from the nodose ganglion, the VN can be anatomically divided into three segments along the rostro-caudal direction: cervical, thoracic and abdominal (26). The cervical VN generates multiple branches, including the superior cardiac and aortic branches (**Figure 1A**) (26, 33–36). Leaving the carotid sheath at the neck base, the VN enters the thorax, and it is referred to as the thoracic VN. Vagal cardiac branches include the superior and inferior cervical cardiac branches and the inferior or thoracic cardiac branch that originates from the thoracic VN (**Figures 1A,B**) (26, 37). The aortic branch or depressor nerve from the left VN contains afferent fibers innervating the aortic arch, while the one from the right VN innervates the bifurcation of the right brachio-cephalic trunk (35, 38). Within the mediastinum, the thoracic VN provides thoracic cardiac branches that are mainly observed between the aortic arch and the pulmonary arterial trunk and innervate the heart along the coronary arteries (37). Together with cardiac nerves from the sympathetic trunk, they contribute to form the cardiac plexus, which is usually divided into a superficial and a deep portion (**Figure 1B**). From a functional perspective, the right-sided nerves innervate mostly the sinoatrial node, while the left-sided nerves innervate mostly the atrioventricular node (36).

Microscopic Anatomy

Similar to other peripheral nerves, vagal fibers are grouped into a variable number of fascicles (39) with high variability among different species and even within the same species (40, 41). Several studies in human cadavers found the mean fascicles number of the cervical VN to oscillate between 5 and 10 (33, 40, 42, 43). The cervical VN in mice, rats, canines and non-human primates displays a less complex fascicular organization than in humans, typically consisting of 1–2 fascicles (43, 44). The porcine VN displays more fascicles than the human, containing 46 ± 10 and 43 ± 8 bundles at cervical and abdominal level, respectively (43, 44). In the somatic nervous system, all fascicles seem to conform a somatotopic organization (45, 46) and whether this occurs also in the ANS needs still to be assessed even if it is highly possible in complex nerves such as the VN (47). In fact, Settell et al. described a distinct bimodal organization of fascicles in the pig cervical VN. Specifically, they observed pseudounipolar cells aggregated in a large "fascicle" in nodose ganglion cross-sections and found a distinct group of fascicles arising from that large "fascicle" in caudal cross-sections of the cervical VN (**Figure 2**). This distinct organization of fascicles disappeared beyond the recurrent laryngeal nerve branching point; thus, they were identified as fascicles pertaining to the recurrent laryngeal

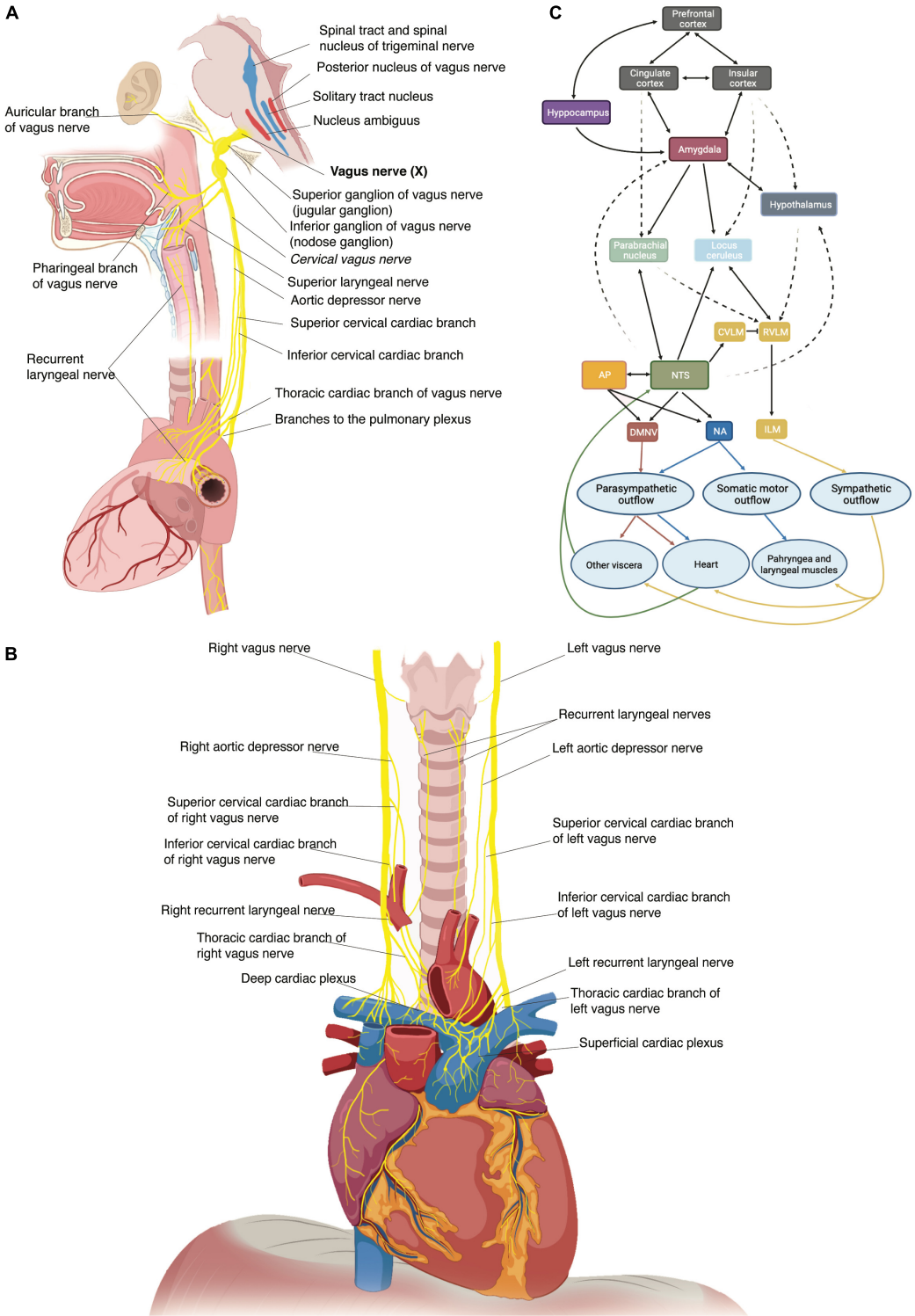

FIGURE 1 | (A) Schematic representation of the origin of the vagus nerve from the medulla, its ganglia and its major branches at the cervical and thoracic levels. **(B)** Thoracic vagus nerves with cervical and thoracic cardiac branches to the deep and superficial cardiac plexi and recurrent laryngeal nerves. **(C)** Schematic representation of the central autonomic network with internuclei connections. An autonomic vagovagal loop comprises visceral inputs to the nucleus tractus solitarii (NTS) which then sends outputs to the dorsal motor nucleus (DMNV), rostral ventrolateral medullary (RVLM), and intermediate lateral medulla (ILM) to adapt autonomic balance to physiological demands. The cross-talk between the NTS and brain regions (hypothalamus, amygdala, cingulate cortex, insula, prefrontal cortex) engaged in neuroendocrine, affective, and cognitive regulation of behavior modulates this autonomic forebrain loop. AP, area postrema; NA, nucleus accumbens.

nerve, separately from those coming from other visceral organs (**Figure 2**) (44). The precise distribution of fibers from the other visceral organs, especially fibers from peripheral cardiovascular targets, remains a matter of study, along with the definition of VN anatomical-functional models to guide the design of future VNS devices and protocols (48).

The VN is a mixed nerve with fibers carrying sensory, motor, and visceral information. It entails mostly afferent nerve fibers (80–90%) with fewer efferent fibers (10–20%) in the majority of mammalian species (26, 49). VN fibers are classified as "A-fibers," "B-fibers" and "C-fibers" in accordance with the classical Erlanger/Gasser classification (50). Among the afferents, C-fibers are thin unmyelinated, Aδ-fibers are thin myelinated and Aβ-fibers are thicker myelinated. Among the efferents, Aα-fibers are the thickest myelinated axons of α-motoneurons that innervate pharyngeal and laryngeal muscles, while B-fibers are tiny, myelinated and carry parasympathetic inputs to visceral organs (51, 52). The diameters of the unmyelinated and myelinated fibers of the VN are in the range of 0.25–1.0 μm and 1–4 μm, respectively, in most animal species (53). Nearly all the large (above 10 μm) and 40–50% of the small (below 4μm) myelinated fibers are efferent (49).

Preganglionic parasympathetic fibers originate from the dorsal motor nucleus of the vagus in the brain stem, branch out of the VN main trunk to join several autonomic plexuses and synapse at cell bodies of postganglionic neurons, generally located in the wall of the target organ. Afferent fibers consist of the T-shaped axons of pseudounipolar sensory neurons, with their neuronal soma residing in the nodose and jugular ganglia (52–54). In the brainstem, central processes of jugular ganglion neurons project to the trigeminal nucleus through the spinal trigeminal tract (26), while the primary relay of vagal visceral inputs from nodose ganglion neurons is the nucleus tractus solitarii in the medulla (55, 56). The nucleus tractus solitarii has direct and indirect connections with a wide range of neural structures, thus endowing the VN with the control of a broad array of processes (**Figure 1C**) (55, 57, 58). An autonomic vagovagal loop encompasses visceral inputs to secondary neurons in the nucleus tractus solitarii, which then contact efferent neurons of the dorsal motor nucleus and sympathetic neurons of the

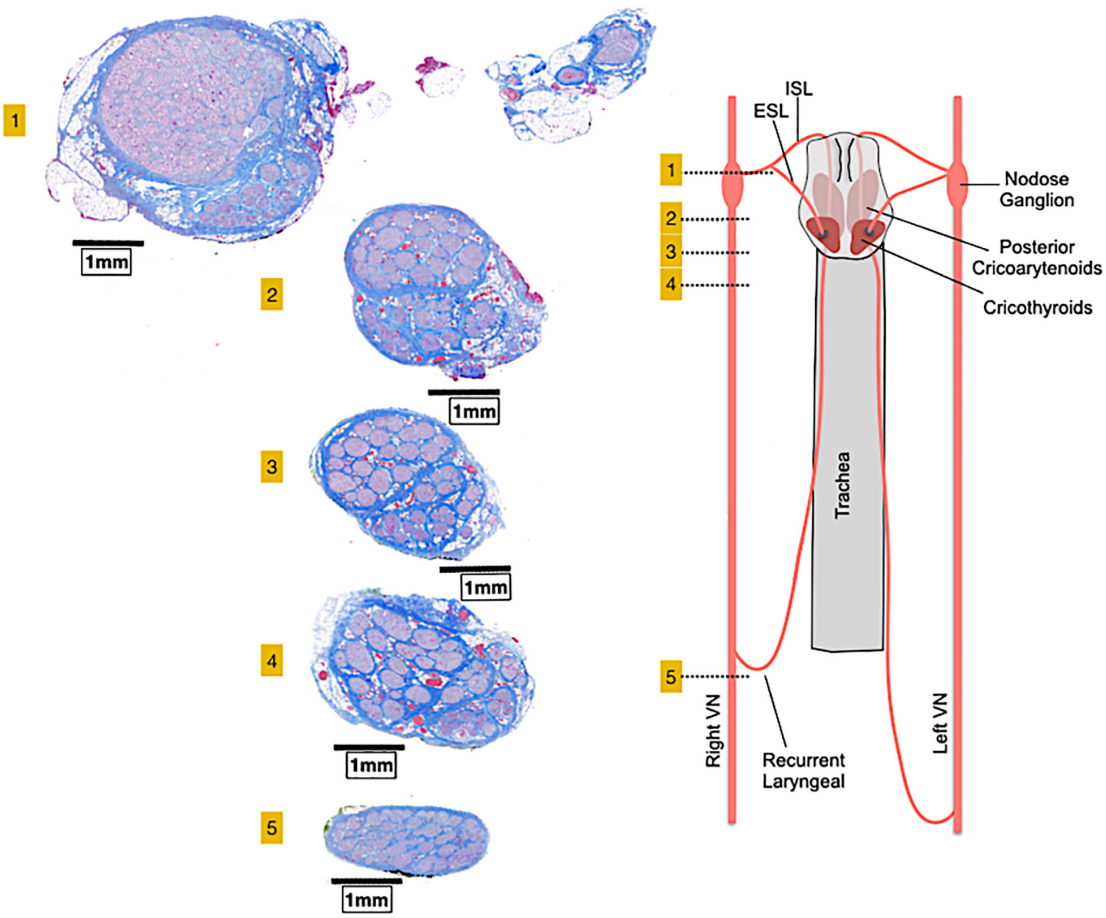

FIGURE 2 | A bimodal arrangement, or vagotopy, can be observed in histological sections collected at various levels along the pig vagus nerve. Section 1 comprises the nodose ganglion and the superior laryngeal branch and show pseudo-unipolar cells grouped in a huge "fascicle" from which arises a smaller group of fascicles in sections 2, 3, and 4. Caudally to the recurrent laryngeal nerve (section 5), the bimodal architecture is no longer visible. ESL, external superior laryngeal nerve; ISL, internal superior laryngeal nerve, VN, vagus nerve. Adapted from Settell et al. (44).

rostral ventrolateral medulla to adapt the autonomic balance to physiological demands (**Figure 1C**) (3).

Vagal Baroreceptors and Chemoreceptors

Vagal sensory neurons densely innervate great thoracic vessels and they include Piezo2 + /TTN3 + mechanosensory fibers, functioning as baroreceptors, and chemosensory fibers that detect arterial blood gas changes in the aortic bodies (**Figure 3**) (59). Afferent fibers from the left nodose ganglion innervate the apex of the aortic arch, while afferents from the right nodose ganglion innervate the right subclavian artery, near its branching from the innominate artery. These fibers run within the aortic depressor nerves forming fascicles that include both high-threshold mechanosensory and chemosensory afferents (59). The majority of vagal baroreceptors are myelinated fibers that convey information on stretch magnitude, pulse frequency and mean arterial pressure (59, 60) and, together with the carotid sinus innervated by the glossopharyngeal nerve, they represent the afferent arm of the arterial baroreflex (61–63).

Vagal Cardiac Receptors

The VN provides both sensory and parasympathetic innervation to the heart *via* cardiac branches. Nodose neurons terminate with chemoreceptors and/or low-threshold mechanoreceptors in cardiac atria, ventricles, and major veins (**Figure 3**). Under normal circumstances, cardiac receptors are necessary for fine-tuning of the cardiovascular system. In CVDs like heart failure (HF), sensory endings undergo pathological activation that causes autonomic imbalance, with sympathetic excitation prevailing over vagal excitation (21, 22, 64).

There are at least two different types of atrial receptors belonging to the VN system of several mammalian species: type B receptors, that fire in response to increased volume (stretch receptors), and type A, that respond to atrial contraction. Both receptor endings correspond to slightly myelinated/unmyelinated fibers located mainly in the endocardium at the pulmonary veins-atrium and caval-atrium junctions and, to a lesser extent, in the free wall and appendage of both atria (65). They function as slowly adapting stretch receptors with low-frequency firing (66). Other vagal afferent fibers from the atria are unmyelinated C-fibers with a diffuse distribution and activity patterns similar to those described for type A or type B receptors (64).

Two types of sensory vagal endings have been described in both cardiac ventricles: myocardial and epicardial receptors (**Figure 3**). Myelinated myocardial receptors are mechanosensitive fibers working as tension/pressure-sensitive receptors and fire at the onset of left ventricular contraction (59, 64). On the other hand, unmyelinated myocardial receptors include 5-HT3R + C-fibers, predominantly functioning as mechanoreceptors (**Figure 3**) (64, 67), and C-fibers predominantly functioning as chemoreceptors which transduce the pain sensation that characterizes angina pectoris (21). Finally, vagal afferent fibers innervating the parietal pericardium are finely myelinated and sensitive to pericardium distension (64).

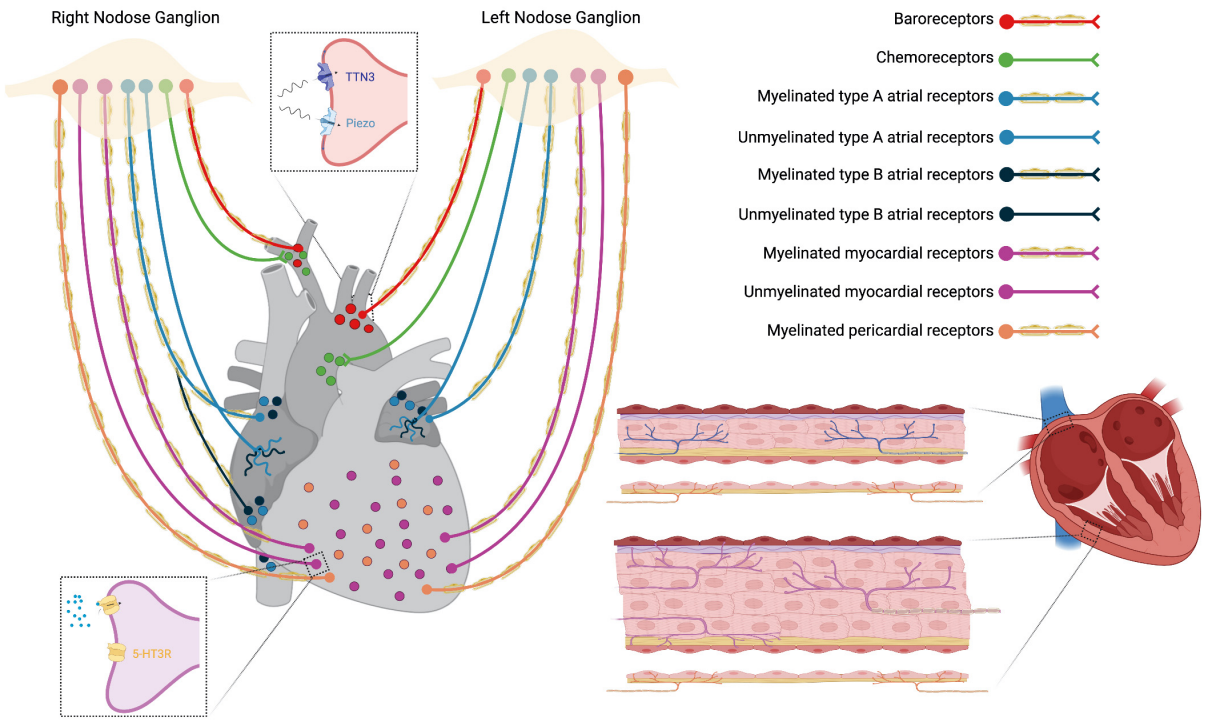

FIGURE 3 | Schematic representation of thoracic cardiovascular afferent fibers running in the vagus nerve. Insets: top, baroreceptors Piezo2 + and TTN3 + terminals; down, unmyelinated 5-HT3R + myocardial receptors.

Vagal Cardiac Efferent Fibers

The VN provides parasympathetic innervation to the heart *via* preganglionic cardioinhibitory neurons mainly located in the nucleus accumbens and to a lesser extent in the caudal dorsal motor nucleus of the vagus (21). Neurons of the nucleus accumbens possess thin myelinated axons with a diameter comprised in the B-fibers range (conduction velocity range 3–15 m/s) (68, 69) and exert strong respiratory- and cardio-modulatory and chronotropic effects. Efferent fibers from the nucleus accumbens of the right VN synapse with postganglionic cholinergic neurons that innervate the sino-atrial node, while fibers of the left VN project to postganglionic cholinergic neurons that innervate the atrioventricular node (21, 63). In contrast, neurons of the dorsal motor nucleus have unmyelinated axons, show little or no respiratory and cardiac modulation, exert smaller effects on HR and possibly stronger dromotropic and inotropic effects, as they project to postganglionic neurons innervating the left ventricle (70, 71). Cardiac neural control is realized *via* tonic interaction between sympathetic and parasympathetic limbs of the ANS, particularly in the mammalian heart, where many terminal fibers lie close to each other and exert reciprocal inhibitory effects at the synaptic level (21, 22, 72).

VAGUS NERVE STIMULATION FOR CARDIOVASCULAR DISEASES

Vagus nerve stimulation was first developed for the treatment of drug-resistant epilepsy and depression, obtaining FDA approval in 1994 (73–77) and 2005 (24, 78–81), respectively. Based on prior animal experiments, the first clinical studies defined the therapeutic range of VNS, the algorithm for stimulation titration up to the threshold of patient tolerance, and the safety and tolerability profile (73, 82). With the discovery of the inflammatory reflex in the early 2000s (83) and the evidence that VNS could attenuate inflammation normalizing the expression of proinflammatory cytokines (such as TNF-α and IL-6), VNS studies increased exponentially (28). At present, research labs worldwide are studying the effects of VNS in a multitude of conditions, spanning from neurological to inflammatory disorders, both in animal models and in patients (84).

The VN can be stimulated in different ways and at different levels. The classical VNS consists in an invasive procedure that is performed as a day case procedure under general anesthesia. The FDA-approved VNS device comprises a spiral anchor and two bipolar helical electrodes with a platinum ribbon functioning as an anode and a cathode. They wrap approximately 270° around the left cervical VN, below the origin of the superior and inferior cervical cardiac branches, and are connected *via* a cable tunneled subcutaneously to a pulse generator that is most commonly positioned in a infra-clavicular pocket (24, 85–90). Despite the fact that VNS is a minimally invasive treatment, surgery remains inherently risky and comes with a number of possible side effects (91, 92). Therefore, alternative non-surgical methods have been developed, such as cervical non-invasive or transcutaneous VNS directed to the auricular branch of the VN (**Figure 4**) (91, 93–95). In the current standard practice, VNS parameters are set individually and tuned periodically for each patient. An "adequate" stimulation is generally set between a minimum level of perception by the patient to a maximum level

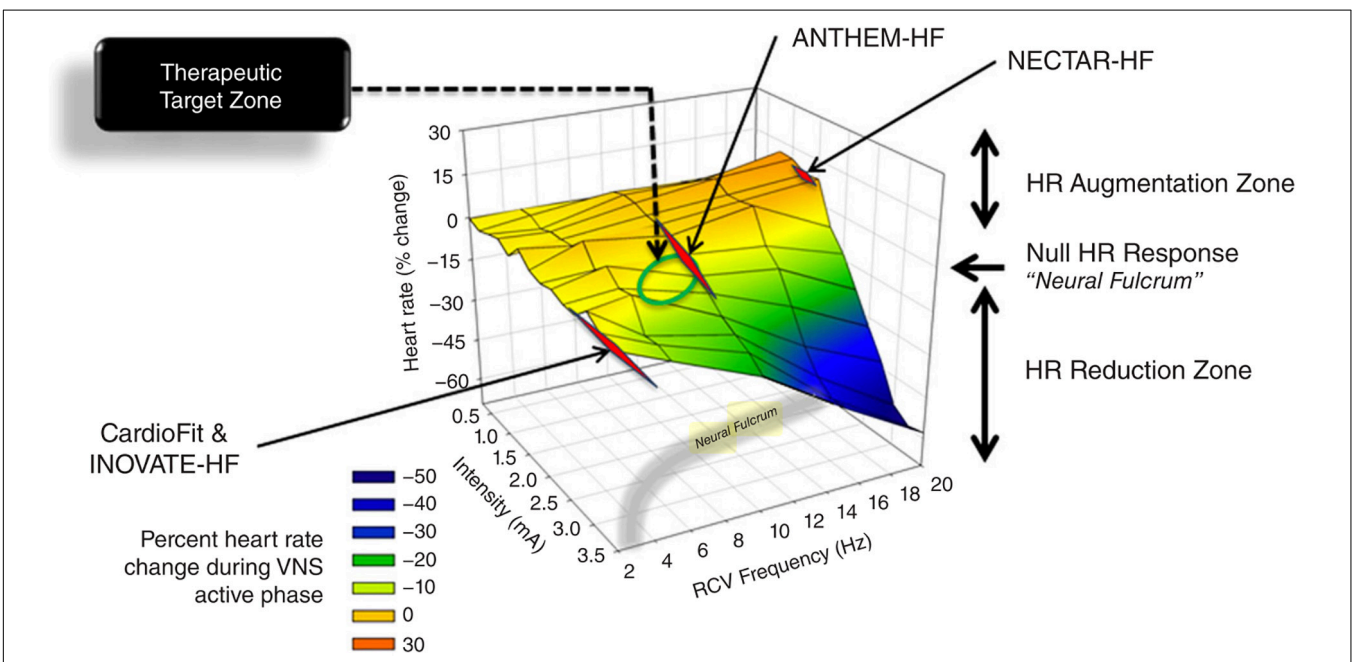

FIGURE 4 | 3D plot of percent change in heart rate against right VNS intensity and frequency, with representation of the neural fulcrum in the yellow-shaded region. Operational points used in the NECTAR-HF, INOVATE-HF, and the ANTHEM-HF studies. Adapted from Ardell et al. (17).

of intolerability due to side effects, both of which are subjective and variable (96).

Vagus Nerve Stimulation Mechanisms on the Cardiovascular System and Cardiovascular Diseases

Vagus nerve stimulation impacts cardiovascular control at multiple levels (97) *via* activation of afferent and efferent pathways and, depending on the frequency, pulse-width, and current intensity, of diverse fibers populations (43, 98).

In general, VNS of efferent cardiac fibers causes a reduction in heart rate (negative chronotropic effect on the sinoatrial node), in atrioventricular conduction (negative dromotropic effect on the atrioventricular node), and in ventricular contractility (negative inotropic effect on ventricular myocardium) (28, 99), with right VNS having mostly chronotropic effects while left VNS mostly dromotropic effects (100). VNS modulates left ventricular function increasing both action potential duration and the effective refractory period, either of which decreases intracellular calcium and ventricular contractility and wall motion (28, 89, 101). Activation of descending efferent projections can also mitigate sympathoexcitation *via* neural interactions within the intrinsic cardiac nervous system, modulate cardio-cardiac reflexes, and impart cardioprotection *via* direct effects on cardiomyocytes (1).

Vagus nerve stimulation of afferent fibers can impact central reflexes, including those that involve sympathetic and parasympathetic efferent outflows to the heart (1). For instance, VNS of vagal baroreceptors reflexively activates vagal cardioinhibitory efferent fibers to reduce heart rate and concurrently inhibits sympathetic efferent activity and down-regulates the renin-angiotensin-aldosterone system (28, 102).

Vagus nerve stimulation effects demonstrated beneficial effects in different animal models of CVDs. First of all, VNS has shown antiarrhythmic effects in several conditions, probably *via* multifactorial mechanisms that include a decrease in heart rate, the release of nitric oxide, anti-inflammatory effects, and antagonism of the sympathetic nervous system (28, 103). It was shown that VNS increases the threshold for ventricular arrhythmias *via* reduction in ventricular excitability and repolarization heterogeneity (effect on ventricular conduction system) (28, 99). In animal models of atrial fibrillation, VNS exhibited antifibrillatory effects by shortening atrial fibrillation duration and prolonging the atrial fibrillation cycle length (19, 28). Moreover, a VNS delivered below the threshold of bradycardia induction can effectively suppress atrial fibrillation in anesthetized dogs (89). VNS effects on the sympathetic nervous system contribute to the prevention of arrhythmias also during cardiopulmonary resuscitation (28). VNS-induced decrease in cardiac motion reduces cardiac metabolic demands during the vulnerable period of ventricular fibrillation, making VNS a potential intervention to improve the efficacy of defibrillation (101).

Vagus nerve stimulation was also shown to decrease infarct size and to halt post-myocardial infarction phenomena such as the remodeling of both the myocytes and the intrinsic cardiac neuronal system. These cardioprotective mechanisms include anti-inflammatory effects, prevention of Connexin 40 and Connexin 43 loss, antioxidative effects, and antiapoptotic effects such as decrease in cytochrome *c* release and in the proapoptotic Bcl-2-associated X protein levels (28, 89, 103–106). When applied during myocardial reperfusion, VNS was shown to improve ventricular function and reduce arrhythmic episodes *via* antagonization of the cardiac sympathetic outflow, reduction of reactive oxygen species and of ventricular excitability (28, 103–106). VNS improves left ventricular ejection fraction post-myocardial infarction restoring subcellular levels of calcium-binding proteins (such as SERCA2a, NCX1, and PLB) and can reestablish baroreceptor reflex to the pre-infarction baseline (28).

Vagus nerve stimulation can slow the progression of myocardial remodeling and atrial and ventricular dysfunction in animal models of chronic HF with reduced ejection fraction (28). VNS beneficial effects in HF can be attributed to improvements in left ventricular mechanics, attenuation of the sympathetic drive, down-regulation of the renin-angiotensin-aldosterone system, reduction of proinflammatory cytokines, normalization of the nitric oxide pathway, increase in myocardial expression of gap junction proteins and capillary density and tempering of myocardial interstitial fibrosis (1, 28, 104, 106–113). Optogenetic stimulation of cardioinhibitory neurons in the dorsal motor nucleus of the vagus can reduce myocardial expression of G-protein-coupled receptor kinase 2 (GRK2) and b-arrestin 2, which both contribute to the progressive decline of myocardial contractile function in HF (104).

Vagus nerve stimulation can ameliorate poststroke recovery *via* enhancement of motor cortex plasticity during rehabilitation, likely favoring the release of acetylcholine, norepinephrine, GABA, and brain-derived neurotrophic factor (28). VNS can also attenuate cerebral edema after brain injury by reducing cerebral blood flow, glutamate excitotoxicity, and inflammation (28).

Finally, VNS in hypertensive rats showed a significant blood pressure reduction, with static stimulation clinically more effective than pulsatile stimulation (28).

Vagus Nerve Stimulation in the Clinical Scenario

To date, despite the vast assortment of CVDs investigated in the pre-clinical scenario, VNS clinical applications in the cardiovascular field have been mostly focused on HF. This syndrome provides a strong rationale for ANS modulation, as its genesis and progression are heavily influenced by autonomic imbalance (21, 22, 114, 115). Preclinical studies have shown that VNS can exert very positive effects on the progression of HF, but clinical trials failed to achieve the same results. Complete clinical trials of VNS for HF with reduced ejection fraction include two randomized controlled trials, i.e., the INOVATE-HF (116) and the NECTAR-HF (117), and two open-label studies, i.e., the ANTHEM-HF (118) and the study by De Ferrari et al. (119). In the study by De Ferrari et al. (119) and in the INOVATE-HF, VNS was delivered using the CardioFit system that senses heart rate (*via* an intracardiac electrode) and delivers asymmetric stimulation at a variable delay (70–325 ms) from the R-wave

(85, 87, 119, 120). The stimulation lead is an asymmetric bipolar multi-contact cuff electrode specifically designed for cathodic induction of action potentials while simultaneously applying asymmetrical anodal blocks, thereby reducing the activation of A-fibers while preferentially activating efferent B-fibers (85, 87, 105). In the NECTAR-HF (117) and the ANTHEM-HF (118) trials the investigators utilized the Boston Scientific VNS device that activates VN fibers bidirectionally with no synchronization with the cardiac cycle (118). All trials recruited similar NYHA class II-III patients with reduced left ventricular ejection fraction and receiving optimal medical therapy (121). These trials did not raise safety issues but showed variable efficacy (116, 118, 121, 122). As highlighted by a recent meta-analysis, these trials showed significant improvement in the functional NYHA class, quality of life, 6-min walking test, and NT-proBNP levels, but VNS did not have any impact on mortality (123). The subjective measures that improved in all trials should be cautiously taken as subjects were not totally blind to the therapeutic procedures, notwithstanding the sham-controlled design (116, 118, 124). Only the two uncontrolled studies (ANTHEM-HF and the study by De Ferrari et al.) showed a positive effect of VNS on cardiac remodeling (118, 119), despite the success of preclinical experiments (118, 120). These trials employed diverse stimulation parameters, and subsequent analyses showed that the ANTHEM-HF study was the only one to achieve the therapeutic stimulation corresponding to the "neural fulcrum" (17). The neural fulcrum is defined as the combination of VNS parameters such as frequency–amplitude–pulse width that results in no heart rate response (**Figure 4**), and it corresponds to a dynamic equilibrium where VNS activates cardiac neural circuits while keeping reflex control of cardiovascular functions. In fact, VNS normally modifies cardiac neural circuits pushing them in one direction that tends to be physiologically counterbalanced by cardiovascular reflexes (17). For instance, low intensity/high frequency (20 Hz or more) stimulation preferentially activates afferent fibers causing tachycardia that leads to secondary activation of both central pathways and vagovagal, vagosympathetic, or vagoadrenal reflexes. On the other hand, higher intensity/lower frequency (10–15 Hz) stimulation activates parasympathetic neurons resulting in bradycardia and mitigation of sympatho-excitation *via* neural interactions within the intrinsic cardiac nervous system (1). Finally, low intensity/very low frequencies (1–2 Hz) stimulation determines little to no cardiomotor effects, as the afferent-driven decreases in central parasympathetic outflow are equivalently counteracted by direct activation of cardiac parasympathetic neurons (125).

ANTHEM-HF utilized the principle of neural fulcrum by determining the autonomic engagement *via* an automatic beat-to-beat pattern analysis throughout the initial phase of VNS titration (122). In the NECTAR-HF trial, the high frequency stimulation provoked patients intolerance and impeded titration to a therapeutic dose; in the INOVATE-HF study, not all patients received adequate stimulation levels (116, 121, 122, 126–128). On the basis of the results of ANTHEM-HF, a large, randomized, controlled trial of right VNS with the use of the same system is now underway (ANTHEM-HFrEF PIVOTAL trial, NCT03425422) (18), along with a novel study in patients suffering from HF with preserved and mid-range ejection fraction (127).

Unresolved Issues in Clinical Vagus Nerve Stimulation for Cardiovascular Diseases

The exploitation of VNS full potential and its transformation into a simple and cost-effective therapy for a wide range of conditions requires the completion of some major steps. A general problem is the still limited knowledge of VN function, with an ensuing lack of understanding of the mechanisms responsible for already established VNS treatments of diseases such as drug-resistant epilepsy (121, 126, 129). Consequently, optimum stimulation parameters tailored for patient-specific clinical characteristics and precise timing remain a matter of debate (74, 75, 79, 128, 129). VNS is normally up-titrated through a series of follow-up visits until a therapeutic dosage is attained without adverse effects and up to the tolerance threshold of the patients (73, 94). Given subjectivity of patients' tolerance and the uniqueness of the electrode-nerve interface, no standard therapeutic dose exist, the effectiveness of parameters adjustment during titration remains dubious, and the response prediction uncertain (92, 121, 122, 129, 130).

Another related problem is the definition of the target population using adequate "predictors" to discriminate between responders and non-responders. These predictors could be markers of autonomic imbalance represented by physiological parameters such as heart rate variability or innovative markers such as those derived from neural decoding of specific ANS circuits (78, 118, 129, 131).

While technology advances at a quick pace, neuromodulation's ultimate potential can be realized when the relationship between nerve activity and physiological function is thoroughly known (4, 6), thus allowing translation of biological information into appropriate engineering specifications (16, 132). The knowledge gap of vagal physiology, functional anatomy and neuromodulation mechanisms inevitably also affects the selectivity of neural interfaces and of neuromodulation protocols (133). First of all, most VNS systems lack functional selectivity, that is stimulation of distinct functional classes of fibers, and are far from mimicking patterns of action potential occurring in healthy nerve fibers (32, 133). Secondly, most VNS systems lack spatial selectivity, that is selective modulation of fibers in the specific anatomical territory innervated by a given fascicle (32). Electrodes commonly used for VNS are not selective enough to achieve targeted neuromodulation in a complex fasciculate nerve like the VN (133). The direct consequences are the failure to achieve therapeutical effects and the onset of side effects that include hoarseness, throat pain, voice alteration, difficulty swallowing, coughing, abdominal and chest pain, nausea, dyspnea, and bradycardia (36, 85–88, 90). The inadvertent stimulation of somatic nerve branches such as the superior and recurrent laryngeal nerve has been addressed as one of the main causes of VNS side effects (36). Selective stimulation of vagal cardiac B and C fibers can be challenging given that their thresholds are 2–100 times greater than A fibers,

as those branching to the laryngeal nerves (89). To achieve such selectiveness, several authors tried the combination of different stimulation parameters or to modify the pulse shape using different techniques such as the anodal block, slowly rising pulses or depolarizing pre-pulses (89). Other strategies are the modification of electrode design to allow preferential activation of efferent fibers (such as in the case of the CardioFit system) or the development of multicontact electrodes that exploit the topographical architecture of human nerves to target organ-specific fascicles (36, 88–90). This last approach would provide better spatial resolution and consequently improve the selectivity both for recordings and stimulation (36, 89). Therefore, new neural interfaces with higher electrode counts and spatial selectivity should be implemented (8, 30), along with advanced signal processing techniques and the use of hybrid models (134, 135) with extensive validation in experimental animals (136). With such technologies, the development of closed-loop VNS based on the combination of selective nerve stimulation and biosensing technologies could be one of the best solutions to overcome the aforementioned limitations emerged during VNS clinical trials (7, 133).

Closed-Loop Strategies for Vagus Nerve Stimulation

The current VNS systems provide stimulation in an open-loop fashion, meaning that parameters are pre-set and are not automatically adjusted according to the patient's clinical characteristics (137). Closed-loop stimulation strategies offer the advantage of providing treatment only in response to detection of altered biomarkers of disease, thus tuning the stimulation according to the patient's condition (138, 139). This approach potentially improves the efficacy of open-loop interventions and decreases the associated side effects (140). Closed-loop devices should continuously monitor internal biological variables to adjust therapy to individual conditions (7), thus allowing not only automatic but also adaptive neurostimulation that could maintain its efficacy over time and overcome the intrinsic plasticity of biological systems (8, 11, 141). In fact, plasticity and memory are crucial characteristics of the cardiac neuraxis, which undergoes profound alterations in chronic CVDs, causing the disruption of homeostatic cardiovascular functional responses (19).

Closed-loop VNS relies heavily on the precise selection and processing of physiological inputs (32, 142). To date, in the clinical scenario, only macro-biosignals like heart rate have been employed as input data for control loops. This concept is well illustrated in the work by Tosato et al. who achieved heart rate regulation with a closed-loop control system that continuously measured the RR interval, recalculated the difference between the measured and the target value and fed it back to the stimulator accordingly (100). Multiple other indirect and non-invasive measures can be used as indexes of cardiac VN activity (115), for instance heart rate variability, baroreflex sensitivity or respiratory sinus arrhythmia (21, 115). However, such clinical vagal indexes should be used with caution as they represent gross markers of the final net effect of parasympathetic and sympathetic action on the heart (72). On the other hand, local control loops can be obtained by recording feedback bio-signals from the same spot where the stimulus is delivered (142). This approach optimizes the spatial and temporal distribution of the local stimuli (142) but requires high-fidelity feedback signals that are not clinically obtainable due to current technological limitations (142, 143). A good example is represented by the lack of instruments to properly follow and locally evaluate in real-time the myriad of metabolic signals or the fluctuating levels of inflammatory stimuli. Nevertheless, all this information is collected by the peripheral sensors of the ANS and converted into electrical signals (neural encoding), a sort of "neural footprints" of physiological processes that can be recorded and decoded (**Figure 5**) (8, 30). Deciphering the neural language through decoding techniques would be essential to understand the underlying mechanisms of many diseases and to develop new methods and technologies that better engage with neural circuities (141). In the case of VNS, the copresence of afferent and efferent fibers in the VN offers the opportunity to build a feedback loop on the same anatomical site, that is to record from and then to stimulate the VN (**Figure 5**) (144, 145). Vagal sensory neurons are equipped with a vast arsenal of receptors to sense and respond to a huge variety of stimuli (27). Consequently, neural signals traveling through the VN represent a peerless source of information, and this helps the implementation of neural decoding strategies that usually benefit from an adequate number of signals (30, 32). Moreover, vagal neural signals offer the advantage of high temporal and spatial resolution (146), helping in the identification and classification of patterns difficult to see using other biosignals (8, 30) and potentially allowing devices to diagnose various conditions before symptoms presentation (32). New neural interfaces with higher electrode densities and spatial selectivity, such as intraneural electrodes, (147) and advanced signal processing techniques (8) will be necessary to take full advantage of the information traveling along with the VN. In the next section, we present the main preclinical studies that aim at the development of VNS closed-loop approaches based on different neural recording and decoding strategies for CVDs.

Decoding Techniques for Physiological Fiber Firing

Neural interfaces used for stimulation can also be used to record neural signals and monitor ANS activity in real-time (13). While innovative neural interfaces with multiple contacts are designed to improve the quality and information content of neural recordings (148–151), comparable efforts are being made to develop advanced signal processing and data analysis methods (152, 153). Several studies recently focused on the extrapolation of neural markers from spontaneous or physiologically enhanced VN activity, employing various decoding techniques. One technique is the coherent electroneurogram averaging that aims at the isolation of the neural activity of interest from the random noise by taking the average of N snippets from a recorded signal in correspondence to an external or an internal trigger, for instance a biological change (154). Using multicontact cuff electrodes, Plachta et al. employed the ECG rising edge as a trigger to remove stochastic noise, isolate baroreceptors activity from VN recordings and perform selective VNS reducing blood

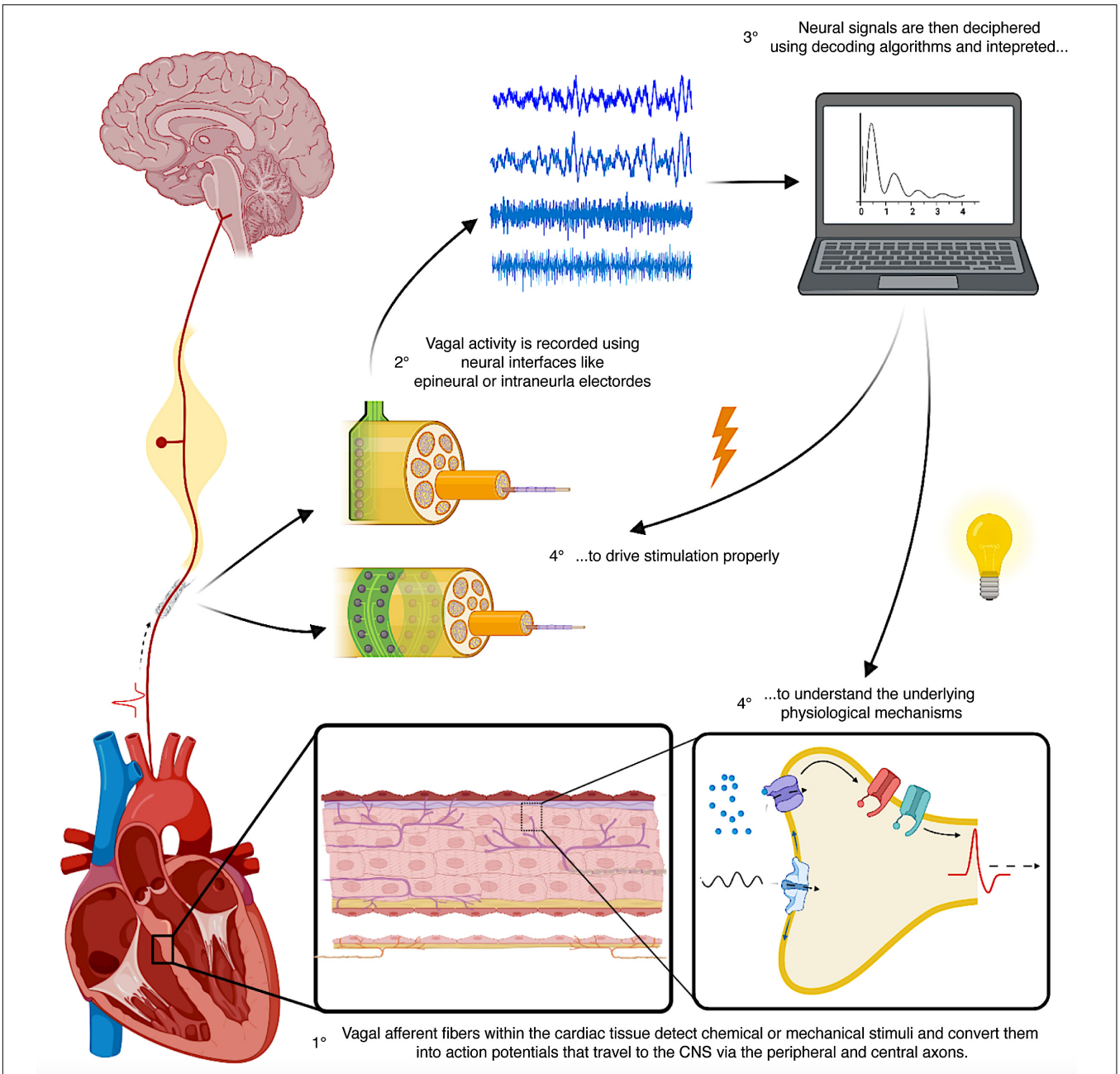

FIGURE 5 | Schematic representation of the principles of closed-loop neuromodulation using neural recordings and decoding strategies. The heart is densely innervated by vagal afferent fibers, which detect chemical and mechanical stimuli, convert them into electrical signals (action potential) and send them to the CNS *via* their peripheral and central axons. Using neural interfaces placed on the vagus nerve, it is possible to record and decode these electrical signals and identify the underlying physiological processes and set the spatiotemporal characteristics of stimulation. CNS, central nervous system.

pressure without producing side effects in rats (145). Sevcencu et al. recorded signals from the porcine left VN to extract intraneural and extraneural profiles resembling the temporal evolution of blood pressure during baseline activity. In particular, systolic peaks and dicrotic waves characterizing blood pressure were reflected by the neural counterpart (144, 155). Rozman and Ribarič employed 33-electrode spiral cuff to record from the left VN of a dog during stimulation of cardiovascular or respiratory stimulations and identified the channel that was best correlated with heart activity using the spectrum estimation technique (156).

Another common approach to extract physiological information from VN electrical activity consists of decoding fiber spike patterns using spikes sorting techniques (157–162). Typically, raw neural signals are band-passed from 200 Hz to a maximum of 10 kHz, robustly denoised, spikes are then

detected using thresholding methods and clustered using feature waveforms (46, 163). In general, there is not a universally adopted low-frequency cutoff as it usually depends on the quality and the nature of the signals. For instance, 200 Hz low-frequency cutoff was used in the context of intrafascicular sciatic nerve recordings (164), 1 kHz for intraneural recordings from the pig VN (136), 700 Hz (165) and 300 Hz (166) in the case of microneurographic recordings from the peroneal nerve and from the human VN, respectively. In the murine VN, spike sorting techniques within decoding frameworks have been employed to decode the activity of different fiber types enhanced by inflammatory stimuli such as particular cytokines (153) or metabolic ones like hypoglycemia/hyperglycemia (167). Spike sorting techniques are potentially usable in human patients as we recently obtained single-fiber recordings from the human cervical VN identifying tonically active neurons that discharged synchronously with the respiratory and cardiac cycles (166). Spike-like signals, as they reflect the activity of individual fibers, are preferable to cumulative signals to obtain maximal functional selectivity (162). Real-time implementations of complex spike sorting algorithms onto low-power off-the-shelf digital signal processors are currently available, as in the case of neuroprosthetic applications where the power consumption enabled more than 24 h processing at the maximum load (162). In the case of closed-loop VNS protocols, this would allow longer operational time scales, such as Holter-like monitoring at a neural level, i.e., a "Neural Holter" with biomarkers extracted directly from neural activity. As pointed out in Raspopovic et al. (136), signals recorded with intraneural electrodes can be classified as a hybrid category between cumulative and single-unit signals. This characteristic allows the development of more robust recording schemes and processing algorithms *via* a combination of decoding strategies developed on both cumulative and single-unit signals (136).

The identification of neural signals elicited by specific physiological stimuli could be extremely useful to distinguish among VN fibers coming from different cardiovascular sites and carrying information on multiple functional parameters that can vary over short time windows, such as cardiac output and blood pressure. Such distinction could be further appreciated using neural multielectrode devices with high spatial selectivity to better interface the potential topographical architecture of the VN (44, 46). However, cuff electrodes can only sense compound nerve action potentials and multi-unit activity and they do not allow access to single-fiber action potentials contrary to intraneural electrodes (89, 168). Intraneural electrodes offer the advantage of higher signal-to-noise ratio and higher spatial specificity compared to epineural electrodes and could allow more effective closed-loop decoding methods to be used (8, 139, 169). Intraneural electrodes such as the Longitudinal Intra-Fascicular Electrode (LIFE) or the Transversal Intra-Fascicular Multi-channel Electrodes (TIME) have provided rich and valuable sensory feedback in human amputees and detailed information from decoding hand movements in somatic nerves (139, 157, 159, 170). The LIFE is a flexible electrode consisting of 25–50 μm diameter Pt or Pt-Ir wires insulated with Teflon or metalized Kevlar fibers insulated with medical-grade silicone. The wire is surgically inserted into the nerve along the fascicle and then pinched out of the nerve again. The recording sites are areas of 0.5–1.5 mm long which are left uninsulated (149). A more recent version of LIFEs is the thin-filmLIFEs (tfLIFE), based on a thin highly flexible micropatterned polyimide substrate filament that can host eight contact sites (46, 149). The TIME consists of a thin, strip-like polyimide substrate with platinum electrode sites. The substrate is folded to align several electrodes and the folded substrate is threaded transversely through the nerve between the fascicles (149). The original design contained 10 sites with interelectrode spacing of 230 μm (148). The TIME was developed to achieve good contact with nerve fibers, selectively addresses several fascicles in a nerve with a single implant, and minimizes the mechanical mismatch between the implanted material and nerve tissue (168). The TIME has shown higher selectivity at low stimulation intensities than the single LIFE and multipolar cuffs (46, 148, 149). Our group combined the use of multichannel intrafascicular electrodes, machine learning principles and hybrid models (136) to study high frequency (>1,500 Hz) VN activity of anesthetized pigs during artificially produced alterations of physiological parameters, simulating increases in respiratory rate, tidal volume and arterial blood pressure (**Figure 6A**) (152). Using a new decoding algorithm that combines wavelet decomposition, dimensionality reduction, and ensemble learning classifiers, we could associate VN signals to specific functional changes (**Figure 6B**). Our approach was a machine learning-driven approach to find informative feature vectors for reliable decoding of cardio-respiratory alterations regardless of their precise nature, and future analysis will serve to get more interpretable features related to units and aggregate activities. Different from epineural electrodes that can only provide a global picture of neural signal trafficking (147, 171), we employed intraneural electrodes to enhance selectivity (147, 169) and to map a possible spatial functional organization of VN fascicles. Thus, we employed a hybrid modeling framework based on histological analysis combined with electrode discrimination ability properties measured *via* a novel quantitative measure called Discriminative Field Potential (DFP) and we obtained distinct spatial configurations of discriminative patterns generated by fascicles during the various functional challenges (**Figure 6C**) (152). This is extremely important for the development not only of timely, but also spatially selective stimulation protocols in a complex nerve with multiple fascicles like the human or porcine VN (152). In this perspective, the precise knowledge of cardiovascular fibers arrangement within the cervical vagal trunk would be crucial to establish effective VNS protocols for the treatment of CVDs. Anatomical models and non-invasive tests should be developed and used to better anticipate which site for neuromodulation would give the best outcomes and to overcome the anatomical variability that limits clinical VNS applications (116, 120, 129).

The biocompatibility and longevity of intraneural electrodes was demonstrated in animal models and preliminarily confirmed in human experiments with long-term stimulation for sensory feedback and chronic neural recordings (139, 172). However, the

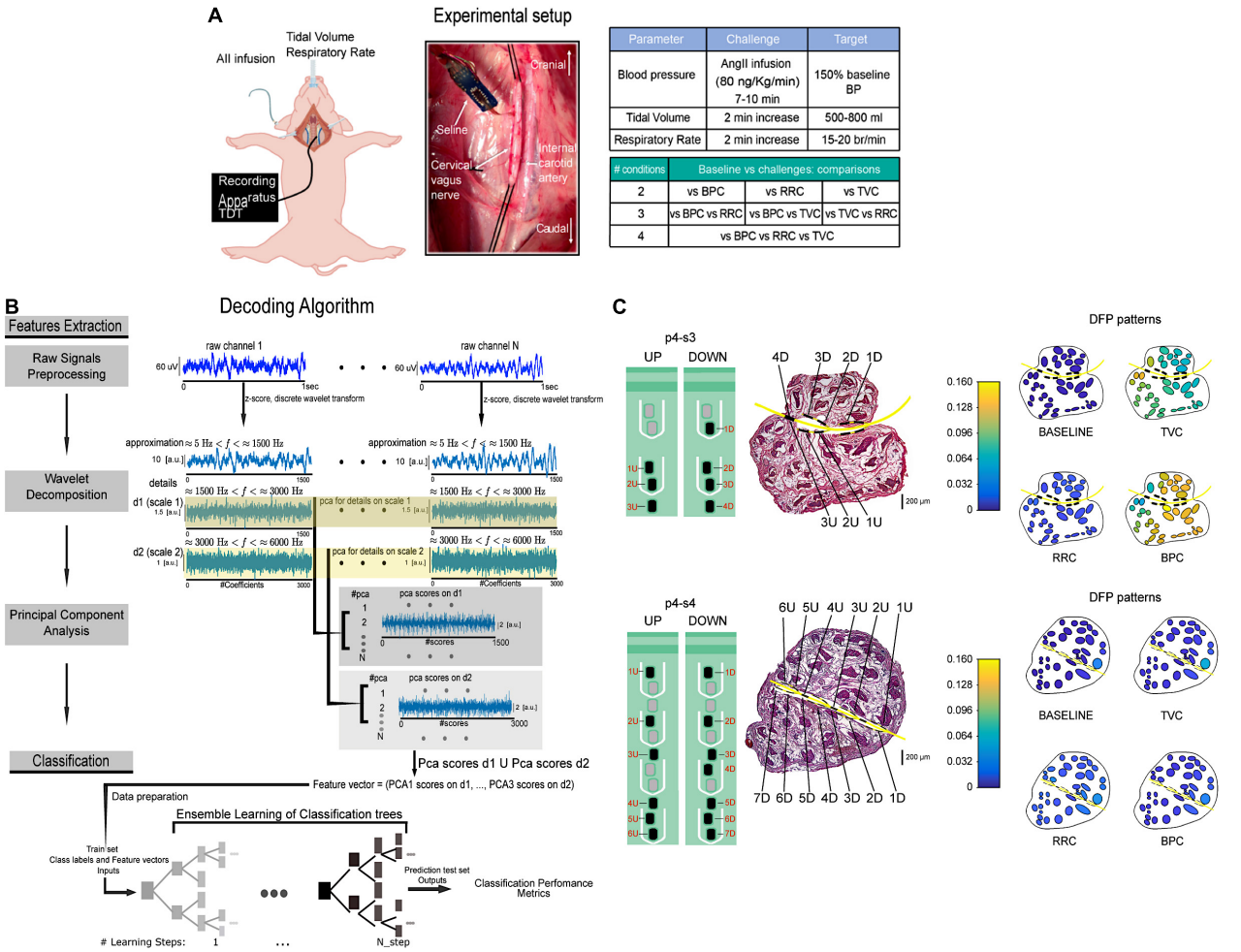

FIGURE 6 | Schematic representation of the experimental setup and decoding algorithm from Vallone et al. (152). **(A)** Recording apparatus, electrode implantation and summary of the *in vivo* protocol and functional challenges comparisons. **(B)** Decoding algorithm. Feature extraction was performed on 1 s portions of raw intraneural signals (dark blue traces) by applying principal component analysis on wavelet details relative to two different scales (1,500 Hz $< f <$ 3,000 Hz and 3,000 Hz $< f <$ 6,000 Hz). Neural signals recorded with intraneural electrodes were a combination of single-unit and compound action potentials. Decoding performed with ensemble learning based on classification trees combined with random undersampling and boosting procedure was applied on feature vectors. Decoding performance was assessed by means of confusion matrices and accuracy level. **(C)** Schematic description of two intraneural electrodes implantation in the VN of one pig and hybrid model simulations of fascicles activation at baseline and in response to functional challenges. The two intraneural electrodes are represented on a VN histological section (central panels). DFP patterns of activity during baseline and each functional challenge (left panels). DFP, discriminative field potentials. Adapted from Vallone et al. (152) with permission.

experience in chronic implantations in humans indicates that there is frequently a reduction in the number of functioning electrode active sites, an in- crease in the stimulation threshold, and a decrease of the signal-to-noise ratio along time. To further improve the usability of the neural electrodes, considerable efforts are being devoted in the engineering field for increasing robustness and flexibility at the same time of miniaturizing the electrodes and in the biological field to increase biocompatibility of the substrates and to modulate the foreign body reaction (46). On the computational side, since the drift in the amplitudes of signals and changes in the signal-to-noise ratio greatly hampers chronic neural recordings and decoding, new algorithms for drift compensation have been developed (173). In the case of the VN, carbon nanotube yarn electrodes have been used to make the first direct chronic measurements of vagal tone in freely moving rats (174). Thanks to their small size, high flexibility, and low impedance, carbon nanotube yarn electrodes have provided stable, high-signal-to-noise chronic recordings in rats VN with high-quality signals continuing up to 4 months after implantation (174).

Decoding Fiber Activity in Electrically Stimulated Nerves

Recording and decoding the spontaneous activity of vagal fibers would be useful to determine the precise timing for VNS delivery, but may prove limited for the definition of precise dosing (133). In this regard, recording and decoding fiber activity during VNS

may represent a complementary method to better define the relationship between stimulation and physiological effects.

The standard VNS dosing method does not rely on any measurement of fiber activation, since the commercial implants lack the capability to record from nerves during stimulation. Consequently, factors like electrode interface and nerve sensitivity are not controlled and VNS effects in a patient are neither uniquely determined over time nor comparable with

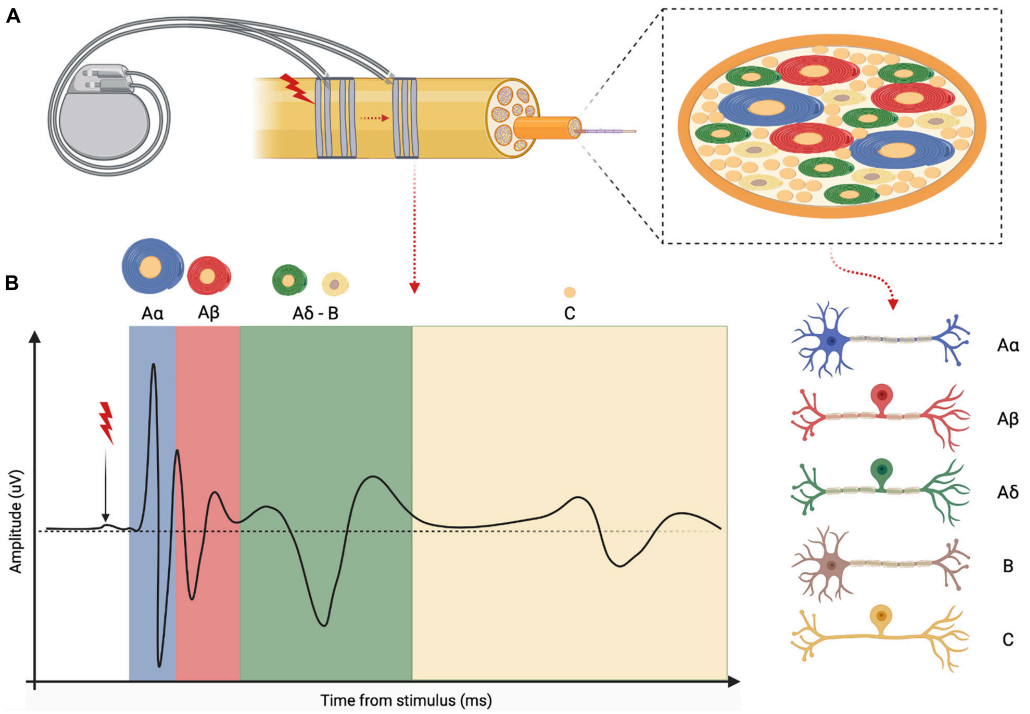

FIGURE 7 | Schematic representation and explanation of the electrically evoked compound action potential (eCAP). **(A)** Example of a VNS device that electrically stimulate the vagus nerve at one site while recording evoked activity at another site. Inset: vagal fascicle containing different fiber types distinguished by myelination degree and fiber caliber. **(B)** Example of a complete vagus nerve eCAP obtained with supra-threshold stimulation of all vagal fibers subtypes. Each peak of the eCAP corresponds to different fiber subtypes as indicated by the different color codes.

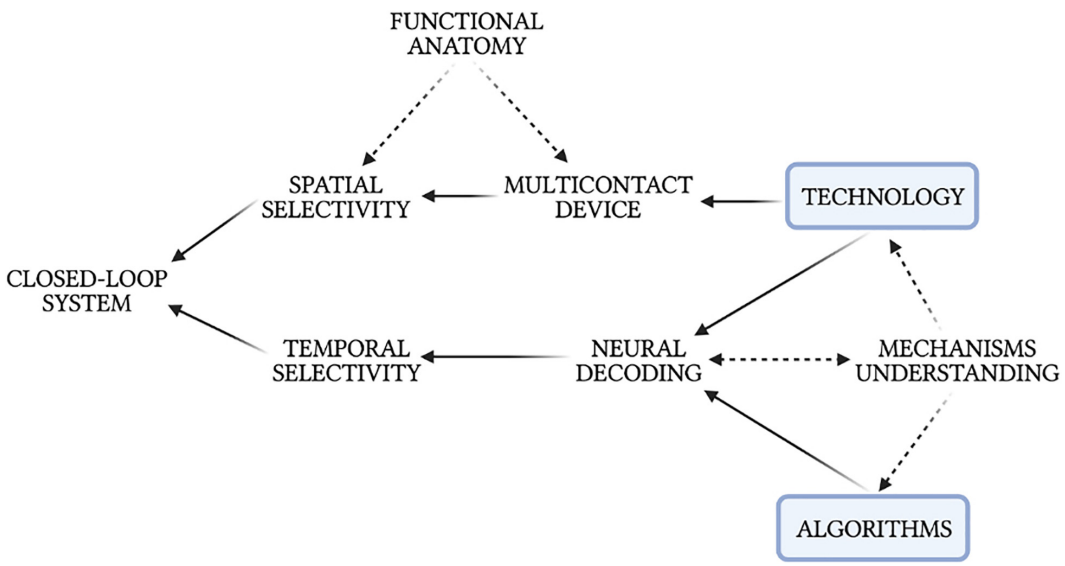

FIGURE 8 | Intercorrelations between neural decoding, technology, algorithms, physiological mechanisms, and the development of a closed-loop neuromodulation system.

other patients (133). The ability to distinguish between stimulated fibers could aid in correlating neural activity to external variables, thus increasing the ability to achieve targeted stimulation (175), with decreased variability in therapeutic responses and increased response rate (96).

In studies that employ extraneural interfaces, nerve activity is frequently analyzed in terms of evoked compound activity by electrical stimulation (eCAP) (176). The diverse conduction velocities of various fiber types, which ultimately depends on fiber caliber and degree of myelination, determine typical patterns and shapes with distinctive latencies and peaks in the eCAP (177), as shown in **Figure 7**. A sophisticated analysis of VN eCAPs would help in the assessment of the relationship between stimulus dose, neural recruitment and physiological effects (178).

Tahry et al. obtained, for the first time, VN eCAPs recordings after implanting the Advanced Nerve Stimulator version 300 (ADNS-300, Neurotech SA, Louvain-La-Neuve, Belgium) in the human VN (179). In preclinical studies, VNS-eCAP were used to optimize stimulation parameters and electrode design during VNS in dogs (176), pigs (180), and rodents (96), showing a strong correlation with the physiological effects of stimulation. For instance, vagal B-type fiber eCAP amplitude was correlated with changes in heart activity (176), indicating that parasympathetic B-fibers are the best predictors of cardiac activity during VNS (96, 177). Ordelman et al. found an indirect component in pig vagal eCAPs during VNS protocols, and they showed that it correlated with the state of the cardiovascular system (181).

CONCLUSION

In conclusion, the VN represents a key component of the cardiac neuraxis and VNS has shown a great potential for the treatment of a wide range of CVDs in the preclinical setting. Results in experimental animals may not be immediately translated into clinical applications, yet they are paving the way for fine-tuning and customized VNS applications. VNS represents a cheaper alternative/complementary solution to pharmacological remedies that lack of efficacy and present significant side effects and astronomical costs (4, 7, 182, 183). Closed-loop VNS would guide earlier, more accurate diagnosis and enable more effective, less costly prevention and intervention compared to pharmacological treatments (183). Moreover, access to personalized bioelectronic data would facilitate greater patient understanding of their conditions and greater engagement with their treatments, higher levels of health literacy, and greater communication and trust between patients and physicians (183). A plethora of promising research is advancing to overcome VNS limitations and develop closed-loop modalities. Advanced neural decoding strategies represent a major candidate. However, automatic closed-loop modalities will require not only advancements in biotechnologies but also improvements in the basic understanding of fundamental biological mechanisms (16). Progress in neural interface technology, big-data analysis methods, and signal processing techniques will accelerate biological breakthroughs that, in turn, will inform additional advancements in technology and methodology, creating a synergistic loop that ensues cross-disciplinary collaboration (**Figure 8**) (8).

AUTHOR CONTRIBUTIONS

MMO conceptualized, wrote the manuscript, and prepared the figures. FV wrote and revised the manuscript. SM and FR conceptualized and revised the manuscript. All authors authorized the submission of the manuscript.

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