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TRANSCUTANEOUS VAGAL NERVE STIMULATION

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Dear ladies and gentlemen,

My whole professional life as a physicist was devoted to medical devices, especially active implants. I developed cardiac pacemakers, the first implantable defibrillator and also neurostimulators. I followed the success of implantable vagus nerve stimulation and saw how these small electrical devices can help impaired people to reduce their symptoms in epilepsy and depression and how they improve their lives. However, I also saw the risks of implants. Surgery, infection, a lot of implants that did not show the desired effect but remained in the body and lots of other unwanted side effects.

In the year 2001 I met the founders of Cerbomed, a young start up in Germany. They asked me, a young engineer at that time, to build the first prototype of an external vagus nerve stimulator, that activates the auricular vagus nerve in the outer ear. From that time on I never forgot this fascinating idea. I followed years of clinical research, the long fight to find the right stimulation region and the right parameter sets. After more than 10 years of research we learned that the cymba conchae region is the most promising area. But how can an electrode be applied to this region. And how can a device been built that is accepted by the patients. Usability, comfort, safety and many other aspects had to be solved.

We believe in non invasive vagus nerve stimulation, because we already saw so many patients who have a better life, less epileptic seizures, less depression. They achieved this state with no risk of implantation and no side effects.
Every day we receive a comment or a short video from a happy patient or his parents who would never reached this level of life quality without our small device. And every day we know why we are doing this job.

And we see so many other indications. Inflammation, anxiety, Parkinsons's disease, stroke, so many neurological disorders that can be improved by transcutaneous vagal nerve stimulation. Small electrical pulses help the brain to emit neurotransmitters and to evolve plasticity thus providing a better life for the patients.
I personally as well as my complete family devoted our life to improve this technology to make it available for everybody on the world.

Our vision is a world where all people on this planet have access to transcutaneous vagus nerve stimulation that helps to improve the life of many people with neurological disorders. We want to help to make this world better.

Prof. Amin Bolz
Founder and CEO , tVNS Technologies

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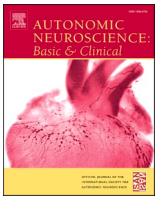

Transcutaneous vagus nerve stimulation in the treatment of drug-resistant epilepsy

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ABSTRACT

Epilepsy is a common chronic neurological disease with a high burden of illness. Invasive vagus nerve stimulation (iVNS) is a well-established treatment option in patients with epilepsy (PWE). More recently, transcutaneous vagus nerve stimulation (tVNS) was introduced, an alternative option which is particularly interesting because it does not require surgery and is instantaneously removable. Here, we thoroughly reviewed clinical data on efficacy and safety of tVNS in epilepsies.

Five prospective trials in 118 patients with drug-resistant epilepsies and 3 randomized controlled trials in 280 patients with drug-resistant epilepsies were carried out. Study protocols were heterogeneous in terms of patients' characteristics, used device, stimulation parameters, study duration and endpoints. Seizure reduction amounted up to 64%, with responder rates (seizure reduction $\geq$50%) up to 65%. Seizure freedom was reached in up to 24%, and even to 31% in a small pediatric study group. Seizure severity scores were provided in 4 studies, showing significant improvement in two of them. Adverse side effects were mostly headache, ear pain and skin alteration and rated as mild to moderate. Drowsiness might be depend on stimulation intensity. Quality of life scores reflecting burden of illness showed significant improvement in two studies.

Efficacy and safety of tVNS in PWE has to be interpreted as promising. Multicenter randomized double-blind clinical trials with standardized stimulation protocols and long-term follow-up studies are necessary to finally assess tVNS treatment outcome in drug-resistant epilepsies.

1. Introduction

An epileptic seizure is defined as a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain (Fisher et al., 2005). Seizures might occur due to acute brain illness (e.g. acute symptomatic seizure due to brain hemorrhages or systemic infection) as well as a symptom of a chronic illness, i.e. epilepsy. According to the recent proposal of the International League against Epilepsy, epilepsy is a disease of the brain with at least two unprovoked (or reflex) seizures, or one unprovoked (or reflex) seizure and a probability of further seizures of at least 60% occurring over the next 10 years or diagnosis of an epilepsy syndrome (Fisher et al., 2014). With a prevalence of 0.5 to 1%, epilepsy is one of the most common neurological diseases with about 50 million patients worldwide (GBD Neurology Collaborators, 2019; World Health Organization, 2019). Although two thirds of affected subjects achieve seizure-freedom with the first two appropriately chosen antiseizure medication (ASM), the other third requires extensive therapy attempts in order to achieve seizure-freedom or at least an acceptable seizure control (Kwan and Brodie, 2000). Failure of two tolerated and appropriately chosen and used ASM (whether as monotherapies or in combination) to achieve sustained seizure freedom is the recent definition of drug-resistance in epilepsy (Kwan et al., 2010). Unfortunately, newly developed ASMs have not resulted in a significantly higher rate of seizure-free patients, even though the tolerability and interaction profile of newer ASM seems to be more favorable (Chen et al., 2018). For some patients resective epilepsy surgery is a hope for seizure freedom or seizure reduction (Baud et al., 2018), nevertheless anesthesia and operation risk as well as postoperative deficits in cognition and vision have to be considered. For those who are not suitable or not willing to undergo resective surgical intervention or in whom surgical intervention failed, alternative treatment options are necessary.

Different methods of neurostimulation for seizure control are available. Invasive methods as deep brain stimulation of the anterior thalamus (DBS) (Fisher et al., 2010) or brain responsive (closed loop) neurostimulation (RNS) (Nair et al., 2020) and invasive (classical) vagus

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nerve stimulation (iVNS) were investigated in several trials (Overview in (Boon et al., 2018)). Due to their invasive nature, anesthesia and operation risk are inherent to all of these treatment opportunities and, if failing, reoperation is necessary. IVNS was approved in the 1990s and more than 100.000 patients with drug resistant epilepsy were implanted to date (Fisher et al., 2020), thus leading to broad experience: efficacy, determined by responder rate (subjects in whom seizure frequency is reduced by at least 50%) amounts up to 60% and seizure freedom was found in up to 8% of implanted PWE (Morris et al., 2013; Elliott et al., 2011; Englot et al., 2016). The overall complication rate ranges from 2.5 to 12%; they can be of technical (as cable break) or surgical nature (for example infection, hematoma, and vocal cord palsy). Surgical complication rate amounts up to 8.6.% (Révész et al., 2016), fortunately most complication symptoms recover well. Apart from operation risk and complication rate, iVNS displays limitations in MRI suitability and need regular onsite appointments for device check.

Given these restrictions and risks, transcutaneous vagus nerve stimulation (tVNS), the non-invasive external stimulation method, is an interesting alternative.

2. Transcutaneous vagal nerve stimulation in epilepsy patients

Different devices are available for transcutaneous vagal nerve stimulation, transcutaneous cervical VNS (tcVNS), percutaneous auricular VNS (paVNS) and transcutaneous auricular VNS (taVNS). Studies performed in PWE were carried out with the taVNS.

A proof of concept trial with taVNS over 9 months in 10 adult PWE showed a reduction of seizure frequency in 5 of 7 patients at 9 month follow-up. None of them reached seizure freedom; none of them was rated to be a responder (at least 50% reduction of seizure frequency). Tolerability was rated 'good' to 'very good' in 6 of 7 patients, for one patient tolerance was acceptable. For adverse effects in detail, one painful stimulation in one patient was mentioned (Stefan et al., 2012).

A prospective pilot trial in 14 children with epilepsy demonstrated efficacy with 7 of 13 children being responders and 4 of 13 children being seizure free after 4 months and persisting after 6 months of stimulation. Reduction of seizure frequency was significant compared to baseline after 4 and 6 months of stimulation ($p < 0.05$). Tolerability was generally good, two children experienced mild skin ulceration (He et al., 2013).

In a single center randomized trial over 12 months 60 children and adults with epilepsy were randomized in treatment group (stimulation in the Ramsay Hunt zone) and control group (stimulation at the ear lobe). Seizure frequency was significantly reduced after stimulation in the treatment group compared to baseline after 6 and 12 months ($p < 0.001$). Significant difference in seizure reduction between treatment group and control group was achieved at 12 months follow-up ($p < 0.001$). Seizure severity, according to questionnaires, was significantly reduced after 12 months taVNS treatment compared to baseline ($p < 0.001$). No such decrease was found in the control group. Interestingly, stimulation intensities up to 8 mA were reported with median intensity of 6 mA in taVNS and control group. Adverse events (AE) in the taVNS was dizziness in one patient (stimulation 6 mA) and drowsiness in 3 patients which lessened after reduction of current intensity (to 6-7 mA) (Aihua et al., 2014).

A multicenter prospective study over 24 months in 50 PWE aged 12 years and older yielded reduction of 50% or greater (responders) in 27 of 47 patients (57%) with seizure freedom in 16% after 6 months of taVNS. Reduction of seizures when comparing with baseline reached significance. Overall, severity of seizures scores significantly improved ($p = 0.017$), even in some patients without reduction of seizure frequency. One patient withdrew from the study due to adverse effects (dizziness), two reported swelling and rush, and fewer (number not given) reported intolerance of stimulation longer than 1 h (Rong et al., 2014b).

The same investigator group conducted a randomized controlled trial over 24 months in 144 PWE. 98 patients were randomized to a taVNS (stimulation of the triangular fossa) and 46 to a non-vagus stimulation (tnVNS, stimulation of the outer ear channel). After eight weeks, all patients converted to taVNS. Follow-up after 8 weeks showed significant differences in seizure reduction ($p < 0.05$). At week 24 both groups (i.e. one group with 24 months with taVNS, the other with 8 weeks tnVNS followed by 16 weeks taVNS) showed similar seizure reduction (about 47.5%). Seizure reduction from baseline to treatment was significant in both groups ($p < 0.01$). Tolerability was generally good, with skin itching in 6.2%, red rashes and swelling in 4.1% and dizziness in one patient (Rong et al., 2014a). It should be noted that in this study design, only the first 8 weeks were placebo-controlled, whereas the remaining 16 weeks compared stimulation over time.

The only multicenter randomized double-blinded clinical trial over 28 weeks was conducted in 76 adult PWE. Treatment-stimulation was carried out with 25 Hz and active control stimulation with 1 Hz. The latter was estimated to be not a sham but an active control group (estimated by 7200 stimuli per day in comparison to 180.000 in the 25 Hz group and 235.000 by iVNS (30 s on, 5 min off, 30 Hz)). Responder rates were similar in both groups (about 25%). In the stimulation group reduction of seizures amounted to 23.4%, whereas seizure frequency tended to increase by 2.9% in the active control group. The results have to be considered under the aspect that even a stimulation at 1 Hz might improve seizure control to some extent. Nevertheless sub-analysis of the patients who completed full treatment period (20 weeks) displayed significant seizure reduction between baseline and study endpoint ($p = 0.034$). In both groups, seizure severity slightly increased, which was not rated clinically relevant. AE were documented thoroughly, treatment-related adverse events were common (60% vs. 39%). AE were mostly rated mild to moderate in both groups (70 vs. 77%). Headache, ear pain, skin erythema, vertigo, fatigue, and nausea were reported in both groups and more often in the 25 Hz group. Three patients in the 25 Hz group and 4 in the 1 Hz group discontinued due to AE. One female patient in the 1 Hz group died (sudden unexpected death in epilepsy (SUDEP)) which was estimated not to be related to stimulation (Bauer et al., 2016).

Another single center study investigated not only efficacy but also different stimulation time. Twenty teenager and adult PWE were included in a single center open label prospective trial. All participants received taVNS for 6 months. On average seizure frequency appeared to decline, but statistical significance was not reached (data not shown). One patient experienced a reduction of seizures between 50 and 79%; the outcome of all other participants was less favorable. Six patients had a seizure reduction $\geq$30% in the first phase of the trial. After a washout period (without stimulation) for two months those 6 proceeded with another 6 months taVNS, with a significant reduction of the seizure frequency as compared to baseline (about 60%, $p = 0.043$) and the end of washout period (about 50%, $p = 0.043$). The second stimulation phase was carried out with shorter stimulation time (2 instead of 4 h) and at least those who seemed to benefit from former taVNS also profited from treatment with shorter stimulation time. These results prompt further investigations addressing stimulation features required for seizure control without disturbing daily living. Adverse events were reported in 4 patients (pain, small abrasions, eczema), one patient reported headache and sense of strangeness, none of them withdrew due to the adverse effects (Barbella et al., 2018).

In another single center prospective study over 6 month including 24 PWE, 17 patients reached study endpoint. One patient discontinued due to AE (dizziness), no other adverse events were reported. Seizure frequency was reduced in 13 patients after 3 months and 16 patients after 6 months of stimulation. Five patients could be rated as responders after 3

months, 11 after 6 months. Seizure freedom could be reached in 4 patients after 6 months of stimulation. Severity of seizures was reduced in 12 and increased in 4 of 17 (Liu et al., 2018).

One case report shared information on long-term seizure freedom for 56 months in formerly drug-resistant epilepsy due to subcortical nodular heterotopias (von Wrede et al., 2019), by now seizure freedom persists for seven years (personal communication).

All above mentioned studies were carried out in drug resistant epilepsies. An additional short term (four weeks) retrospective study was carried out in 52 patients with post-stroke epilepsy. Inclusion criteria did not meet the definition of drug-resistant epilepsy. Twenty-seven PWE received taVNS, 25 PWE on the waiting list were defined as control. TaVNS was applied twice a week for 20 min. Comparing baseline and treatment in the taVNS group as well as comparing taVNS and control group did not show better outcomes (seizure frequency, seizure duration) in the taVNS group. Adverse events (headache, ear pain, nausea, dizziness, fatigue, vomiting, vertigo) were rated minor and acceptable, none of them reaching significance when compared to control group (Song et al., 2018). In fact, the results are not astonishing as the used schedule provides lower stimulation time in total (60 min a week, 240 min throughout whole study) with a the low frequency of 1 Hz which is used as an active control in other studies (Bauer et al., 2016).

Only in one study the time point of stimulation (three stimulation periods: morning, noon and evening) was mentioned (Stefan et al., 2012). For another study stimulation during wakefulness has to be supposed due to approval of used device (Bauer et al., 2016). For all others, all of them using bilateral stimulation, it remains unclear when during the day stimulation was carried out exactly. Used stimulation schedules and parameters are more or less heuristic. Further studies on this issue are needed, as more and more knowledge about cycles in epilepsy offer not only new understanding of epilepsy but, what is more, options for chronotherapy (overview in (Karoly et al., 2018)).

3. Quality of life, cognitive and emotional outcome, usability and handling

Not only seizures, but also side-effects from ASM, co-morbidities, and social consequences of seizures increase the burden of this illness on those affected. Therefore, simply counting seizure does not reflect benefit or risk of an intervention. For example good seizure control with negative cognitive effects leads to lower quality of life which reflects everyday living. Of course, there is no question that each additional seizure can be associated with an increased risk of health problems, but for the patients' daily life, especially for patients with a long-term chronic course, cognition and behavioral side effects or disorders have a higher significance (Gilliam, 2002; Witt et al., 2013). Quality of life can be measured by evaluated questionnaires as QOLIE-89, and its short form QOLIE-31, which deal with emotional and psychological as well as medical and social effects (Cramer et al., 1998; Devinsky et al., 1995).

The QOLIE-89 was used in the proof of concept trial, showing stable scores after 9 month (endpoint) in all seven patients who completed the study (Stefan et al., 2012). Significant improvement of quality of life (assessed by QOLIE-31) was shown in three studies (with $p < 0.001$ (Rong et al., 2014b), $p = 0.001$ (Rong et al., 2014a) and $p = 0.038$ (Aihua et al., 2014)). A trend towards an improved QOLIE-31 was found in two studies (Bauer et al., 2016; Liu et al., 2018), whereas no change or trends in QOLIE-31 were reported after treatment compared to baseline in two other studies (Barbella et al., 2018; Song et al., 2018).

In summary, the influence of taVNS on the quality of life of PWE has not been definitively answered, but preliminary results indicate a positive influence. Therefore, further clinical investigation of taVNS in PWE should definitely address this important parameter as well.

Four studies provided cognitive or emotional outcome data after taVNS in PWE. In one study, Becks Depression Inventory (BDI) was used to reveal stable scores in 5 of 7 patients, and intermittent worsening with improvement afterwards in one and overall improvement in another patient (Stefan et al., 2012). Two studies used the Self-Rating Depression Scale (SDS) with reduction of scores (improvement of experienced depression symptoms) in 11 of 17 patients (Liu et al., 2018) and significant differences ($p = 0.018$) between 12 months of taVNS and baseline in another study (Aihua et al., 2014). One study used the Montgomery-Åsberg Depression Rating Scale (MADRS) showing decline of scores in taVNS 25 Hz group and 1 Hz group ($p = 0.059$ and $p = 0.112$) (Bauer et al., 2016). Two studies used the Self-Rating Anxiety Scale (SAS): One study yielded reduction of scores (improvement of experienced anxiety symptoms) in 15 of 17 patients (Liu et al., 2018), and another study found significant differences ($p = 0.017$) between 12 months of taVNS and baseline (Aihua et al., 2014). Overall, in spite of limited data, taVNS in epilepsy points to an additional positive effect in emotional self-reported symptoms.

With regard to the impact on cognitive outcome, one clinical study reported stable cognitive functions, assessed by a computerized cognitive testing system, and revealed stable or improved outcome in 6 of 7 patients and slight improvement in another patient (Stefan et al., 2012).

In addition to clinical studies with long-term taVNS, one study with short-term taVNS confirmed the absence of negative impact on cognition, behavior, or mood using a more elaborate neuropsychological testing schedule (assessing *attention and executive functions with* Epi-Track®; assessing *verbal memory* using a short version of the Verbal Learning and Memory Test (VLMT); assessing m*ood and depression by* The Neurological Disorders Depression Inventory for Epilepsy (NDDI-E), scoring subjective measures with the Adverse Events Profile (cognition, behavior, physiological symptoms)) (von Wrede et al., 2021).

In one study, handling of the taVNS was rated as good or very good with few patients feeling affected in continuation of their activities. Although most patients found wearing comfort good or very good, some of them felt that the device was rather poorly suited for long or repeated use during one day (von Wrede et al., 2021), similar consideration were the basis of an evaluation of the effects of reduced stimulation before (Barbella et al., 2018) and reported in other trial (Rong et al., 2014b; Barbella et al., 2018).

4. Prediction of seizure reduction by taVNS

All types of intervention – including ASM, epilepsy surgery or neurostimulation - require valid data on outcome features when counseling PWE. Hence, personalized medicine and good medical practice is based on the combination of individual predictor assessment.

Some of the above mentioned studies provide correlations between outcome and patient features. Most studies did not find a correlation between seizure reduction and age (He et al., 2013; Rong et al., 2014b; Aihua et al., 2014), gender (He et al., 2013; Rong et al., 2014b; Bauer et al., 2016; Barbella et al., 2018), duration of epilepsy (Rong et al., 2014b; Aihua et al., 2014; Barbella et al., 2018), baseline frequency (He et al., 2013; Rong et al., 2014b; Bauer et al., 2016; Barbella et al., 2018), seizure duration (He et al., 2013), age at onset of epilepsy (Barbella et al., 2018), seizure type (He et al., 2013; Aihua et al., 2014; Bauer et al., 2016), etiology (Barbella et al., 2018), EEG (Aihua et al., 2014), MRI findings (Aihua et al., 2014), number of ASM (Aihua et al., 2014), concurrent treatment with other drugs than ASM (Bauer et al., 2016) or initial stimulation intensity (Aihua et al., 2014).

One study found positive correlation with age at start of taVNS (Barbella et al., 2018) and another study reported a correlation between seizure reduction and baseline seizure frequency and duration of epilepsy ($p < 0.05$) (Aihua et al., 2014).

Taken together, the above mentioned studies differ by study design, patients' characteristics and used stimulation parameters and some studies are based on a rather small sample size. Therefore, statements on prediction factors are rather limited and have to be drawn with caution. Further randomized double-blind clinical trials in larger patients groups are essential to evaluate potential predictive factors and features (Table 1).

Table 1
Studies in epilepsy, efficacy outcome. *Data not given in detail extrapolated from graph for treatment group, data for control group not given. **Same author. ***Inclusion criteria not meeting drug-resistant epilepsy. ***Negative numbers indicate increase of seizure frequency. n.a. = not available. vs = versus.

	Stefan 2012	He 2013	Liu (Aihua) 2014**	Rong 2014b	Rong 2014a	Bauer 2016	Barbella 2018	Song 2018	Liu 2018**
Patients' characteristics									
Age	Adult	Children (≤12 years)	Children (>4 years) and adults	Children (≥12 years) and adults (≤65 years)	Children (≥12 years) and adults (≤65 years)	Adults (≤65 years)	Children (≥16 years) and adults	Adult poststroke epilepsy	Children (≥12years) and adults (≤65 years)
Seizures	Focal/ generalized	Focal/ generalized	Focal/ generalized	Focal/ generalized	Focal	Focal/ generalized	Focal	Focal***	Focal/ generalized
Number of recruited patients	10	14	60 (30 vs 30)	50	144 (98 vs 46)	76 (37 vs 39)	20	52 (27 vs 25)	24
Number of patients reaching study end	7	13	47 (26 vs 21)	47	133 (93 vs 40)	58 (27 vs 31)	Phase 1: 20 Phase 2: 5	52 (27 vs 25)	17
taVNS parameter									
Stimulation time	3 times a day for 1 h	3 times a day for 30 min	3 times a day for 20 min	Twice a day, 30 min each	Twice a day for 30 min After 8 weeks all stimulated by taVNS	4 h a day	Phase 1: 4 h a day in 2-3 sessions Phase 2: 2 h a day	Twice a week, one session of 30 min	3 times a day 20 min a day
Stimulation parameter (frequency, pulse width, intensity)	Unilateral 10 Hz; 300 μsec; n.a.	Bilateral 20 Hz; n.a., 0.4 → 1 mA	Bilateral 20 Hz; 0.2 s; until below pain (median 6 mA)	n.a. 20-30 Hz, < 1ms; 1 mA	Bilateral 20-30 Hz; ≤1 ms; 1 mA	Unilateral 25 Hz vs 1 Hz; 250 μsec; until below pain (0.5 vs 1 mA)	n.a. n.a. n.a. below pain (0.6–0.8 mA)	Bilateral 1 Hz n.a. n.a.	Bilateral 10 Hz, 200 s; 4 mA
Duration of stimulation period	9 months	24 weeks	12 months	24 weeks	24 weeks	20 weeks	Phase 1: 6 months wash out: 2 months Phase 2: 6 months	4 weeks	6 months
Outcome									
Seizure reduction	5 of 7 median reduction for all: n.a.	After 8 weeks: 32% After 16 weeks: 54% After 24 weeks: 54%	After 6 months: n.a. After 12 months: 40% vs -0.85%***	After 8 weeks: 34% After 16 weeks: 47% After 24 weeks: 51%	after 8 weeks: 43% vs 12.% after 24 weeks: 47% vs 48%	23% vs -3%***	Phase 1:n. a. Phase2: 60% (to baseline) 50% (to end wash out)	No difference treatment/ control No difference baseline/ treatment	After 3 months: 31% After 6 months: 64%
Responder (seizure reduction ≥ 50%)	None	After 8 weeks: 4 of 14 (29%) After 16 weeks: 7 of 13 (54%) After 24 weeks: 7 of 13 (54%)	After 6 months: about 31%* After 12 months: about 42 %*	After 8 weeks: 18 of 47 (38%) After 16 weeks: 23 of 47 (49%) After 24 weeks: 27 of 47 (57%)	After 8 weeks: 41% vs 28% After 24 weeks: 47% vs 48%	27% vs 26%	Phase 1: 1 of 10 (10%) Phase 2:n. a.	None	After 3 months: 5 of 17 (29%) After 6 months: 11 of 17 (65%)
Seizure freedom	None	After 8 weeks: 1 of 14 (7%) After 16 weeks: 4 of 13 (31%) After 24 weeks: 4 of 13 31)	After 6 months: about 12%* After 12 months: about %*	After 8 weeks: 6 of 47 (13%) After 16 weeks: 6 of 47 (13%) After 24 weeks: 8 of 47 (16%)	After 8 weeks: 10% vs 5% After 24 weeks: 15% vs 15%	3% vs 8%	n.a.	None	After 3 months: none After 6 months: 4 of 17 (24%)

5. taVNS and electrophysiology with focus on electroencephalography

Epilepsy is a clinical diagnosis and seizures usually are counted by patients and caregivers. To date, seizure counts are mostly provided with the help of paper diaries. However, new electronic and digital solutions with different wearable technologies, at least for tonic-clonic seizures, offer new possibilities of seizure registration. Diaries are

subject of under- and overestimation, mal-compliance of documentation as well as false documentation by inaccurate event estimation (Hoppe et al., 2007; Karoly et al., 2018). To counteract this problem, additional technical investigations are carried out. Electroencephalography (EEG) measures summed electrical brain activity by recording the voltage fluctuations and provide further information (e.g. confirming diagnosis, diagnosing a specific epilepsy syndrome, seizure recording). Nevertheless, the clinical value of EEG recordings may be overestimated in some instances, as they commonly reflect a short period of time in an dynamic network with many confounders (e.g. biorhythm, state of vigilance) (Baud et al., 2020). Combination of Video and EEG (VEEG) and long-term registration improve diagnostic outcome, but availability as well as technical problems still limit its use in studies on efficacy of different epilepsy treatments. It might even be more critical that interpretation of EEG pattern is highly dependent on experience of the electrophysiologist in charge. Only one of the above mentioned studies (Stefan et al., 2012) used an automatic seizure detection program for computer supported quantification and therefore provided more objective data. These limitations have to be kept in mind while interpreting EEG in clinical studies with taVNS with regard to efficacy (see Table 2).

Apart from limited clinical use of conventional EEG by just counting seizures, EEG is an elegant method for research on epilepsy treatment. As for ASM the so-called "pharmaco-EEG" has provided relevant insights in treatment response, detection of neurotoxic effects of ASM and prediction of ASM treatment response or adverse effects (Höller et al., 2018). Therefore, it is supposed that quantitative analysis of EEG using different approaches can enlighten effects of non-pharmaceutical interventions on brain networks and seizure evolution as well.

It might be assumed that VNS leads to a rather unspecific, global activation of various brain structures, partially reflected by controversial findings of studies addressing EEG features during VNS. For instance opposing effects of VNS on epileptiform activity, synchronization and desynchronization and ambiguous changes in frequency bands were previously reported (Rutecki, 1990; Koo, 2001; de Vos et al., 2011). In a recent study using EEG-derived evolving functional brain networks, short-term taVNS induced stabilizing, robustness- and stability-enhancing modifications in functional brain networks of PWE (von Wrede et al., 2021), strengthening the notion of favorable central nervous effects induced by taVNS in PWE. The action of vagus nerve stimulation in epilepsy, however, is not understood, but may involve

Table 2
Effect of taVNS on EEG features. IED: interictal epileptiform discharges. GSW=generalized spike-waves. vs= versus. n.r., not reported.

	Stefan 2012	Rong 2014a	Barbella 2018	Liu 2018	von Wrede 2019
Studied population					
Age	Adult	Children (≥12 years) and adults (≤65 years	Children (≥16 years) and adults	Children (≥12years) and adults (≤ 65 years)	Adult
Seizures	Focal/generalized	Focal	Focal	Focal/generalized	Focal
Number of recruited patients	10	144 ((98 vs 46)	20	24	1
EEG recording					
Number of patients	7	125 (88 vs 37)	20	17	1
Method	Video –EEG 7 days (baseline, 3 months, 9 months)	Video-EEG	Video-EEG (30 min without taVNS, 20 min with subliminal taVNS and 20 with perceived taVNS)	EEG von 2 h (1 h sleep, 1 h awake)	30 min resting state EEG
Outcome					
Interictal (duration IED) (frequency of IED)	No seizure during baseline:5 patients 3 → 9 months: decrease of duration IED: 2 patients increase of duration IED: 1 patient Not determined: 1 patient GSW, no seizures : 1 patient: 0 → 3 months: increase of duration IED 0 → 9 months: decrease of duration IED Seizure during baseline: 1 patient 0 → 3 months: decrease of duration IED 0 → 9 months: increase of duration IED Pattern resembling discontinuous nonconvulsive status (see below) No seizure during baseline: 5 patients 3 → 9 months: reduction of frequency: 2 patients unchanged/slightly changed of frequency: 2 patients not determined (GSW): 1 patient	Available for one example (reduced interictal discharges)	No change	Frequency of IED: 0 → 3 months: increase: 2/17 decrease: 12/17 no change:3/17 0 → 6 months: increase: 2/17 decrease: 11/17 no change:4/17	No change
Ictal	Seizure during baseline: 1 patient* 0→3 months increase 3→9 months increase Pattern resembling discontinuous nonconvulsive status:1 patient 0 → 3 months: decrease 3 → 6 months: decrease 6 →9 months: leveled off	n.r.	n.r.	n.r.	n.r.

alterations of noradrenergic, serotonergic, GABAergic and anti-inflammatory pathways (for overview see (Farmer et al., 2021)).

None of the above mentioned studies provided data on electrocardiography which for obvious considerations is of interest, especially if taVNS is applied bilaterally, future studies should provide those data as well.

6. Conclusions

TaVNS studies in epilepsy yielded promising results PWE, with improved seizure control and severity. Quality of life increased in most patients (even if seizure control did not improve). Adverse effects are mild or moderate, mostly reversible and well tolerated. Handling is good and usability seems to be dependent on suggested daily stimulation time. Taking into account the currently available data, it remains unclear which patients benefit most from taVNS.

Available studies differ in used taVNS device, study protocol, studied population and outcome parameters so that to date, proof of efficacy of taVNS in patients with epilepsy could not finally drawn. As for research on taVNS (Farmer et al., 2021), standards for technical aspects of the device as well as stimulation parameters should be identified and used in following clinical studies. Multicenter randomized double-blind clinical trials with clear sham stimulation control group are a prerequisite for finally assessing efficacy and safety of taVNS in PWE. These studies should be accompanied by neuropsychological testing, as it is usually done in ASM studies, to investigate additional positive or negative side effects of taVNS on cognition, mood and behavior. Long-term follow-up studies are necessary to evaluate if seizure reduction is long-lasting and possibly increased, in patients with drug-resistant epilepsy as it is discussed for other neurostimulation devices (Schulze-Bonhage, 2018).

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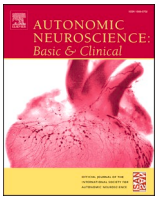

Vagus nerve afferent stimulation: Projection into the brain, reflexive physiological, perceptual, and behavioral responses, and clinical relevance

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ABSTRACT

The afferent vagus nerves project to diverse neural networks within the brainstem and forebrain, based on neuroanatomical, neurophysiological, and functional (fMRI) brain imaging evidence. In response to afferent vagal stimulation, multiple homeostatic visceral reflexes are elicited. Physiological stimuli and both invasive and non-invasive electrical stimulation that activate the afferent vagus elicit perceptual and behavioral responses that are of physiological and clinical significance. In the present review, we address these multiple roles of the afferent vagus under normal and pathological conditions, based on both animal and human evidence.

1. Introduction

The vagus nerve has been extensively studied in animals and humans to understand its role in physiology and behavior, and most recently, to understand the role of the afferent vagal system in perceptual and psychological processes. It is the tenth cranial nerve with both an afferent (sensory) and predominant efferent (motor) component that play a significant role in maintaining homeostasis (Yuan and Silberstein, 2016a, 2016b, 2016c). Herein, we provide an overview of afferent vagal pathways, the reflexive physiological, perceptual, and behavioral effects, and clinical implications of afferent vagus nerve stimulation.

2. Vagus nerve projections: from brainstem to forebrain

There is convergent evidence that the vagus nerve conveys information from thoracic, abdominal, and even pelvic organs to the first synapse in the nucleus tractus solitarii (NTS) in the brainstem (Ortega-Villalobos et al., 1990; Collins et al., 1999; Cheng et al., 2004; Komisaruk et al., 2004). The NTS then relays the input directly and indirectly to diverse brain components (Table 1). Composed of 10 subnuclei (McRitchie and Törk, 1993), the NTS is located medially in the most caudal portion of the medulla oblongata and extends vertically and dorsolaterally into the lower region of the pons. Electrical stimulation of vagal A- and C-fibers directly activates neuronal responses in the nucleus tractus solitarii (NTS) of rat (Beaumont et al., 2017; Snyder and Cantrell, 2017). Projections from the NTS are widespread and recurrent (Sawchenko, 1983; Ruggiero et al., 2000). Within the medulla, the NTS projects directly to the dorsal motor nucleus of the vagus and the nucleus ambiguus, from which originate the preganglionic parasympathetic efferents to the visceral organs. The hypoglossal, facial, and spinal trigeminal nucleus caudalis receive input from the NTS (Sawchenko, 1983; Ruggiero et al., 2000). The NTS also projects to the spinal cord preganglionic sympathetics in the intermediolateral cell column (Sawchenko, 1983). Neuroanatomically, the NTS also projects directly to the parabrachial nucleus (PB). Specifically, the contralateral medial and lateral parabrachial nuclei receive input from the rostral (gustatory) and caudal (visceroceptive) subnuclei of the NTS (Sawchenko, 1983). Additional midbrain projections from the NTS include the locus coeruleus (via the nucleus paragigantocellularis and nucleus prepositus hypoglossi) (Aston-Jones et al., 1991), multiple raphe nuclei, Kölliker-Fuse, and the periaqueductal gray (Berthoud and Neuhuber, 2000; Henry, 2002).

Using the anterograde tracer, Herpes Simplex virus, Rinaman and Schwartz (2004) demonstrated projections from NTS to PB, paraventricular nucleus of the hypothalamus (PVN), bed nucleus of the stria terminalis (BNST), amygdala and posterolateral hypothalamus. Based on injection into the NTS of tritiated leucine and proline in rat, which provides anterograde labeling (Ricardo and Koh, 1978), direct projections were observed to PVN and arcuate nuclei of the hypothalamus, periventricular thalamus, medial preoptic area, BNST, and central amygdala. Direct projections were confirmed via retrograde labeling in NTS after injection of horseradish peroxidase (HRP) into amygdala,

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Table 1
Direct and indirect afferent projections of the Vagus to specific brain regions based on various methodologies.

Origin	Projection to	Method	First author (year, if multiple citations)
Direct projections			
Vagus	Nucleus tractus solitarii (nts)	HRP	Ortega-Villalobos
		DiI, Pseudorabies, Fluorogold	Collins
		fMRI	Komisaruk
		Electrical stimulation	Beaumont Snyder
		DiI	Cheng
NTS	Dorsal motor n, n ambiguus, parabrachial n (PB), hypoglossal n, facial n, spinal trigeminal n, sympathetic preganglionics	Antero- and retro-grade markers	Sawchenko Ruggiero
	Locus coeruleus (LC), multiple raphe ns, periaqueductal gray (PAG), Kolliker-Fuse n	Antero- and retro-grade markers	Aston-Jones Berthoud
	Parabrachial n (PB), paraventricular n hypothalamus (PVN), bed n stria terminalis (BNST), amygdala, post lateral hypothalamus	Anterograde herpes simplex virus	Rinaman
	PVN, arcuate n hypothalamus, periventricular n thalamus, medial preoptic nucleus (MPOA), BNST, central amygdala	Anterograde tritiated leucine and proline	Ricardo
	Amygdala, BNST, MPOA, PVN, periventricular n thalamus	Retrograde horseradish peroxidase (HRP)	
	PB, ventrobasal thalamus, insular cortex	Sequential HRP	Cechetto
PB	PVN, central amygdala, lateral hypothalamus	Sequential HRP	Cechetto
Indirect projections			
Vagus	PVN PB NTS Thalamus, hypothalamus, MPOA, amygdala, insular, orbitofrontal cortices	Electrical evoked potentials	Dyball Cechetto Kannan Dell
	Insular, temporal, occipital, cingulate, frontal cortices, cerebellum, thalamus, postcentral gyrus	Invasive nerve stimulation (iVNS) combined with fMRI	Narayanan Liu Lomarev
	NTS, PB, spinal trigeminal n, vestibular n, PAG, ventral tegmental area, substantia nigra (SN), caudate n, cerebellum, insular, cingulate, and dorsolateral frontal cortices, postcentral gyrus.	Noninvasive over neck	Frangos and Komisaruk (2017)
	NTS, PB, SN trigeminal main and spinal n, LC, red nucleus, hypoglossal n, cerebellum, BNST, amygdala, septum, insular cortex, n accumbens, paracentral lobule of cortex	Transcutaneous (tVNS) electrical stimulation of cymba conchae of ear	Kraus Frangos (2015) Yakunina Sciocco
	Hippocampus (inhibited), hypothalamus (inhibited)	tVNS of ear, neck	Frangos (2015) and Frangos (2017)
	Paracentral lobule of cortex, widespread inhibition in brain	Invasive vagus stim	Nahas

BNST, medial preoptic area, PVN, and periventricular thalamus (Ricardo and Koh, 1978). Also using sequential HRP injections, a pathway from NTS to parabrachial nucleus (PB), to ventrobasal thalamus to insula was demonstrated (Cechetto and Saper, 1987), and from PB to PVN, central amygdala, and lateral hypothalamus (Cechetto, 1987).

The above findings provide evidence of direct synaptic connections between NTS and specific forebrain components. The projection of NTS to these forebrain components has also been demonstrated by less direct, dynamic methods, i.e., evoked potentials and fMRI. These methods demonstrate functional connectivity, though not necessarily via direct, monosynaptic pathways. Thus, evoked responses in PVN have been reported from electrical stimulation of the vagus (Dyball and Koizumi, 1969), PB (Cechetto, 1987) and NTS (Kannan and Yamashita, 1985). Evoked potentials were also recorded, in response to electrical stimulation of the vagus, in thalamus, hypothalamus, medial preoptic area, amygdala, insula, and orbitofrontal cortex (Dell and Olson, 1951). While the advantage of recording evoked potentials, via implanted electrodes, is the anatomical specificity of the site at which the potentials are recorded, a limitation is the restriction to the implanted recording site. This limitation is overcome by using functional MRI to register evoked activity from vagus stimulation, as the fMRI registers activity throughout the brain, and in awake humans.

Separate fMRI studies examining the neural responses to electrical stimulation, applied directly to the vagus nerve or transcutaneously, corroborate the projections described above. Direct electrical stimulation applied to the cervical vagus nerve (i.e., invasive vagus nerve stimulation, iVNS) produced responses within insular, frontal, temporal, and occipital cortices (Narayanan et al., 2002; Liu et al., 2003; Nahas et al., 2007; Lomarev et al., 2002). Among these authors, varying degrees of consistency were also reported for responses in cingulate cortex, cerebellum, thalamus, postcentral gyrus, and frontal cortex. Using non-invasive approaches, in response to electrical stimulation of the surface of the neck overlying the vagus (Frangos and Komisaruk, 2017), activation was observed in NTS, PB, trigeminal nucleus (spinal), vestibular nucleus, periaqueductal gray, ventral tegmental area, substantia nigra, cerebellum, insula, cingulate cortex, caudate nucleus, somatosensory cortex, and dorsolateral frontal cortex. Transcutaneous electrical stimulation of the regions of the external ear innervated by the auricular branch of the vagus produced activation within the medulla region of the brainstem including the NTS in addition to NTS projections that include the PB, substantia nigra, trigeminal nucleus (main and spinal), locus coeruleus, red nucleus, hypoglossal nucleus, cerebellum, BNST, amygdala, septum, insula, nucleus accumbens, paracentral lobule of the cortex, and somatosensory cortex (Kraus et al., 2013; Frangos et al., 2015; Yakunina et al., 2017; Sclocco et al., 2019). Deactivations in response to these transcutaneous approaches have been reported within the hippocampus and the hypothalamus (Frangos et al., 2015; Frangos and Komisaruk, 2017). Widespread deactivation of the brain was reported by Nahas et al. (2007) in depressed patients receiving invasive vagus stimulation.

Demonstrated in the rat, there is a viscerotopic organization via vagal afferents that extends from the NTS to the PB to the ventral posterior and ventrolateral nuclei of the thalamus and then to the insula (Cechetto and Saper, 1987). Further evidence of the viscerotopic organization of the vagal afferent projections is that the cardio-pulmonary field in the posterior insula is adjacent to the trunk representation in

secondary somatosensory cortex (SII), and the gustatory region in the anterior insula is adjacent to the representation of the tongue in the primary somatosensory field (Cechetto and Saper, 1987), a neuroanatomical proximity in the latter case that may facilitate viscero-somatic integration for ingestive behavior.

3. Reflexive physiological and behavioral responses to vagal afferent stimulation

There are a variety of physiological and behavioral reflex responses to vagal afferent stimulation. The reflexes are triggered by mechanical (stretch, pressure) or chemical stimulation of receptors in viscera that are innervated by vagal afferents. These include cardiac (in atrial, ventricular, epicardial, pericardial sites), vascular (aortic, pulmonary arterial sites), pulmonary, esophageal, gastrointestinal (Paintal, 1973) and genital viscera (Komisaruk et al., 1996; Sansone et al., 1997; Bianca and Komisaruk, 2007), as follows.

3.1. Hering-Breuer reflex

This reflex regulates respiration, protecting against hyper-inflation of the lungs. When lung stretch receptors are activated by intense inspiration, vagal afferents convey the activity to neurons in the nucleus tractus solitarii (NTS) that relay the activity to inspiration-inhibitory neurons in the medulla, initiating expiration. The reflex evidently functions in neonates and infants to regulate their normal respiratory rhythm; with normal development, it habituates, playing a lesser role in adults. Pathology of the Hering-Breuer reflex may be involved in obstructive sleep apnea (Vadhan and Tadi, 2021).

3.2. Heymans' vagal-facial inhibitory reflex

Afferent vagal (or vagal/glossopharyngeal) activation in response to increased blood pressure was shown to reflexively inhibit facial respiratory movements in the dog in a series of Nobel Prize-winning studies by Heymans (1945). In a heroic preparation in which a dog's head was attached to its trunk only by the "vagoaortic nerves" (combined fibers of the vagus and glossopharyngeal nerves [de Castro, 2009]), a dog's head was kept alive by connecting its cerebral circulation to a second dog. An increase in blood pressure was induced in the "disconnected head" dog's trunk by injection of epinephrine. Concurrently, the blood pressure in its head was maintained unchanging via the blood supply from the other dog. The increased trunk blood pressure stimulated pressoreceptors in the carotid sinus, which increased activation of the vagoaortic nerves; this inhibited the respiratory system in the brain, temporarily inhibiting the facial respiratory movements, mediated by the facial motor nerve (cranial nerve #7), of the dog's head (Heymans, 1945).

3.3. Baroceptor reflex

In the normal homeostatic, negative feedback mechanism of the baroceptor reflex, vagal afferents from pressure sensitive stretch receptors in the aortic arch respond to an increase in blood pressure. This activates vagal efferent activity, which slows the heart, and concurrently inhibits sympathetic efferent activity. This complementary combination produces a compensatory *decrease* in blood pressure.(Campagna and Carter, 2003). Conversely, when one stands up from a supine position, blood tends to pool in the lower extremities, lowering blood pressure, which is sensed by pressoreceptors in the aortic arch via the vagus nerve and carotid sinus via the glossopharyngeal nerve. This results in increased sympathetic output and reduced vagal output, which generates tachycardia and compensatory *increase* in blood pressure (Fenton et al., 2000).

3.4. Vasovagal reflex-induced syncope

However, in the pathological condition of vasovagal syncope (fainting/loss of consciousness), the above homeostatic negative feedback system fails. In response to rapidly rising from a supine body position, in response to sudden emotional stress, or in an experimental setting being elevated on a tilt table, there is pooling of blood in the lower extremities, vagal output increases, producing bradycardia and reduction in cardiac output. This results in a significant decrease in blood pressure, resulting in reduced blood flow to the brain, and thus, syncope (van Lieshout et al., 1997; Wieling et al., 2016; Jardine et al., 2018). The increase in vagal efferent activity also affects gastric motility, which is one factor that can account for the commonly associated nausea. This abnormal process leading to syncope shares major features with the Bezold-Jarisch reflex (Fenton et al., 2000).

3.5. Bezold-Jarisch reflex

This is a combined response of bradycardia, hypotension, and vasodilation. It was originally elicited in response to injection of an alkaloid (veratridine). The response is blocked by interruption of the afferent c-fibers that comprise 75% of the afferent cardiac branches of the vagus nerves, and thus is a vagal reflex. Under moderate hypovolemia, it functions as a negative feedback, homeostatic mechanism operating synergistically with the baroceptor reflex. However, under severe hypovolemia, paradoxically, it is activated, while at the same time, the baroceptor reflex fails to activate. This combined pathological condition results in bradycardia and further hypotension. It is considered controversial as to whether this process can account for vasovagal syncope (Campagna and Carter, 2003).

3.6. Respiratory sinus arrhythmia

The respiratory sinus arrhythmia reflex is mediated primarily by vagal afferent and efferent function. The reason it is termed "arrhythmia" is that the *interval* between successive heart beats varies over the respiratory cycle, decreasing during inspiration and increasing during expiration. This normal, physiologically adaptive mechanism serves to optimize gas exchange between the lungs and the circulatory system. Thus, during inspiration, when air is flowing into the lungs, the heart speeds up, increasing the opportunity for gas exchange via the higher rate of blood flowing through the lungs. Then, during expiration, gas exchange is less critical, and the heart slows.

Lung inflation stimulates the pulmonary stretch receptors which, via the vagal C-fiber afferents, inhibit cardiac vagal efferent activity, thereby disinhibiting/accelerating the heart rate. Then, during expiration, in response to vagal afferent activity via the arterial chemo- and baroreceptors, the cardiac vagal efferent neurons are stimulated, thereby slowing the heart rate (Yasuma and Hayano, 2004). The most primitive representation of this mechanism is in the dogfish shark, in which there is a "locked phase" synchrony between the beginning of each gulping movement, which forces oxygenated water over the gills, and the beginning of the heartbeat, often in a 1:1 ratio, but also in 1:2, 1:3 or 1:4 ratios. This has the effect of maximizing the rate of blood flowing through the gills with the maximum rate of oxygenated water passing over the gills (Satchell, 1960). The gulp of water stimulates receptors in the pharynx, the afferent fibers of which pass up the branchial branches of the vagus and glossopharyngeal nerves to the medulla and reflexively stimulate the vagus efferents to the heart (Satchell, 1968). The pharyngeal input also triggers reticulospinal neurons that generate swimming movements, resulting in a one-to-one synchrony among a gulp of water, a heartbeat, and an undulation of the tail (Satchell, 1968). "The co-ordination of swimming with respiration [and heartbeat] may be advantageous in ensuring that the mouth opens as the thrust of the trunk and tail forces the fish forward into the water ahead" (Satchell, 1968). Thus, in this "primitive" vertebrate, this reflexive, "robotic",

most direct, synchrony creates the optimization among oxygenation of the blood for the heart and the muscles of locomotion.

As other examples of the optimization of energy delivery by the blood flow to active muscles, synchrony among each heartbeat, each locomotor step, and each breath was reported in zoo animals and the case of a human runner. Thus, Coleman (1921), observing throbbing neck movements as a measure of the heartbeat, reported, "The movements of the whiskers of a resting leopard were perfectly regular and indicated its heart rate as 54; Fog on the breath of polar bears showed one breath for each step; An elephantine tortoise breathed once for each step." And regarding a runner, "One who always became breathless when halfway up a hill felt his pulse and began the climb *breathing and stepping in unison with the pulse* [our emphasis] and climbed the hill without breathlessness, and the rise in blood pressure was only half as great."

These observations suggest that the most efficient (i.e., least energy-wasting) condition is when heart rate, respiration and muscular activity are synchronized, as in the dogfish shark. This suggests that the heartbeat serves as a natural behavioral rhythm pacemaker. A manifestation of this phenomenon in humans is the timing of rhythmical beats in music. Tactus (literally, "time beating" in German) is based on the heartbeat. The duration of the semibreve ("whole note") was originally established as the duration of one pulse beat during quiet respiration (Sachs, 1948, p. 144).

In addition to respiration and respiratory facial (whisker) movements being synchronized, in rats… specifically during their exploratory sniffing behavior, and most prominently if they are standing up on their hind legs along a wall or leaning over the edge of a precipice, …there is a one-to-one synchrony among an inhalation sniff, a forward thrust of their vibrissae, a heartbeat, and one wave of their EEG hippocampal theta rhythm (Komisaruk, 1970). The rat's exploratory behavior is their decision-making behavior, based on the concomitant olfactory and tactile input generated by their sniffing behavior, e.g., to jump, or eat or drink or continue exploring. Perhaps the optimization of the oxygenation of the blood by a respiratory sniff synchronized with a heartbeat, synchronized with the phase of a hippocampal theta wave, optimizes the capacity of the rat's "primitive" brain to make the momentary decision.

Perhaps the primitive vagal afferent-efferent reflexive relationship that links respiration to heart beat is of such fundamental physiological adaptive importance, that it is maintained throughout the phylogenetic tree from fish to humans, and may have evolved to not only optimize energy provision to the muscles of behavior but also to the brain for optimization of cognitive function. While the coupling among respiration, heartbeat, and muscular movement has evolved from a locked-in relationship in fish to increasing de-coupling and independence among these mechanisms in humans, perhaps under extenuating circumstances, physiological reversion to the primitive condition of coupling is manifested, e.g., synchrony among inhalation, heartbeat, and striding during running, which optimizes energy expenditure, and the strength and ease of the effort (Komisaruk, 1982). With the involvement of rhythmical activity of the forebrain (i.e., hippocampus) in addition to that of the brainstem, the intriguing question is raised of which drives which…does vagal afferent activity drive the hippocampal theta, does hippocampal theta drive vagal efferent activity, or is it a two-way interaction? While all these fundamental physiological, neural, and behavioral systems can certainly function asynchronously, when they all lock into synchrony, a unique quality emerges of unity, simplicity, strength, ease of movement, and perhaps optimal perceptual acuity and cognition.

3.7. Vago-vagal reflex: control of satiety

There is a "vago-vagal" reflex from the sensory endings of the vagus nerve in the gastrointestinal (GI) tract to the NTS to the paraventricular nucleus of the hypothalamus (PVN) back to the dorsal motor nucleus of the vagus to the efferent component of the vagus back to the gastrointestinal tract, based on the following. There is substantial evidence that this reflex regulates the physiological and behavior components of satiety. Cholecystokinin (CCK), is released into both the GI tract and systemically by ingested food; it is a "satiety" signal that inhibits food intake; it stimulates NTS neurons (Valassi et al., 2008). The inhibition of feeding by CCK is blocked by section of the vagal innervation of the GI tract (Verbalis et al., 1986). CCK is a neurotransmitter of the afferent vagus, and microinjection of CCK stimulates NTS neurons (Crawley, 1985), which contain CCK receptors. Neurons of the NTS also synthesize CCK (Luckman et al., 1993). There is a direct projection of NTS neurons to the PVN neurons (Ricardo and Koh, 1978), and microinjection of CCK to the PVN stimulates those neurons (Luckman et al., 1993). The PVN neurons, which synthesize oxytocin, project back to the dorsal motor nucleus of the medulla oblongata. Lesions of the PVN produce hyperphagia and obesity (Aravich and Sclafani, 1983). Oxytocin microinjections stimulate the neurons of the dorsal motor nucleus which comprise a component of the vagus efferents to the GI tract, and there decrease gastric tone and motility (Holmes et al., 2013; McCann and Rogers, 1990). In a different context, the stimulatory effect of oxytocin administered directly to the neurons of the dorsal motor nucleus of the efferent vagus in rats, also attenuates exercise-induced tachycardia (Braga et al., 2000); this may be a component of the vasovagal reflex.

3.8. Arnold's nerve reflex (auricular branch of the vagus)

Mechanical stimulation of the external auditory meatus activates Arnold's nerve, i.e., the auricular branch of the vagus, which can trigger the cough reflex. According to Ryan et al. (2014), cough may be provoked from anywhere in the body where there are chemo- or mechanoreceptor c-fiber afferents of the vagus. In many cases, refractory or idiopathic cough may be due to sensory neuropathy of this system. Arnold's reflex can thus be considered a special case of this more general vagus-initiated cough reflex (Ryan et al., 2014; Canning, 2007).

Gupta et al. (1986) proposed the following sequential neural pathways and brain nuclei mediating Arnold's reflex: Auricular branch of the vagus, superior (jugular) ganglion of the vagus, NTS, nucleus ambiguus, spinal trigeminal nucleus and tract, medullary inspiration and expiration "centers", reticulospinal pathway to the spinal phrenic nucleus and nerve, and motor nuclei of the intercostal nerves. Gupta et al. (1986) refer to 3 related reflexes provoked by stimulation of Arnold's nerve: 1) Auriculo-palatal (gag) reflex via nucleus ambiguus, which innervates the muscles of the soft palate, pharynx, larynx, and esophagus; 2) auriculo-vomiting reflex via the dorsal motor nucleus of the vagus, which innervates the abdominal viscera; and 3) auriculo-lacrimal reflex via the facial and greater petrosal nerves to the lacrimal (tear) glands of the eyes.

3.9. Auriculo-genital reflex

Bradford (1938) found that stimulation of external auditory canal elicits stimulus-bound contraction of somatic muscles around the vaginal orifice in cats. In male cats, the stimulation elicits contraction of somatic cutaneous muscles related to the penis and perineum. The response was not elicitable in dogs, rabbits or monkeys. Surgical interruption of the auricular branch of the vagus blocked the response. The efferent innervation is a somatic nerve ("a long nerve", unnamed) that emanates from the brachial plexus and courses down the lateral abdomen and then medial to the genitals.

3.10. "Vago-pupillary reflex": pupil dilatation in response to vaginocervical stimulation

Our first indication of the relationship between vaginocervical stimulation and pupil diameter was during our observations of natural mating behavior in rats. During intromissions and ejaculations, the

females' pupils showed dramatic dilatation (Szechtman et al., 1985). We subsequently observed pupil dilatation in women during vaginal-cervical self-stimulation (Whipple et al., 1992).

In a subsequent study of women with complete spinal cord injury (SCI) at different levels that would block the entry of the several genito-spinal nerves' (pudendal, pelvic, and hypogastric) access to the spinal cord, our most stringent condition was complete SCI at the Thoracic 10 level or above, which would block the access of all three pairs of these nerves' access to the brain. To our surprise, the women with that level of complete SCI could feel the stimulation (Komisaruk et al., 1997). This led us to the hypothesis that the vagus nerve must convey the vaginal/cervical sensory activity to the brain. We tested, and confirmed, this hypothesis using functional MRI, which provided evidence that the NTS was, indeed, activated by vaginal or cervical self-stimulation in these women (Komisaruk et al., 2004).

We sought evidence of a possible genital sensory role for the vagus nerves by analyzing responses to vaginocervical stimulation in rats after spinal cord transection at the mid-thoracic level (Komisaruk et al., 1996). Prior to the surgery, vaginocervical stimulation produced an immediate and marked dilatation of the pupils. After the spinal cord transection, the vaginocervical stimulation still produced the pupillary dilatation, although slightly attenuated in magnitude. However, subsequent transection of the vagus nerves bilaterally abolished the pupillary response to the vaginocervical stimulation, although the pupils still showed the typical constriction in response to light shone in the eyes. As a confirmation of the role of the vagus nerves in this response, we stimulated electrically the cerebral stump of the transected vagus, and observed immediate and marked pupillary dilatation. In order to test whether the response was mediated by the sympathetic autonomic innervation of the iris, we ablated the superior cervical ganglia bilaterally (Bianca and Komisaruk, 2007). This had no effect, as the response to the electrical stimulation of the cerebral stump of the vagus persisted as before the ablation. We then tested the hypothesis of mediation by the more direct pathway from NTS to the Edinger-Westphal nucleus in the midbrain, from which the parasympathetic control of the iris projects. We transected the brainstem at the level of the posterior midbrain. This abolished the pupillary dilatation to the electrical stimulation of the vagus stump, while the pupil still constricted in response to light shone in the eyes. Thus, we concluded that afferent vagus stimulation produces pupillary dilatation by inhibition of the parasympathetic innervation of the iris (Bianca and Komisaruk, 2007). Based on the above studies, we herein term this phenomenon the "*vago-pupillary reflex*."

3.11. *Vago-oxytocin (neuroendocrine) reflex*

There is a surprisingly long research tradition demonstrating that afferent vagal stimulation releases oxytocin from the posterior pituitary into the systemic circulation. Chang et al. (1938) applied electrical stimulation to the afferent vagus in a preparation in which the head of a dog was isolated from the body with just the vagus and the blood supply remaining intact. Upon vagal stimulation, an extract of the venous blood was collected and administered in vitro to uterine muscle of guinea pig. The extract produced uterine contractions. Repeating the procedure after hypophysectomy failed to produce the uterine contractions. The authors concluded, "…the oxytocic principle of the posterior lobe of the pituitary can be released reflexly through the afferent vagus." Prior to this study, in 1927, Kamm isolated substances they termed "pitocin" and "vasopressin" from the posterior pituitary and demonstrated that they induced uterine contractility and blood pressure elevation, respectively (NIMH, 2021). Andersson (1951) followed up on the Chang et al. (1938) study, demonstrating that also in sheep and goats, electrical stimulation of the central end of the vagus produced milk ejection, indicative of oxytocin release. Dyball and Koizumi (1969) demonstrated that electrical stimulation of the central end of the vagus activated the oxytocin-producing neurons of the PVN, which project their axons to the posterior (neural) lobe of the pituitary. The neurons that were activated by the vagal stimulation were identified by antidromic activation from the posterior lobe of the pituitary.

It may seem curious historically as to why the above investigators came to study the connection between the vagus and oxytocin secretion. As Chang et al. (1938) explain their rationale, "Previous studies have shown that the pressor principle of the pituitary [vasopressin] can be reflexly released by afferent vagal stimulation. We have now extended our investigations to ascertain what other principles of the posterior pituitary may be similarly released." Andersson (1951) attempted a (puzzling) speculative rationale for his study described above that electrical stimulation of the vagus induced oxytocin secretion, by stating, "…when the udder and teats were mechanically stimulated by suckling and milking, the sheep and goats usually started ruminating [chewing the cud] after a latent period of about 20 seconds. This indicates that at least some of the somatic afferent paths from the udder and teats are connected with the vagal nuclei." We are not aware of evidence of innervation of the udder and teats by vagal afferents. We can, however, make a stronger case for normal, physiological involvement of vagal afferents in oxytocin release in response to *genital* stimulation.

4. Does the vagus extend to the pelvis? Evidence of vagal afferent innervation of the female genital tract

Ferguson (1941) demonstrated in rabbits that mechanical dilatation of the uterine cervix (or stretching the uterine horns or vagina) would initiate contractions of the uterus, but not after destruction of the posterior pituitary, a process that became known as the "Ferguson reflex", normally involved in parturition. Evidence that this response may be mediated at least in part by vagal afferents was hinted at by Moos and Richard (1975) in rat. They demonstrated that the release of oxytocin in response to vaginal stimulation (as measured by milk ejection) was mimicked by electrical stimulation of the cerebral stump of the vagus nerve. However, they did not report what would seem to be the critical experiment, i.e., to transect the spinal cord and ascertain whether oxytocin release could still be elicited by vaginal stimulation but then blocked by transection of the vagus. Nevertheless, it is likely that the vagus *could* mediate the oxytocin response to vaginal stimulation, on the basis of the following evidence. As mentioned above, vaginocervical stimulation induced pupil dilatation even after the spinal cord was transected at the mid-thoracic level (Komisaruk et al., 1996; Cueva-Rolón et al., 1996) and pupil dilatation was elicited by electrical stimulation of the central stump of the vagus (Bianca and Komisaruk, 2007). We reported that oxytocin is released into spinal cord superfusates as well as into the systemic circulation in response to vaginocervical stimulation in rats (Sansone et al., 2002). Pupil dilatation was elicited immediately upon administration of oxytocin intrathecally, directly to the thoracic spinal cord, evidently by stimulating the spinal cord sympathetic preganglionics that project to the iris of the eye via the superior cervical ganglia (Sansone and Komisaruk, 2001). In that study, administration of an oxytocin receptor blocker intrathecally to the thoracic spinal cord significantly attenuated the pupil dilatation to vaginocervical stimulation. Thus, a) vaginocervical stimulation can elicit pupil dilatation via the vagus nerves after spinal cord transection (Komisaruk et al., 1996), b) pupil dilatation is elicited by direct electrical stimulation of the central end of the vagus (Bianca & Komisaruk), c) electrical stimulation of the vagus stimulates the release of oxytocin (Chang et al., 1938; Andersson, 1951; Moos and Richard (1975), d) oxytocin stimulates pupil dilatation (Sansone and Komisaruk, 2001), e) oxytocin receptor antagonist attenuates (but presumably due to concurrent vagal inhibition of the parasympathetic innervation of the pupil, which also produces dilatation) does not completely block the ability of vaginocervical stimulation to elicit pupil dilatation (Sansone and Komisaruk, 2001), f) electrical stimulation of the central end of the vagus activates neurons of the paraventricular nucleus of the hypothalamus (Dyball and Koizumi, 1969), and g) the NTS projects to the paraventricular nucleus

of the hypothalamus (Ricardo and Koh, 1978). Consequently, it is likely that at least one of the neural pathways of the Ferguson reflex (i.e., oxytocin release in response to stimulation of the birth canal by the fetus at parturition) is: cervix to vagus to NTS to paraventricular nucleus of hypothalamus to posterior lobe of the pituitary to oxytocin release into the systemic circulation (Komisaruk and Sansone, 2003), which directly stimulates uterine smooth muscle contractions, facilitating parturition. The pupil dilatation observed during genital self-stimulation (Whipple et al., 1992) might be a response to the axonal release of oxytocin from the paraventricular nucleus neurons of the hypothalamus, some of which send descending axons to the spinal cord (Swanson and Kuypers, 1980) where they evidently synapse on the sympathetic preganglionics of the spinal cord.

In addition to the evidence of afferent vagal vaginocervical innervation in women (Komisaruk et al., 1997, 2004) there is also evidence of afferent vagal innervation of the cervix, uterus, and possibly vagina, based on neuroanatomical tracer studies in rats. Ortega-Villalobos et al. (1990) injected horseradish peroxidase into the cervix and found evidence of the tracer having been transported to the nodose ganglion, which is the sensory ganglion of the vagus nerve. Confirmatory evidence using a different tracer method was reported by Collins et al. (1999) who injected DiI into the nodose ganglion and reported evidence of the tracer in the cervix and uterus.

Further evidence of vagal innervation of the genital tract is based on functional MRI findings that stimulation of the vagus via electrical stimulation of the cymba conchae of the external ear, which is innervated by the (sensory) auricular branch of the vagus (Butt et al., 2020), activated the paracentral lobule of the sensory cortex in women (Nahas et al., 2007; Frangos et al., 2015). The paracentral lobule is the genital sensory region of the homunculus of Penfield and Rasmussen (1950), and this region was shown, using functional MRI, to be activated by cervical, vaginal and clitoral self-stimulation (Komisaruk et al., 2011). Thus, the activation of genital sensory cortex by afferent vagal stimulation could account for the ability to feel stimulation of the cervix and vagina by women with complete spinal cord injury above the level of entry to the spinal cord of the genitospinal nerves, and to experience orgasm from the stimulation (Komisaruk et al., 1997, 2004).

Thus, the classical view of the vagus nerve extending only as far caudally as the abdominal organs requires revision; there are multiple lines of evidence that the vagus nerve extends to the pelvic level, providing at least afferent innervation of the female reproductive tract. A further implication of these findings is that since afferent vagal activity activates this cortical region, activity in the sensory vagus may thus rise to the level of generating conscious, subjective awareness.

5. Subjective awareness of vagal afferent activity

It is possible that the subjective property of vagal afferent activation is qualitatively different from that produced by other sensory nerves. The cervix responds to pressure stimulation (Kinsey et al., 1953) and in 35% of 128 women, its sensation contributes to their orgasms (Cutler et al., 2000). Total hysterectomy, which removes both the cervix and the uterus was reported to have a greater inhibitory effect on orgasm than subtotal hysterectomy, in which the cervix is retained (Kilkku et al., 1983). In the "Loop Electrosurgical Excision Procedure" (LEEP), which removes a portion of the cervix by cautery, there are reports of women's generalized loss of libido and erotic sensibility throughout the body (Goldstein and Komisaruk, 2020). Women have described orgasms produced by cervical stimulation to feel more abstract — e.g., "universal spaciousness, expanding light in my brain" — than orgasms produced by vaginal or clitoral stimulation (O. Bryant, pers. comm.). Thus, while in addition to the vagus, the pelvic and hypogastric nerves innervate the cervix, it is likely that the vagal component of the cervix makes a unique contribution to the qualities of orgasm, especially, as in the case of the women with complete spinal cord injury, if it is the exclusive remaining genital tract innervation. Furthermore, in the women with complete spinal cord injury, the observation that in some cases the magnitude of analgesia induced by cervical self-stimulation exceeded that of able-bodied women (Komisaruk et al., 1997), suggests that following deafferentation produced by complete spinal cord injury, the vagal projection to the brain can undergo functional reorganization. Such reorganization has been reported in cases of sensory cortical reorganization following limb amputation (Ramachandran and Hirstein, 1998).

In addition to the possibility of conscious awareness of vagal afferent activity per se, there is evidence of vagal-induced referred sensation "phenomena", as described by Engel (1970) as follows.

5.1. Gastro- or pulmono-auricular "phenomenon"

Cases of esophageal hiatus hernia, in which the esophagus bulges through the diaphragm (hiatus), and heartburn, were reported to cause intense itching of the external auditory meatus and eardrum, termed "Gaph". "The reason why Gaph has remained unrecognized may be that a connection between the stomach and the external auditory meatus is regarded as improbable" (Engel, 1970). It seems plausible that the referred sensation could be a consequence of input from the gastrointestinal tract and the auricular branch of the vagus converging on the same individual neurons in the NTS and/or forebrain/cortical neurons.

5.2. Auriculo-uterine phenomenon

Engel terms a case study reported by Vasiliu (1932) as an "auriculo-uterine" phenomenon. Vasiliu described a woman who reported feeling uterine contractions in response to her ear being swabbed. While Vasiliu suggested that it was a "sympathetic response", based on evidence cited above, it was perhaps rather a perceptual response to the uterine contractions resulting from oxytocin release produced by stimulation of the auricular vagus.

5.3. Pulmunoauricular phenomenon

In a case of tuberculosis of the lung, the "skin area of the vagus" was hypersensitive (Engel, 1970).

6. Reflexes mediated by the *efferent* vagus nerve

6.1. Oculocardiac reflex

Pressure on the cornea, traction of an extraocular muscle, strabismus surgery, and other traumatic stimulation of the eye can activate the trigeminal nerve, reflexively activating vagally-mediated severe cardiac arrythmia, bradycardia, or even cardiac arrest, which is sometimes fatal.

6.2. Diving reflex

This reflex classically has been considered to be stimulated by cold water on the face or nasopharyngeal stimulation (Paton et al., 2005), but more recently recognized as triggered by inhibition of respiration per se (Vega, 2017). The diving reflex is characterized by increased vagal afferent activation of the nucleus tractus solitarii from atrial receptors, baroceptors, pulmonary stretch receptors and carotid chemoreceptors (Foster and Sheel, 2005) but also by efferent action on the sino-atrial node, which induces bradycardia. As an exception to the general rule of reciprocal relationship between the sympathetic and parasympathetic divisions of the autonomic nervous system, this reflex is accompanied by cardiac tachyarrhythmias, indicative of concurrent, rather than reciprocal, sympathetic activation (Paton et al., 2005).

7. Clinical applications and implications of afferent vagus stimulation

The examination of vagus nerve stimulation for clinical purposes

dates back over 100 years with mechanistic rationales based on animal studies and, more recently, advanced neuroimaging in humans. A historical summary from auricular acupuncture to invasive and non-invasive VNS is comprehensively presented in a recent international consensus by Farmer et al. (2021). As reviewed above, the afferent vagal system has widespread influence across major neural networks. No longer constrained by the invasive nature of iVNS, researchers have embraced the ease of use and availability of tVNS (auricular or neck) devices to investigate the effects of afferent vagal stimulation on common health conditions and psychological disorders and processes. Several comprehensive reviews on tVNS are now available, focusing on reporting standards and practice (Farmer et al., 2021; Yap et al., 2020), safety and tolerability (Redgrave et al., 2018), anatomic rationale (Butt et al., 2020), engineering (Kaniusas et al., 2019a), and psychologic, physiologic, and mechanistic aspects (Yuan and Silberstein, 2016a, 2016b, 2016c; Cimpianu et al., 2017; Frangos and Komisaruk, 2017; Kaniusas et al., 2019b; Bremner et al., 2020a, 2020b; Colzato and Beste, 2020). The mechanisms that underlie the beneficial effects of vagal stimulation overlap extensively. In brief, the aforementioned projections from the NTS differentially modulate neuronal activity within key regions including the locus coeruleus and dorsal raphe nuclei (Krahl et al., 1998; Dorr and Debonnel, 2006; Cunningham et al., 2008; Giorgi et al., 2008; Furmaga et al., 2012; Carreno and Frazer, 2017). The modulatory effects in the brain of norepinephrine and serotonin, along with gamma-aminobutyric acid (GABA) (Ben-Menachem et al., 1995; Marrosu et al., 2003), each play a role in the anti-seizure or anti-depressive effects. Similarly, anti-nociceptive effects of vagal stimulation are, in part, dependent on the activation of opioid receptors as well as the descending noradrenergic and serotonergic systems within the spinal cord, which act on second order nociceptive neurons to produce nociceptive inhibition (Basbaum and Fields, 1978; Randich and Aicher, 1988; Ren et al., 1990; Randich and Gebhart, 1992). Functional imaging studies on iVNS and tVNS, discussed above, have reported activity within brain regions that not only respond to and modulate pain, e.g., insula, anterior cingulate cortex (Apkarian et al., 2005; Bushnell et al., 2013), but also modulate affect and other psychological factors (e.g., attention), each of which can differentially interact with aspects of the pain experience such as pain intensity vs. pain unpleasantness (Bushnell et al., 2013; Frangos and Komisaruk, 2017).

7.1. Invasive vagus nerve stimulation

At present, direct electrical stimulation of the left cervical vagus nerve is an approved treatment, by the US Food and Drug administration (FDA), for treatment-resistant epilepsy and depression (DeGiorgio et al., 2000; Rush et al., 2000). This method entailed surgical implantation of electrodes that apply direct electrical stimulation to the cervical vagus nerve. Decades later, iVNS still remains the standard despite the promising effects of non-invasive approaches in epilepsy and depression (Toffa et al., 2020). Early indications that iVNS could have additional applications outside of refractory epilepsy and depression, were observed in implanted patients with concomitant migraine or cluster headache. Investigators noted incidental findings of complete relief or decreased severity and frequency of migraine or headache attacks after implantation (Sadler et al., 2002; Hord et al., 2003; Lenaerts et al., 2008). Since then, researchers have also examined the application of iVNS in other conditions, including heart failure (De Ferrari et al., 2011), chronic and experimental pain (Kirchner et al., 2000; Lange et al., 2011; Koopman et al., 2016), and for treatment of weight loss and food cravings (Bodenlos et al., 2007; Pardo et al., 2007).

7.2. Transcutaneous (non-invasive) vagus nerve stimulation

Non-invasive methods of vagus nerve stimulation are a safe, less costly and more accessible alternative to assess the effects of afferent vagal stimulation. An added benefit of non-invasive access to the vagus nerve is that it allows for basic research in non-pathological populations across disciplines. Here, we refer to all forms of non-invasive approaches as *transcutaneous* VNS (tVNS), which can include the following: transcutaneous cervical VNS (tcVNS) applied to the surface of the neck over the cervical vagus nerve, or transcutaneous auricular VNS (taVNS) applied to the external surface of the ear in the areas innervated by the auricular branch of the vagus nerve.

Specifically tcVNS has received FDA approval for the treatment of episodic cluster headaches, migraine, and hemicrania — paroxysmal and chronic (Meglio, 2021; Mwamburi et al., 2018). While other forms of tVNS have not yet received FDA clearance, the application in acute and preventive treatment for migraine and cluster headaches has been assessed with promising results (Silberstein et al., 2020; Goadsby et al., 2014; Barbanti et al., 2015; Straube et al., 2015; Garcia et al., 2017). Similar to its invasive counterpart, beneficial effects of tVNS on depression, anxiety, and mood have been observed (Kraus et al., 2007; Hein et al., 2013; Napadow et al., 2012; Kinfe et al., 2015; Fang et al., 2016; Liu et al., 2016; Rong et al., 2016). Investigators have also assessed the effects of tVNS on the following: experimental and chronic pain aside from migraine and headaches (Napadow et al., 2012; Busch et al., 2013; Laqua et al., 2014; Frøkjaer et al., 2016; Usichenko et al., 2017), tinnitus (Yakunina and Nam, 2021), stroke (Capone et al., 2017), food preference (Öztürk et al., 2020), and cognitive processes such as memory (Jacobs et al., 2015; Beste et al., 2016; Giraudier et al., 2020; Mertens et al., 2020). For a summary of studies assessing the effects of tVNS on other cognitive and psychological processes (see Cimpianu et al., 2017; Frangos and Komisaruk, 2017; Farmer et al., 2021).

Although tVNS is a feasible alternative to iVNS, consistency in intervention success has varied, likely due to the complex nature of its downstream effects, which can be parameter-dependent (Randich and Gebhart, 1992; Borckardt et al., 2005; Yakunina et al., 2017; Badran et al., 2018; Sclocco et al., 2020). Furthermore, as highlighted in recent reviews by Yap et al. (2020) and Farmer et al. (2021), the lack of standardization across paradigms and parameters, and inconsistent reporting of methodological details, has contributed to conflicting reports across studies. Nevertheless, the potential to treat a multitude of clinical diseases and disorders is promising.

8. Conclusion

The widely ramified anatomical and functional projections of the afferent vagus throughout the brain help to account for its diverse homeostatic physiological, perceptual, and behavioral actions, and significant clinical applications. It is likely that further research into the perceptual and behavioral effects of vagal input to the brain will reveal new clinical applications that can take advantage of the remarkable excitatory, inhibitory, and reflexive properties of this crucial afferent/efferent cranial nerve “wanderer.”

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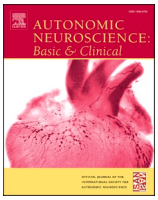

tVNS in the management of headache and pain

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ABSTRACT

First clinical observations of the therapeutic effect of vagus nerve stimulation were of patients who were treated for refractory epilepsy with a fully implanted vagus nerve stimulator, who also reported an improvement of their migraine and cluster headache. With the development of non-invasive vagus nerve stimulation, first clinical studies concerning a possible therapeutic effect in migraine and cluster headache were performed. In a controlled study, transcutaneous cervical vagus nerve stimulation (tcVNS) showed a significant but limited effect in acute treatment of a migraine attack. There was no significant prophylactic effect in episodic migraine. Concerning cluster headache, there was a clear beneficial effect in the prophylaxis of chronic cluster headache and in the attack treatment in episodic cluster headache. There are fewer studies in the literature on the effect of transcutaneous auricular vagus nerve stimulation (taVNS), with a partial overlap with studies on electrical ear acupuncture. In a small controlled clinical trial, there was a significant effect of taVNS in the prevention of chronic migraine. In less defined clinical studies, there were some positive signs that the method may be beneficial in chronic back pain and in unspecific gastro-intestinal pain in adolescents.

Based on the available evidence, it is probable that vagus nerve stimulation can have a clinically meaningful influence on pain syndromes, but there are still several questions (e.g. frequency of the stimulation; duration of the stimulation; differential effects of auricular vagus stimulation and cervical vagus stimulation) to answer before vagus stimulation can be used widely in the clinic.

1. Introduction

The vagus nerve is one of the essential parts of the parasympathetic system and carries motoric nerve fibers for the control of the vocal cords as well as autonomic fibers that control heart and stomach and gut. More than 70% of the fibers in the nerve are afferent fibers and only 20-30% efferent fibers. In most studies, the left cervical vagus nerve is stimulated based on safety concerns, since the left vagus nerve innervates more the atrial-ventricular node and the right the sinus-atrial node (Chen et al., 2015). Due to the current spread and the much lower thresholds for the stimulation of the myelinated afferent fibers, it becomes clear that only the afferent fibers can be stimulated with the non-invasive stimulation. Consequently, the majority of recent clinical studies used bilateral stimulation. Based on these facts, it is not obvious that the vagus nerve is involved in the processing of noxious stimuli. However, physiological investigations in the 1980s and 1990s revealed that stimulation of the left vagus nerve has a modulatory effect on somatic pain and on headache (Ammons et al., 1983; Randich and Gebhart, 1992). Stimulation of the left vagus nerve inhibited the activity of primate spinothalamic neurons related to cardiac pain (Ammons et al., 1983). The effect was eliminated by bilateral cervical vagotomy, in line with the idea that vagal afferents to the brainstem are activated and the effect on the spinothalamic neurons is then carried forward by descending spinal pathways (Ammons et al., 1983). Further, it was shown that the vagal input to the nucleus tractus solitary and other brainstem relays is critical here. This implies the involvement of descending spinal serotonergic and noradrenergic pathways which are known as the descending antinociceptive system (Randich and Gebhart, 1992). Concerning the influence of vagal stimulation on headache, there are several experimental studies, mostly in rats, which show an inhibitory influence of the stimulation of cardiac vagal afferents on the activity of spinal trigeminal nociceptive neurons which respond to the painful stimulation of the orofacial skin and the tooth pulp (Bossut and Maixner, 1996; Lyubashina et al., 2012). Later it was shown that this inhibitory effect was at least

Abbreviations: paVNS, percutaneous auricular vagus nerve stimulation; tVNS, transcutaneous vagus nerve stimulation; taVNS, transcutaneous auricular vagus nerve stimulation; tcVNS, transcutaneous cervical vagus nerve stimulation.

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partially mediated by an opioidergic mechanism and can be blocked by naloxone (Takeda et al., 1998). Continuous vagus nerve stimulation was also able to reduce pain behavior after formalin injection into the mystacial vibrissae in rats and the associated c-Fos expression in the caudal trigeminal nucleus (Bohotin et al., 2003). Based on the experimental results, in 2001 first reports of the effect of vagus nerve stimulation on nociception in humans were reported. In these case series, patients who were implanted with a vagus nerve stimulator for medical refractory seizures reported on a reduction in migraine headache or showed a reduction in pain perception in experimental settings. In ten patients with left vagus nerve stimulation for medically intractable epilepsy, Kirchner et al. (2000) tested pain perception by using quantitative sensory testing (QST). Vagus nerve stimulation diminished the increase in pain perception during trains of pain stimuli ("wind up") and also reduced the pain due to tonic pressure stimuli. There was no effect on pinprick and heat stimulation. In a survey including 62 patients, 4 patients had episodic migraine and all of them reported a reduction of migraine attacks under the vagal stimulation (Hord et al., 2003). In a similar approach, Lenaerts et al. (2008) retrospectively analyzed 34 patients who were initially implanted for seizure treatment. Ten of these patients also had migraine and the attack frequency was analyzed. Eight showed a reduction of attacks of at least 50%; two did not respond. Mauskop (2005) described 6 patients who were treated for headache with an implanted vagus nerve stimulator. Four of them showed excellent or good improvement on the stimulation, two did not benefit.

Based on clinical observations of the benefit of invasive vagal nerve stimulation on migraine and cluster headache in individual cases, first studies investigating the effect of non-invasive vagal nerve stimulation on migraine and cluster headache were initiated. For other chronic pain syndromes, the literature is more anecdotal. In the following, we will discuss the literature on non-invasive vagal stimulation at the neck (nVNS) in the form of transcutaneous auricular stimulation of the sensory vagus nerve (taVNS), transcutaneous cervical stimulation of the sensory vagus nerve (tcVNS) and for the sake of completeness also the minimally invasive form of percutaneous auricular vagus nerve stimulation (paVNS). The review is based on a search in the databank PubMed under the items "non-invasive vagus nerve stimulation AND migraine OR cluster headache OR pain syndrome" and "transcutaneous auricular/cervical vagus nerve stimulation AND migraine OR cluster headache OR pain syndrome". The paper will discuss possible mechanisms by which tVNS influences migraine and cluster headache, as well as clinical experience with non-invasive vagus nerve stimulation in the acute and preventive treatment of headache and somatic pain.

2. Vagus nerve stimulation

2.1. Transcutaneous cervical vagus nerve stimulation (tcVNS)

TcVNS is studied in open uncontrolled case series as well as in controlled studies by using the GammaCore® battery-powered handheld device. In order to stimulate the vagus nerve, the device is placed ventrally to the sternocleidomastoid muscle on the left or right side. When activated by the patient, the device gives an adjustable low-voltage electrical stimulation with a burst of 5 kHz sine waves every 40 ms (25 Hz) for a duration, in most studies, of 120 s. For sham stimulation, the device was set at 0.1 Hz biphasic stimulation. The stimulation was repeated 2–3 times in some studies. The patients were asked to use the maximum tolerated output intensity which normally causes contractions of the platysma muscle.

In migraine, there are studies published concerning acute as well as preventive treatment. In a first open uncontrolled study, 30 patients were enrolled. The patients treated up to four attacks with two 90s stimulation on the right cervical vagal nerve (Goadsby et al., 2014). No severe adverse event was reported; some patients complained about short-lasting redness of the stimulation site or neck twitching or a raspy voice. In the mean across all attacks, the pain-free rate at 2 h, the gold standard in attack treatment studies, was 22%. This rate is comparable to the data in some of the controlled phase 3 studies using triptans or naproxen (Goadsby et al., 2014).

In order to confirm these results, a randomized study (PRESTO) was done (Tassorelli et al., 2018). A total of 248 patients with episodic migraine were included and randomized in a verum and a sham group. The active group stimulated bilaterally for 120 s each and in the sham group the above-described 0.1 Hz stimulation was applied. The stimulation was started within 20 min after beginning of the headache and the effect was assessed after 15, 30, 60 and 120 min and 24 and 48 h. The main outcome was that the active stimulation was superior to the sham stimulation in pain-free responder rates at 30 (12.7% vs. 4.2%) and 60 (21.0% vs. 10.0%) minutes and also with a strong tendency at 120 min (30.4% vs. 19.7%). The secondary endpoints concerning pain relief showed similar results with 40.8% reporting pain relief at 120 min (Tassorelli et al., 2018). The side effects were similar to the open study. Overall, the result confirmed the findings from the open study in which the pain-free data were in the range of the values seen in the studies with medication. The pain relief data are somewhat lower than the percentage in the medication trials. A limitation which is more or less given in all studies using electrical stimulation is establishing a sham stimulation which does surely not have an effect and is also not easily recognized as a sham stimulation. Using different stimulation frequencies does not exclude the possibility that the different stimulation frequencies have a biological effect.

Beside the optimization of acute attack treatment, there is a recommendation in national and international guidelines to start preventive treatment in episodic migraine with more than 4 headache days/month. The PREMIUM trial was a controlled, double-blind, randomized study for the preventive treatment of episodic migraine with tcVNS (Diener et al., 2019). The study protocol started with a 4-week baseline period, followed by a 12-week double-blind period and then a 24-week open-label phase. The intervention was a daily application of two 120 s stimulations at the cervical vagus nerve 3 times a day either with a stimulator or a sham device. The analysis included 332 patients (about 8 migraine days at baseline). There was no difference between the verum and the sham group (reduction of migraine days by 2.26 compared to 1.80). In a post hoc analysis, only including patients with minimal stimulation on 67% of the days showed a significant difference (-2.27 vs. -1.53 days). This result can be interpreted as indicating that there is a dose effect for the vagus nerve stimulation.

In addition to studies on the use of tcVNS in migraine, several studies concerning cluster headache were performed. One of the first studies was published in 2015 (Nesbitt et al., 2015). In this open uncontrolled study, 19 patients (11 chronic cluster headache, 8 episodic) were included. The patients could use the device for acute attack treatment by stimulating 3 times for 120 s ipsilaterally to the cluster attack side and for preventive use 2 to 3 times in the morning and late afternoon. Fifteen patients reported some improvement, four stated that there was no effect. 47% of the attacks were halted within 11 min. In terms of the preventive effect, there was a reduction of the mean attack frequency from 4.5/24 h to 2.6/24 h. These effects were investigated more deeply in controlled, randomized, double-blind studies (ACT1 and ACT2 study) (Silberstein et al., 2016; Goadsby et al., 2018). ACT1 was a double-blind, prospective study including 150 patients with episodic or chronic cluster headache that were randomized for a duration up to one month or five attacks treated either with tcVNS (3x120sec right side of the neck) or sham (which elicited a slight tingling sensation without stimulation of the vagus nerve) (Silberstein et al., 2020). A total of 60 patients (episodic $n = 38$, chronic $n = 22$) were randomized to the verum group, 73 were sham-treated (episodic $n = 47$, chronic $n = 26$). The primary endpoint "response rate" (pain-free 15 min after treatment) was only significant for the episodic cluster headache group compared to sham (34.2% vs. 10.6%; $p = 0.008$), but not for patients with chronic cluster headache. No severe adverse events occurred.

In ACT2, in a 2-week double-blind phase, the patients received non-

invasive vagus nerve stimulation (3x120sec ipsilateral to the cluster headache early at the attack onset) or sham stimulation (see ACT1). A total of 48 patients were randomized to the verum group and 44 to the sham treatment. In both groups, about 1/3 of the patients had an episodic and 2/3 a chronic form. The stimulation again only showed a significant benefit in the subgroup with episodic cluster headache (48% vs. 6%; proportion of pain-free status after 15 min in all stimulated attacks); there was no significant difference for chronic cluster headache. No severe adverse events were reported.

In contrast to the negative finding concerning the acute attack treatment in chronic cluster headache, there was a significant improvement in the prevention of attacks in chronic cluster headache with non-invasive vagus nerve stimulation (Gaul et al., 2016). In this study (PREVA), 97 patients with chronic cluster headache were randomized into two groups after a two-week baseline phase. One group received their standard medical treatment plus the vagal stimulation (3 times 120 s stimulation 2 times a day), the control group went on with their standard care for 4 weeks and after that could also receive the vagus nerve stimulation in a cross-over design. The primary endpoint was the reduction of cluster attacks in the last two weeks of the randomized phase compared to the number of attacks in the 1-week run-in phase. The reduction was also calculated for the 2 last weeks of the extension phase, when the control group also received vagus nerve stimulation. The intervention group showed a significant larger reduction in weekly cluster attacks (-5.9) compared to the group with the usual standard care (-2.1). The number of patients with an improvement of at least 50% was also higher in the group with vagal stimulation (40% versus 8.3%). Further, in the subjects with measurable observations (modified ITT (mITT)) the number of responders (improvement by 50%) increased in the control group in the extension phase when they were allowed to use the vagal stimulation from 8.5% to 21.6%. The net effect of reduction in chronic cluster headache attacks was 3.8 which is nominally larger than the reduction that was seen in the recently published study concerning the preventive effect of prednisone, but which solely included episodic cluster headache patients (−2.4) (Obermann et al., 2021). In a further analysis of the data, it was shown that the improvement was seen starting with the second week of vagal stimulation and that the primary intervention group was better than the control group at all time points. The percentage of patients with a 100% reduction was 8% in the intervention group and 0% in the control group (Gaul et al., 2017). The data were confirmed due to later open case series of 30 patients who were treated in England in the period from 2012 to 2016. Here the attack frequency before start of the stimulation was 26.6 attacks and fell to 9.5 attacks/week afterwards (Marin et al., 2018).

Concerning other trigemino-autonome headaches, there are some single case reports which reported a benefit of ipsilateral stimulation, especially in hemicrania continua (Eren et al., 2017; Tso et al., 2017; Trimboli et al., 2018).

2.2. Transcutaneous auricular vagus nerve stimulation

The scientific rationale behind the stimulation of the auricular branch of the vagus nerve is that the same parasympathetic brainstem center can be assessed as with the vagus nerve stimulation at the neck. Evidence from fMRI studies showed that electrical stimulation of the auricular branch of the vagus nerve causes an activation in the areas of the central vagus projections (nucleus tractus solitary, spinal trigeminal nucleus, dorsal raphe, locus coeruleus, parabrachial area, amygdala and nucleus accumbens as well as cortical activation). The hippocampus and hypothalamus showed a deactivation (Frangos et al., 2015). The auricular branch is the only sensory nerve of the vagus nerve which innervates some surface areas and the skin of the cymba conchae is exclusively innervated by the sensory vagal afferents (Ellrich, 2019). Like the non-invasive vagus nerve stimulation, electrical stimulation of this area activates mostly and exclusively the larger myelinated sensory Aβ fibers (Ellrich, 2019). Most clinical studies used the NEMOS® device. In contrast to the non-invasive neck stimulation, the stimulation was done with 25 Hz or 1 Hz and for 4 h a day with minimum stimulation intervals of 1 h.

There is only a limited number of studies on the clinical benefit of taVNS. In one monocentric controlled, blinded and randomized study, 46 patients with chronic migraine (more than 15 headache days per month and at least 8 of these with migraine-type headache or response to migraine medication) were included (Straube et al., 2015). Forty patients completed the study. After a baseline period of 4 weeks, the patients were randomized to receive 1 Hz or 25 Hz stimulation of the auricular branch of the vagus nerve. The stimulation was delivered by the NEMOS® device using a specially designed electrode to the concha of the left outer ear. The patients were asked to use an intensity which elicited a non-painful tingling sensation and the daily stimulation duration should be 4 h in total. The primary endpoint was the change in headache days in a 28-day period starting 4 weeks before the end of the study compared to the 28 days in the baseline period. Secondary outcome parameter were the percentage of patients with at least 50% improvement and the changes in the MIDAS, HIT-6 questionnaires and the number of adverse events. 46 patients were included in the study, with 6 patients dropping out during the study. Somewhat astonishingly and contrary to the primary hypothesis, the group with 1 Hz stimulation showed a significantly larger reduction in headache days per 4 weeks (19.1; -7.0 days) than the group with the 25 Hz stimulation which is the gold standard for stimulation with implanted stimulators (19.2 days at baseline; -3.3 days). 29.4% of the 1 Hz group and only 13.3% of the 25 Hz group showed a reduction of at least 50%. No serious adverse events were reported (Straube et al., 2015).

The reduction in the 1 Hz group of about 7.0 days (after 12 weeks) is comparable to the effect seen in the large PREEMPT trial for the effect of onabotulinumtoxinA. (-8.4 days after 24 weeks) (Dodick et al., 2010). Unfortunately, no further clinical studies concerning the preventive effect in migraine or cluster headache have been published. In an fMRI study, Zhang et al. (2021) report that the patients with taVNS (1 Hz stimulation) compared to the patients with sham taVNS showed a reduction in intensity and frequency of migraine days after 4 weeks treatment, supporting the results described above.

There is also an effect of taVNS on pain perception beyond the trigeminal supply area. In an experimental setting using QST (quantitative sensory testing), healthy subjects were tested during continuous taVNS stimulation with 25 Hz. Under stimulation there were increased pressure and mechanical pain thresholds as well as reduced pain perception during sustained painful heat stimulation on the hand compared to the sham condition (Busch et al., 2013).

Another study investigated the effect of electrical stimulated auricular acupuncture (compared to classic auricular acupuncture) on chronic low back pain. The study is therefore not a typical tVNS study given the fact that minimally invasive subcutaneous microneedles were used for percutaneous auricular vagus nerve stimulation (paVNS) but gives some indication of a possible effect of tVNS in chronic back pain. In total 61 patients were analyzed: 31 patients received electrical stimulation, 30 served as a control group. In both groups, 3 auricular acupuncture points were inserted with single-use needles and the needles were removed after two days. In the stimulation group, the needles were connected to an electrical stimulator and the stimulation frequency was 1 Hz. Stimulation was done once a week for 48 h for 6 weeks and the patients were followed up until the end of 3 months. Several standard pain questionnaires were used for the assessment. Both groups improved, but the pain reduction was significantly better over the whole study period in the group with the active stimulation. This also translated into less use of analgesics and more patients returning to full-time employment. No severe adverse events were reported (Sator-Katzenschlager et al., 2004).

A frequent problem, especially in adolescents, is unspecific gastrointestinal pain. In a study with 115 patients, the effect of paVNS or sham stimulation over a period of 4 weeks was tested. The stimulated

Table 1
Summary of the clinical studies published for tVNS and paVNS.

Pain syndrome	Stimulation type	Frequency/duration	Study type	N	Result	Literature
Migraine	tcVNS	acute, twice 90s	case	30	Positive: Pain-free rate at 2 h was 22%	Goadsby et al., 2014
Episodic migraine	tcVNS	acute, bilaterally 120 s	RCT	248	Positive: Significant pain-free responder rates at 30 and 60 min, tendency for 120 min (PE). Significant for pain relief (120 min) and percentage of pain intensity reduction (60 and 120 min) (SE)	Tassorelli et al., 2018
Episodic migraine	tcVNS	acute and preventive, twice 120 s 3 times a day	RCT	332	no difference in reduction of migraine days (PE)	Diener et al., 2019
Cluster headache	tcVNS	preventive and acute, 3 times for 120 s and 2 to 3 times a day	Case	19	Positive: reduction of mean attack frequency and painfree in 47% at 11 min.	Nesbitt et al., 2015
Cluster headache	tcVNS	acute, 3 times 120 s	RCT	92	only positive in episodic cluster headache with significant higher proportion of pain-free status after 15 min in all stimulated attacks (48% vs. 6%).	Goadsby et al., 2018
Chronic cluster headache	tcVNS	preventive, 2 times 3 stimulation for 120 s each	RCT	97	Significant reduction in weekly cluster attacks (-5.9 vs. -2.1) and improvement of at least 50% (40% versus 8.3%) in the group with vagal stimulation.	Gaul et al., 2016
Episodic and chronic cluster headache	tcVNS	acute, 3 times 120 s	RCT	150	only positive in episodic cluster headache with significant reduction in response rate (PE). Significant SE were sustained treatment response rate, responder and pain-free rate for >50% of treated attacks, change in attack duration.	Silberstein et al., 2020
Chronic migraine	taVNS	preventive, 4 h daily 1 Hz	RCT	46	Significant reduction in headache days (-7 vs. -3.3.), no difference in SE.	Straube et al., 2015
Episodic migraine	taVNS	preventive, 3 sessions per week for 30 min; 1 Hz	RCT	70	Significant reduction in number of migraine days, pain intensity and migraine attack times after 4 weeks.	Zhang et al., 2021
Chronic low back pain	paVNS	therapy; 48 h per week for 6 weeks; 1 Hz	RCT	61	Significant pain reduction in the group with active stimulation and also significant less use of analgesics. Additionally, improvement in psychological well-being, physical activity, quality of sleep and return to employment.	Sator-Katzenschlager et al., 2004
Functional gastro-intestinal disorders (adolescents)	paVNS	therapy; 4 weeks	RCT	115	Significant larger reduction of worse pain in the active group	Kovacic et al., 2017

paVNS = percutaneous auricular vagus nerve stimulation.
tVNS = transcutaneous vagus nerve stimulation.
taVNS = transcutaneous auricular vagus nerve stimulation.
tcVNS = transcutaneous cervical vagus nerve stimulation.
PE = Primary endpoint.
SE = Secondary endpoint.

Table 2
Study rating.

Study	Selection bias	Study design	Confounders	Blinding	Data collection method	Withdrawals and dropouts	Global rating	Classification of evidence
Goadsby et al., 2014	1	3 (open label single arm)	n.a.	n.a.	1	1	3	IV
Tassorelli et al., 2018	1	1 (RCT)	1	1	1	1	1	I
Diener et al., 2019	1	1 (RCT)	1	1	1	1	1	I
Nesbitt et al., 2015	1	3 (open label single arm)	n.a.	n.a.	1	1	3	IV
Goadsby et al., 2018	1	1 (RCT)	1	1	1	1	1	I
Gaul et al., 2016	1	2 (open label, randomized, controlled, parallel group)	1	n.a.	1	1	2	III
Silberstein et al., 2020	1	1 (RCT)	1	1	1	1	1	I
Straube et al., 2015	1	1 (RCT)	1	1	1	1	1	I
Zhang et al., 2021	1	2 (single blinded crossover design)	1	2	1	1	2	II
Sator-Katzenschlager et al., 2004	1	1 (RCT)	2	1	2	1	2	II
Kovacic et al., 2017	1	1 (RCT)	1	1	1	1	1	I

1 = strong, 2 = moderate, 3 = weak according to the "Effective Public Health Practice Project Quality Assessment Tool" (Armijo-Olivo et al., 2012); n.a. = not applicable. Classification of evidence according to AAN 2017 edition clinical practice guideline process manual and criteria for rating (1.30.17).

group had a larger reduction of the worse pain than the sham group. This reduction was sustained over the treatment period. Some side effects were reported, most often irritation or skin problems at the stimulation site (Kovacic et al., 2017).

3. Discussion

Both vagal stimulations, tcVNS and taVNS, showed a positive effect on pain perception in different pain syndromes or experimental settings in some studies. The studies on both stimulation types included only a limited number of patients. A further limitation is that most clinical syndromes were only investigated in one study, thus there is no

information about the reliability of the results. In recent years, two reviews and meta-analyses of the available studies were published. In a review concerning neuromodulation techniques for acute and preventive migraine treatment, the authors concluded that vagus nerve stimulation had no significant effect and the heterogeneity was high (Moisset et al., 2020). In another review, Lai et al. (2020) analyzed 6 published randomized controlled trials on the effect of tcVNS on migraine and cluster headache. They stated that there is a significant effect of tcVNS on treatment of acute migraine and cluster attacks, but no significant effect on headache days in episodic migraine. Based on the very good tolerability of both stimulation methods (tcVNS and taVNS), some authors stated that the vagal stimulation should be seen as a preferred option in the treatment of acute migraine attacks or cluster attacks in episodic cluster headache as well as an option in the preventive treatment in chronic cluster headache (Silberstein et al., 2020) (Tables 1 and 2).

Several issues have still not been addressed, mainly regarding the stimulation technique itself: 1) the best stimulation frequency, 2) the optimal stimulation duration, 3) whether there is a differential effect of tcVNS and taVNS in a specific pain condition. Answering these questions will be important before vagal stimulation procedures will result in patient specific treatment protocols to find broad clinical acceptance and use. Then, there will be the question of non-responders. Who are they and what is different? Maybe, these will lead to clinical implications and even better stimulation schemes or less use for inappropriate patients.

To achieve this, another point is crucial for further research: The classification of reporting standards to ensure better reporting and easier comparison of findings across different studies (Farmer et al., 2021). This is even more important as the fields of interest go far beyond headaches in various areas of autonomic research and VNS, thus better understanding possibly can complement each other in the future.

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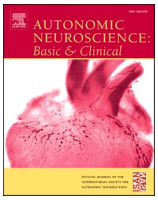

Brain plasticity and vagus nerve stimulation

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ABSTRACT

After damage to the central nervous system, caused by traumatic injury or ischemia, plasticity becomes critically important for functional recovery. When this inherent capacity to adapt is limited despite training, external stimulation may support this process.

Vagus nerve stimulation (VNS) is an effective method to enhance the effect of motor rehabilitation training on functional recovery. However, the mechanisms by which VNS exerts beneficial effects on cortical plasticity are not completely understood. Experimental work suggests that VNS fosters a neurochemical milieu that facilitates synaptic plasticity and supports reinforcement mechanisms. Animal studies, furthermore, suggest that VNS delivery is time-critical and that optima in the parameter space need to be titrated for effect maximization. Human studies suggest that VNS modifies corticospinal excitability. First studies in stroke patients show positive results for invasive, and also promising findings for non-invasive VNS.

The human central nervous system has a remarkable ability to adapt its structure and function in response to experience, learning, or tissue damage, referred to as neural plasticity. While the majority of neuroplastic processes occur inadvertently, in the absence of any explicit intervention, plasticity can be initiated or supported by, e.g., exercise and training [1]. When brain tissue suffers damage, caused by traumatic injury or ischemia, plasticity becomes critically important for functional recovery.

In the following, we will review experimental evidence for VNS as a plasticity-enhancing treatment. This evidence is mainly related to plasticity in the auditory and motor cortex, and treatment of tinnitus and stroke, respectively. In-depth reviews of VNS as a candidate treatment for stroke [2] and tinnitus [3] have been published recently. Compared to those previous reviews, we will give a more compact summary of the literature, with a slightly stronger focus on human studies and the potential of non-invasive VNS.

1. Plasticity after brain tissue damage

During ischemic stroke, neural tissue is deprived from blood supply, causing cell death. Tissue damage is usually gradual, with irreversible damage in the stroke core area, and partial or reversible damage in the surrounding tissue, the so-called penumbra [4]. The middle cerebral artery is a frequent location for ischemic strokes [5], causing tissue damage to the motor cortex and, as a consequence, functional motor impairments. When cortical tissue has suffered irreversible damage, full recovery of motor function requires motor cortex reorganization, such that the function of the irreversibly damaged tissue can be taken over by neighboring, less impaired areas, which has been shown to occur during intensive stroke rehabilitation training [6,7]. Cortical reorganization is not exclusive to stroke, but has also been observed following other types of circumscribed brain tissue damage, e.g., traumatic brain injury [8,9].

There appears to be a critical period of a few weeks post-stroke for plasticity and rehabilitation training [4] (similar for traumatic brain injury [10]), and even with training, a high proportion of stroke survivors suffer from persistent motor impairment [11,12]. Therefore, there is an unmet need for improvement and augmentation of stroke rehabilitation methods. One approach for this relies on combining rehabilitation training with non-invasive external stimulation of motor circuitry, using electrical, magnetic, or mechanical stimulation.

By and large, stimulation is assumed to support motor plasticity by three different mechanisms: First, stimulation can help the patient execute the limb movements that are part of the training, such that an improved movement execution leads to enhanced usage-dependent plasticity in the motor cortex [13]. Examples for this approach are movement-supporting neuromuscular stimulation or robotic exoskeletons [14–16]. The second mechanism aims to strengthen the connection between brain and muscle activation by activating both simultaneously, e.g., by concurrent transcranial magnetic stimulation (TMS) at the motor cortex and electrical muscle stimulation. This

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approach is referred to as paired associative stimulation (PAS) [17]. The third approach uses stimulation to shift the brain towards a more 'plasticity-ready' state. This mechanism is assumed to be the most relevant one for the effect of, e.g., repetitive transcranial magnetic stimulation [18] or vagus nerve stimulation (VNS) on neural plasticity.

2. Vagus nerve stimulation

VNS is electrical stimulation of the vagus nerve (10th cranial nerve). It was originally developed for management of pharmacoresistent epilepsy, and is today under investigation as a candidate treatment for a wide range of conditions [19]. It can be applied invasively or transcutaneously at the neck or the outer ear. VNS has been shown to activate vagal projection areas in the brain, e.g., the locus coeruleus [20]. Furthermore, it shifts the autonomic physiological state, and associated peripheral readouts such as heart rate variability, towards parasympathetic preponderance [21,22].

Numerous animal studies have shown effects of VNS on neural plasticity. However, to the best of our knowledge, VNS is currently not part of the regular clinical treatment of stroke or any other condition related to brain tissue damage, and evidence from human studies is still sparse.

3. VNS and non-motor plasticity

On the cellular and molecular level, modulating effects of VNS on plasticity have been shown repeatedly. One month of VNS treatment increased the expression of brain-derived neurotrophic factor (BDNF) and dendritic complexity in the rat hippocampus [23], and tonic activation of hippocampal serotonergic neurons was found to be enhanced after two weeks of VNS treatment [24]. In addition, an increased availability of hippocampal progenitor cells after two days of moderate-intensity VNS was reported [25], as well as increased hippocampal long-term potentiation (LTP) [26,27]. Also, non-invasive VNS reduced neuroinflammation and restored microglia function in rodent models of endotoxemia [28] and dementia [29]. In human depression patients, altered connectivity patterns in the brain's default mode network were found after one month of non-invasive VNS treatment [30].

The finding that VNS seems to induce plasticity in the hippocampus is accompanied by a number of studies that found VNS effects on learning and memory function. In bulbectomized rats, VNS ameliorated deficits in avoidance learning [31]. Verbal memory retention was enhanced in human epilepsy patients when VNS was delivered after learning [32,33], and associative memory performance was enhanced in healthy, elderly adults when non-invasive VNS was delivered simultaneously with the learning task [34]. Finally, several rodent studies found that VNS can enhance the extinction of learned fear [35–37], and this finding has been reproduced in humans using non-invasive VNS [38–40].

Another branch of research investigated the potential of VNS in the context of auditory plasticity and tinnitus. It has been shown that the representation of frequencies in the rat primary auditory cortex, i.e., the portion of the tonotopically organized auditory cortex that responds to a particular tone frequency, can be enlarged when tones are repeatedly paired with VNS, and that this VNS-induced *targeted plasticity* can be used to restore impaired auditory function following cochlear trauma [41]. Several studies have replicated these findings in rodents [42–44], and some pilot studies translated the method for treatment of human tinnitus patients, albeit with mixed results [45–47].

4. VNS and motor plasticity

Along similar lines, the concept of VNS-induced targeted plasticity has been translated to motor cortex plasticity. Pairing the successful execution of a skilled forelimb task with VNS in healthy rats led to reorganization of the primary motor cortex, i.e., enlargement of the somatotopic cortical representation of the muscles relevant for the task, but no functional improvement [48]. Subsequently, this was successfully translated to rat models of ischemic and hemorrhagic stroke as well as traumatic nervous tissue injuries. In rodent models of ischemic stroke, repeatedly pairing VNS with a lever-pulling task over the course of six weeks led to average limb force restoration close to the pre-lesion level, while performance improved substantially less with lever pull training only [49]. The recovery-enhancing effect of VNS was found in younger and aged rats [50]. This effect was not driven by a higher training intensity in the VNS group. Moreover, it was temporally specific, and administering VNS after lever pull training did not enhance rehabilitation [51,52]. The effects were found to be stable for several months, and to generalize to untrained tasks [53]. In rats with hemorrhagic strokes, pairing a rehabilitative training task with VNS over several weeks improved task performance compared to training only, without changing training intensity or lesion volume, indicating that the effect is related to cortical reorganization [54]. Likewise, studies in rodent models of traumatic injuries of the brain [55] or the spinal cord [56] showed an improved recovery of motor function as well when lever pull training was paired with VNS compared to training only. Again, it was asserted that the effect was not accounted for by VNS effects on lesion size or training intensity. Crucially, one of the studies [56] found that recovery was enhanced when VNS was paired with the most successful trials of the training, but not when it was paired with the least successful trials, indicating that VNS acts as a *neuromodulatory reinforcement*. In sum, these studies found that rehabilitation training was more effective in restoring forelimb motor function when motor training was contingently paired with VNS, compared to motor training alone. In addition, it has been shown that VNS enhances somatosensory recovery after nerve damage when sensory stimuli are paired with VNS [57]. This has been confirmed in a human stroke patient [58].

5. Timing considerations

A common design feature in most of the above studies was time-locking of VNS administration to other events, e.g., motor task execution. This method, related to the concept of PAS, is relatively common in the magnetic or neuromuscular stimulation literature, but it is different from traditional VNS protocols (e.g., for epilepsy), where VNS is usually delivered for a certain amount of time several times a day, without time-locking to any other event.

For the auditory modality, it is obvious that VNS enhances plasticity in a time-critical way, since only the representation of paired but not unpaired tones was enhanced following VNS [41]. Similarly, it has been shown for the motor domain that PAS-VNS administration is important for targeted plasticity, and that VNS administration after completion of motor training, or even adding additional random VNS trains to the task-locked ones, significantly reduces the beneficial effect on motor rehabilitation [59]. In addition, one study found that pairing the most successful trials of a motor task (highest force) with VNS enhanced motor rehabilitation, whereas pairing the least successful trials (lowest force) was not better compared to rehabilitation training alone [56], which further supports the idea that VNS might act on motor plasticity by neuromodulatory reinforcement [56], rather than making the brain tonically susceptible for plasticity. On the other hand, human studies [60–62] have shown that corticospinal excitability, as probed by TMS, was modified by VNS in the absence of a motor task, suggesting that there might also be a tonic component of VNS effects on the motor cortex.

6. Stimulation parameter considerations

Choosing the right stimulation parameters for VNS is a non-trivial task. Parameters include, among others, stimulation side, frequency, intensity, thus, the parameter space is high-dimensional and comprehensive mapping of all possible parameter combinations is prohibitive.

With respect to stimulation current intensity, it has been shown repeatedly that plasticity-enhancing effects of VNS in the auditory and motor cortex preferentially occurred at moderate intensities, and were weaker or absent at high or low intensities [63–67]. For the rodent models used in these studies, an intensity of 0.8 mA turned out to be an optimal, moderate intensity.

Two studies additionally considered the interaction between pulse width and current intensity, and found that both can partially compensate each other: Acute brainstem activation was found to be approximately proportional to the product of pulse width and current intensity [20], and plastic changes in the auditory cortex were found to be weak when 0.2 mA stimulation was delivered in short (100μS) pulses, stronger when 0.2 mA stimulation was delivered in long (500μS) pulses, and strongest when 0.8 mA stimulation was delivered in short pulses, i.e., the u-shaped relationship mentioned above was roughly replicated [68].

Moreover, stimulation rate was found to be relevant for plastic VNS effects, i.e., plastic effects disappeared when intervals between paired stimulation trains were too long (180 s compared to 30s) [69]. With respect to VNS train duration and intra-train frequency, u-shaped relationships were found as for intensities: Auditory plasticity effects were stronger for medium than for short or long train durations (0.5 s compared to 0.125 s and 2 s) [70], and stronger for medium than for high or low intra-train pulse frequencies (30 Hz compared to 7.5 Hz and 120 Hz) [71].

In sum, all investigated stimulation parameters showed non-monotonic relationships with plasticity outcomes, i.e., each parameter had a ‘sweet spot’ for plasticity enhancement, and deviation in either direction decreased the outcome. Whether such inverted U-shaped relationships can also be replicated in humans, what the average optimal parameters are, and how variable the sweet spots are between humans, are open questions.

7. Mechanisms of action

The mechanisms by which VNS exerts beneficial effects on cortical plasticity are, like the mechanisms of action of VNS in general, not completely understood. A relatively consistent finding is that VNS activates several brainstem nuclei, in particular, the nucleus of the solitary tract and the locus coeruleus, leading to increases in noradrenergic neurotransmission [20]. A few studies have investigated the neuromodulatory mechanisms that drive the effect of VNS on motor plasticity. They found that the VNS effect vanished when rats were pharmacologically depleted of either noradrenergic, serotonergic, or cholinergic neurotransmission [72,73], suggesting that VNS fosters a neurochemical milieu that facilitates synaptic plasticity, without a single neurotransmitter being responsible for the effect.

8. Clinical translation

In two pilot studies, ischemic stroke survivors were implanted with VNS electrodes and carried out rehabilitation training paired with VNS trains. These studies found paired VNS treatment to be safe and feasible, and have shown trends towards an enhanced rehabilitation success, as quantified by the Fugl-Meyer upper extremity assessment, a standard rating instrument for stroke survivors [74,75]. The first large-scale clinical trial was published very recently [76]. In this trial, patients were implanted with VNS stimulators and received moderate-intensity stimulation (0.8 mA at 30 Hz pulse frequency and 100 μs pulse width) paired with movements during in-clinic rehabilitation training three times a week across six weeks, with an additional home exercise program. The study found that patients in the VNS group recovered significantly better than patients in the control group, where the stimulator was implanted but no current was delivered during exercises. A clinically meaningful increase in Fugl-Meyer upper extremity score was reported for 47% of the VNS group, compared to 23% of the control group.

In addition, two pilot studies have investigated the use of non-invasive auricular VNS paired with rehabilitation training, on motor [77] and sensory [78] recovery. Both studies report non-invasive VNS to be safe and feasible, and consider the clinical effects to be promising enough to be investigated in a larger-scale study.

9. Summary

VNS enhances the effect of motor rehabilitation training on functional motor recovery. Animal studies suggest that VNS delivery is time-critical and that optima in the parameter space need to be titrated for effect maximization. First clinical studies in human stroke patients show positive results for invasive, and promising first results for non-invasive VNS.

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Review

A two-week course of transcutaneous vagal nerve stimulation improves global sleep: Findings from a randomised trial in community-dwelling adults☆

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ABSTRACT

Short sleep duration and poor sleep quality are common in the general population. This study tested if a 2-week course of daily transcutaneous vagal nerve stimulation (tVNS) improves sleep in community-dwelling adults. Participants were n = 68 men and women aged 18–75 years randomised into four groups: early and sham tVNS and late and sham tVNS. Early groups underwent daily 4 h stimulation between Day 0 and 13, while late groups underwent daily 4 h stimulation between Day 14 and 28. tVNS was performed with transcutaneous electrical nerve stimulation (TENS) on the left tragus, and sham tVNS (control conditions) was applied on the left earlobe. Sleep was measured with the Pittsburgh Sleep Quality Index. Analysis of prespecified contrasts (C), based on linear mixed modelling, revealed that for tVNS there were significant improvements in global sleep scores over time between Day 0 and Day 13 in the early stimulation phase (C = −1.90; 95% CI = −2.87 to −0.94), and between Day 14 and Day 28 in the late phase (C = −0.87; 95% CI = −1.41 to −0.32). No such differences were found under sham tVNS (applied early or late). However, global sleep scores showed no significant improvement under tVNS when compared against control groups during both the early ($\chi 2 = 0.83$, $p = 0.36$), or late stimulation phase ($\chi 2 = 0.24$, $p = 0.63$). We showed that two weeks of tVNS improves global sleep scores, but the change in sleep was not significantly different to control groups. Further studies are warranted to test the utility of tVNS in alleviating sleep complains in community-dwelling adults.

1. Introduction

Poor sleep quality and too short sleep duration, typically defined as ≤5–6 h per night, are common in the general population (Hisler et al., 2019; van de Straat and Bracke, 2015). For example, data from over 80.000 British men and women showed that as many as 43.3% slept for <6 h (Zhu et al., 2019). A study based on over 40.000 men and women from 9 countries reported a 9.2% prevalence rate of poor sleep quality (defined as a combination of difficulties with falling asleep, waking up frequently in the night and waking up too early in the morning), with countries like Poland and India reporting a 17% and 15% prevalence, respectively (Koyanagi et al., 2014). This is noteworthy given the well-established links between too short sleep and poor sleep quality and heighten risk of many non-communicable diseases such as cardiovascular outcomes and mortality (Cappuccio et al., 2011; Sofi et al., 2014), type 2 diabetes, obesity, hypertension (Itani et al., 2017; Schmid et al., 2015), and cancer (Chen et al., 2018). Reduced cognitive performance (Jackowska and Cadar, 2020) and cognitive decline (Lo et al., 2014), as well as depression (Zhai et al., 2015), are also predicted by unhealthy sleep patterns including both too short sleep and low sleep quality.

Given the deleterious consequences of poor sleep on physical and mental health in community-dwelling adults, efforts are warranted to improve people's sleep. For mild to moderate sleep difficulties, improvements in sleep hygiene (e.g., timing of exercise, alcohol use, napping, regular bedtime) are often recommended, yet empirical support for their effectiveness in the general population have been criticised and

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remain mainly restricted to experimental or clinical data (Irish et al., 2015). Severe and chronic sleep disturbances, in particular insomnia disorder, can be treated with pharmacotherapy (e.g., benzodiazepines, hypnotics) and cognitive behavioural therapy for insomnia (CBT-I) (e.g., see Miller et al. (2014) for a review). There is also growing evidence that CBT-I delivered online can be efficacious (Zachariae et al., 2016). However, pharmacotherapy is limited to short-term improvements, and is associated with side effects and potentially addiction (Miller et al., 2014). There is also a shortage of therapists specialising in CBT-I, and many individuals struggle to complete a full course of CBT-I (Matthews et al., 2013).

Interestingly, a recent review reported preliminary but promising effects of two non-invasive brain stimulation techniques on sleep difficulties. Briefly, Herrero Babiloni et al. (2021) found evidence for repetitive transcranial magnetic stimulation (rTMS), which utilises magnetic pulses, to be particularly useful for ameliorating sleep disturbances in conditions including major depressive disorder, Parkinson's disease, and chronic pain. One way through which rTMS may improve sleep is by decreasing cortical hyperarousal, achieved by lowering hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-thyroid (HPT) axis activity. The review also found transcranial direct current stimulation (tDCS), which uses electrical currents, to be effective in insomnia, and it has been proposed that in this technique sleep improves by altering electroencephalographic (EEG) activity (see Herrero Babiloni et al., 2021 for more details). However, findings of this review are limited to individuals with neurological and neuropsychiatric conditions, or individuals with insomnia disorder, thus do not generalize to the general population.

Another non-invasive technique that could potentially improve sleep is transcutaneous vagal nerve stimulation (tVNS). In tVNS, stimulation is applied to the ear where the auricular branch of the vagus nerve is located (see Yap et al., 2020 for a detailed review). In healthy sleepers, the transition from wake to sleep states is linked to a decrease of sympathetic activity and an increase of parasympathetic modulation of the heart, which is facilitated by the vagus nerve (Meerlo et al., 2008; Stein and Pu, 2012). Therefore, stimulating the vagus nerve may potentially help to maintain a flexible balance of the autonomic nervous system, needed to facilitate wake and sleep states. Increasing vagal nerve activity may also help to improve sleep via a reduction of inflammation and cortisol levels, both of which are implicated in poor sleep (Abell et al., 2016; Irwin, 2019). Briefly, stimulation of the vagal nerve activates neurons in the nucleus of the solitary tract (NST), and efferent vagal nerves that project from the NST to various organs in the body (e.g. the heart or lungs) release acetylcholine, which offsets pro-inflammatory cytokines (Thayer and Sternberg, 2010).

So far, tVNS has been mainly used to treat depression, epilepsy, tinnitus, pain and migraine (Yap et al., 2020). To date, very few studies have tested the effects of tVNS on sleep, and the therapeutic impact of non-invasive vagal nerve stimulation on this key health behaviour is yet to be recognised. Luo et al. (2017) showed that stimulation of the auricular concha led to improvements in sleep in patients with insomnia disorder, however since this study lacked a control group its findings are challenging to interpret. Another study, also based on patients with insomnia disorder, found that tVNS was associated with improvements in sleep, but this effect was not superior to sleep changes seen in a control group (Jiao et al., 2020). Importantly, this experiment as well as the study by Luo et al. (2017), were based on clinical populations, limiting a generalization to community-dwelling adults, who nonetheless often report a high prevalence of short sleep of low quality. Bretherton et al. (2019) showed that in community-dwelling adults, who were free of insomnia disorder, tVNS applied daily over a two-week period led to improvements in sleep quality. Notably, this trial was based on n = 26 participants (of whom n = 9 responded to the stimulation) and lacked a control group, while sleep was measured with an unvalidated questionnaire. This leaves the utility of tVNS in alleviating non-clinical sleep complaints in the general population uncertain. Therefore, given the growing interest in non-invasive and portable devices stimulating vagus nerve (Gidron et al., 2018), and the need to test novel techniques that could improve sleep in community-dwelling adults, this study tested the hypothesis that a two-week course of tVNS would lead to improved global sleep ratings, when compared with placebo stimulation.

2. Method

2.1. Design

This study was a single-blinded randomised control clinical trial (NCT04070547) that tested the effect of a 2-week course of tVNS on cognitive function and health-related variables including global sleep scores (focus of the present manuscript). Participants were randomised into early or late (waitlist) group (first step randomisation). In the early group, participants were further randomised into tVNS (active/actual intervention) and a control condition (sham tVNS) that started immediately after baseline measures (Day 0 to 13); two weeks after tVNS and control stimulation ended participants further provided follow-up assessments (Day 14 to 28). In the late (waitlist) group, participants randomised into active and sham tVNS groups began their conditions two weeks after baseline assessments (Day 14 to 28). In total, participants in the 4 arms detailed above remained in the study for 4 weeks.

Our rationale to randomise participants into early and late groups was to enable us to test changes in variables of interest between the pre- and postintervention phase in active intervention and placebo groups, as well as to test changes in variables of interest after active and sham stimulation ceased (Day 14 to 28) in the early groups, and whether any change occurred during waiting time in the late groups (Day 0 to 13).

2.2. Participants

Participants were n = 78 men and women aged 18–75 years recruited through advertisement from Ostrava University, Czech Republic, and neighbouring institutions. The exclusion criteria were as follows: cardiovascular disease (e.g., arrhythmia, history of coronary heart disease, history of stroke), severe mental condition (e.g., clinical depression, schizophrenia, anxiety disorder), severe neurological condition (e.g., epilepsy, brain tumours, significant migraine, traumatic brain injury), brain surgery, and pregnancy. Sample size was not determined based on formal a-priori power analysis but in line with existing research (e.g., Bretherton et al., 2019). The study was approved by the Ethnical Committee of University of Ostrava. Participants providing written informed consent and who completed the study received a small honorarium of 1000 CZK.

2.3. Procedure

Following eligibility screening, participants were allocated into early (n = 38) and late groups (n = 40). Written consent was obtained during the first visit to the research laboratory. Sociodemographic data and income information were measured via questionnaire during screening, at which stage participants were also asked in detail about their health and prescribed medication. Blood pressure, heart rate variability (HRV) (not described here), and height and weight measures were taken, followed by a battery of cognitive and emotion testing (not described here). Following these assessments, a second step randomisation allocation was applied whereby participants were allocated into active (actual) tVNS or active sham (placebo) tVNS conditions. Participants were explained how to fit and use the tVNS stimulator, and were given a manual. Information on adherence to tVNS device was submitted daily as well as after the stimulation ended (see Section 2.4 Transcutaneous vagus nerve stimulation below, for full details). Online questionnaires (e.g., sleep scale or depressive symptoms described below) were completed at home on the day of the laboratory measurement (in most cases prior the lab session) (Day 0). Late group had an identical lab

session 2 weeks later (Day 14). Online questionnaires, including the sleep scale, were repeated at Day 14 (postintervention assessment for active/sham early groups, beginning of active/sham stimulation for late groups), and at Day 28 (follow-up assessment for early groups, post-intervention assessment for late groups).

2.4. Measures

2.4.1. Background measures

As mentioned, sociodemographic data were collected at screening stage. In our study, we included age and sex, education and employment status (see Table 1) as measures of sociodemographic and economic information. Height and weight were used to calculate body mass index (BMI, kg/m^2). We also included information on whether participants were taking prescribed medication including antihypertensives, diabetes, depression and sleep medication (see statistical analysis and Table 1). Depressive symptoms were measured with a shortened version of the Center for Epidemiologic Studies Depression Scale (CES-D) (Radloff, 1977), which has been deemed valid and reliable (Andresen et al., 1994). In this study, we used a double back translation method as to obtain the scale in Czech language. In this questionnaire, participants are asked to give information on negative affect and somatic symptoms, and items are rated on a scale ranging from 0 to 3. Higher scores are indicative of greater depressive symptoms. In our sample, at baseline, the Cronbach alpha was 0.73, reflecting an acceptable internal consistency.

2.4.2. Sleep

Global sleep was measured with the self-reported Pittsburgh Sleep Quality Index (PSQI) (Buysse et al., 1989), and a Czech version of the validated English version was obtained by a double back translation method into Czech language. Briefly, the PSQI consists of 19 items which measure 7 components: subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleeping medication, and daytime dysfunction; the sum of these components yields a global sleep score. In this study the scale was answered with regards to the past two weeks. Higher scores are indicative of greater sleep disturbances, and at baseline scores ranged from 1 to 13. Cronbach alpha was 0.71 at baseline. Each participant was asked to complete the PSQI at 3 time points: the early groups at preintervention (baseline), postintervention and at follow-up; the late group filled in the scale at beginning of the 2-week waiting period, then at preintervention and postintervention stage. For comparison purposes with other studies, we also present baseline sleep duration and efficiency, both derived from PSQI data; mean sleep duration was 6.9 (SD 1.1), and mean sleep efficiency was 90.5% (SD 9.4). These data suggest that our participants were, on average, good sleepers.

2.4.3. Transcutaneous vagus nerve stimulation

tVNS was delivered via modified transcutaneous electrical nerve stimulation (TENS) devices (Parasym, Parasym Ltd., London, UK) with 2 electrodes on a clip. The active stimulation group underwent 14 days of auricular tVNS placed on the left tragus as this ear location was shown to be 45% innervated via auricular vagus nerve (Yakunina et al., 2017). The sham (control) group underwent 14 days of sham tVNS placed on the left earlobe, which is not innervated via the vagus nerve (Yakunina et al., 2017), and therefore it is thought to be an appropriate ear location that may be used as an active placebo location (Farmer et al., 2021; Burger et al., 2020). Every participant was instructed to use the stimulator for 4 h (240 min) a day, in several time segments, for a period of 14 days in their own home. Participants were asked to set the intensity of stimulation with a constant current, based on an individual level of sensitivity, with a pulse width of 200–300 μs at a frequency of 25 Hz with no on/off cycles. Before placement of the clip on the ear, participants were instructed to spray a conductive liquid with electrolytes on the electrodes. After 14 days of tVNS intervention (active or sham) participants were asked to demonstrate how they set the tVNS device every day, and where on the ear they placed the electrodes. Only in 1 case the setting was incorrect therefore the participant was excluded from the study. To monitor protocol adherence, we used an online daily diaries, in which participants were asked to indicate duration of usage of the tVNS stimulator, and in how many separate time segments it was worn. Additionally, participants were asked to write on a piece of paper (white for active tVNS and yellow for sham tVNS) how many days of the intervention they used the tVNS device, and how many hours on average they used the stimulator every day. Then they were asked to drop the paper into a cardboard box, which was sealed until the end of the study. The settings of the tVNS device were designed in accordance with existing studies and recommendations of the recently established tVNS consensus group (see Farmer et al., 2021).

2.5. Statistical analysis

Of the n = 78 participants that were enrolled, n = 68 were included into our analytical sample. We had to exclude participants due to their personal circumstances (n = 1), and an incorrect wear of the tVNS

Table 1
Baseline characteristics of participants in the four experimental conditions.

	Early group		Late group		p-Value
	Active tVNS (n = 15) Means (95% CI/frequency (%))	Sham tVNS (n = 16) Means (95% CI/frequency (%))	Active tVNS (n = 22) Means (95% CI/frequency (%))	Sham tVNS (n = 15) Means (95% CI/frequency (%))	
Age	47.3 (37.3 to 57.3)	51.8 (42 to 61.5)	50 (43.1 to 56.9)	42 (30.6 to 53.4)	0.46
Sex (men)	7 (46.7)	7 (43.8)	9 (39.1)	5 (35.7)	0.93
High educational level (degree)	5 (33.3)	10 (62.5)	14 (60.9)	8 (57.1)	0.32
Employment status					0.39
Student	3 (20)	2 (18.2)	2(10.5)	4 (33.3)	
Employed	8 (53.3)	7 (63.6)	16(84.2)	6 (50.0)	
Unemployed	4 (26.7)	2 (18.2)	1 (5.26)	2 (16.7)	
Body mass index	25.3 (22.5 to 28.1)	26.9 (24.5 to 29.3)	25.7 (23.8 to 27.6)	26.3 (23.6 to 28.9)	0.96
CESD-10 depressive symptoms	6.7 (4.2 to 9.3)	5.1 (3.6 to 6.5)	6.2 (4.6 to 7.8)	7.1 (4.3 to 10.03)	0.50
Prescribed medication use	5 (33.3)	6 (37.5)	9 (39.1)	4 (28.6)	0.92
Antihypertensives	1 (6.7)	5 (31.3)	5 (21.7)	1 (7.1)	0.20
Diabetes medication	1 (6.7)	1 (6.3)	1 (4.4)	0 (0)	0.81
Global sleep score[a]	5.9 (4.3 to 7.4)	4.7 (3.3 to 6.1)	5.3 (3.6 to 7.0)	4.9 (3.6 to 6.3)	0.71
Sleep duration[b]	7.0 (6.5 to 7.6)	7.1 (6.5 to 7.6)	6.9 (6.4 to 7.3)	6.8 (6.2 to 7.4)	0.89
Sleep efficiency (%)[b]	89.3 (84.4 to 94.2)	90.6 (85.8 to 95.3)	92.3 (88.1 to 96.4)	88.9 (83.4 to 94.4)	0.73

[a] Baseline difference in PSQI between active tVNS and sham tVN in early and late groups was tested separately.
[b] Derived from PSQI data.

stimulator (n = 1). Given that this study focuses on sleep and its longitudinal change, we also excluded participants who were taking hypnotics (n = 1), antidepressant medication (n = 3), and those who completed the sleep questionnaire only at 1 time point (n = 4). Of n = 68 participants (our analytical sample), n = 57 provided their global sleep ratings at all 3 time points, and n = 11 rated their sleep at 2 time points.

Participants characteristics were described as frequencies or means and 95% confidence intervals (95% CI), as appropriate. Differences in sociodemographic and economic characteristics, depressive symptoms and baseline sleep scores between the active and sham tVNS groups were tested using *t*-tests, univariate ANOVAS and chi-square tests, as appropriate. Mixed linear regression models were computed to examine main effects of the fixed effect of time (T0, T1, T2), group (early, late) and condition (active tVNS, sham tVNS), as well as their interaction. Mixed linear models use all available data over the follow-up period, and take into account the fact that repeated measures on the same individual are dependent. In these analyses, both the intercept and the slope were fitted as random effects, allowing individuals to have different sleep scores at baseline, and different rates of change in sleep over the follow-up period. The basic models included the following terms: sleep measure, time (0, 1 and 2), group (active tVNS and sham tVNS), phase (early and late), sex, age and two-way interaction terms between TIME × GROUP, TIME × PHASE and GROUP × PHASE; and a three-way interaction term between TIME × GROUP × PHASE (STATA syntax used to perform this analysis can be found in the supplementary materials). In our analyses, first, contrasts were derived a priori from the respective model, investigating significant changes between adjacent time points (T0 vs T1, T1 vs T2) for each interaction of group and condition. Using contrasts has been suggested as an effective way to test a priori expectations based on statistical models such as those implemented here. Planned comparisons between specific conditions (groups) or clusters of conditions are recommended to be implemented as contrasts (see Hays, 1973; Schad et al., 2020). More specifically, in experimental designs including ours, tests such as analysis of variance (ANOVA) F-statistics offer limited information about the source of effect or interaction with regards to a factor of interest. Furthermore, in situations when ANOVA yields a significant main effect the magnitude and source of effect remains vague without proper post-hoc tests. It is common that in experiments including ours, researchers have a priori expectation about the pattern of means. We could have implemented subsequent individual *t*-tests, but given the complexity of our design and the number of study groups, this could have resulted in a loss of power and would not have considered all observations in our data set. Therefore, based on the recommendations by Schad et al. (2020), instead of performing multiple comparisons that are not recommended for complex models including linear mixed-effects models we used contrasts. In the case of significant differences for one condition between adjacent time periods per expectation in the group (i.e., when actual stimulation was applied), superiority of the effect was tested against the other active stimulation condition. Men and women were combined in the analyses as the interaction term between time (0, 1 and 2), group (active tVNS and sham tVNS), phase (early and late) and sex (male and female) suggested no significant differences in sleep scores over time between men and women (p = 0.131).

All analyses were conducted using STATA 14. Results are presented as contrasts (C), 95% confidence intervals (CI) and p-values.

3. Results

3.1. Descriptive statistics

Table 1 shows baseline characteristics of the study participants in active tVNS and sham tVNS groups. Participants did not differ across the four groups in any of the variables that may impact vagal nerve activity or sleep, such as age, sex, BMI, or depressive symptoms.

In terms of adherence to wearing the tVNS device, data from the anonymous box showed that all participants used the tVNS device for a minimum of 12 out of 14 days of intervention. There were 95% of participants in active tVNS and 92% in sham tVNS groups who reported using the tVNS device every day. The average daily mean time of using the tVNS device was 3.8 h for active tVNS groups, and 3.9 h for sham tVNS groups. (Interestingly there were 67% and 72% of participants in active and sham tVNS groups, respectively, who reported anonymously using the tVNS device daily for the full 4 h; usually in 2 to 3 sessions).

3.2. Main analysis

On average, results from the mixed models indicated a significant main effect of TIME in global sleep scores, specifically, on average PSQI scores decreased over time (p = 0.0006).

Analysis of prespecified contrasts revealed that for the active tVNS group there was a statistical difference in the estimated means between time 0 and time 1 in participants who underwent stimulation "early" (see Fig. 1, blue line) (C = −1.90; 95% CI = −2.87 to −0.94), and between time 1 and time 2 in participants who received stimulation "late" (see Fig. 1, red line) (C = −0.87; 95% CI = −1.41 to −0.32) (see Table 2). Furthermore, as shown in Fig. 1 (see blue line), the improvement in global sleep scores achieved in the early tVNS group between time 0 and time 1 reduced over the subsequent follow-up period between time 1 and time 2, but the reduction was not statistically significant (C = 0.6; 95% CI = −0.21 to 1.41) (see Table 2). Similarly, in the late active tVNS group (see Fig. 1, red line), global sleep scores did not change over the first 14 days of waiting between time 0 and time 1 (C = 0.37; 95% CI = −0.63 to 1.36) (see Table 2). For the sham tVNS (control) group, the difference in the estimated means between time 0 and time 1 in participants who underwent stimulation "early", and between time 1 and time 2 in participants who underwent sham stimulation "late" was not significant (see Table 2). In the sham early (Fig. 1, green line) group, global sleep scores did not change significantly in the follow-up period between time 1 and time 2; and participants in the sham late tVNS group (Fig. 1, yellow line) did not report significant changes in global sleep scores in the waiting period between time 0 and time 1 (see Table 2). In supplementary materials we further present results of our linear mixed model with random intercepts for the effect of

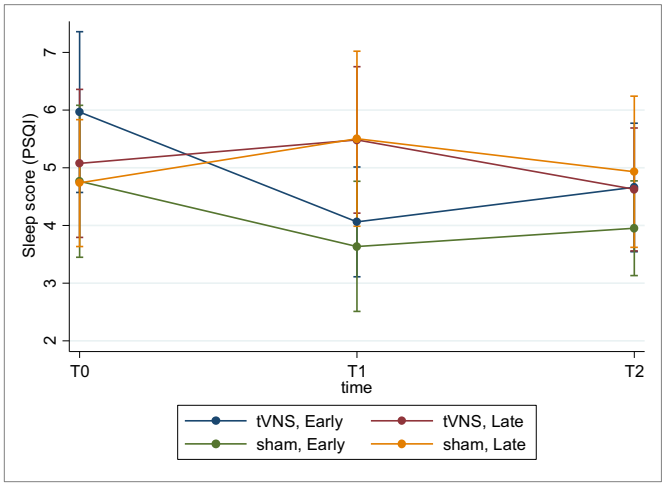

Fig. 1. Change in global sleep scores in 4 intervention groups over the course of the study.
Predicted change in global sleep scores (PSQI) with 95% confidence intervals in 68 men and women aged 18–75 years who underwent 14 days of daily active tVNS (blue and red lines) or daily sham tVNS (green and orange lines). Estimates for each timepoint for each group were predictions from mixed model including global sleep scores, time, group, phase, age, gender and the following interaction terms: time × group; time × phase; group × phase; time × group × phase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Analysis of contrasts between active tVNS and sham tVNS groups.

Time I group × phase	Contrast	Std. error	95% CI	p-Value
(1 vs 0) active tVNS early	−1.90	0.49	−2.87 to −0.94	<0.001
(1 vs 0) sham tVNS early	−1.13	0.70	−2.50 to 0.25	0.11
(2 vs 1) active tVNS early	0.6	0.41	−0.21 to 1.41	0.15
(2 vs 1) sham tVNS early	0.31	0.47	−0.61 to 1.23	0.51
(1 vs 0) active tVNS late	0.37	0.51	−0.63 to 1.36	0.47
(1 vs 0) sham tVNS late	0.77	0.59	−0.38 to 1.92	0.19
(2 vs 1) active tVNS late	−0.87	0.28	−1.41 to −0.32	0.02
(2 vs 1) sham tVNS late	−0.54	0.60	−1.72 to 0.64	0.31

time, group and phase on global sleep score in 68 participants (see Supplementary Table 1).

In the main analysis, tVNS was not superior to sham in both early ($\chi^2 = 0.83$, $p = 0.36$) and late stimulation phases ($\chi^2 = 0.24$, $p = 0.63$).

3.3. Sensitivity analyses

As illustrated in the Supplementary Table 2, analyses restricted to participants with complete global sleep scores at all 3 time points (T0, T1, T2) showed that these participants did not differ at baseline in terms of any variables except antihypertensive medication. The effects of time seen in global sleep scores in active tVNS and sham tVNS groups were replicated. Specifically, analysis of prespecified contrasts revealed that for the active tVNS group there was a statistical difference in the estimated means between time 0 and time 1 in participants who underwent stimulation "early" (C = −1.93; 95% CI = −2.90 to −0.96), and between time 1 and time 2 in participants who received stimulation "late" (C = −0.94, 95% CI = −1.58 to −0.30). In line with the main results, no significant changes in global sleep scores were found during the follow-up period (time 1 to time 2) for the early active tVNS group, or in the waiting period (time 0 to time 2) for the late active tVNS group (data not shown). For both sham groups, changes in global sleep scores were not statistically significant in any phase of the study (data not shown), in line with our analyses based on n = 68 participants.

4. Discussion

This study tested whether a two-week course of tVNS would lead to improved global sleep scores, when compared to respective control groups. We found that sleep improved significantly in participants who received active tVNS (early and late phase of the trial), while global sleep scores did not improve in participants who underwent sham stimulation (early or late phase). However, our study's hypothesis was not supported since we did not find tVNS to be superior to sham; participants in the active tVNS arms of the intervention (early and late phase) did not have their sleep improved over and above sleep ratings of participants in the control group (early and late sham).

Our findings support data from a recent study by Bretherton et al. (2019), where, like in our trial, participants who received tVNS over a 2-week period also reported improvements in self-reported sleep. Unlike in the study of Bretherton et al. (2019) that did not have any control group, we showed that the change in global sleep scores in participants who received active tVNS was not significant in comparison with placebo groups. To the best of our knowledge no other (published) study has demonstrated this effect so far in the general population, but some preliminary data exist in clinical populations including patients with insomnia disorder (Jiao et al., 2020; Luo et al., 2017) or sleep disordered breathing (Marzec et al., 2003).

Stimulation of vagal never may improve sleep via a number of plausible biological pathways. One possibility includes reducing arousal by restoring a more flexible balance of the autonomic nervous system whereby activity of the parasympathetic branch, which promotes restorative processes including sleep, is increased and activity of the sympathetic branch that is linked to arousal is decreased (Stein and Pu, 2012; Yap et al., 2020). Another possibility is that an increase in vagal nerve activity reduces circulating levels of inflammatory markers and cortisol, both of which impede sleep, via its activation of neurons in the nucleus of the solitary tract (Thayer and Sternberg, 2010). Clearly, these biological pathways need to be confirmed with studies using techniques such as functional magnetic resonance imaging (fMRI) or electroencephalography (EEG) (Yap et al., 2020).

It is notable that tVNS improved global sleep ratings, but our study cannot yet offer evidence that tVNS can improve sleep when compared with appropriate control conditions. The most obvious explanation could be that a 2-week course of tVNS is not sufficient to trigger substantial changes in sleep, especially if participants' sleep difficulties are of chronic rather than acute nature. Another explanation could be that our participants had too modest sleep difficulties (PSQI mean at baseline was 5.5, SD 2.8), and more severe sleep problems could have offered more room for improvement, and responded more strongly to stimulation. Finally, our selection of the stimulation site, namely the tragus, could have also impacted on our findings; anatomical studies show that tragus is rather not exclusively innervated by the auricular branch of the vagus nerve (Farmer et al., 2021). There is currently no consensus which site should be used for external vagus nerve stimulation (Yap et al., 2020). However, we followed current recommendations with regards to current strength (see Farmer et al., 2021; Burger et al., 2020), and used current strength matched to each participant's individual level.

Our findings need to be interpreted in light of our study limitations. Sleep was measured with a well described sleep questionnaire, but sleep ratings are affected by range of biases (Jackowska et al., 2011), and are only modestly correlated with objective assessments (Lauderdale et al., 2008). Therefore, this study should be replicated with the use of objective sleep monitoring such as actigraphy. Our participants' age ranged from 18 to 75 years, and it is well established that sleep vary by age (Ohayon et al., 2004). To address this issue all analyses were adjusted for age, and participants randomised into four groups did not differ on age (see Table 1). Sample size was determined in line with existing studies on sleep and tVNS (e.g. Bretherton et al., 2019) as well as by asking experts in the field, but we did not conduct a formal a priori power analysis. We had sleep data from n = 68 participants who provided sleep reports at two time points, and n = 57 participants rated their sleep at all three sleep assessments. Importantly, findings were broadly similar across these two groups of participants, as shown in sensitivity analyses. Albeit participants provided longitudinal sleep data spanning over at least two weeks apart (Day 0, Day 14, Day 28), it is still a relatively short period of time. While participants were not invited to take part in this study if they had cardiovascular disease, severe mental or neurological condition or reported pregnancy (see method section for full details), we did not consider sleep disorders as an exclusion criteria. Finally, adherence to the tVNS protocol was assessed via self-reports, which makes it prone to a social desirability bias. Although we took great care to ensure participants were not aware whether they were undergoing active or sham tVNS, an objective monitoring of adherence would have been a more valid tool to indicate whether the tVNS device was worn as requested. Our study has a number of strengths. We included an active sham control group in order to control for effects of participants' expectations, and our strong study design enabled us to test both the within-subject and between-subject effects. Our analyses were controlled for important confounding factors relevant to sleep and vagal nerve function, in particular, age, sex, sleep medication and depression medication, and individuals with health conditions, potentially impacting vagus nerve activity, have not been invited to participate. To model within-individual changes over the 4 weeks of participating in the study, we used mixed-effects models which are considered largely robust.

In conclusion, the benefits and application of non-invasive vagal nerve stimulation, such as tVNS, go beyond treating clinical populations, and could be scaled-up to a population level to prevent global burden of non-communicable diseases (see Gidron et al., 2018). Poor sleep habits

are a significant contribution to a number of non-communicable diseases including cardiovascular disease, diabetes or cancer. We showed that two weeks of active tVNS led to a significant improvement of global sleep ratings, while 2 weeks of sham tVNS had no effect on sleep. However, the change in sleep pattern was not significantly different when directly comparing the groups. Further well-powered studies are urgently needed to test the utility of tVNS in alleviating sleep complains in community-dwelling adults.

Declaration of competing interest

All authors declare no conflict of interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.autneu.2022.102972.

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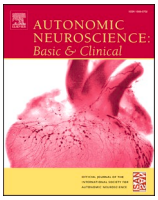

Transcutaneous vagus nerve stimulation (tVNS) in stroke: the evidence, challenges and future directions

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Rehabilitation

ABSTRACT

Stroke is one of the leading causes of death and disability globally. A significant proportion of stroke survivors are left with long term neurological deficits that have a detrimental effect on personal wellbeing and wider socioeconomic impacts. As such, there is an unmet need for novel therapies that improve neurological recovery after stroke. Invasive vagus nerve stimulation (VNS) paired with rehabilitation has been shown to improve upper limb motor function in chronic stroke. However, invasive VNS requires a surgical procedure and therefore may not be suitable for all stroke patients. Non-invasive, transcutaneous VNS (tVNS) *via* auricular vagus nerve stimulation in the ear (taVNS) and cervical vagus nerve stimulation in the neck (tcVNS) have been shown to activate similar vagal nerve projections in the central nervous system to invasive VNS. A number of pre-clinical studies indicate that tVNS delivered in acute middle cerebral artery occlusion reduces infarct size through anti-inflammatory effects, reduced excitotoxicity and increased blood-brain barrier integrity. Longer term effects of tVNS in stroke that may mediate neuroplasticity include microglial polarisation, angiogenesis and neurogenesis. Pilot clinical trials of taVNS indicate that taVNS paired with rehabilitation may improve upper limb motor and sensory function in patients with chronic stroke. In this review, we summarise and critically appraise the current pre-clinical and clinical evidence, outline the major ongoing clinical trials and detail the challenges and future directions regarding tVNS in acute and chronic stroke.

1. Introduction

Stroke remains one of the leading causes of mortality and adult-onset disability globally (Thrift et al., 2016). A significant proportion of chronic stroke survivors are left with long term disability despite physiotherapy and rehabilitation (Johnson et al., 2019). These neurological deficits include weakness, sensory impairment, loss of coordination, spasticity, dysphasia, dysphagia, visual field dysfunction and cognitive impairment (Hurford et al., 2020). The wider socioeconomic impact of stroke includes both direct costs such as healthcare expenditure and indirect costs including lost economic productivity and carer burden (Rajsic et al., June 2018). As such, there is an unmet need for neuroprotective agents in acute stroke and novel therapeutic strategies that promote neuroplasticity in chronic stroke.

Data from trials demonstrate that invasive vagus nerve stimulation (VNS) paired with rehabilitation improves upper limb impairment in people with long term arm weakness after ischaemic stroke (Dawson et al., 2016; Kimberley et al., 2018). However, invasive VNS requires a surgical procedure under general anaesthetic and carries potential procedure-related risks such as cardiac arrhythmias, peri-tracheal haematomas and vocal cord dysfunction (Ma et al., 2019). In chronic stroke, the presence of severe disability, antiplatelet/anticoagulant use and cardio-respiratory co-morbidities may reduce accessibility of this intervention. Furthermore, in acute stroke, the combination of rapid onset critical illness and a time-sensitive requirement for revascularisation through thrombolysis (Berge et al., 2021) and/or mechanical

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thrombectomy (Mokin et al., 2019) precludes the feasibility of implanting a vagus nerve stimulator acutely.

Transcutaneous VNS (tVNS), typically carried out through auricular vagus nerve stimulation (taVNS) in the ear or transcutaneous cervical branch vagus nerve stimulation in the neck (tcVNS), enables stimulation of the vagus nerve non-invasively (Yap et al., 2020). It is safe and well tolerated in research studies to date (Redgrave et al., 2018a) and has been shown to activate similar vagal nerve projections and vagus nerve mediated pathways as invasive VNS (Yakunina et al., 2018; Zhang et al., 2021). These factors make tVNS an attractive strategy to investigate the effects of VNS in people with stroke; both in terms of replicating the established effects of invasive VNS on upper limb recovery, particularly in the treatment of individuals in whom invasive VNS may be unsafe, and the effect on other neurological impairments including aphasia and lower limb weakness.

Here we will discuss the pre-clinical and clinical evidence for tVNS in acute and chronic stroke. We will then outline the ongoing clinical trials of tVNS and critically appraise the unanswered questions, challenges to translation into clinical practice and suggest directions for future research.

2. Pre-clinical evidence

The majority of pre-clinical studies of tVNS and stroke involve the application of tVNS in rodent models of acute middle cerebral artery occlusion (MCAO). This was originally performed by Ay et al. (2015) where 30 s trains of taVNS (pulse width 0.5 ms, pulse frequency 20 Hz, pulse amplitude 0.5 mA) at the left cavum concha were delivered at 5 min intervals for 1 h starting 30 min after unilateral transient middle cerebral artery occlusion in adult male Wistar rats (Ay et al., 2015). They found that taVNS in acute MCAO was associated with a 28% reduction in infarct volume and improvement in neurological outcome at 24 h (Ay et al., 2015). Interestingly, the neurological outcome did not improve at 3 h post-intervention suggesting that the therapeutic effects of taVNS in acute stroke may involve signalling cascades and adaptive changes that operate over hours rather than seconds to minutes. In this study, unilateral taVNS was associated with increased nucleus tractus solitarius (NTS) and locus coeruleus (LC) c-Fos staining bilaterally, indicating the activation of brainstem centres which are also seen in invasive cervical VNS (Cunningham et al., 2008). Whilst magnitude of reduction in infarct size was lower than that seen in invasive VNS (Ay et al., 2011), this may be partially explained by the fact that optimal stimulation parameters for taVNS have not been determined.

Further studies of tVNS in rodent models of acute MCAO have helped characterise the underlying mechanisms, temporal response and effect size of tVNS (Table 1). Taken in collaboration with the data available for invasive VNS (Engineer et al., 2019), there are several potential interdependent mechanisms through which tVNS may exert beneficial effects in acute ischaemic stroke. Given the contention regarding the efficacy of tVNS (particularly taVNS) in reliably activating the same pathways seen in invasive VNS (Engineer et al., 2019; Burger and Verkuil, 2018), here we detail neurobiological effects of tVNS in animal models of stroke. These include reduced systemic inflammation (Jiang et al., 2016), increased M2 microglial polarisation (Zhao et al., 2019), reduced spreading depolarisation (Lindemann et al., 2020), reduced blood-brain barrier breakdown (Yang et al., 2018), increased angiogenesis (Li et al., 2020a), and improved axon regeneration and reorganisation (Li et al., 2020b) (Fig. 1).

2.1. Mechanisms

2.1.1. Anti-inflammatory effects

The α7 nicotinic acetylcholine receptor (α7nAChR) is a neurotransmitter gated ion channel which is expressed widely in the brain and on immune cells including macrophages (Wang et al., 2003, Lukas et al., 1999). The cholinergic anti-inflammatory pathway refers to a mechanism by which the vagal efferent fibres, *via* enteric neurons, activate α7nAChR on peripheral macrophages which consequently leads to a reduction in the systemic release of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and increased release of pro-angiogenic factors (Bonaz et al., 2016). Further, the afferent vagus nerve transmission can increase cholinergic activity in the basal forebrain which could increase α7nAChR activation centrally (Kalkman and Feuerbach, 2016).

There is an array of evidence indicating protective benefits of VNS and α7nAChR activation in stroke. Firstly, pharmacological activation of the α7nAChR has been associated with reductions in infarct size, oxidative stress and pro-inflammatory macrophages in a mouse model of ischaemic stroke (Han et al., 2014). Secondly, α7nAChR agonists have been shown to reduce cerebral oedema in a mouse model of intracerebral haemorrhage (Krafft et al., 2012). Thirdly, invasive VNS has been shown to reduce infarct volume and improve neurological outcomes in a rat model of MCAO which are prevented with α7nAChR blockade (Lu et al., 2017). In keeping with this, Li et al. (2020b) found that taVNS reversed the MCAO-induced reduction in α7nAChR expression in the peri-infarct cortex after 14 days and that taVNS-related neuroprotective effects were abolished by α7nAChR antagonism (Li et al., 2020b).

Microglia are the resident macrophages in the central nervous system and carry out a range of functions including synaptic organisation, phagocytosis of apoptotic cells and regulating neuronal excitability (Sasaki, 2017). Ischaemia is a potent trigger for activation of microglia which, in a simplified scheme, can operate in a 'pro-inflammatory' (M1) or 'anti-inflammatory' (M2) phenotype (Zhang, 2019). Activated M1 microglia can propagate the inflammatory cascade causing secondary cell death in acute stroke whereas M2 microglia can downregulate the pro-inflammatory mileu leading to reductions in secondary cell death and increased brain repair (Zhang, 2019). Agonism of the α7nAChR has been associated with increased M2 polarisation suggesting a potential mechanism through which tVNS may promote anti-inflammatory effects (Zhang et al., 2017). In keeping with this, Ay et al. (2016) showed that tcVNS was associated with reduced microglial activation and a reduction in cells containing TNF-α (expressed by M1 microglia) cells by 3 h post MCAO (Ay et al., 2016). Zhang et al. (2020) further demonstrated that tcVNS in acute MCAO was associated with M2 polarisation, improved neurological outcomes and reduced infarct size (Zhao et al., 2019). These benefits were associated with reduced levels of the pro-inflammatory IL-17A and abolished by the administration of recombinant IL-17A. Similarly, taVNS has also been associated with increases in brain-derived neurotrophic factor (BDNF) (Jiang et al., 2016) and peroxisome proliferator- activated receptor gamma (PPAR-γ) (Li et al., 2020a) which are both associated with the M2 microglial phenotype (Jiang et al., 2020).

2.1.2. Blood-brain barrier integrity

Disruption of the blood-brain barrier (BBB) is a hallmark of ischaemic stroke and is associated with secondary brain damage and heightened neurological dysfunction (Zhang et al., 2020). This occurs through several mechanisms including the activation of matrix-metalloproteinases (MMP) by pro-inflammatory cytokines such as TNF-α (Zhang et al., 2020). Yang *et al* (2018) demonstrated that tcVNS reduced MMP-2 and MMP-9 expression in reactive astrocytes around injured vessels in ischaemic hemisphere and reduced BBB leakage on dynamic contrast enhanced MRI at 24 h (Yang et al., 2018). IL-17A has also been demonstrated to increased BBB breakdown (Huppert et al., 2010) therefore, the aforementioned inhibition of IL-17A production *via* tcVNS (Zhao et al., 2019) may be an alternative mechanism through which tVNS reduces infarct size.

2.1.3. Excitotoxicity

Glutamate-mediated excitotoxicity is a sequelae of acute stroke and leads to secondary, post-ischaemic neuronal injury (Belov Kirdajova et al., 2020). The effect of VNS on excitotoxicity in stroke is not well

Table 1
Animal studies of transcutaneous VNS (tVNS) in stroke.

Author (year)	Animal	tVNS stimulation parameters							Main findings
		Site	Timing	Pulse width	Frequency	Intensity	Interval	Duration	
Ay et al. (2015)	Adult male Wistar rats	Left cavum concha	30 min post MCAO	0.5 ms	20 Hz	0.5 mA	30 s trains every 5 min	1 h	No change in regional cerebral blood flow Significant reduction in infarct volume Significant reduction in Bederson scale at 24 h (but not 3 h) Bilateral c-Fos staining in the NTS and LC
Jiang et al. (2016)	Adult male SD rats	Left cavum concha	30 min post right MCAO	0.5 ms	20 Hz	0.5 mA	30 s trains every 5 min	1 h, twice daily for 21 days	Improved neurological deficit scores, beam-walking test and staircase test (*i.e.* sensory and motor function improved) at 7 days and 21 days. Reduced infarct volume at 24 h Less brain pathology and more surviving neurons on microscopy Increased angiogenesis in the peri-infarct region at 21 days Increased BDNF, P-eNOS and VEGF expression in the penumbra at 21 days
Ma et al. (2016)	Adult male SD rats	Left cavum concha	30 min post MCAO	0.5 ms	20 Hz	0.5 mA	30 s trains every 5 min	1 h, twice daily for up to 7 days	Transient decrease in blood pressure and heart rate during stimulation (not sustained). No overall effect on regional cerebral blood flow. taVNS associated with higher mNSS score at 24 h and improvement in adhesive removal test at 3 days post-MCAO. Reduced infarct volume Increased plasma GDF11 protein and GDF11 positive cells in the peri-infarct cerebral cortex (peak at 3 days). Increased ECs in peri-infarct cortex and increased ALK5 expression in ECs in peri-infarct cortex
Ay et al. (2016)	Adult male spontaneously hypertensive rats	Right cervical vagus nerve	30 min post right MCAO or 4 h post right MCAO	1 ms	25 Hz	12 V 350 Ω	2 min trains every 10 min	1 h	Reduced infarct volume, higher neurological scores and forelimb grip strength at Day 7 when treated at 30 min post MCAO. Smaller infarct volume and improved neurological score if treated 4 h post MCAO. Increased c-Fos positive cells in NTS. More HMGB1-positive cells at 24 h. Fewer Iba-1-positive, TNF-α and CD68-positive cells at 24 h.
Yang et al. (2018)	Adult male spontaneously hypertensive rats	Left cervical vagus nerve	30 min post right MCAO	1 ms	25 Hz	15 V	2 min trains every 10 min	50 mins	Reduced infarct size on MRI Reduced BBB leakage in infarcted area at 24 h on DCE-MRI. Protected TJP ZO-1 in endothelium Reduced expression of MMP-2 and MMP-9 in reactive astrocytes around injured vessels in ischaemic hemisphere
Zhao et al. (2019)	Male C57BL/6 mice	Right cervical vagus nerve	1 day pre right MCAO	1 ms	25 Hz	15 V	2 min trains every 10 min	1 h	tcVNS decreased infarct volume, improved neurological outcomes, reduced apoptotic neurons, promoted M2 microglial polarisation and attenuated the rise in IL-17A protein expression after MCAO. Recombinant IL-17A nullified the tcVNS induced microglial M2 polarisation and abolished the neuroprotective effect of taVNS.
Li et al. (2020a)	Adult male SD rats	Left cavum concha	30 min post right MCAO	0.5 ms	20 Hz	0.5 mA	30 s trains every 5 min	1 h, twice daily up to 28 days	taVNS increases PPAR-γ expression in the peri-infarct cortex at day 14 and 28.

(*continued on next page*)

Table 1 (*continued*)

Author (year)	Animal	tVNS stimulation parameters							Main findings
		Site	Timing	Pulse width	Frequency	Intensity	Interval	Duration	
									Inhibition of PPAR-γ *via* siRNA reduces the improvement in neurological scores from taVNS and abrogates the neuroprotective effects of taVNS on neuronal damage and infarct volume.
Li et al. (2020b)	Adult male SD rats	Left cavum concha	30 min post right MCAO	0.5 ms	20 Hz	0.5 mA	30 s trains every 5 min	1 h, twice daily up to 28 days	taVNS reversed the reduction in α7nAChR mRNA and protein expression in the peri-infarct cortex at 14 days and was associated with increased levels at 28 days. taVNS prevented neurological impairment (mNSS and adhesive removal test) with continuous improving trends from day 14–28. taVNS enhanced axon regeneration and re-organisation. Attenuation of taVNS-related improvements in neurological scores, ta-VNS related increases in the BDNF-cAMP-PKA-p-CREB pathway and axonal plasticity after the administration of an α7nAChR blocker
Lindemann et al. (2020)	Adult male Wistar rats	Left cervical vagus nerve	30 min post transient/ permanent left MCAO	1 ms	25 Hz	12 V	2 min train repeated once after 15 min	15 min	tcVNS reduces spreading depolarisation frequency in permanent MCAO tcVNS reduces cortical infarct volume but not subcortical infarct volume in transient MCAO No difference in Garcia neurological score or Grid Walk performance between tcVNS and sham VNS at 3 days.

Key
α7nAChR – Alpha-7 Nicotinic Acetylcholine Receptor
BBB – Blood Brain Barrier
BDNF – Brain-derived Neurotrophic Factor
cAMP – Cyclic Adenosine Monophosphate
|DCE – MRI – Dynamic Contrast Enhanced Magnetic Resonance Imaging
EC – Endothelial Cell
LC – Locus Coeruleus
MCAO – Middle Cerebral Artery Occlusion
mNSS – Modified Neurological Severity Score
NTS – Nucleus Tractus Solitarius
PPAR-γ – Peroxisome Proliferator-Activated Receptor Gamma
PKA – Protein Kinase A
SD – Sprague-Dawley
siRNA – small inhibitory RNA
taVNS – Transcutaneous Auricular Vagus Nerve Stimulation
tcVNS – Transcutaneous Cervical Vagus Nerve Stimulation
TNF-α – Tumour necrosis factor alpha
tVNS – Transcutaneous Vagus Nerve Stimulation

characterised. Invasive VNS has been shown to reduce ischaemia-induced glutamate release and increase hippocampal neuronal survival in a gerbil model of ischaemia (Miyamoto et al., 2003). Spreading depolarisations (SDs) occurs spontaneously after a stroke and are associated with dysregulated blood flow and altered metabolism that leads to infarct propagation (Taş et al., 2019). Elevated glutamate is a proposed trigger for SDs (Taş et al., 2019). Lindemann et al. (2020) found that invasive cervical VNS and tcVNS reduced cortical SD frequency in the peri-infarct region (Lindemann et al., 2020). Interestingly, they found that tcVNS reduced cortical infarct volume but not subcortical infarct volume suggesting that the mechanisms of VNS preferentially protect cortical neurons. It is important to note that, in this study, only a short duration of tcVNS was employed; it would be interesting to determine if there is a dose-dependent relationship between tVNS and reduced SD frequency.

2.1.4. Angiogenesis, neurogenesis and neuroplasticity

Given the association of tVNS with reduced infarct size in acute stroke, it would be tempting to assume that tVNS increases collateral blood flow and improves perfusion in the ischaemic penumbra. However, studies of tVNS have consistently shown no acute change in regional cerebral blood flow (Ay et al., 2015; Ay et al., 2016). In contrast with this, longer durations of tVNS have been associated with increases in PPAR-γ (Li et al., 2020a), BDNF (Li et al., 2020b) and growth differentiation factor 11 (GDF-11) (Ma et al., 2016) – promoters of angiogenesis and neurogenesis.

PPAR-γ is a multifunctional nuclear transcription factor, expressed in neurons, endothelial cells and microglia, which has numerous

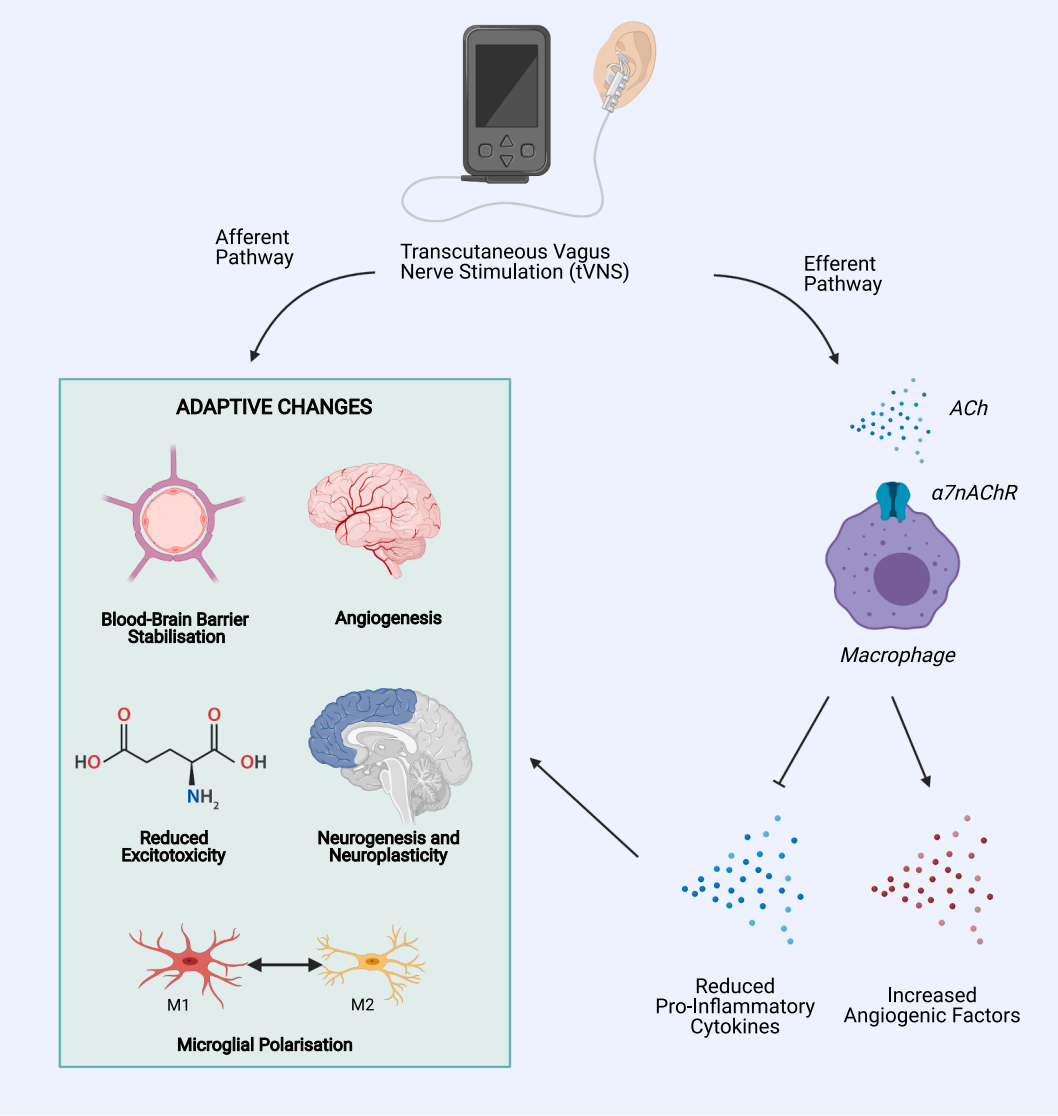

Fig. 1. Effects of tVNS in animal models of stroke.

neuroprotective and anti-inflammatory actions that make it a promising potential target in cerebral ischaemia (Culman et al., 2007). Li et al (2020) demonstrated that twice daily taVNS led to increased PPAR-γ expression in the peri-infarct cerebral cortex 14 and 28 days post-MCAO and was associated with higher levels of BDNF, phosphorylated endothelial nitric oxide synthase (eNOS) and vascular endothelial growth factor (VEGF), higher microvessel density and proliferating endothelial cells in the peri-infarct region, reduced infarct size and improved neurological outcomes (Li et al., 2020a). Crucially, inhibition of PPAR-γ prevented these improvements indicating that PPAR-γ mediates the pro-angiogenic effects of taVNS. This is in keeping with findings of studies of invasive VNS (Jiang et al., 2015).

In addition to neuroprotective effects in the acute phase of stroke, invasive VNS paired with rehabilitative therapies has been shown to improve neurological deficits in the subacute and chronic phases of stroke (Khodaparast et al., 2013). Whilst there are no animal studies of tVNS paired with rehabilitation, several studies of tVNS in acute stroke in rodent models employ protocols where tVNS is administered for up to 28 days allowing us to delineate some of the neural mechanisms through which tVNS may promote neuroplasticity (Table 1). For example, as discussed above, taVNS has been shown to upregulate BDNF from M2 microglia, at least partly *via* the α7nAChR (Li et al., 2020a). BDNF is a growth factor that has been shown to promote neurogenesis and regulate and maintain synaptic plasticity (Liu et al., 2020). In keeping with this, sustained taVNS (up to 28 days following acute MCAO) has been shown to improve axon regeneration and re-organisation (Li et al., 2020b). Similarly, taVNS increases GDF11 expression in the peri-infarct cortex (Ma et al., 2016). GDF11 is a member of the transforming growth factor beta superfamily; delayed treatment with recombinant GDF11 7 days after ischaemic stroke has been shown to increase markers of neurogenesis, angiogenesis and improve neurological outcome (Lu et al., 2018). Therefore, it is feasible that taVNS may stimulate neurogenesis *via* a GDF11-dependent mechanism.

3. Clinical evidence

In contrast to the pre-clinical studies of VNS in stroke, all the currently published clinical research on the use of invasive and transcutaneous VNS relates to chronic stroke (Table 2). The potential for invasive VNS in chronic stroke was first demonstrated by Dawson et al. (2016) who randomised 21 patients with ischaemic stroke more than six months prior and residual upper limb impairment to either VNS paired with rehabilitation (three 2 h sessions per week for 6 weeks) or rehabilitation alone. They found invasive VNS was safe and, in the per-protocol analysis, that the participants treated with VNS had a significantly greater improvement in UFM scores. A subsequent study by the

Table 2
Clinical studies of tVNS in stroke.

Authors	Type of study	Population	N	Stimulation parameters						Main Findings
				Site	Pulse width	Frequency	Intensity	Interval	Duration	
(Capone et al., 2017)	RCT	Ischaemic or haemorrhagic stroke at least one year prior	14	Left auricular vagus nerve	0.3 ms	20 Hz	Mean 2.8–7.2 mA	30 s trains every 5 min	60 min daily for 10 days	taVNS immediately prior to robotic rehabilitation is safe and tolerable taVNS associated with higher percentage improvement in UFM than sham
(Redgrave et al., 2018b)	Pilot study	Anterior circulation ischaemic stroke at least 3 months prior	12	Left auricular vagus nerve	0.1 ms	25 Hz	Median (range) 1.4 (1–3.2 mA)	During RTP	60 min session 3 sessions per week for 6 weeks	taVNS paired with concurrent RTP is safe, tolerable and associated with a significant increase in UFM scores One patient noticed some light-headedness which was possibly related to taVNS.
(Baig et al., 2019)*	Pilot study	Anterior circulation ischaemic stroke at least 3 months prior	12	Left auricular vagus nerve	0.1 ms	25 Hz	Median (range) 1.4 (1–3.2 mA)	During RTP	60 min session 3 sessions per week for 6 weeks	taVNS paired with concurrent RTP is associated with improvement in sensory function.
(Wu et al., 2020)	RCT	Ischaemic stroke between 0.5 and 3 months prior	21	Left auricular vagus nerve	0.3 ms	20 Hz	Mean 1.66 mA	30 s trains every 5 min	30 mins daily for 15 days	taVNS prior to upper limb rehabilitation was associated with greater improvements in UFM and WMFT than sham taVNS. This is sustained at 12 weeks. One patient developed skin redness which subsided.
(Subrahmanyamc and Suresh, 2020)	RCT	Previous stroke and post-stroke urinary incontinence	30	Left auricular vagus nerve	0.25 ms	25 Hz	Not specified	Continuous	60 mins daily for 60 days	taVNS alongside Kegel exercises associated with increased Barthel Index but higher Overactive Bladder Symptom Score at 60 days

Key
RCT – Randomised Controlled Trial
RTP – Repetitive Task Practice
UFM – Upper Limb Fugl – Meyer score
WMFT – Wolf Motor Function Test
* post-hoc analysis of Redgrave et al. (2018b).

same group found that similar in-clinic therapy followed by self-delivered VNS paired with rehabilitation at home was feasible and effective (Kimberley et al., 2018; Dawson et al., 2020). In a recent pivotal, randomised, blinded, sham-controlled trial, done in 19 stroke rehabilitation services in the UK and the USA, VNS paired with rehabilitation was found to be superior to sham stimulation paired with rehabilitation (Dawson et al., 2021). 108 people were included and randomised. The primary outcome was the change in Fugl-Meyer upper extremity assessment score (UFM) between baseline and the first day after completion of 6 weeks in-clinic therapy. The mean UFM score increased by 5·0 points (SD 4·4) in the VNS group and by 2·4 points (3·8) in the control group ($p = 0{\cdot}0014$). At 90 days after in-clinic therapy, a clinically meaningful response on the UFM score was achieved in 23 (47%) of 53 patients in the VNS group *versus* 13 (24%) of 55 patients in the control group ($p = 0{\cdot}0098$) (Dawson et al., 2021). These studies provide a clinical correlate to the animal studies of invasive VNS which indicated that iVNS paired with motor rehabilitation can drive task-specific plasticity (Hays et al., 2013). This is of immense clinical importance given the fact that these studies were carried out in stroke patients who had passed the timeframe in which spontaneous improvement typically occurs (Cramer, 2008).

Given the fact that invasive VNS requires surgical implantation of a device, many stroke patients with co-morbidities and chronic disability may be unwilling or unsuitable for this as a rehabilitative therapy. It is therefore imperative to determine whether tVNS paired with rehabilitation can also promote neuroplasticity and improve clinical outcomes in chronic stroke patients. Accordingly, to date, there have been five studies of tVNS in chronic stroke (Table 2). These have all used taVNS rather than tcVNS.

Capone et al. (2017) found that taVNS delivered for 60 min prior to robotic rehabilitation for 10 days in patients with chronic ischaemic or haemorrhagic stroke was safe and feasible. They reported a higher percentage improvement in the UFM scores in the active taVNS group compared to sham. However, an important caveat to this study is that baseline UFM scores were lower in the taVNS group therefore each incremental increase in raw UFM score would be associated with a higher percentage increase in UFM in the taVNS group compared to sham. One of the reasons that may underlie the modest benefit seen in this study is that taVNS was delivered *prior* to rehabilitative physiotherapy. Given that it has been established that the synchronous pairing of VNS with specific motor tasks is what drives task-specific neuroplasticity (Hays et al., 2013), the timing of the taVNS in this study was therefore potentially less likely to be associated with clinical improvements. Furthermore, a shorter duration of taVNS was performed in comparison to the studies of invasive VNS in which the interventions spanned at least 6 weeks (Dawson et al., 2016).

The effects of taVNS paired with concurrent upper limb rehabilitation were first demonstrated by Redgrave et al. (2018b). In that study, patients with ischaemic stroke more than 3 months prior (median time 1.16 years post-stroke) underwent three 60 min sessions per week for 6 weeks. They carried out motor rehabilitation through repetitive task practice and were administered pulses of taVNS at the start of each movement. This was found to be safe, tolerable and associated with a mean increase of 17. 1 point in the overall UFM score. A subsequent post-hoc analysis demonstrated that, even in the absence of specific sensory rehabilitation, taVNS was associated with improvements in sensory components of the UFM score as well as motor scores (). This is in keeping with a case report from Kilgard et al. which showed that invasive VNS can improve sensory rehabilitation (Kilgard et al., 2018).

As we discuss below, the optimal timing of taVNS to promote neuroplasticity after stroke is unknown. Wu et al. (2020) found that taVNS delivered prior to rehabilitation in subacute ischaemic stroke (15 days–3 months) for 15 days was associated with greater increases in UFM scores than sham treatment (Wu et al., 2020). Caveats to this study include that spontaneous or rehabilitation-driven improvements in neurological outcome can be expected in this relatively early phase after stroke, and, that the blinding to treatment group is not possible due to the fact that taVNS stimulation is perceptible by participants. However, it is important that studies are carried out in this intermediate phase after stroke to determine whether tVNS can potentiate rehabilitation-driven neuroplasticity and improve clinical outcomes.

4. Upcoming clinical studies

There are several active clinical trials which are investigating the use of tVNS in acute and chronic stroke (Table 3). The results of these will develop our understanding of the efficacy and optimal parameters of tVNS.

4.1. Acute stroke

There is a significant mismatch between the abundance of promising

Table 3
Registered clinical trials of transcutaneous vagus nerve stimulation (tVNS) in stroke.

Study name/ registration number	Location	Type of study	Population	N	tVNS parameters	Key outcome measures	Estimated completion date
Acute stroke							
NOVIS trial NCT04050501 (van der Meij et al., 2020)	Netherlands	RCT	Acute anterior circulation ischaemic stroke (< 12 h from onset)	150	tcVNS (gammaCore Sapphire™) 25 Hz, 0–24 Volts, 120 s, every 15 mins for 3 h then 8 hourly until Day 5 or discharge.	MRI Infarct Volume on Day 5 NIHSS on Day 5 or day of discharge Proportion of patients with <50% penumbra turned into ischaemic core Degree of BBB leakage on CT perfusion on Day 3 mRS at 90 days	January 2022
TR-VENUS NCT03733431	Turkey	RCT	Acute ischaemic or haemorrhagic stroke (within 6 h of onset or no ischaemia on FLAIR imaging)	60	tcVNS *via* gammaCore™ 7 × 2 min trains every 10 mins for 1 h +/− repeated cycle after 3 h	Safety Feasibility NIHSS at 24 h Change in MRI infarct volume at 24 h	February 2021
Sub-acute or chronic stroke							
NCT03592745	USA	RCT	First unilateral ischaemic supratentorial stroke >6 months prior with UFM 12–44.	35	Left taVNS Delivered alongside robotic arm therapy for 60 mins, 3 times a week for 3 weeks.	Mean change in EMG activation of biceps and triceps at 3 weeks Median change in UFM at 3 weeks	March 2021
NCT02878720	Italy	RCT	Ischaemic or haemorrhagic stroke >1 year prior with hand function impairment	30	Left taVNS 20 Hz, 0–8 mA and 0.3 ms pulse width for 30 s every 5 mins for 60 mins; repeated daily for 10 days alongside robotic rehabilitation.	UFM post-intervention, 1 month and 3 months.	December 2022
NCT03292159	USA	RCT	Supratentorial ischaemic or haemorrhagic stroke 4–30 days prior and upper limb NIHSS score 1 or 2	30	Left, respiratory-gated taVNS alongside motor arm training. 10 × 30 min sessions over 2 weeks.	UFM post-intervention and at 3 months.	January 2020 (suspended)
NCT04088578	USA	RCT	First ever ischaemic or haemorrhagic stroke at least 6 months prior	30	taVNS Unspecified parameters 3 testing and 8 training session	Time on target score using a force transducer linked to a computer monitor	December 2025
NCT04088565	USA	RCT	First ever ischaemic or haemorrhagic stroke at least 6 months prior	30	taVNS with paired-associative stimulation Unspecified parameters/duration	Evoked potentials at 1 week	December 2025
NCT04129242	USA	RCT	First ever ischaemic stroke at least 6 months prior and UFM ≤ 58	25	Closed-loop taVNS with motor rehabilitation Unspecified parameters 3 sessions a week for 4 weeks	UFM post-intervention, 2 weeks and 8 weeks	October 2021

Key
BBB – Blood-Brain Barrier
RCT – Randomised Controlled Trial
taVNS – Transcutaneous auricular vagus nerve stimulation
tcVNS – Transcutaneous cervical vagus nerve stimulation
UFM – Upper Limb Fugl-Meyer Score

pre-clinical studies and the absence of any published human studies of tVNS in acute stroke. Added to this, given the impracticality of delivering acute invasive VNS in hyperacute stroke, the registered studies of acute tVNS are of great importance. The NOVIS trial (NCT04050501) in the Netherlands is the largest of these studies (van der Meij et al., 2020). One hundred and fifty patients with anterior circulation ischaemic stroke will be randomised to tcVNS delivered *via* Gammacore™ for up to 5 days or best medical therapy. The investigators will assess the degree of infarct growth in the ischaemic penumbra at 3 days *via* CT perfusion, final infarct volume on MRI at 5 days and neurological outcome at 5 days. This highly anticipated study will determine whether tcVNS delivered in acute stroke can confer neuroprotective benefits as has been shown in animal models. Additionally, the use of CT perfusion scans will enable the investigators to determine the degree of BBB leakage and confirm whether any protective effect of tcVNS is partially mediated by BBB integrity as suggested by the animal studies (Yang et al., 2018). The TR-VENUS study (NCT03733431) in Turkey will similarly use tcVNS in acute stroke using different protocol of stimulation parameters and will also investigate the effects of tcVNS in acute intracerebral haemorrhage.

4.2. Subacute and chronic stroke

There a number of ongoing smaller randomised controlled trials of tVNS in subacute and chronic stroke (Table 3). These will recruit between 25 and 35 participants and all utilise tVNS to assess the effects on markers of upper limb function. These will be of value to see if the findings in previous clinical studies are replicated in disparate populations. However, to our knowledge, there are no definitive large multicentre trials of tVNS in chronic stroke currently registered. Moreover, whilst taVNS is being investigated in chronic stroke, we are not aware of any registered studies investigating the use of tcVNS in chronic stroke. An appropriately powered study is imperative to determine whether tVNS paired with rehabilitation promotes plasticity and improves clinical outcomes. Furthermore, there are a number of other unanswered questions related to tVNS and stroke that need to be addressed through well designed clinical trials which we will discuss below.

5. Barriers and future directions

Larger, multi-centre clinical trials will determine whether tVNS is a cost-effective ancillary therapy in acute and chronic stroke. However, as we discuss below, there are still a number of challenges and barriers to the implementation of tVNS in stroke that need to be addressed through well designed pre-clinical and clinical research studies.

5.1. Optimizing animal models

The pre-clinical studies of tVNS in stroke have established protective effects and elucidated some of the underlying neurobiological mechanisms. Moreover, the studies outlined above meet several criteria from the Stroke Therapy Academic Industry Roundtable (STAIR) Preclinical Recommendations for acute stroke therapies (Fisher et al., 2009) with regards to identifying a likely therapeutic window, incorporating physiological monitoring during MCAO occlusion, including histological and behavioural endpoints, showing reproducibility of effect in different laboratories and analysing serum biomarkers that can be tested in human studies. However, there are several limitations of the animal models of tVNS that are important to discuss in order for the pre-clinical evidence base to fulfil the STAIR criteria. First, the majority of studies use a model of transient MCAO with ischaemia then subsequent reperfusion; whilst an increasing number of stroke patients are able to access urgent revascularisation *via* intravenous thrombolysis and mechanical thrombectomy, a significant proportion of patients do not attend hospital fast enough to receive these (Faiz et al., 2013). It is not known to what extent VNS mitigates against reperfusion injury, therefore it is important that there are models of permanent vascular occlusion that recapitulate a common stroke phenotype in clinical practice. Second, all the animal models are of proximal middle cerebral artery occlusion therefore the effects of tVNS in small vessel occlusion and posterior circulation infarction are not known. Third, as discussed by Ay et al. (2016), the MCAO model risks injury to the vagus nerve which may blunt the effect of tVNS. Fourth, there is a paucity of models of tVNS in chronic stroke and rehabilitation; as such it has not been demonstrated whether tVNS paired with rehabilitative therapies increases cortical plasticity and functional recovery in chronic stroke as has been shown for invasive VNS (Hays et al., 2013). Fifth, all the animal studies are performed in males; given there is evidence of sexual dimorphism in the mechanism of stroke-induced cell death (Manwani and McCullough, 2011) there is a responsibility to evaluate the mechanisms of tVNS in female animal models in order to develop a reliable evidence base for best medical practice for all. Sixth, as can be seen in Table 1, only some of the pre-clinical studies are performed in animal populations that model the vascular and neural phenotype of stroke patients. It has been demonstrated that pre-clinical studies of acute stroke treatments in predominantly young healthy male animals have low external validity given the clinical stroke population is that of predominantly older adults with medical comorbidities (Schmidt-Pogoda et al., 2020). There is therefore a necessity to perform studies in animals with co-morbidities that are frequently seen in stroke populations such as aged mice, spontaneously hypertensive rats, animals withwith experimentally-induced diabetes or concomitant use of common medications.

5.2. Timing

In acute stroke, the majority of pre-clinical studies investigate the use of tVNS delivered around 30 mins after the induction of ischaemia (Table 1). Ay et al. (2016) demonstrated that tVNS delivered 4 h after ischaemia was still associated with a reduction in infarct size but that this protective effect was not present when delivered 5 h post-infarct (Ay et al., 2016). Given the presence of pre-hospital delays in stroke patients accessing urgent stroke services (Faiz et al., 2013), it is important to characterise the response to tVNS initiated at various time points post-stroke in animal models. Furthermore, in the larger studies of acute tVNS in stroke such as the NOVIS study (van der Meij et al., 2020), it will be of interest for the investigators to present data on the relationship between timing of tVNS initiation and subsequent clinical and radiological outcomes. The results from this could help rationalise resources and ensure appropriate patient selection for tVNS in clinical practice. If it is found that early initiation of tVNS is safe and efficacious, then it would be important to consider pre-hospital trials of tVNS in acute stroke as have been done with remote ischaemic conditioning (Blauenfeldt et al., 2019).

The published clinical studies of tVNS in stroke focus on promoting neuroplasticity by delivering tVNS alongside rehabilitation. The evidence from studies of VNS and stroke rehabilitation indicates that this is optimised through “pairing” a specific task to VNS in order to promote task-specific plasticity (Dawson et al., 2020). The majority of clinical studies (Table 2) and registered clinical trials (Table 3) are trialling tVNS in patients who had a stroke more than 6 months prior to radnomisation. Whilst this minimises the confounding factor of spontaneous recovery of neurological function from the clinical trials, there is a potential concern that there may be a more optimal window of recovery that is being missed. A larger randomised study of tVNS in the first few weeks after stroke alongside intensive physiotherapy would help determine whether earlier delivery of tVNS is associated with improved clinical outcomes.

5.3. Stimulation parameters and treatment duration

There are multiple parameters of tVNS that vary between studies including site, laterality, respiratory-gating, pulse frequency, pulse width, amplitude, train duration, inter-train interval (on-off cycle) and duration of treatment.

5.3.1. Auricular vs cervical stimulation

There have been no head to head comparisons of taVNS and tcVNS in either animal models or clinical research of tVNS in stroke. Whilst Ay et al. (2016) found that the magnitude of reduction in infarct size was higher in their study of tcVNS compared to their study of taVNS (Ay et al., 2015), it is important to note that different animal models were used (spontaneously hypertensive rats and Wistar rats, respectively). It is also possible that the stimulation parameters required to optimise afferent vagus nerve activation in the ear and the neck may vary therefore studies using a range of different stimulation parameters are required before a direct comparison of taVNS and tcVNS can be made. The anatomy and cutaneous nerve supply of the ear is complex; the cymba concha and inner tragus appear to be optimal sites for stimulation for auricular vagus nerve activation (Butt et al., 2020) therefore it is important that future studies specify the site and distribution of stimulating electrodes used in protocols of taVNS.

5.3.2. Laterality

The standard convention, as seen in the majority of studies detailed above, use left sided tVNS as right sided vagal nerve efferents innervate the sinoatrial node and could potentially cause bradycardia. Whilst Ay et al. (2015) demonstrated that left sided tVNS led to c-Fos activation in the NTS and LC bilaterally (Ay et al., 2015), it has previously been demonstrated that left sided taVNS increased gamma-aminobutyric acid A ($GABA_A$) activity in the right but not left motor cortex (Capone et al., 2015). When reviewing the pre-clinical studies of tVNS, it is clear that the majority of studies investigate left sided tVNS in models of right MCAO (Table 1). If the protective effects of tVNS are lateralised to the contralateral cortex, then the animal models are not readily applicable to a stroke population. It would be of interest for a post-hoc analysis of published clinical studies to demonstrate whether the lateralisation of stroke affected response to tVNS. The development of carefully monitored studies of right sided or bilateral tVNS may be necessary to translate this into clinical practice.

5.3.3. Respiratory-gating

One of the principal synaptic targets of vagus nerve afferent fibres is the NTS (Sclocco et al., 2019). It is known that the NTS receives facilitatory influence during exhalation thereby raising the possibility that tVNS delivered during exhalation rather than inhalation would be associated with a greater effect (Sclocco et al., 2019). Sclocco et al. (2019) demonstrated that taVNS delivered during exhalation rather than inhalation was associated with greater activation of the ipsilateral NTS on 7T MRI (Sclocco et al., 2019). Clinical studies of respiratory-gated tVNS such as NCT03292159 will help determine whether tVNS can be optimised through the utilisation of this physiological principle. If shown to be effective, automated devices could be developed that stimulate at appropriate times in the respiratory cycle to maximise the effect of tVNS.

5.3.4. Pulse width, frequency and amplitude

The optimal stimulation parameters for tVNS in acute or chronic stroke are not known. Hulsey et al. (2017) investigated the effect of varying invasive VNS current amplitude, pulse width, pulse frequency, train durations on activation of neurons in the LC (Hulsey et al., 2017). They found that a broad range of each of these parameters was associated with LC activation, however, higher current amplitude and longer pulse widths increase LC neuron firing whilst pulse frequency affects the timing but not total phasic LC activity. However, due to the multiplicity of excitatory and inhibitory projections of the brainstem nuclei activated by VNS, the cortical and clinical benefits of VNS in stroke may not be linearly correlated to the magnitude of VNS activation. Accordingly, there is some evidence from invasive VNS studies that moderate amplitudes of 0.8 mA were associated with better recovery of forelimb function than smaller (0.4 mA) or larger (1.6 mA) amplitudes in a rat model of ischaemic stroke (Pruitt et al., 2021). The presence of this inverted-U-shaped relationship between amplitude has not been studied for tVNS in models of stroke. In fact, in clinical studies of tVNS, the amplitude of stimulation is often determined by the patient at the maximally tolerated level (Redgrave et al., 2018b); if the optimal amplitude to promote cortical plasticity is below this level, then tVNS could potentially be most effectively delivered at intensities that are more tolerable. Similarly, whilst higher pulse frequencies have been associated with greater activation of brainstem nuclei in invasive (Hulsey et al., 2017) and tVNS (Sclocco et al., 2020), an inverted U-shaped relationship between pulse frequency and cortical plasticity has also been reported (Buell et al., 2018). It is imperative that future studies of tVNS systemically vary the stimulation parameters and determine the optimal range to influence functional outcome rather than simply focusing on achieving the highest level of vagus nerve activation alone.

5.3.5. Treatment duration

It is unclear whether there is a ceiling effect from prolonged courses of VNS in stroke. In a study of invasive VNS, individuals who carried out VNS paired with rehabilitation at home sustained improvements in upper limb function at 12 months (Dawson et al., 2020). As further clinical studies of tVNS in stroke take place we will develop longitudinal data on whether neurological recovery is long-lasting and whether continued improvements can be made after years of VNS with rehabilitation.

5.4. Biomarkers

The ascertainment of biomarkers for vagus nerve activation and response in tVNS are essential for several reasons. First, it could identify responders *versus* non-responders and help with allocation of scarce resources. Second, it may enable optimisation of tVNS parameters and treatment duration and, ultimately, individualisation of tVNS parameters at a patient-level. Third, it can confirm whether tVNS, particularly taVNS, is activating the same neural pathways as invasive VNS. Fourthly, it may help delineate the underlying mechanism of tVNS in acute and chronic stroke and aid in the development of drug targets for neuroprotection and neuroplasticity.

Biomarkers of tVNS can be conceptualised into a non-binary framework of biomarkers associated with the degree of vagus nerve activation or biomarkers associated with improved clinical outcome. These may take the form of physiological, blood-borne, neurophysiological or radiological signifiers of a response to tVNS. Four major biomarkers have been studied in healthy volunteers: heart rate variability, pupillary response, salivary alpha-amylase and P300 event related potentials (Burger et al., 2020). There is limited evidence that any of these are associated with the degree or effectiveness of tVNS (Burger et al., 2020). It is also important to consider that biomarkers of tVNS in healthy volunteers may not necessarily be transferable to a clinical population with neurological dysfunction.

It seems appropriate that biomarkers of tVNS should build upon the pre-clinical evidence base for tVNS in stroke. Potential avenues for discovery of biomarkers may include blood tests of pro- and anti-inflammatory cytokines associated with the cholinergic anti-inflammatory pathway, high-field functional MRI imaging of brainstem nuclei, PET-MRI of microglial activation or imaging of post-ischaemic angiogenesis.

5.5. Optimal patient selection

In the absence of established biomarkers, further research is needed to determine factors which reduce responsiveness to tVNS. These may include patient-related factors (*e.g.* age, sex, comorbidity and medications) or stroke-related factors (*e.g.* stroke location, stroke mechanism). Large, multi-centre studies with a diverse casemix of patients and stroke-subtypes will help determine whether certain groups are less likely to benefit from tVNS. For instance, in a recent rat model of ischaemic

stroke, acute tVNS improved cortical but not subcortical stroke volume (Lindemann et al., 2020); the upcoming clinical trials of acute tVNS will inform whether this is replicated in humans. If so, it is possible that there may be a lower effect size in subcortical strokes *e.g.* lacunar syndromes. Given the prevalence of diabetes in stroke sufferers (Chen et al., 2016), clinical trials should document the presence or absence of symptoms of vagal neuropathy (*e.g.* gastroparesis) in the baseline data collection to help establish whether diabetic autonomic neuropathy precludes favourable outcomes from tVNS. It remains to be seen whether use of drugs affecting central noradrenergic activity *e.g.* beta blockers and tricyclic antidepressants influence the response to tVNS. Similarly, nicotine is an agonist for the α7nAChR (de Jonge and Ulloa, 2007) therefore clinical trials should aim to report smoking status and the use of nicotine replacement therapy to determine the impact of nicotine as a confounder to the downstream signalling pathways of tVNS.

5.6. Looking beyond motor rehabilitation

The focus of tVNS in stroke has been largely isolated to two domains: improving neurological outcomes in hyperacute stroke and improving upper limb motor function in chronic stroke. It has already been demonstrated that tVNS is associated with improvements in sensory function in chronic stroke () and that invasive VNS paired with tactile training improved sensory dysfunction in a stroke survivor (Kilgard et al., 2018). Given the fact that sensory dysfunction is common and a barrier to rehabilitation after stroke (Bolognini et al., 2016), tVNS paired with focused sensory training should be a priority in the coming years.

It will be of interest to evaluate whether tVNS can improve other cortical-based neurological deficits after stroke including dysphasia, dysphagia, cognitive impairment and visual field dysfunction. Furthermore, with evidence for tVNS use in epilepsy (Barbella et al., 2018), depression (Kong et al., 2018) and migraine (Straube et al., 2015), it is tempting to hypothesise that tVNS could be applied to post -stroke epilepsy, post-stroke depression and post-stroke pain. Finally, given the role of microglial activation in the development of neuro-cardiogenic injury post-stroke (particularly those involving the insular cortex) (Sposato et al., 2020), the effect of tVNS on cardiac structure, electrophysiology and contractile function after stroke is a key future area of interest.

5.7. Practical considerations

In acute stroke, there are several time sensitive processes that need to be coordinated among several practitioners including clinical assessment, urgent CT scanning, decision for thrombolysis or mechanical thrombectomy and blood pressure control. As such, it may be challenging to introduce an additional therapy such as tVNS in an efficient and safe manner. The GammaCore™ device is a handheld device that would require a healthcare practitioner to manually deliver pulses at set intervals whilst the NEMOS® device uses a secured ear electrode which could be adapted to an automated cycle to deliver tVNS at regular interval. The upcoming clinical studies of tVNS in acute stroke will inform whether tVNS delivery is associated with delays in other aspects of acute medical care in stroke.

For chronic stroke, one of the challenges of tVNS is that the studies performed thus far have been in highly monitored environments with tVNS delivered by researchers and often paired with rehabilitative exercises. Whilst there is data on patient-delivered invasive VNS in the home environment (Dawson et al., 2020), this has not yet been trialled for tVNS. In chronic stroke patients where there may be significant upper limb dysfunction, it may be difficult for an individual to deliver a pulse of tVNS to coincide with each movement in a rehabilitation program. The next stage for tVNS in stroke rehabilitation should ideally include an exploration of how tVNS could be upscaled for home-based rehabilitation. This may include training family members or carers of stroke patients in using tVNS or the development of movement-activated tVNS therapies to automatically pair repetitive task practice with tVNS.

5.8. Nomenclature

A recent systematic review identified that there were 97 different combinations of full and abbreviated names given to transcutaneous vagus nerve stimulation in the published literature (Wang et al., 2020). Moving forward, the standardisation of nomenclature will aid researchers in identifying relevant studies and developing the evidence base for tVNS in stroke.

6. Conclusion

Transcutaneous vagus nerve stimulation (tVNS) has been shown to improve neurological outcomes in pre-clinical models of stroke and in early clinical studies of stroke rehabilitation. We are rapidly moving toward an exciting phase where tVNS could be used in stroke patients to fulfil an unmet need for novel therapies that provide clinically meaningful benefits. However, there are still unanswered questions about how best to utilise tVNS and its underlying mechanisms that should be addressed through continued, well-designed pre-clinical and clinical research.

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Fig. 1 Created with BioRender.com

Declaration of competing interest

None.

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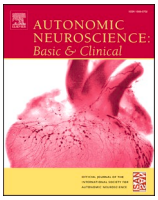

Transcutaneous vagus nerve stimulation (tVNS) as a potential therapeutic application for neurodegenerative disorders – A focus on dysautonomia in Parkinson's disease

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ABSTRACT

The understandings of pathogenic processes in major neurodegenerative diseases has significantly advanced in recent years, with evidence showing pathological spread of intraneuronal proteinaceous inclusions as a fundamental factor. In Parkinson's disease (PD), the culprit protein has been identified as α-synuclein as the main component for mediating progressive neurodegeneration. With severe pathology evident in the autonomic nervous system prior to clinical manifestations of PD, pathogenic spread can occur from the peripheral nervous system through key nuclei, such as the anterior olfactory nucleus and dorsal motor nucleus of the glossopharyngeal and vagal nerves, gradually reaching the brainstem, midbrain and cerebral cortex. With this understanding and the proposed involvement of the vagus nerve in disease progression in PD, notably occurring prior to characterized clinical motor features, it raises intriguing questions as to whether vagal nerve pathology can be accurately detected, and importantly used as a reliable marker for determining early neurodegeneration. Along with this is the potential use of vagus nerve neuromodulation for treatment of early disease symptoms like dysautonomia, for modulating sympatho-vagal imbalances and easing severe comorbidities of the disease. In this article, we take a closer look at the pathogenic transmission processes in neurodegenerative disorders that impact the vagus nerve, and how vagus nerve neuromodulation can be potentially applied as a therapeutic approach for major neurodegenerative disorders.

1. Introduction

The rapid and continued up-rise of patients suffering from neurological disorders is a prominent global health challenge that impacts the socio-economic status (DiLuca and Olesen, 2014; Olesen and Leonardi, 2003) with brain disorders currently representing approximately 1/3 of all costs of diseases in the world (Olesen and Leonardi, 2003). In Europe, disorders of mood and dementia are the greatest cause of economic burden within brain diseases, from accounted figures for direct/other related health expenses and patient production losses (Olesen et al., 2012). From a global perspective, the cost of care for dementia, most commonly caused by neurodegenerative disorder Alzheimer's disease (AD), reached over $800 billion USD in 2015 and is estimated to exceed $2 trillion USD by 2030 (Wimo et al., 2017). With increased life expectancy of the global population, incidences of brain diseases are expected to surge, making these imminent health and economic challenges (DiLuca and Olesen, 2014).

Although disease-modifying agents for debilitating neurodegenerative disorders are not yet available to cure patients, important steps have been made in recent years for uncovering the underlying pathological processes towards reaching this goal (Braak et al., 2006a; Gelpi et al., 2014; Recasens et al., 2014; Kim et al., 2019). With greater understanding of disease pathogenesis at this current time, advanced rehabilitation and treatment approaches can be adopted for effectively managing different clinical stages over the disease course. The complexity of having a diverse range of clinical symptoms that present in a progressive manner is likely to require a combinative approach with easy-to-apply treatments that can help achieve mosaic symptomatic management. In this article, we provide an overview of the current understanding of the underlying processes of neurodegeneration, with focus on Parkinson's Disease (PD) pathology, and specific involvement of the vagus nerve and the autonomic nervous system. We then discuss the clinically used non-invasive neuromodulation therapy, transcutaneous Vagus Nerve Stimulation (tVNS) for treatment of sympatho-vagal imbalance and its future use for neurodegenerative disorders, highlighting the clinical opportunities in

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this era of neuromodulation therapy.

2. Neurodegeneration

2.1. An insight into Parkinson's disease

Since the turn of the millennium, the incidence of PD has been projected to double by 2030 (Dorsey et al., 2007). In Europe, the costs for PD at the start of the last decade was close to €14 billion, with over half attributed to direct medical costs (Olesen et al., 2012). Currently, PD is the 2nd most common neurodegenerative disorder, affecting 1% of the population over the age of 55 years and has highest prevalence in ages of over 85 years (de Rijk et al., 1997). It is commonly characterized by a clinical syndrome of motor symptoms (bradykinesia, postural deficits and resting tremor) (Marsden, 1994) that occurs due to extensive loss of nigrostriatal dopaminergic neurons (Ehringer and Hornykiewicz, 1960) and subsequent depletion of neurotransmitter dopamine, leading to the dysregulation of the basal ganglia motor circuit (Obeso et al., 2008). It remains important to recognize that in the years prior to the manifestation of these characteristic motor symptoms, which occurs only after approximately 70% of dopaminergic neurons have degenerated (Ehringer and Hornykiewicz, 1960), the majority of patients (i.e. up to two-thirds) commonly suffer from non-motor symptoms, in a so-called 'pre-motor' stage of PD. This phase includes presentation of core symptoms such as olfactory dysfunction (anosmia), sleep abnormalities (REM sleep Behaviour Disorder, RBD), dysphagia, cardiac sympathetic denervation, cognitive impairment, anxiety and depression, pain, and gastrointestinal irregularities (constipation, reduced gastric motility), which are seen before any other neurological detriment (Edwards et al., 1992; Knudsen et al., 2018). These non-motor symptoms arise from progressive neuronal loss of other important neurotransmitter systems, including noradrenergic, serotonergic and cholinergic neurons (Jellinger, 1991), and can precede clinical parkinsonism by 10-15 years (Pfeiffer, 2003; Hardoff et al., 2001). This has been supported by clinical reports showing the presence of PD biomarkers, such as α-synuclein staining in bowel biopsy samples many years prior to presentation of PD motor signs (Shannon et al., 2012).

2.2. Prion-like pathological spreading

As with other well-known α-synucleinopathies, such as Dementia with Lewy Bodies (DLB) and Multiple System Atrophy (MSA), PD is characterized by widespread clusters of aberrant forms of α-synuclein (Dodel et al., 2008). In PD, these intraneuronal proteinaceous inclusions, known as Lewy Bodies (LB) are mainly composed of α-synuclein, a 14 kDa endogenous protein of 140 amino acid length, and can be found in perikaryal of neurons within the central and peripheral nervous systems. It is now established that the main 'culprit' linked to progressive neuronal degeneration in PD is α-synuclein, where the pathological structure is a misfolded conformation of oligomeric or fibrillary nature, of phosphorylated form (phosphor-Ser129) (Fujiwara et al., 2002; Spillantini et al., 1997).

The neurodegenerative spread in a prionoid-like manner describes the transfer of α-synuclein-positive intracytoplasmic inclusions after initial seeding of pathogenic α-synuclein. In PD, pathological neuronal transmission was first indicated from post-mortem analysis showing host-to-graft transmission, where LB formations were found in embryonic mesencephalic neuron grafts in the striatum decades following brain implantation (Kordower et al., 2008). Since then, pathogenic α-synuclein is found in monomeric, oligomeric and phosphor-Ser129 forms in PD patient cerebrospinal fluid and plasma, supporting a process of transmission across the nervous system in disease sufferers (Borghi et al., 2000; El-Agnaf et al., 2003; Foulds et al., 2012; Mollenhauer et al., 2011; Tokuda et al., 2010). With its release into the extracellular space, pathogenic α-synuclein is then taken up by healthy neurons and through subsequent self-propagation, α-synuclein acts as a permissive template for misfolding of endogenous α-synuclein proteins (Karpowicz et al., 2019). This propagates the spread of neurodegeneration across interconnected brain regions mediating widespread disruption of intracellular processes i.e. neurotransmission, mitochondrial activity, mitophagy, vesicular transport and protein degradation. Indeed, experimental models have demonstrated that the injection of α-synuclein preformed fibrils (PFFs) into striatum of mice causes accumulation of phosphor-Ser129 α-synuclein and dopaminergic cell death in the substantia nigra pars compacta (SNc), demonstrating retrograde transmission of pathogenic species in the brain (Luk et al., 2012; Mao et al., 2016). Since the original descriptions of a progressive spread of neurodegeneration in PD, prionoid-like mechanisms have now been proposed in other major neurodegenerative diseases including AD, Huntington's Disease and Amyotrophic Lateral Sclerosis (Aguzzi and Rajendran, 2009; Brundin et al., 2010; Polymenidou and Cleveland, 2011).

2.3. Neurodegenerative pathogenesis – Braak staging

The stereotypic progression of PD in distinct stages was first put forward by Braak et al. (Braak et al., 2003a, 2006b) following their assessments of the regional distribution of α-synuclein immunoreactive structures in detailed post-mortem analyses. A 'bottom-up' approach was initially suggested, starting in the peripheral autonomic nervous system where topographic distribution of LBs were identified in the gut (Braak et al., 2003a; Braak et al., 2004). It was also later suggested that the 'unknown pathogen' enters the human body through the nasal cavity and is swallowed, reaching the gastrointestinal (GI) tract. This was used to explain intrusion into the epithelial lining and anterior olfactory nucleus, before pathological spreading to the dorsal motor nucleus of the glossopharyngeal and vagal nerves. From the vagus nerve, the proposed non-random anterograde progression reaches the midbrain and cerebral cortex (Braak et al., 2003a; Braak et al., 2006b). Indeed, autopsy of PD patients commonly demonstrate LBs and Lewy Neurites (LNs) in the enteric nervous system (ENS) (Beach et al., 2010), vagal motor neurons and in the colon and submandibular gland (Braak et al., 2006b; Wakabayashi et al., 1990).

The initial access of LB pathology to the CNS is via trans-synaptic spread (Braak et al., 2003b) through the lower brainstem in the dorsal motor nucleus of the vagus nerve (DMV) and relates to the earliest stages in clinical PD (Braak et al., 2004). The progressive pathogenic spread is suggested to move rostrally to brain regions including the medulla, pontine, tegmentum, midbrain and basal forebrain, before reaching the cerebral cortex (Braak et al., 2003a) (Fig. 1), with severity of lesions corresponding to clinical manifestations (Braak et al., 2005). It has been shown in rodent experimental studies, the injection of specific viral vector (rAAV) expressing human wild-type α-synuclein into the left vagus nerve results in the expression of human α-synuclein in the medulla, before a caudo-rostral spread through brain regions (Ulusoy et al., 2013). Moreover, an injection of pathological α-synuclein extract into the gut of rodents, from PD brain lysate samples or recombinant human α-synuclein fibrils, are found transported through the vagus nerve in a time-dependent manner (12, 48, 72 h after injection), reaching the DMV after 6 days (Holmqvist et al., 2014).

Although Braak proposed 6 pathological stages (3 premotor stages with α-synuclein present in the ENS and lower brainstem, followed by 3 later stages with pathology affecting motor and cognitive areas), some experimental studies have reported discrepancies with this staging classification. For example, occurrence of a 'top-down' brain-to-gut transmission has been since proposed (Beach et al., 2010; O'Donovan et al., 2020). The similar descriptions, however, of an underlying non-random progressive development via vulnerable neuroanatomical regions that are likely mediated through thin, poorly myelinated axons are now conceived, greatly helping an understanding of the pathogenesis of certain neurodegenerative diseases (Braak et al., 2003b).

For age-related neurodegenerative disorders such as AD and PD,

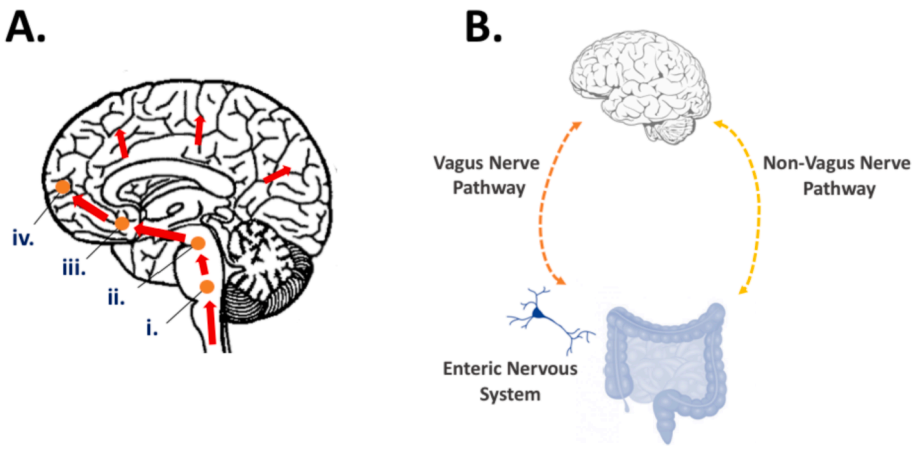

Fig. 1. The spread of Lewy Body pathology according to the Braak staging model. A. An illustration of Parkinson's disease progression in a stereotypic temporal pattern from trans-synaptic retrograde spread, starting from the peripheral enteric nervous system to the brainstem in the dorsal motor nucleus of the vagus nerve to areas including i. the medulla oblongata ii. pontine tegmentum iii. hypothalamus, thalamus, basal mid- and forebrain iv. mesocortex, allocortex. B. An illustration of the proposed bidirectional pathogenic transport of α-synuclein from gut to brain by the enteric nervous system to the vagus nerve, and/or non-vagus nerve pathways, such as bloodstream and lymphatic tissue.

notable pathology is observed with loss of neurons in the locus coeruleus of the brainstem, being one of the first areas affected. In early stages of AD, the locus coeruleus shows primary hallmarks being one of the first brainstem structures expressing neurofibrillary tangles (NFTs), which are the aggregates of microtubule-associated protein Tau, and can be found decades prior to any other established pathology (Braak et al., 2011; Braak et al., 2012). Interestingly for PD, α-synuclein is also identified in the locus coeruleus in early disease stages, which can be 10 years before clinical diagnosis (Baloyannis et al., 2006; Hawkes et al., 2010) and is a brain region severely affected over the total disease time-course. Although a complete temporal relationship from (A) potential compromise of the GI tract, which increases in likelihood with aging (Attems et al., 2015; Bowman et al., 2018) and is a high-risk factor for idiopathic PD (Reeve et al., 2014), to (B) pathogenic entry and spread through the vagus nerve and (C) full clinical manifestation of PD, is not yet fully characterized, it is suggested that it may occur over a period of 10-20 years (Savica et al., 2010). At this time, however, from a more detailed understanding of some fundamental mechanisms in neurodegenerative disease progression, there are new opportunities now for developing strategies directed at alleviating symptoms at different disease stages.

2.4. *Gut-brain connection – the vagus nerve*

Using the Braak model for a framework in understanding neurodegeneration pathogenesis, the DMV represents the major neuroanatomical site where the pathogenic agent could enter the central nervous system. As the ENS provides bi-directional communication between the brain and the gut, mainly mediated through the vagus nerve, a compromised functionality of gut microbiome e.g. dysbiosis, has been suggested to increase the risk of developing certain neurological disorders such as anxiety, depression, autism, AD and PD (Hsiao et al., 2013; Pellegrini et al., 2018). In PD, it is proposed that the unmyelinated preganglionic neurons that innervate the ENS, from DMV viscero-motor projections (Braak et al., 2003b) may be the route for retrograde transport of the pathogen into the brain, and are actually found to be among the earliest cells with LB pathology (Braak et al., 2003b).

The vagal nuclei that includes the nucleus ambiguous (NA), nucleus tractus solitarius (NTS) and DMV, are located in the caudal brainstem and give rise to vago-vagal neurocircuits (Travagli and Anselmi, 2016). The DMV and NA mainly project efferent vagal motor fibres that modulate target organs in normal physiology. For the NTS, it receives a range of vagal afferents and integrates brainstem, limbic and hypothalamic sensory information, coordinating autonomic and visceral functions i.e. for gastric mobility. In PD, neurodegeneration affecting vagal neurocircuitry is seen with PD patients and may attribute to the delay of gastric emptying that can occur at all stages of the disease, with such symptoms appearing in up to 90% of PD patients (Pfeiffer, 2003; Hardoff et al., 2001). While pathological studies have identified LB in the GI tract from early to late stages of PD (Shannon et al., 2012; Braak et al., 2006b; Lebouvier et al., 2010), patients also show vagus nerve dysfunction (Knudsen et al., 2018; Fedorova et al., 2017). Interestingly, in large cohort studies conducted in Europe, full truncal vagotomy – that is the complete resection of the vagus nerve clinically used for in gastric disease treatment (Lagoo et al., 2014) - is associated with a reduced risk of developing PD compared to age-matched controls (Svensson et al., 2015; Tysnes et al., 2015). The involvement of the vagus nerve in PD pathogenesis has also been supported from elegant experimental studies in rodents (Kim et al., 2019). The data showed that PD pathology and motor behaviour deficits induced by injection of α-synuclein fibrils into the muscularis layer of pylorus and duodenum of the gut in mice, are blocked following vagotomy and prevent a temporal stereotypic pattern of gut-to-brain propagation of the pathological phosphor-Ser129 α-synuclein (Kim et al., 2019).

3. Dysautonomia – non-motor features

In PD, dysfunction of the autonomic nervous system accounts for the non-motor features seen years before the onset of motor symptoms (Braak et al., 2003a; Hawkes et al., 2010; Jellinger, 2008). In a healthy state, the autonomic nervous system controls vital physiology for maintaining homeostasis, connecting to each major organ and providing regulation. Following LB pathology that affects peripheral ganglia of the autonomic nervous system (Hawkes et al., 2010), there is subsequent dysfunction which causes a distinct range of autonomic symptoms. Indeed, autonomic regulatory disorders are a prominent feature in PD, preceding motor symptoms and can dominate the clinical picture over the disease course (Ziemssen and Reichmann, 2010), being a major detriment in quality of life (QoL) (Sauerbier and Ray Chaudhuri, 2014; Martinez-Martin et al., 2011). It is also worth highlighting that dysautonomia can be triggered and/or exacerbated by drug treatments for motor symptoms through effects on the autonomic nervous system. With overlapping features of autonomic failure, for example between PD and MSA, and the potential drug treatment induced side-effects, it remains difficult to separate PD from non-PD disorders in vivo (Hughes et al., 1992) only based on autonomic features, requiring more detailed clinical evaluations for accurate disease diagnosis.

In PD, LB pathology can be found in the peripheral cardiovascular autonomic network, such as the superior sympathetic ganglia, which innervates the heart (Ziemssen and Reichmann, 2010; Jain et al., 2012). Studies have indicated cardiac sympathetic denervation in early stages of PD through 123-I-meta-iodobenzylguanidine (MIBG) scintigraphy (Treglia et al., 2012), with most deficiencies seen in the left ventricular myocardium (Goldstein et al., 2003). PD patients commonly show the

development of orthostatic hypotension (Senard et al., 1997) and this occurs in 4-60% of patients (Micieli et al., 1987). Orthostasis can also occur in PD patients and can be experienced as dizziness, following a drop-in blood pressure on standing (Low, 2008). Moreover, PD patients may experience orthostatic hypotension within a few minutes of orthostasis (Jamnadas-Khoda et al., 2009) indicating features of autonomic failure. As well as the associated motor disability in PD patients, such non-motor symptoms can further increase the risk of falling and injury (Hiorth et al., 2013).

In observational studies, PD patients show dysfunction of the diurnal autonomic cardiovascular regulation (Haapaniemi et al., 2001), particularly during sleep where drug naïve patients have shown defective cardiac autonomic control (Ferini-Strambi et al., 1992; Kallio et al., 2004). The Heart Rate Variability (HRV) parameter analyses used to evaluate autonomic system activity has been studied in PD patients in comparison to control subjects, demonstrating differences in specific parameters. For example, the Low Frequency (LF)/High Frequency (HF) ratio (an index for sympatho-parasympathetic balance) is lower in early stage untreated PD (Asahina et al., 2014) indicating a predominant parasympathetic drive to the heart, while there are no significant changes found in LF (0.004–0.15 Hz, sympathetic modulation of the sinoatrial node) or HF (0.15-0.4 Hz, parasympathethic vagal modulation). When comparing PD and MSA, patients show differences in the vagal tone, with changes associated with the natural circadian rhythm. More specifically, PD patients show a reduced vagal tone predominantly with sleep, while MSA patients show a depression of the sympathetic tone during daily activities (Iodice et al., 2011). Although previous studies have eluded that HRV may not be used as a definitive indicator of premotor PD (Jain et al., 2012), the advanced characterization of the pathophysiological basis of cardiovascular autonomic dysfunction across disease stages aids a basis for using target therapies and improving management strategies in patient care. A previous study analysed the time-domain analysis of HRV in PD patient groups across progressive stages by continuous ECG recordings (24-h spectral analysis). These groups included patients of a. early stage, minor impairment without L-DOPA drug treatment, b. mild impairment with L-DOPA, and c. advanced PD with motor fluctuations following L-DOPA treatment. It was found that in later stages of PD (groups b and c) there was decreased diurnal LF and LF/HF power compared to controls, and nocturnal vagal indicators (HF and pNN50) were reduced in the most advanced disease state (Devos et al., 2003).

It is important to note that the majority of clinical treatments to date for PD are for the alleviation of motor symptoms after clinical manifestation. In clinical neurology, detecting autonomic dysfunction in PD, which notably can occur years to decades prior to characteristic motor symptoms, may be fundamental for early detection of neurodegeneration. With potential disease modifying therapies on the horizon, it remains of vital importance for early stage detection given the best clinical outcomes would be expected with introduction at the earliest possible stages for slowing or changing the disease course. Although it is unlikely this could be resolved with a single screening tool, the regular assessments of cardiovascular autonomic dysfunction in PD could be advantageous for rapid and efficient screening, where recurrent use can also support the monitoring of disease progression.

4. Transcutaneous vagus nerve stimulation (tVNS) for neurodegenerative diseases

With complex multi-stage development in neurodegenerative diseases, it is conceivable that a combinative approach could best achieve effective symptomatic management and improve clinical prognosis. For PD, current treatment therapies remain largely unchanged with focus on motor symptom treatment using dopamine replacement drug therapies. Although these are highly effective for motor symptom relief during the initial years of treatment, the majority of PD patients inevitably experience drug-induced complications, such as motor fluctuations (Fahn, 1974; Duvoisin, 1974), 'wearing off' of therapeutic efficacy (Shoulson et al., 1975; Marsden and Parkes, 1976) and L-DOPA-induced dyskinesia (Cotzias et al., 1969). More recent suggestions of combining several different potential therapies as a future clinical approach has been put forward (Paolone, 2020). This includes i. treatment with neurotrophic factors like glial cell line-derived neurotrophic factor, ii. deliverance of immunotherapeutic interventions such as monoclonal antibodies against pathogenic α-synuclein (Baecher-Allan et al., 2018), iii. an intestinal gel pad for stable and continued release of L-DOPA, and iv. rehabilitation methods for drug-refractory complications, such as core posture control (Gandolfi et al., 2019; Demartini et al., 2020). The inclusion of therapies for reducing autonomic dysfunction could also provide a range of additional benefits, such as improved abnormal gut absorption rates for reaching greater effectiveness of drug treatments. A reduction of autonomic dysfunctions throughout the course of the disease, especially for treating sympatho-vagal dysfunction, is likely to substantially add to an overall clinical improvement. Given the involvement of the vagus nerve in the pathogenesis of PD, the effects of vagus nerve stimulation (VNS) on central nervous system neural circuitry, like the noradrenergic system, could potentially provide direct clinical benefits, as well as potential indirect benefits, for example in promoting neuroprotective mechanisms (Janitzky, 2020; Farrand et al., 2020).

4.1. Neuromodulatory effects of vagus nerve stimulation (VNS)

VNS is an established clinical neuromodulation therapy for treatment of neurological disorders including epilepsy and depression (Ben-Menachem et al., 2015; Toffa et al., 2020; Aaronson et al., 2017; Johnson and Wilson, 2018). VNS can be achieved through non-invasive or invasive applications, with the latter requiring neurosurgery under general anesthesia. In this procedure, a spiral electrode is secured to the left cervical vagus nerve in the neck that is then connected via a subcutaneous cable to a pulse generator implanted into the chest cavity (Beekwilder and Beems, 2010). Alternatively, non-invasive VNS can be achieved through stimulation of the auricular branch of the vagus nerve (ABVN) which is applied through the skin, and is more commonly known as transcutaneous vagus nerve stimulation (tVNS) (Verma et al., 2021). fMRI imaging studies have shown that tVNS activates key nuclei such as the NTS of the vagal nuclei complex, bilateral spinal trigeminal nucleus, dorsal raphe, locus coeruleus, parabrachialis nucleus, amygdala and nucleus accumbens (Fig. 2) (Frangos et al., 2015). These are notably similar brain areas activated following invasive VNS which occurs along the central vagal projections (Frangos et al., 2015;

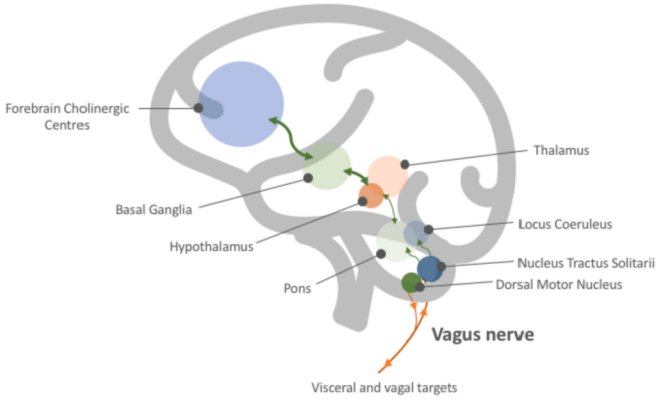

Fig. 2. A schematic diagram showing the effects of Vagus Nerve Stimulation (VNS). VNS activates ascending neural pathways leading to modulation of neural activity in the brainstem, midbrain and cortex, as shown from previous brain imaging studies. Activated regions include the dorsal motor nucleus of the vagus nerve, nucleus tractus solitarii, thalamus, hypothalamus, basal ganglia and forebrain regions.

Narayanan et al., 2002).

The application of tVNS is most commonly used for drug-resistant neurological disorders including epilepsy, migraine and depression/anxiety (JYY et al., 2020). With a favorable risk-benefit profile from its non-surgical application and ease-of-use, clinical effectiveness is typically seen over several weeks to months, with high-level patience compliance being a major factor for producing a clinical benefit (Bauer et al., 2016). Studies in epileptic patients have found the mean reduction frequency of seizures to be between 34 and 64% in comparison to baseline (He et al., 2013; Stefan et al., 2012; Rong et al., 2014). Reports of long-term seizure free states have also been reported (von Wrede et al., 2019), and reductions of seizures is further supported with improvements in neurophysiological brain activities seen from EEG recordings (Rong et al., 2014).

4.2. Treating neuropsychiatric comorbidities in neurodegenerative diseases

With an overall clinical view, the total burden of non-motor symptoms has been previously suggested to have greater impact on QoL than motor symptoms in early and advanced stages of PD (Martinez-Martin et al., 2011; Zis et al., 2014). A previous study characterizing non-motor symptoms in over 100 drug-naïve PD patients (PRIAMO study), found that anxiety and depression occurred at the highest prevalence (66%) (Barone et al., 2009). Depression also tends to be seen prior to motor symptoms in PD, with this being the case for 91% of drug-naïve PD patients (Santamaria et al., 1987).

It is well documented that invasive VNS improves drug-resistant depression and has been approved in the USA for clinical use since 2005. A recent open-label 5-year observational study of 795 patients with major depression demonstrated that adjunct VNS provides significantly improved clinical outcomes in comparison to 'treatment-as-usual' patients, with a higher cumulative response rate (67.7% cf. 40.9%) and significantly higher remission rates (43.3% cf. 25.7%) (Aaronson et al., 2017). The application of tVNS in recent studies also shows efficacy in depression with improved symptoms of anxiety, psychomotor disability, sleep disturbance and hopelessness (Kong et al., 2018; Rong et al., 2016; Hein et al., 2013). Interestingly, short term use of tVNS over a 2-week period in MDD patients, demonstrates sustained clinical improvements in depression with >50% reduction in clinical ratings of Hamilton 17-item (HAMD) scores (Trevizol et al., 2016).

The specific changes in neural circuitry for achieving anti-depressant effects from clinical neuromodulation therapies remains to be fully elucidated (Choi et al., 2018; Riva-Posse et al., 2018). With VNS therapy, the anti-depressant effects in patients are likely attributed from afferent vagal fibres projecting to the NTS, resulting in modulated activity of key brain structures including the amygdala and insula, and other key limbic structures in mood regulation (Fig. 3) (Dandekar et al., 2018). Early studies using BOLD-fMRI by Kraus et al., (2007) showed that tVNS reduces activity in limbic and temporal brain structures, including the amygdala, hippocampus, parahippocampal gyrus, and middle and superior temporal gyrus, while elevating the activity in other brain regions including the insula, precentral gyrus and thalamus (Kraus et al., 2007). These changes may be the main mechanisms to the mood enhancing effect caused by tVNS in depressed patients. A recent fMRI brain imaging study also demonstrated that the responsiveness of the left anterior insula following the first tVNS treatment may be a potential biomarker for predicting clinical improvements. Indeed, major depression patients that exhibited this marker reached a 42% decrease in HAMD scores following continued tVNS therapy (Fang et al., 2017). The mood enhancing effects of tVNS treatment has also been demonstrated in different age groups, with beneficial effects recently indicated in adolescents (Koenig et al., 2021) and also in aging (>55 years of age) (Bretherton et al., 2019). In the latter age group, the authors proposed the responsiveness in subjects to tVNS therapy may be related to individuals with a higher baseline of LF/HF ratio, indicating a shift from sympathetic to parasympathetic prevalence (Bretherton et al., 2019).

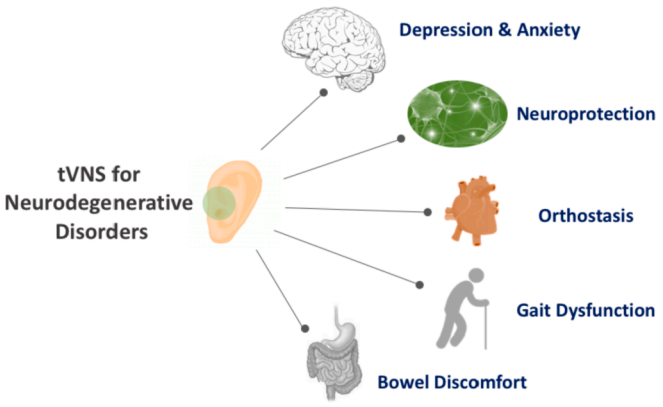

Fig. 3. The potential use of transcutaneous Vagus Nerve Stimulation (tVNS) for troublesome dysautonomia in neurodegenerative diseases such as Parkinson's Disease. tVNS activates the Auricular Branch of the Vagus Nerve (ABVN) in the ear, which may have potential therapeutic effects including neuropsychiatric symptoms, neuroprotection, orthostasis and orthostatic hypotension, gait dysfunction and bowel discomfort.

4.3. Treating gastrointestinal disturbances

As mentioned above, a large majority of PD patients suffer from GI disturbances throughout disease progression (Tanaka et al., 2011; Ozawa et al., 2011). So far, the application of non-invasive VNS (nVNS) has been conducted in a small pilot trial of PD patients for treatment of GI dysfunction. nVNS is utilized by application of a hand-held device placed at the left neck region (below the mandibular angle, medial to the sternocleidomastoid muscle and lateral to the larynx) for delivering a series of short electrical pulses through the skin. In a randomized pilot trial in PD patients (n = 19) receiving nVNS four times per day over a four week period, neurostimulation elicited modest improvements in GI symptoms (Kaut et al., 2019). Further trials are now required with a larger number of patients that would help elucidate the potential benefit of VNS for GI disturbances in PD (Fig. 3).

4.4. Specific motor symptoms in PD

A previous clinical case report showed that invasive VNS can provide significant alleviation of PD motor symptoms (Bokkala-Pinninti et al., 2008). In a 64-year old female PD patient who presented colocalized symptoms of complex partial epilepsy and parkinsonism, the titration of VNS resolved slow resting tremor and improved bradykinesia, with a significant reduction in Unified Parkinson's Disease Rating Scale (UPDRS) scores. This also coincided with effective alleviation of epileptic seizures. Authors of this report also noted that PD symptoms were resolved before epilepsy, with improvements sustained even at 6 months follow-up (Bokkala-Pinninti et al., 2008).

In the approach to clinical treatment, certain parkinsonian motor features are unresponsive to the main treatment applications of dopamine replacement or deep brain stimulation, such as gain dysfunction (Sharpe et al., 2020), occurring from specific pathological degeneration. For example, gait disturbance has been attributed to cholinergic neuronal loss in the forebrain (nucleus of Meyert) and the Pedunculopontine Nucleus (PPN) of the brainstem, and remains difficult to clinically treat and manage (Debû et al., 2018). Interestingly, recent application of nVNS in PD patients has indicated beneficial effects for Freezing Of Gait (FOG) (Morris et al., 2019; Mondal et al., 2019). Following 2D spatiotemporal gait parameter analysis, and measurements of stride during walking, there were benefits seen in a small group of PD patients tested (n = 19). More specifically, acute nVNS (2 × 120 s application with a 15 min interval between treatments) improved the

number steps taken while turning and reduced UPDRS III scores in FOG PD patients (Mondal et al., 2019). In a larger trial, acute nVNS (1 × 120 s) in PD patients (n = 31) improved gait and also significantly reduced step length variability after a single treatment (Morris et al., 2019). Furthermore, in a recent randomized, double-blind, sham-controlled cross-over study conducted by Mondal et al. (2021) in PD patients (n = 33), it was found that repeated nVNS improved gait and promoted neuroplasticity. Following the application of daily nVNS (6 × 120 s) over one month, PD patients demonstrated improved motor function and gait, from measurements in walking speeds, stance, step-length, along with MDS-UPDRS III scores (Mondal et al., 2021). Interestingly, the analysis of serum biomarkers after nVNS showed reduction in neuroinflammation (TNF-α) along with increased brain-derived neurotrophic factor (BDNF) in PD (Mondal et al., 2021). Together, data from these preliminary studies support the utilization of VNS applications for treatment of gait and eliciting potential disease modifying effects (Fig. 3).

4.5. Rationale for VNS in neurodegenerative disorders for achieving potential disease modifying effects

Modulation of noradrenergic neurotransmission in the locus coeruleus from VNS is one of the key neural pathways modulated for eliciting therapeutic benefit in epilepsy (Fornai et al., 2011). Such effects are implicated through the NTS (Follesa et al., 2007), where noradrenergic neural activity is increased relative to VNS, elevating noradrenergic neurotransmission (Dorr and Debonnel, 2006). As the locus coeruleus is one of the first brain regions affected in the pathology of major age-related neurodegenerative disease including AD and PD, significant loss of locus coeruleus neurons is a prominent pathological hallmark. For instance in AD, extensive neuronal loss is identified specifically in the rostral and dorsal areas of the locus coeruleus, with pathology also in the locus coeruleus cortical projections (Braak et al., 2011; Braak and Del Tredici, 2011; Stratmann et al., 2016; Theofilas et al., 2017). In post-mortem studies of PD, LB pathology is identified in the locus coeruleus in the premotor stage (Braak II) prior to characteristic LB pathology in the SNc (Braak III) (Braak et al., 2003a). While disease pathology of the locus coeruleus in AD and PD are typically seen prior to their characteristic pathological hallmarks, it is suggested that these neurons have higher vulnerability to neurodegeneration in age-related diseases. A contributing factor to the susceptibility of neurodegeneration of these neurons could be from excessive energy demands with high mitochondrial activity for endogenous pace-making functionality (Janitzky, 2020). In addition, the phenotypic structure of noradrenergic neurons may also be a critical factor i.e. being long, extensively branched and thinly myelinated or unmyelinated axons (Braak et al., 2003b), which may favor the early neurodegeneration in α-synucleinopathies. Interestingly, a recent study by Butkovich et al. (2020) identified the degeneration of the locus coeruleus in PD mice models as an early catalyst for neurodegenerative spread, with loss of central noradrenergic transmission contributing to chronic neuroinflammation in PD progression (Butkovich et al., 2020). Dysfunction of locus coeruleus neurophysiology has also been suggested in PD pathophysiology, with impaired phasic discharge and a shift to persistent high tonic activity causing exacerbation of chronic neuroinflammation (Janitzky, 2020). This is may be due to the findings that under normal physiology, locus coeruleus noradrenergic transmission elicits neuroprotective effects, with extra-synaptic release of noradrenaline mediating anti-inflammatory effects on surrounding neurons, glial cells and microvessels (Aston-Jones and Cohen, 2005) i.e. enhancing astrocytic functions, microglial neurotrophic factor production and reducing pro-inflammatory processes (O'Donnell et al., 2012; Jiang et al., 2015). Indeed, in healthy cells, the extra-synaptically released noradrenaline can mediate decreased inflammation caused by aberrant proteins like β-amyloid (Heneka et al., 2002; Heneka et al., 2010; Counts and Mufson, 2010).

As neuromodulation therapy can be used to engage the locus coeruleus for increased noradrenergic release (Fig. 2), the subsequent effect may potentially provide neuronal protection against neurotoxicity and neuroinflammation (Biggio et al., 2009; Furmaga et al., 2011; Tofaris and Buckley, 2018). It has been found that low frequency invasive VNS can mediate activation of systemic anti-inflammatory pathways, providing therapeutic benefit in patients suffering from rheumatoid arthritis and inflammatory bowel disorder (Bonaz et al., 2017; Koopman et al., 2017). With specific relevance to neurodegenerative disorders, studies using the neurotoxin-induced experimental rat model of PD showed that invasive VNS reduced neuroinflammation and behavioral motor deficits (Farrand et al., 2020). Moreover, the effects of VNS in this particular study extended to dampening pathological hallmarks, reducing the loss of dopamine neurons in the SNc and intrasomal α-synuclein accumulation (Farrand et al., 2020), which importantly indicates the potential disease altering effects of VNS therapy.

It is also plausible that VNS therapy, which stimulates the NTS, could directly excite the locus coeruleus via the monosynaptic excitatory projections (Fornai et al., 2011) and modulate short latency spiking (Hulsey et al., 2017; Ruffoli et al., 2011). The neuromodulation could therefore be used to elicit phasic release of noradrenaline in neurodegenerative diseases, where VNS induces significant increase in the percentage of locus coeruleus neurons to burst firing, as reported previously (Dorr and Debonnel, 2006; Manta et al., 2009; Manta et al., 2013). Indeed, short trains of VNS increases the firing rates of locus coeruleus neurons and noradrenaline concentration in the cortex and hippocampus (Groves et al., 2005; Roosevelt et al., 2006), driving rapid phasic neural activity (Hulsey et al., 2017). With subsequent elevations in noradrenergic transmission, this could potentially promote endogenous anti-inflammatory and neuroprotective effects for combatting the neurodegenerative processes (Fig. 3).

4.6. Exploratory avenues or monitoring neurodegenerative diseases

In clinical neurology, a major task remains for establishing early detection in neurodegenerative disorders that can translate to a therapeutic window for implementing future disease-modifying therapies. The utilization of evoked potentials from vagal nuclei may help in early detection of degeneration of brainstem nuclei. So far, several preliminary studies have assessed brainstem activity with vagus somatosensory evoked potentials (VSEP) which are rapid, reproducible and do not require an invasive approach (Fallgatter et al., 2003). VSEP are attained after stimulation of the ABVN, with measurements of far-field potentials taken from EEG electrodes. Although there has been no standardization characterized so far, pilot studies in AD patients (57–78 years of age) show VSEP have significantly longer latencies compared to healthy age-matched controls (Polak et al., 2007). The prolonged latencies are likely to originate from neurodegeneration of the vagal nuclei, which can be found present in AD but not in major depression (Polak et al., 2014). An ongoing observational study (the 'Vogel' study) is now observing 3 time points over a decade period in 604 subjects for the early detection of brainstem pathology with VSEP latencies in AD (Polak et al., 2017), which could eventually help support early detection procedures. So far, studies utilizing VSEP in PD patients have reported contrasting data. It has been demonstrated that longer VSEP latencies can be found in PD indicating the potential use for identifying premotor manifestations (Polak et al., 2011). However, a cross-sectional observational study in PD patients failed to find differences to matched controls (Weise et al., 2015), requiring further clinical investigations to elucidate the use of VSEP as a marker in PD.

Building up a clinical picture of early disease manifestation in neurodegenerative diseases, such as α-synucleinopathies, may be optimized with measurements of dysautonomia (Fig. 4). In combination with clinical signs such as sleep disorders like RBD, the reduction of HRV parameters could be used to detect cardiac autonomic denervation, with recurrent assessments for monitoring disease progression. It is known

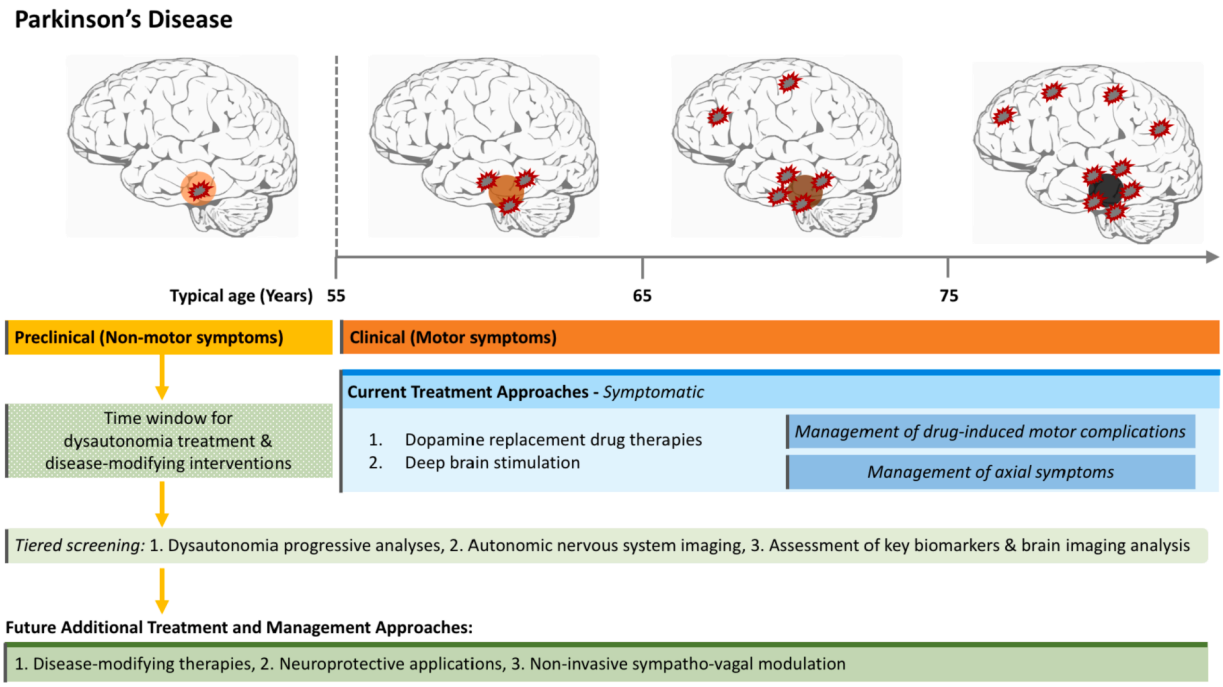

Fig. 4. The progressive spread of Lewy Body pathology in the brain from pre-clinical to clinical stages of Parkinson's disease (PD). The severity of midbrain dopaminergic neuron loss (depicted with a darkening colour) corresponds to the disease time-course with prominence in the clinical phase. Current symptomatic treatments are focused in the clinical phase, which includes dopaminergic drug replacement therapies (i.e. L-DOPA, dopamine D2 receptor agonists) and invasive neuromodulation (Deep Brain Stimulation, DBS), often followed by advanced PD motor symptom management. The proposal for tiered screening tools aims for early detection of PD from evaluations of 1. Related dysautonomia (i.e. anosmia, sleep abnormalities, anxiety, constipation, orthostasis, orthostatic hypotension) and vagus nerve neurophysiology (i.e. VSEP, HRV), that will be continually monitored for disease progression in clinical practice; 2. Autonomic nervous system imaging (^{123}I-MIBG scintigraphy, CT assessment of cholinergic gut innervation integrity); and 3. Detailed imaging analyses and biomarker detection (11C-MeNER and 18F-DOPA PET imaging, CSF sampling). The integration of future treatments in pre-clinical stages aim to optimize treatment effects of slowing disease progression with disease modifying and neuroprotective therapies, as well as alleviation of PD dysautonomia at the pre-clinical stage (i.e. glial derived neurotrophic factors, immunotherapies and non-invasive VNS for treating sympatho-vagal imbalance).

that people with RBD are at high risk of developing cognitive features of LB disorders, with 50% of individuals eventually developing parkinsonism (Schenck et al., 1996) in a span of approximately 12 years (Iranzo et al., 2006). Moreover, people with RBD show reduction of HRV parameters (LF, HF power, and pNN50), which suggests the sympathetic and parasympathetic influence on cardiac function are reduced (Valappil et al., 2010). It has been revealed that RBD sufferers have severe pathology in the autonomic nervous system and locus coeruleus i.e. noradrenergic denervation, without any detectable impairments in dopaminergic storage capacity, prior to any development of a severe parkinsonian syndrome (Knudsen et al., 2018).

A combined approach of detailed imaging for detection of early neurodegeneration would help support the implementation of early treatment and disease management (Fig. 4). The identification of behavioral deficits and the presence of dysautonomia, as well as the screening of the autonomic nervous system function and vagus nerve neurophysiology, can be supported with detailed imaging analysis from MRI and PET imaging, as well as cerebrospinal fluid sampling. The initial screening tools of the autonomic nervous system and the monitoring of physiological changes could be incorporated in routine examinations in aging populations, having the advantage of being both rapid and efficient. As such, structuring a multi-tiered approach may be most practical for examinations, with a first-tier screening criteria for dysautonomia followed by second-tier detailed imaging, such as ^{123}I-MIBG cardiac scintigraphy for assessment of cardiac sympathetic innervation, and CT assessment of cholinergic gut innervation integrity (in the enteric and parasympathetic synapses) (Fedorova et al., 2017; Treglia et al., 2012); to a third-tier for brain scans of 11C-methylreboxetine (MeNER) PET for assessing noradrenergic nerve terminals, and also 18F-Dihydroxyphenylalanine (DOPA) PET to assess nigrostriatal dopamine storage integrity (Knudsen et al., 2018). Although such a process remains to be established, combining these methods would ultimately increase the accuracy of early disease detection and provide an opportunity for implementing disease-modifying strategies for achieving better patient care and management (Fig. 4).

5. Conclusions

With a deeper understanding of neurodegenerative disease pathogenesis, new opportunities are available for establishing effective therapeutic and rehabilitation approaches to these disorders, for the complex range of disease symptoms that occur over a typically long progressive time course. From evidence of a severely affected autonomic nervous system in major neurodegenerative diseases, and considerations of pathological disease spread across the nervous system, we propose that vagus nerve neuromodulation for rebalancing sympatho-vagal abnormalities and brainstem neuro-signaling at an early stage, could provide improved clinical outcomes for disease sufferers, especially for troublesome dysautonomia. In addition, screening processes that are rapid and efficient for measuring the autonomic nervous system and brainstem integrity could be further employed to determine early neurodegeneration, and remain vital for enhancing the successes of any potential future disease-modifying agent or application.

Declaration of competing interest

D.K. has equity stake in Neuropix Company Ltd.

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The LC-NE system as a potential target for neuromodulation to ameliorate non-motor symptoms in Parkinson's disease

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ABSTRACT

Parkinson's disease (PD) is associated with severe motor symptoms but also with several non-motor symptoms (NMS). A substantial reduction of norepinephrine (NE) levels in various brain regions reflecting an extensive loss of innervation from the LC has been assumed as causal for the development of NMS and specifically of attentional impairments in PD. Transcutaneous auricular vagus nerve stimulation (taVNS) is a new, non-invasive neurostimulation method supposed to modulate the LC-NE system in humans. In the current opinion paper, we introduce taVNS as a systemic approach to directly affect NE neurotransmission in healthy as well as clinical populations and discuss its potential as therapeutic option for the treatment of NMS, specifically attentional deficits, in patients with PD. Here, we first describe the LC-NE system and discuss how LC-NE dysfunction might affects cognition in PD before detailing the mode of action of taVNS and proposing its use to modulate cognitive deficits in these patients.

1. Introduction

Parkinson's disease (PD) is generally characterized by a substantial degeneration of dopamine (DA) neurons in the substantia nigra *pars compacta* (*SNc*), and its related motor symptoms including bradykinesia, rigidity, and tremor. Recently, it has been well accepted that in addition to motor deficits PD is also characterized by severe non-motor symptoms (NMS). These NMS include autonomic dysfunction, sensory and sleep difficulties, but also cognitive, and neurobehavioral problems, including attentional deficits, dementia, depression and fatigue. Importantly, NMS can appear years before the onset of motor symptoms in PD (Lemke et al., 2004) and depletion of DA alone cannot simultaneously elicit both motor and non-motor deficits in a rat model of PD. This strongly argues for a multi-system causality underlying PD that includes a profound loss in other neurotransmitter systems. Indeed, animal data show that a specific loss of DAergic and NAergic innervation of the limbic system is associated with cognitive and neurobehavioral problems, including dementia (Cash et al., 1987), depression (Cummings, 1992; Remy et al., 2005), anxiety (Lauterbach et al., 2003; Stein et al., 1990) and attentional deficits (Riekkinen et al., 1998). Moreover, there is growing evidence that additional loss of norepinephrine (NE) neurons of the locus coeruleus, the principal source of NE in the brain, is involved in the clinical expression of PD.

In the following, we first review the locus coeruleus-norepinephrine (LC-NE) system, including its anatomical and functional properties and its role in modulating alertness, arousal, and attention before discussing LC-NE dysfunction in patients with PD. We then review data strongly suggesting that the ongoing loss of norepinephrine cells in the LC during the course of PD and the resulting progressive decline in NE activity may be causally related to the development of cognitive and, in particular, attentional impairments in PD. Finally, we introduce transcutaneous auricular vagus nerve stimulation (taVNS) as a noninvasive neurostimulation method that directly modulates the LC-NE system in humans and highlight its potential as an adjunctive therapy for cognitive deficits in PD.

2. The LC-NE system

The locus coeruleus (LC) is a compact nucleus consisting of approximately 50,000 neurons in adult humans and is located bilaterally in the pontine tegmentum in the brainstem (Aston-Jones and Cohen, 2005a). The LC is present in all mammalian species and is considered the main source of NE synthesis in the brain (Mann, 1983). The LC-NE system modulates a variety of brain functions through the release of

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NE into widely distributed brain areas including the neocortex, hippocampus, thalamus, subthalamic nucleus, and substantia nigra (Aston-Jones and Cohen, 2005b) and has a major role in the regulation of arousal and attention (Berridge and Waterhouse, 2003).

The LC-NE operates in a tonic and a phasic mode (Aston-Jones and Cohen, 2005b). Tonic discharges of the LC - characterized by a sustained and highly regular discharge pattern - modulate the individuals' alertness and arousal state. Accordingly, stimulation of central NEergic receptors leads to changes in vigilance, and environmental stimuli that trigger attention and exploratory behavior induce sustained increases in tonic discharge rates. During focused attention, LC neurons reduce their tonic firing and respond phasically. Moderate tonic discharge rates are most effective in facilitating these phasic discharges of LC neurons in response to relevant stimuli in the environment. The LC in its phasic mode is thought to promote selective attention, whereas the tonic mode enables behavioral flexibility (Aston-Jones et al., 1999; Nieuwenhuis et al., 2005; Polich, 2007). Accordingly, several animal data have confirmed the importance of the LC-NE system for attentional functions, but also for cognition, arousal, anxiety, depression, pain, and locomotor control (Aston-Jones and Bloom, 1981; Beas et al., 2018; Carter et al., 2010; Chandler et al., 2014; Delaville et al., 2011; Sara and Bouret, 2012). Although arousal and attention are regulated by several neurotransmitter systems, norepinephrine is one of the most important (Smith and Nutt, 1996). It has been consistently shown that decreases in central NE have deleterious effects on attention (Smith and Nutt, 1996), whereas increases in central NE improve performance in attentional tasks (Bunsey and Strupp, 1995; Sirviö et al., 1993), suggesting that increased NE activity facilitates the function of cortical circuits that promote behavioral activation, vigilance, and attention (Aston-Jones et al., 1999). Moreover, NE interacts with other catecholamines like DA to support these functions, with NE also playing a regulatory role in DA signaling. For example, chemical modulation or electrical stimulation of the LC increases the extracellular concentrations of both NE and DA (Smith and Greene, 2012). Finally, alterations of the LC-NE system have been implicated in several neuropsychiatric pathologies such as sleep and arousal disorders, post-traumatic stress disorder, anxiety, depression, schizophrenia, and mechanisms of opioid addiction, as well as in attention deficit hyperactivity disorder (ADHD) and PD (Benarroch, 2009).

3. LC – NE dysfunction in PD

Whereas historically the focus of pathophysiology in PD was on SN degeneration and associated motor symptoms, there is now consistent evidence that NE cells in the LC also degenerate in PD (Delaville et al., 2011; Ehringer and Hornykiewicz, 1998; Greenfield and Bosanquet, 1953). In fact, LC-NE pathology occurs in the preclinical or prodromal stages of the disease even before the degeneration of DAergic neurons of the SNc (Braak and Del Tredici, 2008), and up to 10 years before the clinical diagnosis of PD (Baloyannis et al., 2006; Hawkes et al., 2010). Moreover, in PD patients, neurodegeneration is even greater in the LC (83%) than in the SNc (78%) (Zarow et al., 2003). Consistent with these observations, significant depletions of over 80% of NE concentration have been reported in the brain of PD patients (Gaspar et al., 1991), particularly in the frontal cortex, cerebellum, striatum, thalamus, and hypothalamus (Pavese et al., 2011), all of which receive projections from the LC. Moreover, the severity of motor symptoms of PD correlates with both the severity of DA and NE depletion (Marié et al., 1995). In the cognitive domain, however, only the extent of LC cell loss significantly correlates with attentional and vigilance problems in PD patients (Bédard et al., 1998; McNamara and Durso, 2006), suggesting that the impaired LC-NE system, rather than the deficient SN system, contributes significantly to cognitive NMS in PD. It is worth noting that both, non-cognitive NMS and motor symptoms in PD have been associated with pathological NE deficiency. NMS such as orthostatic and postprandial hypotension, as well as disruption of circadian rhythm and arousal/wakefulness cycles, are likewise associated with NE deficiency (Berridge and Waterhouse, 2003; Kaufmann and Goldstein, 2013). Motor symptoms in PD patients such as freezing of gait have been directly associated with decreased CSF concentrations of NE as well, and administration of an NE precursor has been shown to improve gait (Tohgi et al., 1993). Thus, studies in animal models (mainly in a rat model of PD) and human patients strongly suggest that the impairment of the LC-NE system contributes to both the motor and essentially to the non-motor symptoms of PD (see (Delaville et al., 2011) for a comprehensive overview). Accordingly, it can be concluded that dysfunction of the NE system is a key feature of PD. As mentioned above, these deficits are revealed in post-mortem studies showing loss of LC-NE neurons, in in-vivo structural brain imaging as atrophic changes in structures connected with the LC, and finally in functional imaging as changes in network activity and connectivity (Goldstein et al., 2011; Janitzky, 2020; Kelberman et al., 2020). Finally, pharmacological intervention studies with NE reuptake inhibitors underline the role of NE in remediating aberrations in functional connectivity and deficits in cognitive performance (Jankovic, 2009; Peterson and Li, 2018; Weintraub et al., 2010).

Interestingly, PD and Alzheimer's disease (AD) share the common feature of an early impairment of the LC. The LC shows prodromal accumulation of hyperphosphorylated tau in AD (Braak et al., 2011; Pletnikova et al., 2018) and alpha-synuclein aggregation in PD (Del Tredici and Braak, 2013) long before other structures such as the hippocampus, cortex (AD), and basal ganglia (PD). In addition, AD and PD share clinical symptoms associated with LC dysfunction, such as sleep disturbances (Ju et al., 2013; Suzuki, 2021), and attention deficits (AD (Berardi et al., 2005), PD (Dujardin et al., 2013)), which seem to be temporally coupled with the development of LC pathology (Ehrenberg et al., 2018; Grinberg et al., 2010).

Finally, growing evidence suggests that in PD besides the DA and the NE system further neurotransmitter systems are affected as well, including the serotonergic system. Serotonergic (5-HT) neurons in the dorsal raphe nuclei project mainly to the basal ganglia, but also to the frontal cortex and the limbic system. The serotonergic system is thought to be involved in the modulation of various cognitive and physiological processes, such as mood, emotion, sleep, and appetite. Thus altered serotonergic neurotransmission is likely to be implicated in both motor and non-motor disturbances observed in PD (Chaudhuri et al., 2006b; Politis and Loane, 2011). Postmortem, animal, and especially functional imaging studies (Kish, 2003; Kish et al., 2008; Politis et al., 2010a) have demonstrated serotonergic dysfunction in PD and relate these pathophysiology to motor symptoms such as the severity of tremor (Loane et al., 2013) but also non-motor symptoms such as depression (Politis et al., 2010b), fatigue (Hagell and Brundin, 2009; Pavese et al., 2010), and others (for a recent comprehensive review see: Pagano and Politis, 2018).

4. Cognitive deficits in PD

Although motor symptoms are clearly paramount in PD, the NMS are devastating. Several studies show that the global burden of NMSs has a greater impact on patients' quality of life than their motor symptoms (GPDS, 2002; Löhle et al., 2009). Depression, poor sleep, dribbling of saliva, severe constipation, urinary discomfort and attention deficits have been reported to severely impact health-related quality of life (Chaudhuri et al., 2007; Martinez-Martin et al., 2009). Despite the relevance of NMSs in PD, studies have shown that clinicians often fail to identify them during consultations (Chaudhuri et al., 2006a). Among severe NMS, cognitive impairment without dementia is frequently observed in PD, even in early stages of the disease (Muslimovic et al., 2005). Even though James Parkinson claimed in 1817 that the disease does not affect the mind and psyche of sufferers, recent data consistently shows that problems with attention and concentration are common and debilitating. One-third of PD patients also develop dementia during the course of the disease. However, while this dementia usually occurs in the

late stages of the disease and almost exclusively affects patients of advanced age, cognitive dysfunctions are already present earlier in the course of the disease. It has been shown that up to 57% of patients with early PD had mild cognitive impairments (Williams-Gray et al., 2007). Typically, the cognitive deficit presents as a dysexecutive syndrome in which impairments in attention play a central role (Brown and Marsden, 1988; Dujardin et al., 2013). Accordingly, severe deficits in sustained (Koerts et al., 2010), focused (Machado et al., 2009) as well as divided attention (Malapani et al., 1994) have been reported in PD. Moreover, there is also compelling evidence for an impairment in the voluntary control of attention in PD patients (Cameron et al., 2010; Cools et al., 2010). Finally, PD patients are impaired in attentional set-shifting, as well as in planning and spatial working memory tests (Lange et al., 2016; Owen et al., 1992).

Classically, a disruption of the associative and limbic circuits connecting the striatum to the frontal and prefrontal areas by dopamine depletion has been considered as a main cause of attention and executive function impairments in PD (Cools et al., 2010). Accordingly, some data indicate that aspects of cognitive dysfunction might improve with dopaminergic therapy (Cools et al., 2002; Mattay et al., 2002). However, dopaminergic treatment can actually worsen cognitive functions, particularly in patients with advanced disease (Chaudhuri and Odin, 2010). Thus, while DA deficits might be involved in some aspects of cognitive impairments (Gotham et al., 1988; Lange et al., 1992), reduced noradrenergic activity seems to substantially contribute to impaired attentional processing in PD patients (Riekkinen et al., 1998). Finally, pharmacological reduction of NE activity disrupts attention in mild PD patients to the same level as seen in severe PD patients. In this vein, it has been shown that PD patient have a marked reduction in noradrenergic innervation reflected by significantly reduced NE levels in widespread brain regions (Buddhala et al., 2015). The reduction of NE levels in various brain regions reflects the extensive loss of innervation from the LC in PD. Thus the development of norepinephrine cell loss in the LC during the course of PD and the resulting progressive decrease in NE activity (Chan-Palay and Asan, 1989; Mann, 1983) can be assumed to be causally involved in the development of attentional impairments in PD (Riekkinen et al., 1998). To sum up, PD is canonically a dopaminergic disorder, but DA-resistant non-motor symptoms, including attention deficits, (1) occur earlier in the disease course, (2) are mostly unaffected by DA replacement therapies, and (3) correlate with the extent of LC cell loss. Accordingly, notable LC-NE pathology may be thought to underlie non-motor symptoms in PD, particularly attentional cognitive deficits. Several preclinical studies have already shown that prefrontal LC-NE projections play a crucial role in the executive control of attention (Vazey and Aston-Jones, 2012).

5. Treatment of cognitive deficits in PD

First line treatments for PD involve dopaminergic medications (i.e., levodopa or dopamine agonists) to primarily address motor symptoms (Rogers et al., 2017). Most NMS, however, are not susceptible to these treatments. For alternative treatments of MCI or cognitive impairment short of dementia in PD, the evidence is limited, with a reported insufficient efficacy for dopaminergic or cholinergic medications and an unclear role of DBS surgery (Seppi et al., 2019). Earlier studies suggested that PD patients who also suffer from cognitive deficits can benefit from neuropsychological training. Consequently, earlier treatment guidelines (e.g. of the German Society for Neurology for Idiopathic Parkinson) proposed that neuropsychologically based therapy could be used to address problems in the areas of attention, memory and problem solving in PD sufferers. However, the 2018 report of the International Parkinson and Movement Disorder Society Evidence-Based Medicine Committee, which regularly publishes recommendations for the management of non-motor symptoms in PD, concluded that while the evidence base for the treatment of a number of NMS in PD has grown substantially, effective treatment options remain significantly limited given the high prevalence and overall negative impact of these disorders (Seppi et al., 2019). In particular, only *rivastigmine* has been designated as clinically useful for the treatment of Parkinson disease dementia while *donepezil* and *galantamine* are designated as possibly useful because of limited evidence to support their efficacy in Parkinson disease. Furthermore, there is no evidence to support *memantine*. Importantly, there is a lack of a sufficient therapy for cognitive and attentional deficits in PD, and especially non-pharmacological approaches have insufficient evidence for use in PD (Sun and Armstrong, 2021). Consequently, the development of an effective treatment that efficiently alleviates cognitive and attentional deficits should become an imperative for PD research and clinical practice. An appropriate treatment option for cognitive deficits in general and attention deficits in particular has the potential to substantially improve everyday functioning in PD and to significantly reduce the impact of the disease on the daily lives of individuals with PD.

6. Transcutaneous auricular vagus nerve stimulation (taVNS)

Recently, transcutaneous auricular vagus nerve stimulation (taVNS) has emerged as a noninvasive neurostimulation method for the direct modulation of the LC-NE system in humans (Ellrich, 2019; Farmer et al., 2021). This technique involves applying a small, weak electrical current to the cymba conchae, typically the left auricle, which is innervated exclusively by the auricular branch of the vagus nerve (Peuker and Filler, 2002). The direct link between electrical stimulation of the afferent vagal fibers and NE release has been repeatedly demonstrated using invasive stimulation of the vagus nerve in animal models. Intracranial single cell recordings and microdialysis in rodents consistently show that activation of vagal afferents via invasive VNS (iVNS) causes an increase in the firing of NE neurons in the LC (Hulsey et al., 2017) as well as an augmentation of NE release in the neocortex including the hippocampus, the basolateral amygdala, and the prefrontal cortex (Hassert et al., 2004; Manta et al., 2013). Finally, imaging studies in patients with depression showed that iVNS resulted in an increased regional cerebral blood flow in the left DLPFC following treatment (Henry et al., 1998). Findings from animal models further support the notion that VNS has the potential to support cognition and memory. Rats showed memory enhancements when they received VNS immediately after training (Clark et al., 1998). In humans, iVNS enhanced recognition memory (Clark et al., 1999; Ghacibeh et al., 2006) and modulated decision making in epilepsy patients (Martin et al., 2004). In healthy humans, the efficacy of non-invasive taVNS to activate the LC and thus to increase NE availability in the neocortex has been demonstrated as well (Burger et al., 2020; Butt et al., 2020; Colzato and Beste, 2020). Thus, taVNS can activate afferents of the vagal pathway (Badran et al., 2018a, 2018b) and affect central brain functions related to NE, such as executive functions (Keute et al., 2020), working memory (Beste et al., 2016) and associative memory (Jacobs et al., 2015), fear conditioning (Burger et al., 2016), as well as motor learning in preterm neonates (Badran et al., 2020; Badran et al., 2018a, 2018b) Importantly, taVNS can enhance attentional processes evident in increased resource allocation during selective attention paradigms (Rufener et al., 2018) and an attenuation of occipital alpha activity (Sharon et al., 2021). However, the efficiency of taVNS depends to a large extent on the optimization of critical stimulation parameters, in particular pulse width, frequency and intensity, but also on the optimal site for stimulation within the ear or at the neck (Farmer et al., 2021). The current lack of knowledge about the optimal - ideally individualized - stimulation parameters can still be considered a general limitation of this field of research (Ludwig et al., und. rev.), although there has been a number of excellent research on this topic in the recent past (Badran et al., 2019; Thompson et al., 2021).

Interestingly, beside the proposed effects of taVNS on the LC-NE system, the afferent path of the vagus affects other structures and neurotransmitter systems that are relevant to PD. Effects on serotonergic neurotransmission have also been implicated as a key mechanism of the beneficial effects of taVNS. A recent study in rodents with invasive VNS

found an increased firing rate of serotonergic neurons and this upregulation appeared to be mediated by serotonin release (Manta et al., 2009). Finally, long-term vagus nerve stimulation increased basal firing in both the noradrenergic LC and the serotonergic dorsal raphe nucleus (DRN), with increasing the LC firing rate significantly earlier than the firing of the dorsal raphe serotonergic neurons. As the LC has an excitatory influence on the DRN, it has been assumed that the increased DRN firing rate is secondary to an initial increased LC firing rate from VNS (Dorr and Debonnel, 2006).

While research on the effectiveness of taVNS in PD is in its infancy, several studies have evaluated the potential of taVNS treatments in other neuropsychiatric disorders in the last few years (for a comprehensive overview see Farmer et al., 2021; Farmer et al., 2016). They consistently reported positive effects in reducing the frequency of epileptic seizures (Bauer et al., 2016) and improving depressive symptoms (Fang et al., 2016). Beside cognitive deficits, recent studies are already showing the benefit of tVNS for motor deficits in Parkinson's disease. Notably, one report showed improvement in gait already after a single application of cervical tVNS (Morris et al., 2019), while a recent randomized, double-blind, sham-controlled crossover trial demonstrated the efficacy of repetitive, cervical tVNS in treating freezing of gait in patients with PD (Mondal et al., 2021). Three-times-daily tVNS for one month significantly improved gait and motor function and reduced serum inflammatory markers. Finally, the clinical potential of taVNS for a concomitant therapy in AD (Jacobs et al., 2015) or PD (Janitzky, 2020) patients has been proposed.

7. Summary

Beside a well-documented reduction of SN dopamine underlying motor symptoms in PD a substantial reduction of NE levels in various brain regions reflecting the extensive loss of innervation from the LC has been assumed as causal for the development of attentional impairments in PD. State-of-the-art neurostimulation technology, namely taVNS can efficiently modulate LC-NE system functioning in both healthy and clinical populations and could therefore be a promising therapeutic option for the treatment of NMS, specifically attentional deficits in PD. Moreover, non-motor symptoms have been also related to alterations in serotonin signaling in PD and there are data suggesting that tVNS may also affect serotonergic neurotransmission. However, to conclude that taVNS can be an effective approach for the treatment of impaired attention in a clinical setting, further data are needed that convincingly demonstrate that (I) a single session of taVNS can positively affect attention processes (transient effects), (II) multisession taVNS can stabilize and/or enhance this effect, (III) these stimulation regimens lead to long-term effects of adequate duration, and (IV) specific conditions for a pronounced and successful home-application can be identified.

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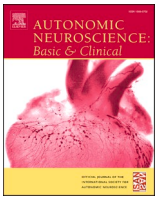

Transdermal auricular vagus stimulation for the treatment of postural tachycardia syndrome

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ABSTRACT

Postural Tachycardia Syndrome (POTS) is a chronic disorder characterized by symptoms of orthostatic intolerance such as fatigue, lightheadedness, dizziness, palpitations, dyspnea, chest discomfort and remarkable tachycardia upon standing.

Non-invasive transdermal vagal stimulators have been applied for the treatment of epilepsy, anxiety, depression, headache, and chronic pain syndromes. Anti-inflammatory and immunomodulating effects after transdermal vagal stimulation raised interest for applications in other diseases. Patients with sympathetic overactivity, reduced cardiac vagal drive and presence of systemic inflammation like POTS may benefit from tVNS.

This article will address crucial methodological aspects of tVNS and provide preliminary results of its acute and chronic use in POTS, with regards to its potential effectiveness on autonomic symptoms reduction and heart rate modulation.

1. Introduction

1.1. Postural tachycardia syndrome

Postural Tachycardia Syndrome (POTS) is a chronic disorder characterized by symptoms of orthostatic intolerance such as fatigue, lightheadedness, dizziness, palpitations, dyspnea, chest discomfort and remarkable tachycardia upon standing. Positional symptoms occur without concomitant orthostatic hypotension (Freeman et al., 2011; Furlan et al., 1998; Jacob et al., 2000; Mar and Raj, 2020; Raj, 2013; Robertson, 1999). Patients affected by POTS have pronounced orthostatic distress which affects their daily living, eventually resulting in poor quality of life (Dipaola et al., 2020) and reduced work ability (Barbic et al., 2020).

Notably, a subtype of POTS patients, defined as hyperadrenergic, may display a sympathetic over-activity and a concomitant vagal impairment when supine (Furlan et al., 1998). This results in the presence of excessive catecholamine plasma titers and related signs and symptoms, such as palpitations, dyspnea, chest discomfort and tremors mostly upon standing. Such a hyperadrenergic profile along with low parasympathetic activity, leads to the hypothesis that any intervention resulting in an enhancement of the parasympathetic modulation and/or a decrease of the cardiovascular sympathetic modulation, might diminish symptom intensity. Additionally, it is likely that an increase in parasympathetic activity or modulation might also promote an anti-inflammatory effect in POTS. In this syndrome, chronic and inappropriate sympathetic activation was associated with elevated plasma titers of the systemic inflammatory marker Interleukin-6 (IL-6) compared to

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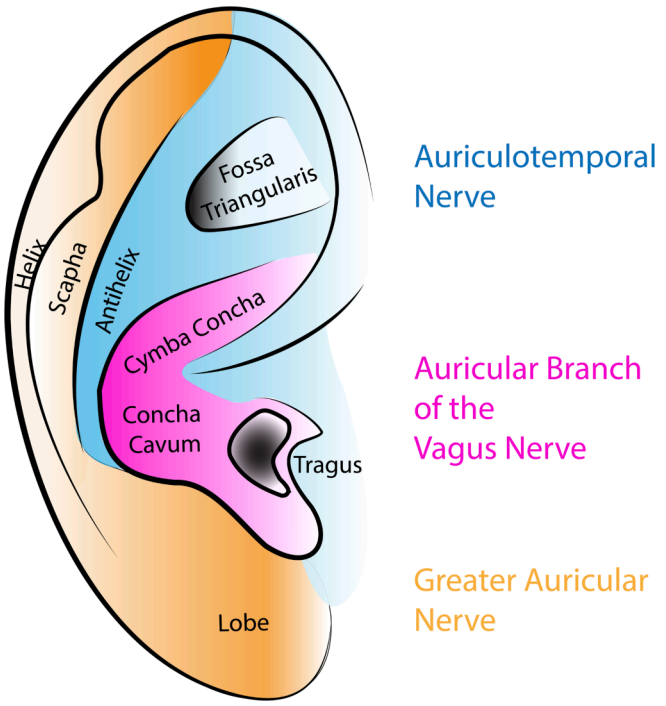

Fig. 1. Simplified schematic of the innervation of the ear. The Cymba Concha and Concha Cavum are innervated by the Auricular Branch of the Vagus Nerve and therefore preferred anatomical regions for transdermal vagal stimulation (tVNS). The tragus and fossa triangularis have also been proposed as sites for tVNS.

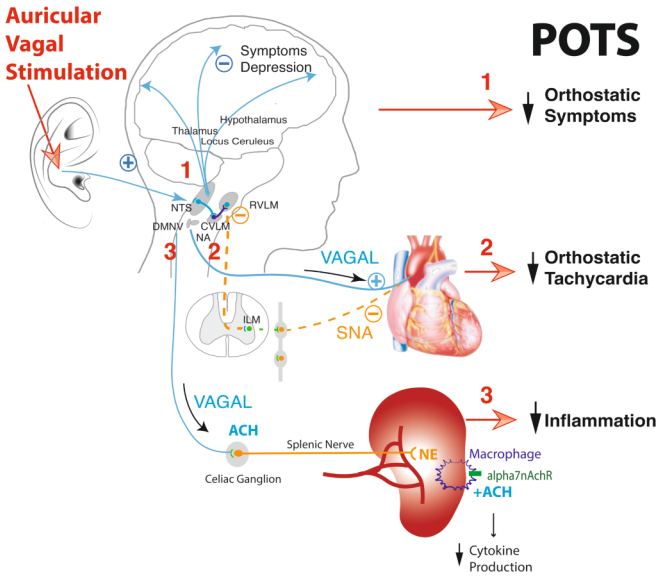

Fig. 2. Transdermal stimulation of the auricular branch of the vagal nerve of the ear modulates vagal afferent inputs which activate regions of the brain such as the locus coeruleus, the thalamus, the prefrontal cortex, the postcentral gyrus, the posterior cingulate gyrus and the insula cortex. This can modulate perception and mood (1). Projection via nucleus tractus solitary (NTS) increases activity of neurons in the caudal ventrolateral medulla (CVLM), increases inhibition of sympathetic pacemaker neurons in the rostral ventrolateral medulla (RVLM), and increases vagal activity in the nucleus ambiguous (NA). Increased vagal activity and reduced sympathetic neural activity (SNA) to the sinus atrial node reduces heart rate (2). Vagal stimulation mobilizes the cholinergic anti-inflammatory pathway through sympathetic axons supplying the spleen. It increases norepinephrine (NE) release in the spleen. NE activates beta2 receptors expressed in splenic macrophages and attenuates cytokine production when signaling through nicotinic acetylcholine receptor 7 subunit (alpha7nAchR) is present (3). All these effects are beneficial for patients with postural tachycardia syndrome (POTS).

healthy individuals, although a causal relationship between inflammation and POTS symptoms has not yet been elucidated (Furlan et al., 2000; Okamoto et al., 2015).

2. Vagal stimulation

The idea of applying controlled electrical currents to specific human body locations with therapeutic aims has fascinated physicians and researchers since the first report of surgically implantable devices enabling the stimulation of the vagus nerves in canines (Zabara, 1992). Early clinical trials investigating the use of implanted Vagus Nerve Stimulation (iVNS) devices in pharmaco-resistant epilepsy (Penry and Dean, 1990; Uthman et al., 1993) showed the efficacy of that methodology in humans, as assessed by a remarkable reduction in seizure frequency. However, the invasiveness of that technique, clinical side effects related to direct electrical nerve stimulation, late complications due to surgery and significant costs associated with the implantable stimulator restricted its application to otherwise untreatable Central Nervous System disorders. More recently, a non-invasive technique based on transdermal electrical vagus nerve stimulation (tVNS) was developed (Yuan and Silberstein, 2016). Briefly, tVNS treatment involves the application of low-voltage electrical currents to readily accessible anatomical regions innervated by vagus nerve afferents, i.e., outer-ear cymba conchae (Fig. 1) or the neck, resulting in their activation.

Several devices were approved by American and European Regulatory Agencies for the treatment of various conditions, including epilepsy (Ardesch et al., 2007), anxiety and depression (Hein et al., 2013), primary chronic headache (Garcia et al., 2017) and chronic pain syndromes (Kirchner et al., 2000).

Recently, anti-inflammatory and immunomodulating properties of tVNS have raised interest for its possible applications in autoimmune diseases, such as Rheumatoid Arthritis and Crohn's disease (Drewes et al., 2021; Koopman et al., 2016; Tracey, 2007). In this context, the cholinergic anti-inflammatory reflex model was hypothesized based on evidence that a potent anti-inflammatory action of acetylcholine exists and it depends on 1) activation of central cholinergic projections 2) integrity of the vagus nerve 3) effective peripheral nicotinic receptors functions (Bernik et al., 2002; Borovikova et al., 2000; Pavlov et al., 2006) 4) and intact spleen (Huston et al., 2006). The unifying model suggested that vagal activation can decrease proinflammatory cytokine production by activating nicotinic receptors present on splenic macrophages. However, consensus regarding the precise mechanism is far from being reached as evidence of vagal splenic innervation is scarce (Rosas-Ballina et al., 2008), and the hypothesis of di-synaptic connections involving vagal efferent fibers and postganglionic sympathetic fibers located in the celiac ganglion has been challenged by Bratton et al.'s (2012) experiments. The same research group postulated that the efferent arm of the anti-inflammatory reflex might be composed by the sympathetic nervous system alone (Martelli et al., 2019, 2014), challenging the traditional view of a direct correlation between sympathetic activity and inflammation (Cervi et al., 2014; Furlan et al., 2006). In light of these considerations, the effects of directly stimulating the vagus (iVNS) or stimulating vagal afferents (tVNS) may result in profound differences in anti-inflammatory effects (Fig. 2).

An additional effect of vagal stimulation might be the modulation of persistent cardiac sympathetic overactivity. Previous studies documented that a persistent prevalence of cardiac sympathetic modulation is a relevant maladaptive mechanism resulting in increased cardiovascular morbidity and mortality (Barretto et al., 2009; De Ferrari et al., 2011; Malliani and Montano, 2004; Zoccali et al., 2002). Indeed, well-known and documented associations exist between excessive cardiovascular sympathetic tone and essential hypertension (Mancia and Grassi, 2014), persistent atrial fibrillation (Chen et al., 2014), ischemic

Table 1
Reported effects of implanted vagal stimulators on autonomic cardiovascular parameters, inflammation, and symptoms in humans.

Author	Sample	Site & parameters	Main outcomes						
			HR	Vagal indices	LF/HF	BP	MSNA	Infl	Symp
Nearing et al. (2021)	Heart Failure $N = 21$	Right vs left cervical impl. 10-50 Hz, 250 μs pw, 1.5-3 mA	↓	↓	=	.	.	.	.
Bonaz et al. (2016)	Chron disease N = 7	Left cervical impl. 10 Hz, 500 μs pw, 0,25 mA	.	↑	=	.	.	↓	↓
Koopman et al. (2016)	Rheumatoid arthritis $N = 17$	Left cervical impl. 20 Hz, 500 μs pw, 0,25-2,0 mA	.	.	.	.	.	↓	↓
De Ferrari et al. (2014)	Heart failure $N = 32$	Right cervical impl. 1 pulse/hb, 5.5 mA	↓	↑	.	=	.	.	↓
Sperling et al. (2010)	Depression $N = 9$	Left cervical impl.	↓	↑	.	.	.	.	=
Barone et al. (2007)	Epilepsy $N = 8$	Left cervical impl. 30 Hz, 500 ms pw, 0.75-1.75 mA	=	=	=	.	.	=	.
Ronkainen et al. (2006)	Epilepsy vs healthy $N = 14$ vs 28	Impl. left cervical 30 Hz, 500 μs pw,	=	=	=	.	.	.	↓
Setty et al. (1998)	Epilepsy $N = 10$	Impl. left cervical 30 Hz, 750 μs pw,	=	=	=	.	.	.	.
Kamath et al. (1992)	Epilepsy N = 8	Impl. left cervical 30 Hz, 500 μs pw VS 2 Hz, 130 μs pw	=	↑	↓	.	.	.	.

HR, heart rate; LF/HF, ratio between the low and high frequency components of heart rate variability; BP, blood pressure; MSNA, muscle sympathetic nerve activity; Infl, inflammation; Symp, symptoms; pw, pulse width; Impl, implant; ↑ increased; = unchanged; ↓ decreased.

heart disease (Malliani and Montano, 2004) and heart failure (Florea and Cohn, 2014). In this context, disorders potentially characterized by sympathetic overactivity, reduced cardiac vagal drive and presence of systemic inflammation, including POTS (Furlan et al., 2006; Okamoto et al., 2015), may theoretically benefit from tVNS as illustrated in Fig. 2.

Many detailed reviews about the mechanism and application of vagal stimulation in patients with epilepsy, chronic pain, and other diseases are available (Beekwilder and Beems, 2010; Ellrich, 2011; Farmer et al., 2021; He et al., 2012; Johnson and Wilson, 2018; Kaniusas et al., 2019b, 2019a; Yap et al., 2020). In this article, we will address crucial methodological aspects of tVNS and provide preliminary results of its acute and chronic use in POTS, with regards to its potential effectiveness on autonomic symptoms reduction and heart rate modulation.

3. Meta research

We reviewed current literature on the topic using the main scientific research engines: PubMed, UpToDate, Google Scholar, CiteSeer, GetCITED, Microsoft Academic Research, Bioline International, Directory of Open Access Journals, PLOS ONE, BioOne, Science and Technology of Advanced Material.

The following keywords were used: vagal nerve stimulation (VNS), VNS effects on cardiovascular system, VNS effects on heart rate and VNS effects on blood pressure. The initial search revealed 1103 papers.

After reviewing the abstracts, we used exclusion parameters such as duplicate articles, review articles, abstract only papers, animal model studies and non-English language published studies, to reduce the number of articles to 169.

During our analysis of those 169 original works, we ultimately focused on those presenting data on heart rate, blood pressure, heart rate variability, vagal, sympathetic, and inflammatory markers. These papers concerning the effects of VNS (26 in total) contained information on both tVNS and invasive VNS (iVNS) delivery. Results are summarized in Tables 1 (iVNS) and Table 2 (tVNS).

3.1. Invasive vagus nerve stimulation

Among the 9 articles exploring the iVNS technique, only 7 focused on the effects on heart rate (Barone et al., 2007; De Ferrari, 2014; Kamath et al., 1992; Nearing et al., 2021; Ronkainen et al., 2006; Setty et al., 1998; Sperling et al., 2010). Out of these, 3 articles successfully provided evidence of mean heart rate decrease after stimulation (De Ferrari, 2014; Nearing et al., 2021; Sperling et al., 2010) while the remaining 4 studies described no changes in mean heart rate after iVNS (Barone et al., 2007; Kamath et al., 1992; Ronkainen et al., 2006; Setty et al., 1998). It is important to emphasize that all three studies with evidence of decreased heart rate during stimulation included patients with heart failure or depression, whereas the negative studies dealt with subjects affected by epilepsy. Moreover, only one article investigated the effect of VNS on blood pressure (De Ferrari, 2014): no changes in blood pressure following iVNS were observed. We could not find studies on the effects of iVNS on muscle sympathetic nerve activity (MSNA).

The effects of iVNS on heart rate variability were addressed in 6 articles (Barone et al., 2007; Bonaz et al., 2016; Kamath et al., 1992; Nearing et al., 2021; Ronkainen et al., 2006; Setty et al., 1998). Only one showed changes in heart rate variability parameters, namely a decrease in ratio of low and high frequency power (LF/HF) of heart rate variability after iVNS (Kamath et al., 1992).

The possible effect of iVNS on inflammation has been investigated in 3 studies (Barone et al., 2007; Bonaz et al., 2016; Koopman et al., 2016). Two of them showed a decrease in the inflammatory markers (Bonaz et al., 2016; Koopman et al., 2016) while in the remaining one no effects were observed (Barone et al., 2007).

From the clinical standpoint, 5 articles focused on potential symptoms changes after iVNS (Barone et al., 2007; Bonaz et al., 2016; De Ferrari, 2014; Koopman et al., 2016; Sperling et al., 2010). Out of those, 4 studies showed a decrease in symptoms intensity (Barone et al., 2007; Bonaz et al., 2016; De Ferrari, 2014; Koopman et al., 2016), whereas one investigation showed no change in disease-related symptoms (Sperling et al., 2010). Importantly, symptoms changes refer to the different diseases taken into account in each single study.

In two case reports patients with POTS underwent implantation of a vagal stimulator for the treatment of epilepsy (Early and Stankovic, 2018; von Wrede et al., 2019). Results of iVNS concerning the chronic effects of vagal stimulation are provided below.

3.2. Transdermal vagus nerve stimulation

Review of the literature about the effects of tVNS on heart rate showed a decrease in heart rate in 10 articles (Antonino et al., 2017; Badran et al., 2018; Clancy et al., 2014; Colzato et al., 2018; Gauthey et al., 2020; Paleczny et al., 2021; Stavrakis et al., 2015; Tobaldini et al., 2019; Zamotrinsky et al., 2001). In studies demonstrating a reduction in mean heart rate after tVNS, the stimulation frequency was set around 25-30 Hz. Only 5 papers presented data dealing with potential effects of tVNS on blood pressure (Antonino et al., 2017; Gauthey et al., 2020;

Table 2
Reported effects of transdermal vagal stimulation on autonomic cardiovascular parameters, inflammation, and symptoms in humans.

Author	Sample	Site & parameters	Main outcomes						
			HR	Vagal indices	LF/HF	BP	MSNA	Infl	Symp
Aranow et al. (2021)	SLE $N = 18$	Left concha/posterior ear 30 Hz, 300 μs pw, max tolerated	.	.	.	.	.	=	↓
Paleczny et al. (2021)	Healthy controls $N = 12$	25 Hz, 1000 μs/phase, 30 μs interphase interval,10 μA, 80% pain threshold	↓	=	=	=	.	.	.
Drewes et al. (2021)	Rheumatoid arthritis $N = 36$	Cervical vagus nerve 1 ms, 5 sine waves, 200us; 1burst/40 ms, 25 Hz, 60 mA, 24 V	.	↓	.	.	.	↓	↓
Gauthey et al. (2020)	Healthy males $N = 28$	Right cymba 5-20 Hz, 200 μs pw, sensory perception Right Lobe (sham): 5 Hz, 200 μs pw, sensory perception	↓	=	↑	=	↓	.	.
Borges et al. (2019)	Healthy controls $N = 61$	Left cymba conchae 25 Hz, 200–300 μs, 0.1-1.0 mA, on-off 30s cycle, different intensities	.	↑	.	.	.	.	.
Tobaldini et al. (2019)	Healthy controls $N = 13$	Left cymba conchae 25 Hz, 200 μs pw, 1-6 mA	↓	=	=	=	.	.	.
Badran et al. (2018)	Healthy controls $N = 15$	Left tragus 1/10/25 Hz; 100/200/500 μs; 60s; 200% pain threshold	↓	.	.	.	.	.	.
Colzato et al. (2018)	Healthy males N = 32	Left cymba conchae 0.5 mA 200–300 μs at 25 Hz, 30 min	↓	.	.	.	.	.	.
Antonino et al. (2017)	Healthy males N = 13	Left tragus 30 Hz, 200 μs pw 10-50 mA	↓	=	↓	=	.	.	.
De Couck et al. (2017)	Healthy controls $N = 30$	Left cymba conchae vs right cymba conchae 25 Hz, 250 μs pw, ±1 mA 30s; 50% pain threshold	.	↑	↑	.	.	.	.
Wang et al. (2015)	Myocardial Infarction $N = 42$	Left tragus vs right tragus 30 Hz, 200 μs pw, 10-50 mA	.	.	↓	.	.	.	.
Stavrakis et al. (2015)	Atrial Fibrillation N = 42	Right tragus 20 Hz; 250-200 ms/cycle	↓	.	.	.	.	↓	.
Clancy et al. (2014)	Healthy controls $N = 48$	*Tragus* 30 Hz, 200 μs pw, 10-50 mA	↓	=	↓	.	↓	.	.
Popov et al. (2013)	Coronary artery disease N = 48	Bilateral cymba concha 3 Hz, 0.2-1.5 mA, 1.5 ms,	↓	.	.	.	.	.	↓
Rong et al. (2012)	Depression $N = 49$	Cymba concha 20 Hz, 1 mA	.	.	↓	.	.	.	.
Zamotrinsky et al. (2001)	Preoperative coronary artery disease $N = 38$	Bilateral cymba concha 0.2-1.5 mA, 1.5 ms, 3 Hz	↓	.	.	↓	.	.	↓
Zamotrinsky et al. (2001)	Preoperative coronary artery disease $N = 20$	Bilateral cymba concha 0.2-1.5 mA, 1.5 ms, 3 Hz	.	.	.	.	.	.	↓

HR, heart rate; LF/HF, ratio between the low and high frequency components of heart rate variability; BP, blood pressure; MSNA, muscle sympathetic nerve activity; Inflam, inflammation; Symp, symptoms; pw, pulse width; ↑ increased; = unchanged; ↓ decrease.

Paleczny et al., 2021; Tobaldini et al., 2019; Zamotrinsky et al., 2001). Out of them, four studies demonstrated a clear effect of tVNS on blood pressure (Antonino et al., 2017; Gauthey et al., 2020; Paleczny et al., 2021; Tobaldini et al., 2019) while one showed a decrease in blood pressure (Zamotrinsky et al., 2001).

The effects of tVNS on heart rate variability were studied in 8 different investigations (Antonino et al., 2017; Clancy et al., 2014; De Couck et al., 2017; Gauthey et al., 2020; Paleczny et al., 2021; Rong et al., 2012; Tobaldini et al., 2019; Wang et al., 2014). Out of these, two studies showed no change under electrical stimulation (Paleczny et al., 2021; Tobaldini et al., 2019), two studies showed an increase in parameters reflecting cardiac vagal modulation (De Couck et al., 2017; Gauthey et al., 2020) whereas the remaining four studies showed a decrease in the same vagal related indices (Antonino et al., 2017; Clancy et al., 2014; Rong et al., 2012; Wang et al., 2014). Furthermore, tVNS was associated with a decrease in the LF/HF ratio in two different studies when the electrical stimulation was applied on the tragus (Antonino et al., 2017; Clancy et al., 2014), while two studies showed an increase in the LF/HF ratio when the electrical stimulus was delivered at the cymba site (De Couck et al., 2017; Gauthey et al., 2020).

tVNS and direct recordings of muscle sympathetic nerve activity (MSNA) were investigated in two studies only. Both found a decrease in MSNA (Clancy et al., 2014; Gauthey et al., 2020).

The effects of tVNS on inflammatory marker plasma titers were addressed in three studies. One study observed no effects (Aranow et al., 2021). The other two investigation showed a decrease of inflammatory markers (Drewes et al., 2021; Stavrakis et al., 2015).

Regarding the potential changes induced by tVNS on pain and related symptoms intensity, five studies found a significant decrease of symptomatology after tVNS (Zaotrinsky et al., 1997; Zamotrinsky et al., 2001; Aranow et al., 2021; Drewes et al., 2021).

In summary, our meta research suggests that vagal stimulation may have beneficial effects on symptom intensity and may reduce mean heart rate and plasma titers of systemic inflammation biochemical markers in different disorders. Presently, these aspects have never been considered in a unitary and systematic manner in POTS.

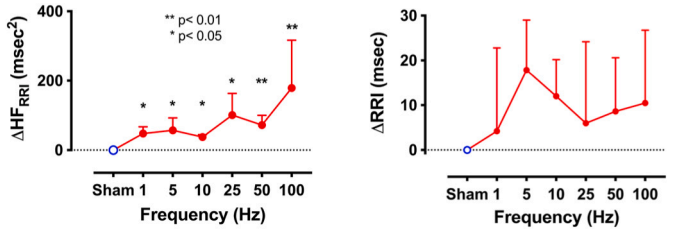

Fig. 3. Frequency response of high frequency component of heart rate variability (HF RRI) and R-R intervals (RRI) to randomized transdermal stimulation of the right auricular branch of Vagus nerve at subsensory levels in patients with POTS and low resting HF component. Values are expressed as mean ± SD.

4. Methodology of transdermal vagus nerve stimulation

4.1. Site of stimulation

Although cardiac parasympathetic innervation seems to be equally distributed between the right and left vagus nerves, experiments performed on conscious dogs (Ardell and Randall, 1986) showed that low-voltage electrical stimulation of the right vagus nerve produced a greater degree of bradycardia (Randall et al., 1986).

These findings were later supported by human studies reporting that 1) neuro-cardiovascular sympathetic interactions are predominantly modulated by the right cerebral hemisphere and 2) the expression of peripheral vascular sympathetic activity exhibits right predominance, at least in right-handed healthy individuals (Diedrich et al., 2009). Evidence suggesting right-sided lateralization of autonomic functions is crucial, as the choice of a specific stimulation site may be necessary to achieve specific therapeutic effects.

In addition, the finding of diverging changes in the indices of autonomic activity observed in previous studies may highlight the importance of the site of stimulation, for example the cymba conchae versus the tragus (Antonino et al., 2017; Clancy et al., 2014; De Couck et al., 2017; Gauthey et al., 2020). This suggests that site specificity must be taken into account when planning the stimulation procedure. However, a systematic methodological study of this issue is still lacking.

4.2. Stimulation mode

Frequency and pulse width are important variables of vagal stimulation on brain activation. Mu et al. (2004) found that a short pulse width of 130 μs produced significantly less overall activation in the human brain than longer pulse widths of 250 μs and 500 μs. Several authors used transdermal stimulation at frequencies from 1 to 30 Hz and pulse width from 250 to 1000 μs. The strength of stimulation (current in mA) also plays an important role (Tables 1 and 2). Stimulation can be delivered at subsensory level, sensory level, or maximal tolerance. Stimulation at 25 Hz, 250 μs pulse width, 30 s on/30 s off, for 4 h a day are typically applied to reduce the number of seizures in patients with epilepsy (Hamer and Bauer, 2019). It is unknown what stimulation mode might be optimal for patients with POTS.

4.3. Optimal stimulation frequency to enhance cardiac vagal modulation

Diedrich et al. systematically studied the response to stimulation with different frequencies on heart rate variability using randomized transdermal electrical stimulation of the right auricular branch of the vagus nerve at subsensory levels in 14 patients with POTS (age 31 ± 12 years, BMI 22.6 ± 3.9 kg/m^2) (Diedrich et al., 2018). The sensory threshold was determined by increasing the current intensity (starting from 0 up to 4.5 mA) until the subject sensed a tingling sensation without pain and discomfort. This process was repeated to validate the threshold value. The sub-sensory threshold was determined by lowering the current until the stimulation was not felt by the subject. The threshold was verified when the subject could no longer distinguish between no stimulation (sham) and a sub-sensory stimulation. A protocol consisting of 5-minute recording blocks of subsensory stimulations at stimulation frequencies between 1 and 100 Hz (rectangular waveform, pulse width 300 μs, length 1 ms) was performed in randomized order in the supine resting position. This approach allowed us to control for any placebo and time effects of stimulation. Fig. 3 shows that the high frequency band (HF, 0.15-0.4 Hz) of heart rate variability (HRV) obtained by power spectrum analysis increased with stimulation frequency. There was a non-significant prolongation of mean R-R interval. Blood pressure did not change. This pilot study showed that it is possible to increase cardiac vagal modulation, as assessed by the HF component of RR variability, during transdermal stimulation in POTS.

4.4. Safety and adverse effects

Early et al. reported a case of a 58-year-old female patient who experienced sensorineural hearing loss (SNHL) after prolonged application of vagal transdermal stimulation. SNHL reversed to normal hearing after discontinuing the use of the vagal stimulator. This adverse effect could be caused by comorbidities of preexisting nonfluctuating SNHL. It was also discussed that SNHL was caused by a small current imbalance of the bipolar stimulation (Early and Stankovic, 2018). Other data obtained in patients without a history of cardiac disease and suffering from tinnitus suggest that long-term tVNS application of up to 6 months may be considered safe (Kreuzer et al., 2014).

4.5. Comparison between invasive vagal stimulation and transdermal vagal stimulation

Common invasive vagal nerve stimulation (iVNS) therapy requires the surgical implantation of electrodes, which seems safe and well tolerated. However, adverse events such as infection and dysrhythmias during the surgical procedure or dysrhythmias, voice alteration, paresthesia, cough, headache, dyspnea, pharyngitis and pain during stimulation have been reported (Beekwilder and Beems, 2010; Ben-Menachem, 2001; Ben-Menachem et al., 1994). Non-invasive transdermal vagal stimulation (tVNS) is an alternative delivery option that eliminates the need for surgical implantation and its associated risks. tVNS eliminates the adverse effects related to *on–off* stimulation cycles of implantable devices (Goadsby et al., 2014; Jürgens and Leone, 2013) and is a less expensive procedure. tVNS could be an alternative treatment in patients with comorbidities that exclude them from surgical procedures.

The vagal nerve consists of 80% afferent and 20% efferent fibers (Grimonprez et al., 2015). Therefore, iVNS has direct efferent effects on end organs (i.e.., heart) and direct afferent effects to the brain. Auricular tVNS has mainly direct afferent effects to the brain.

Efficacy cannot be compared between the two modalities of VNS stimulation at the current time as the delivery systems are in different stages of development. One common observation for implanted VNS is a consistent improvement over a period of about 18 months (Ben-Menachem, 2001; Morris et al., 2013; Nahas et al., 2005; Ryzí et al., 2013; Siddiqui et al., 2010). No long-term efficacy data are available for tVNS.

5. Clinical studies with tVNS in POTS

Presently, the following three clinical trials exploiting tVNS in POTS have been registered at clincaltrials.gov.

1. Transdermal Vagal Stimulation for POTS (NCT02281097, PI Diedrich, Vanderbilt University, Nashville, TN). This is a pilot study. In a randomized order, tVNS and sham stimulations are acutely applied. (Diedrich et al., 2018).

   Outcomes: Heart rate, heart rate variability and symptom intensity

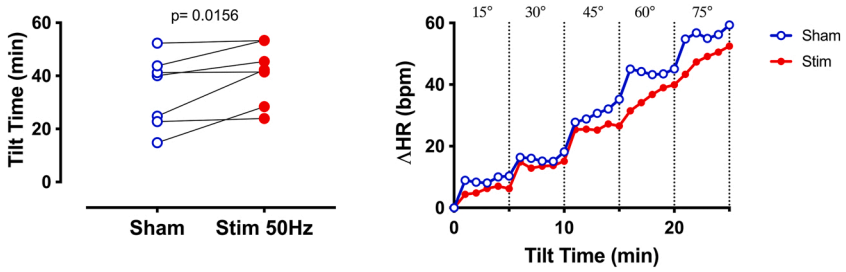

Fig. 4. Improvement of orthostatic intolerance (Tilt Time, left panel) and tendency of reduction of heart rate response (ΔHR) after transdermal stimulation of the right auricular branch of the vagus nerve at subsensory levels in patients with POTS and low resting HF component.

changes during graded stepwise 15° head-up tilt after tVNS or sham stimulation.

2. Vagal Stimulation in POTS: The Autonomic Inflammatory Reflex (Pilot 3) (NCT03124355, PI Biaggioni, Vanderbilt University, Nashville, TN). This clinical trial applies tVNS with placebo or in combination with two medications (galantamine and pyridostigmine) acutely in POTS.

   Outcomes: The effect on orthostatic response on symptoms and inflammatory markers plasma titers are studied. Modification in the high frequency component of heart rate variability during head-up tilt, before and after intervention.

3. Long-term Effects of Transcutaneous Vagus Nerve Stimulation on Postural Orthostatic Tachycardia Syndrome (POTS-VAG) (NCT04632134 Study Director Raffaello Furlan, PI Dana Shiffer, Humanitas Research Hospital, Rozzano, Italy).

   This clinical trial aims to apply tVNS chronically, i.e. for 4 h a day for 14 days, in POTS. The modifications induced by tVNS in the response of heart rate, blood pressure and MSNA to a gravitational challenge (15° stepwise head-up tilt, up to 75°) are under investigation.

   Outcomes: changes in symptom intensity, in the hemodynamic and in the autonomic profile (i.e., in heart rate variability, plasma catecholamines titers and MSNA).

6. Acute stimulation in POTS

While most researchers focus on the hyperadrenergic features of POTS, there is evidence of parasympathetic cardiovagal impairment in these patients. For instance, a diminished vagal marker of heart rate variability and abnormalities in the cardiovagal component of the Composite Autonomic Scoring Scale (CASS) have been previously reported (Diedrich et al., 2018; Jacob et al., 2019; Okamoto et al., 2015) A rational hypothesis would be that transdermal electrical stimulation of the auricular branch of the vagus nerve will enhance cardio-vagal modulation, reduce heart rate and upright symptoms, and improve orthostatic tolerance in POTS.

Diedrich et al. (2018) studied 14 patients with POTS. Sham or transdermal electrical vagal stimulation below perception threshold was applied in random order to the auricular branch in the right ear while supine and during a graded tilt. Patients with low vagal modulation (high frequency $HF_{RR} < 200$ ms^2) responded to vagal stimulation (Kruskal Wallis $p = 0.01$, $n = 7$) with significant increase in HF power where the most consistent effect was found at 50 Hz (Fig. 2, stimulation at 50 Hz, delta: $+51 \pm 10$ ms^2, $p = 0.0032$). Vagal stimulation during upright tilt tended to reduce orthostatic tachycardia and the overall orthostatic symptom score. tVNS improved tilt time significantly (delta: $+5.3 \pm 2.6$ min, $p = 0.0156$) and there was a tendency toward blunted heart rate increase during standing (Fig. 4). Patients with higher baseline vagal modulation (HF ≥ 200 ms^2) did not respond to vagal stimulation (interaction $p = 0.41$). This proof-of-concept study indicates that auricular transdermal vagal stimulation improves supine cardio-vagal function in POTS patients with low vagal modulation. Further research will determine if this approach can be used therapeutically, alone or in combination with other therapies.

7. Chronic stimulation in POTS

Previous studies reported that acute stimulation could improve orthostatic tolerance in POTS (Diedrich et al., 2018; Shiffer et al., 2019). However, it is still unestablished whether such a positive outcome persists for a longer time period and if chronic application would be effective without the occurrence of major side effects. Initial attempts evaluating the clinical effectiveness of chronic stimulation in POTS were based on the use of *implanted Vagus nerve stimulators*. In this context, Lankford et al. (2015) reported the case of a nine-year-old female with history of intractable epilepsy, dysautonomia and developmental delay related to confirmed genetic abnormalities. The patient underwent implant of the vagus stimulator for treating epilepsy. An improvement in dysautonomia symptoms with the stimulator switched on was found. Importantly, these beneficial effects were abolished when the stimulator was turned off (Lankford et al., 2015). Gazde and colleagues (Petelin Gadze et al., 2018) reported the case of a patient with resistant epilepsy, who was referred for the iVNS procedure. As part of pre-operative workup, a head-up tilt was performed showing orthostatic symptoms and excessive tachycardia consistent with a possible POTS diagnosis. A second tilt test, performed after Vagus nerve stimulator implantation, showed disappearance of orthostatic intolerance symptoms. These case reports indicate the potential effectiveness of Vagus stimulation in improving orthostatic intolerance symptoms.

After evaluating the acute effects of tVNS on orthostatic tolerance and mean heart rate increment during orthostatic stress (Shiffer et al., 2019), Shiffer et al. expanded their study to evaluate the effects of a *chronic* right Vagus nerve stimulation in patients affected by the hyperadrenergic type of POTS. They studied 9 patients undergoing chronic electrical tVNS of the right cymba conchae by means of the Nemos© device (Cerbomed, Germany). Subjects were enrolled and evaluated at baseline (PRE) by continuously recording the ECG, non-invasive beat-by-beat blood pressure, respiratory activity both at rest and during a 75° head-up tilt test. Thereafter, patients underwent a stimulation protocol consisting of 1-hour stimulation blocks, 4 times a day, for 14 days. After completion, they came back for a follow-up evaluation (POST) identical to PRE.

Notably, stimulation was applied using a pulse width of 200 μs and a squared impulse waveform with the stimulator switched on for 30 s and off for 30 s. The stimulation frequency was 25 Hz and the electrical current was set to the highest intensity without causing patient discomfort (population mean 1.8 mA ± 0.2).

A strength of this study is that the authors objectively quantified the whole spectrum of dysautonomia symptoms POST vs PRE using the validated Composite Autonomic Symptom Score (COMPASS-31) tool. Briefly, the COMPASS-31 scoring system yields individual autonomic domains symptoms burden and a total score, which is considered as a general summary index. Single domains and total score are expressed as a percentage from 0 to 100, the lowest indicating symptom absence and

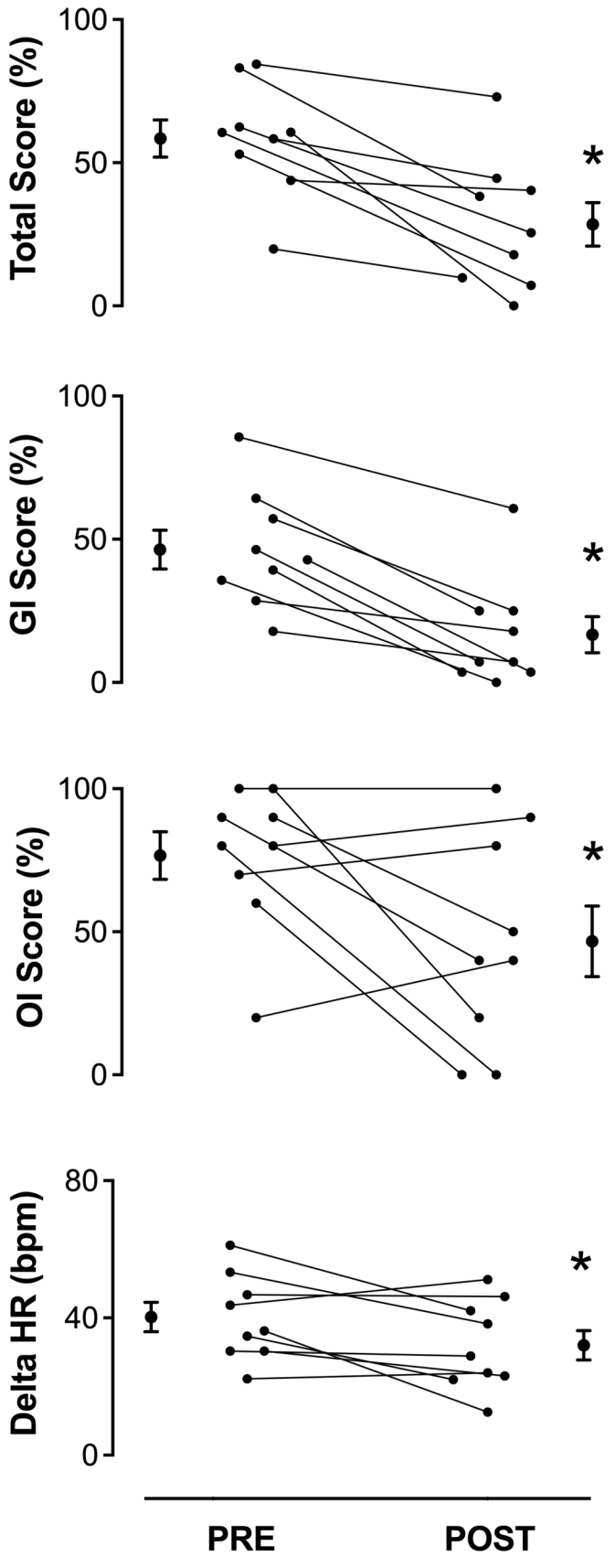

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Fig. 5. Dysautonomia symptoms and HR changes (Delta) after chronic tVNS. Individual values of COMPASS-31 domains and delta HR after 14-day tVNS are shown together with their mean ± SEM values. The reduction in dysautonomia symptom intensity is significant in Total Score and in Orthostatic Intolerance and Gastrointestinal symptom domains. The individual difference between supine and orthostatic heart rate was blunted, although not significantly, during POST evaluation. GI indicates gastrointestinal domain. OI, orthostatic intolerance. Delta HR, mean value of the individual differences between orthostatic and clinostatic heart rate. PRE, pre-treatment. POST, post-treatment. *$P < 0.05$ POST vs PRE; data are expressed as mean ± SEM.

the highest indicating the greatest symptom intensity.

Analysis of POST vs PRE questionnaires revealed a statistically significant reduction ($P < 0.05$) of orthostatic intolerance and gastrointestinal symptom domains percentages and of total COMPASS score, after chronic tVNS. Fig. 5 shows individual and mean values of COMPASS-31 domains and total score before and after treatment. In addition, the effects of vagal stimulation on heart rate were assessed as mean heart rate difference between the 75° head-up tilt and the supine position. Of note, after 14 days stimulation, gastrointestinal, orthostatic and total scores were lower than those observed at baseline condition, suggesting the efficacy of chronic tVNS in decreasing symptoms intensity. Moreover, the magnitude of increase in heart rate induced by the upright posture tended to be blunted after chronic tVNS compared to baseline, although this finding was not statistically significant. These results are encouraging but will need to be validated by randomized controlled studies.

8. Summary

In this paper, we performed a systematic review that addressed the topic of non-invasive tVNS as an emerging tool targeting the vagal impairment and hyperadrenergic state occurring in POTS (Furlan et al., 2000; Okamoto et al., 2015). In addition, activation of the vagus nerve may theoretically decrease the concomitant chronic inflammatory state by activating the cholinergic anti-inflammatory pathway (Bonaz et al., 2016; Drewes et al., 2021; Furlan et al., 2006; Koopman et al., 2016; Tracey, 2007). We highlighted that several variables such as stimulation site, pulse width, frequency and amplitude of the electric current are believed to critically influence efferent parasympathetic activation and therefore tVNS efficacy.

In addition, preliminary results from studies evaluating acute stimulation in POTS (Diedrich et al., 2018; Shiffer et al., 2019) suggest that tVNS may effectively increase cardiac vagal modulation in patients characterized by cardiovagal impairment. Of note, these findings are coupled with an increase in tilt tolerance time and an improvement in orthostatic intolerance symptoms.

Similarly, initial observations from an ongoing chronic tVNS study confirmed the cardiovascular modifications induced by acute tVNS. The study also showed that stimulation for 4 h/day for 14 days was associated with a reduction of overall dysautonomia symptoms. Chronic tVNS was well tolerated and it was not associated with significant side effects.

These promising results suggest that tVNS might be a useful and safe treatment tool in POTS with reduced vagal activity. Additional studies are necessary to explore its combination with existing treatments and to define optimal stimulation parameters.

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Transcutaneous auricular vagus nerve stimulation and heart rate variability: Analysis of parameters and targets

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ABSTRACT

Objectives: Transcutaneous auricular vagus nerve stimulation (taVNS) modulates central and peripheral neurophysiology. Specifically, taVNS increases heart rate variability (HRV) indicating a shift in autonomic function towards parasympathetic predominance. However, knowledge on the influence of stimulation parameters and targets is scarce. We hypothesized that the location and charge per phase of taVNS influences HRV.

Materials and methods: In thirteen healthy subjects, six different stimulation targets were investigated, i.e., cymba conchae, cavum conchae, outer tragus, inner tragus, crus helicis, and fossa triangularis. At each target, 24 parameter combinations were studied: Eight different electrical charges per phase were evaluated by investigating three pulse durations and eight charge-balanced current intensities, i.e., 100 μs (0.250-2 mA in steps of 0.250 mA), 260 μs (0.096–0.769 mA in steps of 0.096 mA), and 500 μs (0.050–0.400 mA in steps of 0.050 mA). In a parallel group design, left and right taVNS were compared to each other. 30 bursts at each parameter combination were applied with a periodicity of 1 Hz. Each burst consisted of five pulses applied at 25 Hz.

Results: HRV increased in a charge-dependent way with significant differences between the right and left ear. The targets with the strongest effects were the cymba conchae and fossa triangularis, and to a lesser extent the inner tragus.

Conclusions: HRV is suitable to define taVNS parameters and targets for research and therapeutic purposes. Bursts of taVNS with a pulse duration of 100 μs and a current intensity of 2 mA are comfortable for the participants and effective in increasing HRV when applied at specific auricular locations. These findings need to be replicated in larger cohorts, and with longer stimulation and off-periods between conditions. Since results may differ in conditions with an impaired autonomic tone, future studies should also consider aged and patient populations.

1. Introduction

Invasive vagus nerve stimulation (iVNS) is an approved treatment for pharmacoresistant epilepsy and depression, but may be associated with side effects and potential surgical complications. By contrast, electrical stimulation of the external ear, referred to as transcutaneous auricular vagus nerve stimulation (taVNS), is a non-invasive and well tolerated intervention that is currently investigated for its physiological and behavioral effects and potential therapeutic applications in neurological, psychiatric, cardiovascular, immunological and metabolic disorders (Redgrave et al., 2018). (Ta)VNS modulates central and peripheral neurophysiology and can induce, e.g., anti-depressive, anti-epileptic, cardiac and pain-modulating effects (Yap et al., 2020; Sinkovec et al., 2021; von Wrede and Surges, 2021). Autonomic changes may be captured by different measures such as functional magnetic resonance imaging (fMRI) (Kraus et al., 2013; Frangos et al., 2015; Yakunina et al., 2016; Badran et al., 2018b; Tu et al., 2018; Sclocco et al., 2019), electroencephalography (EEG) (Fallgatter et al., 2003; Leutzow et al., 2013; Hagen et al., 2014), electrocardiography (ECG) (Antonino et al., 2017; Badran et al., 2018c), microneurography (Clancy et al., 2014) and

Abbreviations: ABVN, auricular branch of the vagus nerve; ATN, auriculotemporal nerve; AVN, artrioventricular node; DMN, dorsal motor nucleus of the vagus; ECG, electrocardiography; EEG, electroencephalography; fMRI, functional magnetic resonance imaging; GAN, greater auricular nerve; HRV, heart rate variability; LON, lesser occipital nerve; NTS, nucleus of the solitary tract; SAN, sinoartrial; taVNS, transcutaneous auricular vagus nerve stimulation; VSEP, vagus somatosensory evoked potentials.

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pupillometry (Desbeaumes Jodoin et al., 2015).

Notably, there are still considerable uncertainties concerning the auricular stimulation targets (Badran et al., 2018a; Burger and Verkuil, 2018) and underlying mechanisms (Leutzow et al., 2013, 2014; Polak et al., 2014) that may limit the clinical application. As the vagal innervation of the heart is asymmetric, the stimulation side (right vs. left) needs to be specifically considered during cervical VNS for neuropsychiatric and cardiovascular indications (Capilupi et al., 2019); resulting in different cardiac effects, i.e., reduction in heart rate (negative chronotropic action on the sinoartrial node (SAN) with right stimulation), and reduction in artrioventricular conduction (negative dromotopic action on the artrioventricular node (AVN) with left stimulation). For VNS of the auricular branch of the vagus nerve (ABVN), studies indicate less influence of the stimulation side, since the afferent input is likely integrated at the level of the brainstem in the nucleus of the solitary tract (NTS) and dorsal motor nucleus of the vagus (DMN) before leaving the central nervous system on the right and left side of the body (Chen et al., 2015). However, there is still a debate about the influence of the stimulation side during taVNS.

Moreover, it is still an open question which auricular stimulation locations (Yakunina et al., 2016) and parameters (Badran et al., 2018c) are best suited for modulation of the autonomic nervous system. In this context, changes of heart rate variability (HRV) during auricular stimulation have been revealed as a suitable biomarker and indicated a shift in autonomic function towards parasympathetic predominance (Clancy et al., 2014; De Couck et al., 2017; Sclocco et al., 2019). We hypothesized that the location and charge per phase of taVNS influences its impact on HRV. To research this hypothesis, we systematically varied auricular stimulation targets and parameters.

2. Material and methods

2.1. Subjects

Thirteen healthy subjects (age = 24 ± 3 [mean ± SD], 8 female) took part in this study. All participants were right-handed and reached a score equal or above 75 in the Edinburgh Handedness Inventory. None of the subjects had any history of habitual drug or alcohol consumption, cognitive or psychiatric impairments or neurological disorders. Furthermore, patients were asked to avoid substances that alter autonomic activity (e.g., caffeine or alcohol) on the day of measurement for at least 2 h before the examination. Subjects gave their written informed consent before participation and were not compensated for their participation. The study was approved by the local ethics committee of the medical faculty of the University of Tuebingen.

2.2. Experimental procedure

In a parallel group design, participants were pseudo-randomized in two groups (stimulation at right [$n = 7$; age = 24.3 ± 2.8; 3 females] and left ear [$n = 6$; age = 24 ± 3.1; 5 females]) and participated in one session. In each session, we investigated six different stimulation targets, i.e., cymba conchae, cavum conchae, outer tragus, inner tragus, crus helicis, and fossa triangularis (Fig. 1). Subjects were seated comfortably and upright in a reclining chair. At each stimulation target, we started with the lowest electrical charge per phase in three different charge-balanced parameter combinations [i.e., 100 μs (0.250 mA), 260 μs (0.096 mA), and 500 μs (0.050 mA)] using symmetrical pulses. This resulted in 90s (i.e., 3 × 30s) of stimulation at the same charge per phase and stimulation target. Then, the next higher charge was applied [i.e., 100 μs (0.500 mA), 260 μs (0.192 mA), and 500 μs (0.100 mA)]. After applying 2–3 different charges at one target, there was a short break of around 1 min before moving to the next target. After stimulating all targets with the same charges, the next stimulation round with higher charges ensued. Altogether, we studied six targets with eight different charges by investigating three pulse durations (100 μs, 260 μs, and 500 μs) and eight charge-balanced current intensities, i.e., 0.250-2 mA in steps of 0.250 mA with 100 μs, 0.096–0.769 mA in steps of 0.096 mA with 260 μs, and 0.050–0.400 mA in steps of 0.050 mA with 260 μs. At each of these altogether 144 parameter combinations, we applied 30 bursts at a periodicity of 1 Hz. Each burst consisted of five pulses at 25 Hz (Fig. 2). We used a multichannel stimulator (STG-4000 series, multichannel systems, Harvard Bioscience Inc.) that was triggered by a customized python program, and a bipolar spherical probe (GVB-geliimed GmbH) with a probe diameter of 2 mm and an inter-probe distance of 5 mm. During stimulation, the examiner holds the probe continuously at the respective stimulation site before moving to the next target. The examiner placed the arm on an armrest during stimulation to keep the application pressure and, thereby, also the current as consistent as possible. The participants were asked to indicate uncomfortable sensations whenever they occurred during stimulation. Stimulation was terminated, if the sensations became uncomfortable for the subjects. However, the stimulation intensity was not adjusted according to the individual perception threshold as applied in other studies (Clancy et al., 2014; De Couck et al., 2017; Badran et al., 2018d; Borges et al., 2020), because i) the adjustment was used very inconsistent in these studies and ii) our aim was to stimulate the Aβ-fibers and not the Aδ- and C-fibers, which primarily convey pain stimuli. The electrocardiography (ECG) signals were digitized at a sampling frequency of 1000 Hz (Brain Products Amplifiers, Brain Products GmbH, Gilching, Germany). In this study, we analyzed and report the heart rate variability (HRV) that was acquired from ECG recordings with electrodes at the sternum and below the left clavicle, and with the right olecranon as ground. HRV was analyzed individually for each stimulation condition (i.e., 30s).

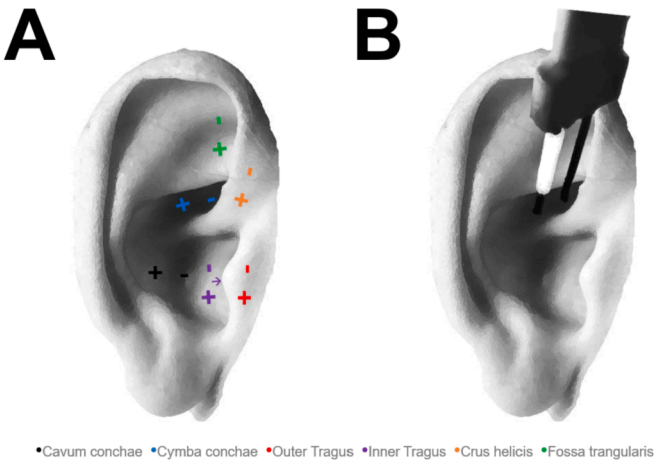

Fig. 1. Stimulation targets at the ear. (A) Stimulation targets at the ear: cavum conchae (black), cymba conchae (blue), inner tragus (purple), outer tragus (red), crus helicis (orange) and fossa triangularis (green); (B) stimulation probe at the cymba conchae. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

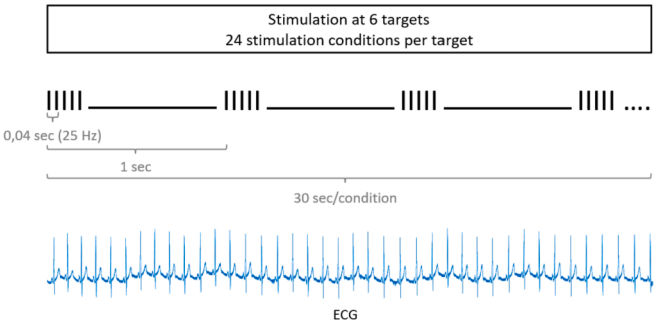

Fig. 2. Overview of the stimulation procedure.

2.3. Data analysis and statistics

Electrophysiological analyses and statistical tests were performed using MATLAB (MathWorks, Inc., Natick, MA, USA) and compatible toolboxes. After a manual visual artifact rejection, the heart rate and various parameters of heart rate variability (RR mean, SDNN, RMSSD, pNN50, SD1, SD2, rrHRV relative; Table 1) were determined for each of the subjects and conditions using the MarcusVollmer/HRV toolbox (Vollmer, 2019). Using this toolbox, a further artifact rejection was performed followed by the estimation of the HRV parameters described by Vollmer and colleagues; due to preprocessing and artifact rejection, some data values were rejected, which made the data not suitable for spectral HRV analysis (Vollmer, 2015). Subsequently, a mixed model ANOVA with the factors *target*, *current intensity*, *pulse duration*, *side of the stimulation* (right vs. left) and *subject* was carried out, followed by a second ANOVA, in which the factors *current intensity* and *pulse duration* were collapsed to a common factor *charge per phase*. Furthermore, in both analyses the order of stimulation conditions was considered as another factor to avoid order effects. *P*-values <0.05 were considered significant. W-values were calculated as weighting factors; they correspond to the slope/coefficient of a linear equation and describe the effect and direction within the ANOVA (increasing/decreasing HRV). Finally, a Tukey-Kramer post hoc test was performed after the second ANOVA. We investigated differences between targets, charge per phase combinations and stimulation sites for SDNN and RMSSD findings which represent the overall HRV and short-term/parasympathetic activity, respectively. *P*-values <0.05 were considered significant.

3. Results

3.1. Adverse effects

Stimulation was performed in all participants without major adverse effects. In particular, there were no major cardiac incidents such as palpitation, bradycardia or arrhythmia. Temporarily, there was a slight redness at the stimulation site in a few subjects. Only at the highest charge levels, an unpleasant sensation at the stimulation site was occasionally reported, which was related rather to longer pulse durations than higher current intensities. As a result, the stimulation condition with the highest charge was not finished in one subject.

Table 1
Overview of different parameters of the heart rate variability.

Analysis	Parameter	Abbreviation	Description
Time domain measures	Standard deviation of normal-to-normal (NN/RR) interval	SDNN	Overall variability (short and long term; parasympathetic and sympathetic)
	Root Mean Square of Successive Differences	RMSSD	Short term HRV; parasympathetic
	Difference between consecutive RR-intervals with a difference of more than 50 ms	NN50	
	Proportion of NN50/ number of NN (RR) intervals	pNN50	(Ultra-)short term; parasympathetic
Non-linear domain measures	Standard deviation of the scatterplot perpendicular the line of identity	SD1	Short term; parasympathetic; correlated with RMSSD
	Standard deviation of the scatterplot along the line of identity	SD2	Long term; parasympathetic
	Relative RR interval	rrHRV relative	Short and long term

3.2. Influence of pulse duration, current intensity and charge on HRV

The pulse duration had a significant impact on the HRV parameters SDNN and SD2. With increasing pulse duration, there was a significant decrease of SDNN and SD2. Increasing the current intensity resulted in higher SDNN, pNN50, SD1 and SD2 (Tables 2 and 3), while increasing the charge per phase resulted in higher SDNN and SD2 (Tables 4 and 5). Tukey-Kramer post hoc analysis revealed no HRV differences at each stimulation site with different parameter combinations when the applied stimulation was charge-balanced with regard to current intensity and pulse width.

3.3. Influence of stimulation side on HRV

The stimulation side (right vs. left) showed a significant influence on SDNN, RMSSD, SD1 and SD2 ratio (Tables 2 and 4). Notably, the HRV parameters SDNN, RMSSD, SD1 and SD2, were increasing and decreasing after stimulation of the right and left ear, respectively (Tables 3 and 5). Post hoc analysis showed significant differences between right- and left-sided stimulation for the SDNN ($p < 0.001$, Tukey-Kramer) and RMSSD ($p < 0.001$; Tukey-Kramer).

3.4. Influence of stimulation location on HRV

HRV increases were related to stimulation locations at the cymba conchae and fossa triangularis, and to a lesser extent to stimulation at the inner tragus (Tables 5 and 6, Fig. 3). Post hoc analysis revealed that cymba conchae stimulation differed from cavum conchae (SDNN: $p = 0.002$, RMSSD: $p < 0.001$, Tukey-Kramer), outer tragus (SDNN: $p < 0.001$, RMSSD: $p = 0.002$, Tukey-Kramer) and crus helicis stimulation (SDNN: $p = 0.037$, RMSSD: $p = 0.100$, Tukey-Kramer) (Table 7). Fossa triangularis stimulation differed from cavum conchae (SDNN: $p = 0.041$, RMSSD: $p = 0.018$, Tukey-Kramer) and outer tragus stimulation (SDNN: $p = 0.003$, RMSSD: $p = 0.076$, Tukey-Kramer). Inner tragus stimulation differed from cavum conchae (SDNN: $p = 0.301$, RMSSD: $p = 0.040$, Tukey-Kramer) and outer tragus stimulation (SDNN: $p = 0.049$, RMSSD: $p = 0.142$, Tukey-Kramer).

4. Discussion

This study showed that location, charge per phase and stimulation side of transcutaneous electrical stimulation of the external human ear determine its influence on HRV.

4.1. Auricular stimulation parameters

In previous work that investigated stimulation parameters (Badran et al., 2018c), 60s blocks of 1 Hz, 10 Hz and 25 Hz stimulation were applied to the tragus at pulse durations of 100 μs, 200 μs, and 500 μs and mean intensities (derived in relation to the perceptional level) of 9.28 ± 2.56 mA, 5.32 ± 1.60 mA, and 3.0 ± 0.93 mA, respectively. Larger effects on heart rate were observed for higher frequencies (10 Hz, 20 Hz) and pulse durations (500 μs). Since a larger charge per phase (i.e., product of pulse duration and current intensity) was applied in the 500 μs condition as compared to the 100 μs and 200 μs conditions, the effects of shorter, charge-balanced pulse durations (e.g., 100 μs), i.e., with proportionally higher current intensities remained to be clarified. In the present study, we demonstrated charge-dependent HRV increases for all investigated pulse durations when the current intensities were proportionally adapted to achieve equal charges per phase for the different conditions. Notably, shorter pulse durations were more effective than longer ones, when charge-balanced stimulation was applied. Since different nerve fiber types (A-fibers [~1-22 μm diameter], B-fibers [≤1.5-3 μm diameter], C-fibers [~0.2–1.5 μm diameter]) may be activated by different pulse durations (Helmers et al., 2012), varying this parameter (with corresponding changes of current intensity) might be a

Table 2
Influence of pulse duration and current intensity (ANOVA: P-values; factors target, pulse duration, intensity, stimulation side and subject).

	Mean RR	HR	rrHRV relative	SDNN	RMSSD	pNN50	SD1	SD2	SD1/SD2 ratio
Target	0,0000*	0,1744	0,5560	0,0000*	0,0000*	0,0002*	0,0000*	0,0000*	0,5263
Pulse duration	0,3200	0,2119	0,2788	0,0005*	0,1283	0,1932	0,0783	0,0003*	0,0225*
Current intensity	0,2678	0,0788	0,0697	0,0045*	0,0507	0,0411*	0,0402*	0,0353*	0,4947
Stimulation side	0,0000*	0,0000*	0,8714	0,0053*	0,0000*	0,2446	0,0000*	0,0325*	0,0000*
Subject	0,0000*	0,0000*	0,0360	0,0000*	0,0000*	0,0000*	0,0000*	0,0000*	0,0000*

*<0.05.

Table 3
Influence of pulse duration and current intensity (ANOVA: W-values; factors target, pulse duration, intensity, stimulation side and subject).

	Mean RR	HR	rrHRV relative	SDNN	RMSSD	pNN50	SD1	SD2
Cavum conchae	**-20,6654**	**1,2515**	**-0,9024**	−3,0286	**−2,8621**	**−2,1854**	**−2,0834**	−3,3279
Cymba conchae	**14,6858**	**−1,4146**	−0,4690	**5,4689**	**3,1435**	**2,3760**	**2,1654**	**6,7199**
Outer tragus	−8,9840	0,3867	**1,5038**	**−5,3359**	−2,1908	−1,8750	−1,5955	**−7,0320**
Inner tragus	12,0480	0,5956	−0,5680	1,2513	1,0932	1,0490	1,0311	2,1473
Crus helicis	−8,4443	0,4696	−0,6980	−1,5048	−0,5396	−0,6966	−0,4252	−2,4139
Fossa triangularis	11,3598	−1,2888	1,1337	3,1492	1,3559	1,3320	0,9075	3,9065
Pulse duration	−3,6206	0,7646	−0,8808	−3,5911	−0,9516	−0,7619	−0,7897	−4,9884
Current intensity	0,0063	−0,0017	0,0023	0,0045	0,0019	0,0019	0,0014	0,0045
Left stimulation	−23,1760	1,8872	0,0798	−1,7374	−2,1896	−0,4132	−1,5505	−1,7810
Right stimulation	23,1760	−1,8872	−0,0798	1,7374	2,1896	0,4132	1,5505	1,7810

W-values: weighting factors, which describe the effect and directions within the ANOVA (increasing/decreasing HRV).
Bold numbers mark the targets with the most positive and negative values, respectively.

Table 4
Influence of charge per phase (ANOVA: P-values; factors target, total charge, stimulation side and subject).

	Mean RR	HR	rrHRV relative	SDNN	RMSSD	pNN50	SD1	SD2
Target	0,0000*	0,2149	0,8222	0,0000*	0,0000*	0,0000*	0,0000*	0,0000*
Charge per phase	0,8651	0,5930	0,7094	0,0118*	0,2563	0,4247	0,3329	0,0305*
Stimulation side	0,0000*	0,0000*	0,9278	0,0033*	0,0000*	0,1189	0,0000*	0,0218*
Subject	0,0000*	0,0000*	0,0378*	0,0000*	0,0000*	0,0000*	0,0000*	0,0000*
Target × charge per phase	1,0000	0,2475	0,7229	0,9411	0,9977	0,9967	0,9974	0,7733
Target × charge per phase × stimulation side	1,0000	0,0610	0,8274	0,9975	1,0000	1,0000	1,0000	0,9881

*<0.05.

Table 5
Influence of charge per phase (ANOVA: W-values; with factors target, total charge, stimulation side and subject).

	Mean RR	HR	rrHRV relative	SDNN	RMSSD	pNN50	SD1	SD2
Cavum conchae	**−20,4314**	1,1360	**−0,7445**	−3,4353	**−2,9718**	**−2,2972**	**−2,1442**	−4,0763
Cymba conchae	**14,0362**	**−1,5318**	−0,2785	**5,4696**	**3,1730**	**2,4774**	**2,1998**	**6,8950**
Outer tragus	−9,2014	0,2379	**1,0704**	**−5,2369**	−2,3279	−1,8094	−1,6835	**−6,8299**
Inner tragus	13,2782	**1,2805**	−0,4098	1,2776	1,1916	1,1183	1,0304	1,9579
Crus helicis	−8,4810	0,2833	−0,5280	−1,3771	−0,5248	−0,8068	−0,3953	−2,1417
Fossa triangularis	10,7995	−1,4059	0,8904	3,3021	1,4600	1,3178	0,9928	4,1949
Left stimulation	−23,5246	2,0921	0,0451	−1,6868	−2,1557	−0,4436	−1,5145	−1,8025
Right stimulation	23,5246	−2,0921	−0,0451	1,6868	2,1557	0,4436	1,5145	1,8025

W-values: weighting factors, which describe the effect and directions within the ANOVA (increasing/decreasing HRV).
Bold numbers mark the targets with the most positive and negative values, respectively.

Table 6
Impact of the stimulation target.

	rrHRV relative	SDNN	RMSSD	pNN50	SD1	SD2
Cavum conchae	↓	↓	↓	↓	↓	↓
Cymba conchae	↓	↑	↑	↑	↑	↑
Outer tragus	↑	↓	↓	↓	↓	↓
Inner tragus	↓	↑	↑	↑	↑	↑
Crus helicis	↓	↓	↓	↓	↓	↓
Fossa triangularis	↑	↓	↑	↑	↑	↑

suitable approach to more selectively disentangle and modulate specific auricular nerves in future studies. Longer (as compared to short) pulse durations allow for less selective fiber recruitment, i.e., the combination of 500 μs pulse duration and 1.5 mA current intensity activated all fibers with a diameter of ≥1 μm, whereas a shorter pulse duration allowed a better threshold selection between different nerve fibers (Gorman and Mortimer, 1983; Helmers et al., 2012). Specifically, larger diameter fibers, which also mediate the VNS effects, will already be activated with shorter pulse durations. Therefore, a pulse duration of 100 μs may be both more selective and better tolerable during taVNS.

Notably, the current intensities applied in the present study during the 30s stimulation blocks, i.e., a maximum of 2 mA, 0.769 mA and 0.400 mA (for pulse durations of 100 μs, 260 μs and 500 μs, respectively), were relevantly lower than those used in previous work; and may allow the stimulation of a more specific auricular area and a better tolerability of the intervention. This effectivity of lower current intensities might be related to the burst stimulation pattern that was

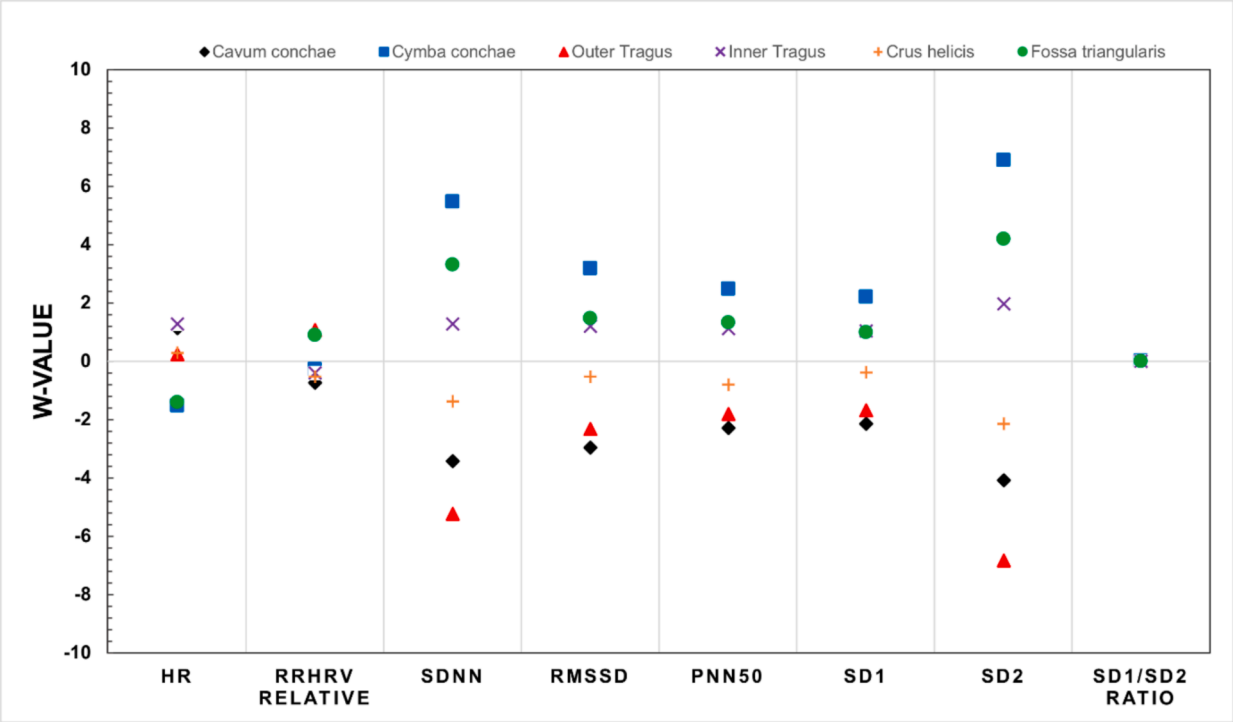

Fig. 3. Comparison of different VNS targets and HRV parameters.

Table 7
Pairwise post hoc analysis.

Targets		SDNN		RMSSD	
		p-Value	Difference group means	p-Value	Difference group means
Cavum conchae	Cymba conchae	**0,002**	**−8,810**	**0,000**	**−6,123**
Cavum conchae	Outer tragus	0,971	1767	0,997	−0,691
Cavum conchae	Inner tragus	0,301	−4,691	**0,040**	**−4,125**
Cavum conchae	Crus helicis	0,940	−2,098	0,497	−2,471
Cavum conchae	Fossa triangularis	**0,041**	**−6,619**	**0,018**	**−4,496**
Cymba conchae	Outer tragus	**0,000**	**10,577**	**0,002**	**5,432**
Cymba conchae	Inner tragus	0,453	4,120	0,716	1,998
Cymba conchae	Crus helicis	**0,037**	**6,712**	0,100	3,652
Cymba conchae	Fossa triangularis	0,929	2,192	0,860	1,627
Outer tragus	Inner tragus	**0,049**	**−6,458**	0,142	−3,435
Outer tragus	Crus helicis	0,528	−3,865	0,806	−1,780
Outer tragus	Fossa triangularis	**0,003**	**−8,386**	0,076	−3,806
Inner tragus	Crus helicis	0,864	2,592	0,851	1,655
Inner tragus	Fossa triangularis	0,958	−1,928	1,000	−0,371
Crus helicis	Fossa triangularis	0,352	−4,520	0,709	−2,026

Bold numbers demonstrate pariwise combinations of SDNN and RMSSD with significant p-values <0.05.

applied here in contrast to continuous protocols used previously. Specifically, standard auricular stimulation protocols apply cyclic stimulation (e.g., 30s STIM ON/30s STIM OFF) with a continuous stimulation frequency (usually 1 Hz or 25 Hz) during the 30s STIM ON blocks. We chose a pragmatic approach by combining 1 Hz and 25 Hz stimulation in a novel paradigm. Specifically, during the 30s STIM ON blocks, we applied short 25 Hz bursts at a periodicity of 1 Hz. This was inspired by previous experimental work that exposed the vagus nerve in the rabbit neck, and showed that intermitted (0.5-1 Hz) but not continuous (5–25 Hz) stimulation mimicked physiological vagal input to the heart (Iwao et al., 2000). However, future work in human subjects is necessary to compare these different stimulation approaches (intermittent vs. continuous stimulation) directly to each other in terms of central and peripheral neurophysiological effect sizes. In the current study design, the stimulation parameters were systematically increased, and the same stimulation intensities (and corresponding charges) were applied at all targets before moving to the next stimulation intensity level. This resulted in habituation effects, which were taken into account during the ANOVA analysis. Future studies, however, may avoid such order effects by applying the stimulation intensities (and according charges per phase) in randomized order.

4.2. *Auricular stimulation locations*

An anatomical study of the auricular nerve supply in human cadavers revealed that the ear is innervated by the lesser occipital nerve (LON), greater auricular nerve (GAN), auricular branch of vagus nerve (ABVN) and the auricotemporal nerve (ATN, i.e., branch of the trigeminal nerve) (Peuker and Filler, 2002). Auricular stimulation is supposed to activate the ABVN in order to exhibit its effects on central and peripheral neurophysiology. However, it remains unclear at which auricular locations the ABVN may be best targeted. The most consistent ABVN

innervation patterns were described for the cymba conchae (100%), antihelix (73%), cavum conchae (45%), and to a lesser extent the crus helicis (20%) and crura antihelicis (9%) (Peuker and Filler, 2002). The ABVN innervation of the tragus remains unclear (45% vs 0%) due to inconsistent reporting in the respective anatomical work (Peuker and Filler, 2002), which could not be clarified and initiated a debate (Badran et al., 2018a; Burger and Verkuil, 2018).

In this context, cymba conchae and tragus are currently the two most applied stimulation targets showing both physiological and behavioral effects (Fallgatter et al., 2003; Clancy et al., 2014; Yakunina et al., 2016; Badran et al., 2018b). However, direct comparisons between these targets are rare: fMRI revealed activation of vagal brainstem nuclei during stimulation of both targets, but with significant increases in comparison to sham for the cymba conchae only (Yakunina et al., 2016). In contrast, EEG recordings showed evoked potentials, referred to as vagus somatosensory evoked potentials (VSEP), during inner tragus but not cymba conchae stimulation (Fallgatter et al., 2003). These EEG responses disappeared, however, under a pharmacological neuromuscular block (Leutzow et al., 2013). This observation suggests rather a peripheral muscular than a central neural origin and has fueled a controversy (Leutzow et al., 2014; Polak et al., 2014). Furthermore, a study incorporating microneurography and HRV measurements detected no differences, when stimulating the tragus, conchae or their combination (Clancy et al., 2014). However, it should be noted that the approach for stimulating the tragus is different between studies, i.e., applying the charge across the tragus (Clancy et al., 2014), or on either side of the tragus (present study).

The present work stimulated both the cymba conchae and the tragus (among other targets) and revealed a stronger influence on HRV of the former. This finding is in line with the mentioned anatomical (Peuker and Filler, 2002) and fMRI findings (Yakunina et al., 2016) on the vagal innervation of the cymba conchae and previous HRV observations during taVNS (De Couck et al., 2017). Even though to a lesser extent, tragus stimulation led also to HRV increases as observed in previous work (Clancy et al., 2014) leaving the possibility of vagal innervation open. Notably, microneurography recordings during tragus stimulation revealed a decrease in frequency and incidence of muscle sympathetic nerve activity (Clancy et al., 2014), thereby, suggesting potential alternative mechanisms of the observed HRV effects.

Along these lines, recent research in the rat probed the underlying neural pathways of tragus stimulation by tracer injections and nerve transections (Mahadi et al., 2019). This experimental work revealed the cervical dorsal horn of the spinal cord and the paratrigeminal nucleus within the spinal trigeminal tract as prominent projections sites from the tragus, but only very few to the nucleus tractus solitarii, which is considered the major target of the ABVN. Importantly, stimulating the tragus resulted in inhibition of sympathetic nerve activity recorded from the spinal sympathetic chain which disappeared after cervical nerve transections (Mahadi et al., 2019). Together, the findings of these human and experimental studies suggest that auricular stimulation effects may be mediated by complementary autonomic mechanisms dependent on the stimulated locations and activated pathways.

We found relevant increases of HRV also when stimulating the fossa triangularis, an auricular area that has not been in the focus of previous taVNS research. This area is located between the crura antihelicis (91% GAN) and the spine helicis (91% ATN) and is probably innervated by the trigeminal nerve (ATN) and greater auricular nerve (GAN), and to a lesser extent potentially also by the vagal nerve (9% ABVN at crura antihelicis) (Peuker and Filler, 2002). Interestingly, this area has previously been targeted in the context auf auricular acupuncture/acupressure to reduce anxiety (Wang and Kain, 2001; Kober et al., 2003), which is closely related to the autonomous nervous system and HRV (Paniccia et al., 2017). Thus, the findings of the present study regarding the fossa triangularis suggest that non-vagal afferents may have a relevant influence on HRV during auricular stimulation.

4.3. Left and right auricular stimulation

The vagal innervation of the heart is asymmetric. The sinoartrial (SAN) and the artrioventricular node (AVN) are innervated by the right and left efferent vagus nerves, respectively. Therefore, stimulating these efferent fibers at the cervical level may result in different cardiac effects, e.g., reduction in heart rate (negative chronotropic action on SAN with right stimulation), and reduction in artrioventricular conduction (negative dromotopic action on AVN with left stimulation). Therefore, the stimulation side (right vs. left) is specifically considered during cervical VNS for neuropsychiatric and cardiovascular indications (Capilupi et al., 2019). However, for auricular VNS of ABVN fibers such side-specific effects are less apparent, since this afferent input will be integrated at the level of the brainstem in the nucleus of the solitary tract (NTS) and dorsal motor nucleus of the vagus (DMN) before leaving the central nervous system on the right and left side of the body (Chen et al., 2015). Along these lines, there were no side-specific (right vs. left) increases in cardiac adverse effects during taVNS (Redgrave et al., 2018) or differences of evoked potential in the EEG (Polak et al., 2009).

In this context, it was surprising that the present study showed significant differences between the right and left ear with regard to HRV. Specifically, right and left ear stimulation led to an increase and decrease, respectively, with regard to different HRV parameters such as SDNN, RMSSD, pNN50, SD1, and SD2. Therefore, a side-specific influence of taVNS on HRV necessitates further investigations.

4.4. Parameters of heart rate variability

In the present work, we applied both time-domain and non-linear methodologies for the estimation of heart rate variability. Time domain analysis, which is based on the inter-beat interval, included different parameters (i.e., SDNN, RMSSD, pNN50 and TINN) with the SDNN reflecting an overall HRV parameter, and RMSSD and pNN50 reflecting the parasympathetic nervous system. Within the non-linear methods, the SD1 and SD2 are considered as measures of short-term variability of the parasympathetic nervous system and long-term variability of both the para- and sympathetic nervous systems, respectively (Geitel, 2016; Shaffer and Ginsberg, 2017). In the present study, there were HRV changes in both the time-domain and non-linear evaluations, confirming the previously reported autonomic shift towards parasympathetic predominance (Clancy et al., 2014; De Couck et al., 2017; Sclocco et al., 2019). On the basis of our findings, RMSSD, SD1 and SDNN may be particularly suitable biomarkers for taVNS adaption. If an increase in HRV is desired, our findings suggest stimulation at the right cymba conchae with shorter pulse durations (Tables 3 and 5) might be a preferable approach. This effect of increased HRV appears to be linked with a decrease in HR. Vice versa, a decrease in HRV can be found when stimulating at the cavum conchae or the outer tragus.

4.5. Limitations

This study evaluated six stimulation locations with increasing electrical charges that were balancing intensity and pulse duration. Thereby, we examined a large parameter space with different (even though not independent) conditions within one screening session. As these stimulation parameters were tested in a relatively small number of participants, future studies need to consider larger cohorts. This may also allow for more specific comparisons between different conditions with regard to HRV levels. Furthermore, our approach allowed a relatively tight experimental protocol with short HRV measurement periods of 30s for each condition only. In this context, the observed effects may be non-stationary, but rather correspond to dynamic responses. Future work may, therefore, consider longer stimulation and Off-periods between conditions. A randomization of stimulation intensities might, furthermore, avoid habituation effects. Thus, findings that are consistent across different HRV parameters (Table 5), i.e., those with regard to the

stimulation location, may be considered as robust, while other observations, i.e., those with regard to the stimulation side (right vs left), need to be reexamined in further studies. Although even (ultra) short HRV recordings have been shown to be valid, future studies may select specific stimulation parameters and locations for direct comparisons in longer HRV measurement periods on the basis of the present work (Munoz et al., 2015; Shaffer and Ginsberg, 2017).

There is a possibility that the lower range of intensities used in this study (although applied in a charge-balanced way) were not strong enough to provide a significant activation of ABVN receptors and therefore failed to provide activation of this vagal afferent pathway. Given that these are receptors capturing somatosensory afference, it could be helpful in future studies to capture information about the sensation that the subjects experienced during the different stimulation parameter combinations and potential differences. Examining these parameters on different examination days or including wash out periods between conditions may, furthermore, avoid potential carry-over effects. In addition, future studies may consider a randomization of the stimulation sequence of each session, as well as possible confounders such as the respiratory rate and volume. Furthermore, future studies may monitor the actual current to control for the applied pressure during the manual probing.

In this study, we examined the cymba conchae and the inner tragus as the standard VNS targets, and stimulated four additional sites (i.e., cavum conchae, outer tragus, fossa triangularis, crus helicis). This screening algorithm revealed the fossa triangularis as a new stimulation target for HRV effects, whereas the cavum conchae, outer tragus, and crus helicis may serve as active control stimulation locations. We consider them more realistic control stimulation sites than the usually stimulated earlobe due to their topographic vicinity to the targets cymba conchae (control site: cavum conchae), inner tragus (control site: outer tragus), and fossa triangularis (control site: crus helicis). However, future work may also examine the earlobe to facilitate comparisons with previous work. Finally, the observations made in healthy young subjects need to be confirmed in agedas well as patient populations and, since results may differ in conditions with an impaired autonomic tone.

5. Conclusion

Suitable auricular stimulation parameters and targets may be determined on the basis of short-term HRV measurements. Intermittent stimulation is effective in increasing HRV at low current intensities (e.g., 2 mA) and with short pulse durations (e.g., 100 μs) when applied at specific auricular locations; the cymba conchae, fossa triangularis, and inner tragus are particularly promising in this context.

Author contributions statement

KM contributed to the conception and design of the study, the acquisition, analysis and interpretation of data, and writing of the manuscript. LB and RG contributed to the data acquisition and analysis, interpretation of data and review of the manuscript. AG was responsible for the conception and design of the study, interpretation of data, and writing of the manuscript.

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Data availability statement

All data and material are available and can be provided on request.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Effects of transcutaneous auricular vagus nerve stimulation on cardiovascular autonomic control in health and disease

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ABSTRACT

Autonomic nervous system (ANS) dysfunction is a well-known feature of cardiovascular diseases (CVDs). Studies on heart rate variability (HRV), a non-invasive method useful in investigating the status of cardiovascular autonomic control, have shown that a predominance of sympathetic modulation not only contributes to the progression of CVDs but has a pivotal role in their onset. Current therapies focus more on inhibition of sympathetic activity, but the presence of drug-resistant conditions and the invasiveness of some surgical procedures are an obstacle to complete therapeutic success. On the other hand, targeting the parasympathetic branch of the autonomic nervous system through invasive vagus nerve stimulation (VNS) has shown interesting results as alternative therapeutic approach for CVDs. However, the invasiveness and cost of the surgical procedure limit the clinical applicability of VNS and hinder the research on the physiological pathway involved.

Transcutaneous stimulation of the auricular branch of the vagus nerve (tVNS) seems to represent an important non-invasive alternative with effects comparable to those of VNS with surgical implant. Thus, in the present narrative review, we illustrate the main studies on tVNS performed in healthy subjects and in three key examples of CVDs, namely heart failure, hypertension and atrial fibrillation, highlighting the neuromodulatory effects of this technique.

1. Introduction

The autonomic nervous system (ANS) plays a key role in the development of several pathologies, in particular cardiovascular diseases (CVDs), such as hypertension, arrhythmias, coronary artery disease, and heart failure (Malliani and Montano, 2002; Hadaya and Ardell, 2020). As a matter of fact, sympathetic hyperactivity seems to be an important mediator in both the onset and the progression of cardiovascular diseases (Malpas, 2010; Malliani et al., 1991), and in chronic conditions, a predominant cardiovascular sympathetic modulation is linked to poor clinical outcomes and life-threatening complications (Grassi et al., 2015). On the other hand, cardiac parasympathetic control seems to protect from CVDs and related mortality both in healthy subjects and already affected patients (Thayer et al., 2010; Thayer and Lane, 2007).

Sympathetic and parasympathetic control on the cardiovascular system determines the heart rate and blood pressure oscillatory responses to both exogenous and endogenous stimuli. These rhythmical components can be analyzed and quantified using Heart Rate Variability

Abbreviations: ANS, autonomic nervous system; CVDs, cardiovascular diseases; HRV, heart rate variability; IBI, inter-beat interval; SDNN, standard deviation of the normal sinus-initiated inter-beat interval; RMSSD, root mean square of successive differences between normal beats; VLF, very low frequency band; LF, low frequency band; HF, high frequency band; tVNS, transcutaneous stimulation of the auricular branch of the vagus nerve; ABVN, auricular branch of vagus nerve; MSNA, muscle sympathetic nerve activity; SAPV, systolic arterial pressure variability; HR, heart rate; BRS, baroreflex sensitivity; NTS, nucleus tractus solitarius; RVLM, rostral ventrolateral medulla; CHF, congestive heart failure; AF, atrial fibrillation; HFrEF, heart failure with reduced ejection fraction; LVEF, left ventricle ejection fraction; QoL, quality of life; ART, autonomic regulation therapy; LLTS, low-level tragus stimulation; HTN, chronic hypertension; RHT, resistant hypertension; BP, blood pressure; RAVANS, respiratory-gated auricular vagal afferent nerve stimulation; GP, ganglionated plexi; POAF, post-operative atrial fibrillation.

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(HRV). HRV is the most objective, reproducible and validated tool for investigating autonomic functions (Thayer et al., 2010; Shaffer and Ginsberg, 2017). It is known that HRV is altered in a variety of pathological conditions and reduced HRV predicts increased risk for all-cause mortality (Thayer et al., 2010; Tsuji et al., 1994). In their meta-analysis, Hillebrand et al. showed that individuals with low HRV have ~40% increased risk of a first fatal or non-fatal cardiovascular event compared with individuals with higher HRV (Hillebrand et al., 2013). The core of all these studies is that a lower HRV suggests impaired autonomic cardiovascular control and a less reactive and adaptive autonomic system underlying CVDs.

1.1. Heart rate variability analysis

HRV analysis can be performed through various analytical approaches and is based on the extrapolation of time intervals between each R wave peak (Shaffer and Ginsberg, 2017). The most commonly applied methods 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 (IBI) employing statistical measures, such as the standard deviation of the normal sinus-initiated IBI (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 total spectrum can be identified: i) the spectral power in the very low frequency band (VLF), below 0.04 Hz, which seems to be influenced by thermoregulatory mechanisms, physical activity and circadian rhythms; ii) the low-frequency band (LF) between 0.04 and 0.15 Hz in humans, a marker influenced by baroreflex (Furlan et al., 2019; Barbic et al., 2019), sympathetic and parasympathetic modulation; iii) 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 (Shaffer et al., 2014; Montano, 2009). The total power of the spectrum, obtained from the sum of the three main spectral components, is an index of global HRV. Thus, LF and HF components can be expressed in absolute values (ms^2) and in relative power or normalized units (LFnu and HFnu) obtained from the ratio between the power of the band and the total power without the VLF component (Malliani and Montano, 2002). The LF/HF ratio is an index of instantaneous sympatho-vagal modulation to the sino-atrial node discharge (Shaffer and Ginsberg, 2017; Montano, 2009). Finally, non-linear approaches represent more recently developed methods of analysis. Among these, the symbolic analysis makes it possible to detect non-reciprocal changes of sympathetic and parasympathetic modulation on cardiovascular autonomic control and appears to be a better investigation model in the pathological conditions characterized by low HRV (Carandina et al., 2021; Guzzetti et al., 2005a; Zamunér et al., 2019). To perform the symbolic analysis, inter-beat time series are converted into a sequence of symbols and then divided into 3-beat patterns. Patterns will be classified into 4 main families: a) 0 V, patterns with no variation, all 3 symbols are equal (e.g., 4-4-4); b) 1 V, patterns with 1 variation, 2 consecutive symbols are equal forming a 2-beat plateau, while the remaining one is different (e.g., 3-3-5); c) 2LV, patterns with 2 like variations, all symbols are different from the previous one and they are in ascending or descending order (e.g., 2-4-5); d) 2UV, patterns with 2 unlike variations, all symbols are different from the previous one but not in a consequent order (e.g., 1-4-3). The occurrence percentage of the patterns 0 V is a marker of cardiac sympathetic modulation and 2UV or 2LV are markers of cardiac vagal modulation (Guzzetti et al., 2005a) (Fig. 1).

Image taken from "Symbolic Dynamics of Heart Rate Variability: A Probe to Investigate Cardiac Autonomic Modulation" by Guzzetti et al. (2005a).

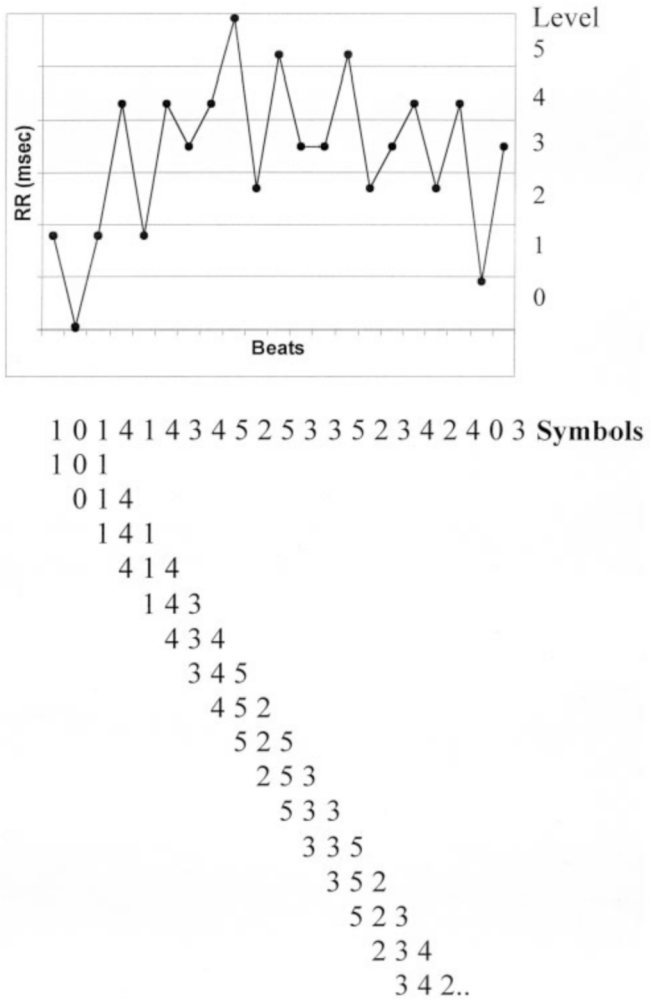

Fig. 1. Methodological aspects of symbolic analysis. A 6-level grid is applied to the inter-beat time series. The IBI are converted into a sequence of symbols and then 3-beat/symbols patterns are constructed.

1.2. Vagus nerve stimulation: methodological aspects

In recent years, more and more studies have investigated several methods for restoring altered sympathovagal balance. To counteract the ANS impairment, pharmacological therapies have been directed at decreasing sympathetic overactivity and β-blockers have for many years represented the reference standard for the modulation of sympathetic cardiovascular control. However, the possibility of targeting the parasympathetic branch of the ANS has appeared over the years as a potential alternative. Unfortunately, only a few drugs act directly on vagal activity without significant side effects (He et al., 2015; Liu et al., 2019). In contrast, recent developments in biomedical engineering have ensured the opportunity to develop intriguing devices that allow direct stimulation of the vagus nerve.

The VNS is an encouraging non-pharmacological therapeutic approach, approved by the Food and Drug Administration (FDA) to treat refractory epilepsy (since 1997) and depression (since 2005) (Ben-Menachem et al., 2015). The potential use of VNS for other conditions has gained much interest over the years given its pleiotropic effects. However, since the first VNS techniques required an invasive and expensive surgical procedure to implant the subcutaneous generator device and the unidirectional wire connected around the cervical vagus nerve, the clinical use has been limited and associated with side-effects (i.e., dysphonia, vocal hoarseness, dyspnea, paresthesia, and pain) (Ben-Menachem et al., 2015; Spuck et al., 2010). Instead, the most recently

developed transcutaneous stimulation of the auricular branch of the vagus nerve (tVNS) is considered to be an important non-invasive alternative, without the side effects of the surgery procedures but with similar efficacy as to the invasive device (Murray et al., 2016).

The tVNS devices consist of an electrode placed on the skin of the external ear and connected to a stimulating box (Redgrave et al., 2018; Butt et al., 2020). There is contrasting evidence on the optimal anatomical application area of tVNS, since the exact distribution of vagal fibers at the external ear (ABVN) has yet to be adequately delineated. Peuker and Filler identified two main areas innervated by ABVN: the tragus 45% innervated by ABVN, 46% by the great auricular nerve, and 9% by the auriculotemporal nerve, and the cymba concha 100% innervated by the ABVN (Peuker and Filler, 2002). A study comparing the modifications on functional MRI of tVNS application in three different areas (i.e., the tragus, the ear canal, and the cymba conchae) concluded that tVNS delivered on the cymba conchae provoked the strongest activation of the vagal afferent pathway directed to the brainstem (Yakunina et al., 2017).

As in all types of stimulation, the tVNS parameters can be modulated (Fig. 2). The main parameters referred to in the present narrative review are: the pulse width, i.e. the determined time period (μs) from the beginning to the end of one pulse stimulus; the stimulation frequency, i.e. the number of pulse cycles that are generated in 1 s (Hz); the stimulation intensity or current intensity, i.e. the magnitude of current relative to the isoelectric baseline, expressed in milliamperes (mA) (Koenig et al., 2020).

2. tVNS modulates cardiovascular autonomic control: physiological substrates in healthy subjects

The first study aimed at investigating the effects of this new type of non-invasive VNS on cardiovascular autonomic control in healthy humans was conducted by Clancy et al. (2014). Electrodes were placed on the tragus and the tVNS was applied continuously for 15 min (pulse width of 200 μs and pulse frequency of 30 Hz). The current amplitude was adjusted to the level of a sensory threshold between 10 and 50 mA. The effects on ANS were assessed by HRV analysis and muscle sympathetic nerve activity (MSNA) recordings. Data were recorded during 3 periods of 15 min each: at baseline, during tVNS, and recovery. The group observed a significant modification of HRV indicating a shift in cardiac autonomic function towards parasympathetic predominance and a reduction in sympathetic nerve outflow mediated by tVNS. The effect persisted even during the 15-minute recovery. Furthermore, of particular interest, linear regression analysis revealed a greater response to tVNS in those subjects who had a more pronounced shift of sympathovagal balance towards a sympathetic predominance (higher LF/HF ratio) (Clancy et al., 2014). In another study, Antonino and colleagues measured HRV and systolic arterial pressure variability (SAPV) in young healthy men in sitting position during rest, tVNS and recovery (Antonino et al., 2017). Spontaneous cardiac baroreflex sensitivity was also assessed by the sequence technique and calculated through regression analysis as an index of coherence between increased systolic blood pressure/lengthening of the IBI and reduction of systolic pressure/shortening of the IBI. The applied tVNS parameters were the same as in the study described above. The authors found an acute effect of tVNS performed for 15 min, that was able to shift LF/HF ratio to a vagal predominance, reducing the heart rate (HR) and improving spontaneous cardiac baroreflex sensitivity (Antonino et al., 2017).

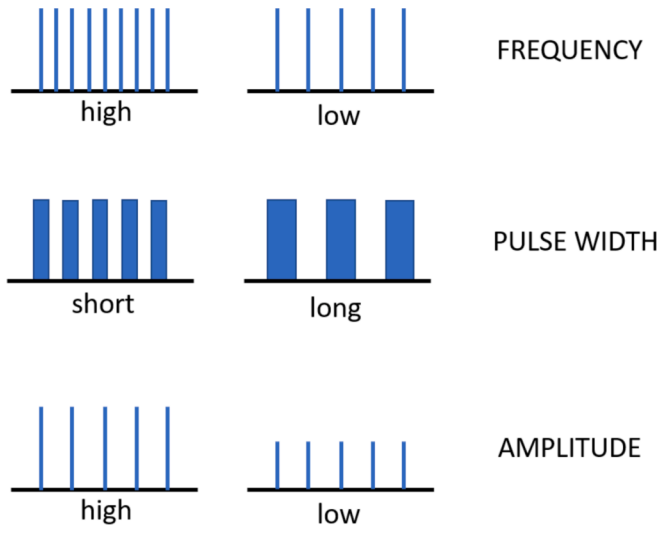

Fig. 2. Stimulation Parameters.

To better investigate the cardiovascular effects of different types of tVNS, De Couck and colleagues compared different sets of short simulation duration (10 min) and assessed the effects of prolonged tVNS (1 h) in healthy subjects (De Couck et al., 2017). tVNS was applied to the cymba conchae area of the external left or right ear with 25 Hz frequency for 10 min and stimulation intensity was once again adjusted based on each subject's sensory threshold. Results revealed significant increases in SDNN time-domain index, a marker of global variability following right-ear acute tVNS. Moreover, they found that the cardiovascular autonomic control did not change as a function of stimulation duration in the entire sample. Similarly, Badran et al. explored the effects of different tVNS parameters on HR, applying different pulse widths (100 μs, 200 μs, 500 μs) and frequencies (1 Hz, 10 Hz, 25 Hz) and creating nine different combinations (Badran et al., 2018). The intensity was set at 200% of each participant's perceptual threshold and the stimulated area was the left tragus for all the active tVNS protocols. The study showed that the parameters that most modulated HR were the most energy-dense, specifically 500 μs 10 Hz and 500 μs 25 Hz.

In a study by our research group, in addition, to evaluate the cardiovascular autonomic control modulation determined by tVNS at rest, we also tested the response of autonomic branches to an orthostatic challenge during tVNS in healthy subjects (Tobaldini et al., 2019). We found that acute tVNS applied on the left cymba conchae (200 μs pulse width, 25 Hz frequency, and from 1 to 6 mA intensity) decreases cardiac and peripheral sympathetic modulation in the rest condition, assessed by non-linear symbolic analysis of HRV and SAPV respectively. Interestingly, the orthostatic test revealed that acute tVNS could increase the responsivity of the sympathetic vasomotor modulation to postural challenge.

To our knowledge, just one study was designed to investigate the potential benefits of long-term daily tVNS in healthy adults (≥55 years) (Bretherton et al., 2019). Two weeks of 15-min tVNS (pulse width of 200 μs, pulse frequency of 30 Hz, and amplitude of 2-4 mA) administered every day was associated with increased vagal-mediated HRV indexes and improved baroreflex sensitivity (BRS) at rest. Interestingly, participants with higher LF/HF at baseline showed a greater reduction in LF/HF at the end of the protocol.

Finally, Paleczny et al. evaluated the cardiovascular effects of a new tVNS approach coupled with respiratory activity (Paleczny et al., 2021). Healthy subjects underwent expiratory-gated tVNS, inspiratory-gated tVNS and classic non-respiratory-gated tVNS during controlled breathing protocol. Other stimulation parameters were similar in the three treatments (1000 μs pulse width, delivered at 25 Hz and stimulation intensity adjusted on the individual perceptual threshold). The authors found that expiratory-gated and non-respiratory-gated tVNS exerted comparable cardioinhibitory effects in healthy subjects, whereas the effect of inspiratory-gated tVNS seemed to be rather stimulatory than inhibitory. Furthermore, a negative relationship between the HR changes in response to tVNS and the baseline HR emerged, indicating a greater response to tVNS in more tachycardic subjects.

Taken together, these findings on healthy volunteers suggest that acute tVNS favorably affects HRV promoting a shift of the

sympathovagal balance towards a parasympathetic predominance, improves BRS, and determines a sympathetic withdrawal (e.g., decreased MSNA). These effects not only occur during stimulation but persist over time (Clancy et al., 2014). The hypothesis is that tVNS, in addition to the stimulation role, also acts as a neuromodulation technique via vagal afferent projections. According to the literature, tVNS seems to have an indirect effect on the cardiovascular system and more broadly on the peripheral autonomic system through the nucleus tractus solitarius (NTS), the first synaptic station of the autonomic afferent projections in the central nervous system (Frangos, 2015). The NTS recruitment from tVNS could cause activation of excitatory inputs to the caudal ventrolateral medulla which in turn inhibits the rostral ventrolateral medulla (RVLM), the source of excitatory drive to sympathetic efferent (Fig. 3). The fact that the phenotypic effects on cardiovascular autonomic control persist over time suggests an enhancement of these pathways and therefore, a persistent neuromodulatory effect. Moreover, the almost ubiquitous finding of sympathetic predominance at baseline as a favorable predictor of the response to tVNS, supports the possibility of using tVNS as a therapeutic approach in those disease conditions characterized by an autonomic impairment.

Some questions remain unanswered and require further studies to understand the actual duration of acute tVNS effects on cardiovascular autonomic control and the optimal stimulation parameters.

3. tVNS as a non-invasive therapeutic approach for cardiovascular diseases

In 2015, CVDs were estimated to cause 30% of all deaths worldwide, representing the main cause of morbidity and mortality (Mensah George et al., 2019; Leal et al., 2006) and the most expensive healthcare condition among chronic diseases (Leal et al., 2006; Trogdon et al., 2007). Despite the immense effort dedicated to the development of new pharmacological and surgical treatments and in raising awareness on the importance of primary prevention, CVDs remain a hot topic in terms of social and clinical management.

Autonomic dysfunction is known to play an important role in the onset and progression of CVDs. An alteration of the sympathovagal balance and a reduction in HRV represent risk factors for the development of cardiovascular diseases, both independently and secondarily as a pathophysiological mechanism consequent to other cardiovascular risk factors such as aging, obesity, and diabetes (Messerli et al., 2019; Greiser et al., 2005). For example, a reduction over time in SDNN time-domain index is an independent predictor of mortality in patients with congestive heart failure (CHF) (James et al., 1998; Kearney et al., 2002). Moreover, modification of HRV patterns has been associated with CHF progression. In particular, both in 24 h and in short-term HRV analyses, an increase of the amplitude of LF oscillations was observed in the early stages of CHF, whereas the reduction of the LF spectral component of HRV has been strongly related to sudden death in the end stages of the disease (La Rovere et al., 2003; Guzzetti et al., 2005b; Guzzetti et al., 1995). These results stand for a detrimental escalation where the chronic increased sympathetic activity in CHF lastly determines a complete loss of HRV rhythmic properties, compromising any possibility of adaptation of the heart to internal and external stimuli (Shaffer et al., 2014; Guzzetti et al., 1995).

Patients with essential hypertension show progressive increase of the indexes of cardiac and vascular sympathetic modulation assessed by spectral analysis of HRV with increasing severity of the disease, as well as reduced responsiveness to orthostatic challenge and an enhanced LF component of SAPV (Pagani and Lucini, 2001). The available evidence supports the hypothesis that in these patients there is an increased sympathetic and a parallel reduced vagal cardiac modulation coupled with an enhancement of vasomotor sympathetic drive. Indeed, treatment with antihypertensive drugs, in newly diagnosed patients with essential hypertension that is responsive to medications, determines a rebalancing of the autonomic modulation (Pavithran et al., 2010).

With regard to atrial fibrillation (AF), it has been seen that both a sympathetic predominance or a vagal predominance may precede an arrhythmic event (Boon et al., 2016). In such condition, it seems that the autonomic imbalance itself and not just the sympathetic predominance may cause the arrhythmic event.

Considering both the evidence on the role of autonomic dysfunction in CVDs and the results of studies on non-invasive VNS in healthy subjects, targeting the altered sympathovagal balance with tVNS could represent a new therapeutic approach at low costs and with no side effects. In the following subsections of our review, we illustrate the proof-of-concept studies supporting this hypothesis through three key examples of cardiovascular diseases (Fig. 4).

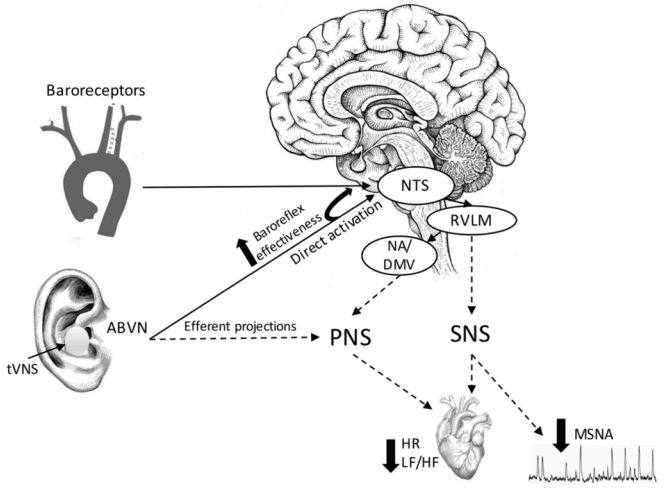

Fig. 3. The hypothesized model of physiological underlying mechanisms of acute tVNS and baroreflex interactions considering cardiovascular reflexes. Continuous arrows represent afferences and dashed arrows represent efferences. Abbreviations: PNS: Parasympathetic nervous system; SNS: Sympathetic nervous system; NTS: nucleus tractus solitarius; ABVN: auricular branch of the vagus nerve; NA: nucleus ambiguous; DMV: dorsal motor nucleus of the vagus: RVLM: rostral ventrolateral medulla; tVNS: Transcutaneous Auricular Vagal Nerve Stimulation; MSNA: muscle sympathetic nerve activity; LF/HF: sympathovagal balance; HR: heart rate.

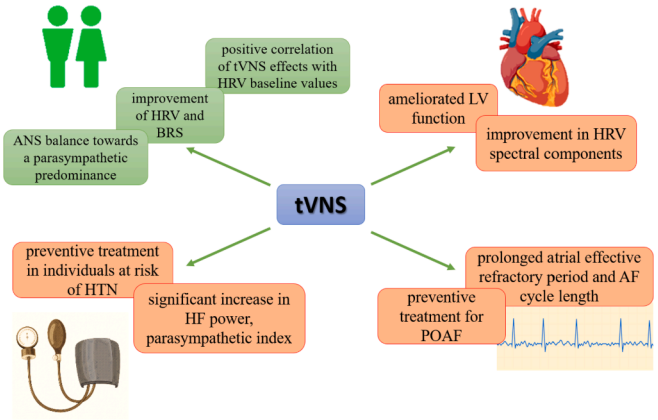

Fig. 4. tVNS effects on cardiovascular autonomic control in healthy subjects and patients affected by heart failure, hypertension, and atrial fibrillation. Abbreviations: tVNS: transcutaneous auricular vagus nerve stimulation; ANS: autonomic nervous system; HRV: heart rate variability; BRS: baroreflex sensitivity; LV: left ventricle; HTN: hypertension; HF: high frequency; AF: atrial fibrillation; POAF: post-operative atrial fibrillation.

3.1. Heart failure

As described above, it has long been recognized that ANS imbalance, characterized by vagal withdrawal and increased sympathetic activity, plays a major role in the worsening of both CHF and its prognosis. More in detail, the hyperactivity of the sympathetic nervous system causes afferent sympathetic signaling, which leads to the tonic and reflex inhibition of the vagal cardiac efferent activity. The loop of the sympathetic nervous system hyperactivation, generated from this mechanism, plays an important role in worsening CHF, contributing to left ventricular diastolic dysfunction and an increase in cardiovascular risk (Toschi-Dias et al., 2017; Kishi, 2012). Therefore, modulation of ANS to break this sympathetic positive feedback loop is sparking interest in CHF therapy.

Preclinical studies demonstrated the benefit of invasive VNS to reduce mortality, improve LV remodeling and hemodynamics, and decrease inflammation in a variety of animal models of CHF (Li et al., 2004; Zhang et al., 2009; Sabbah et al., 2011). Moreover, Hamman et all demonstrated that VNS must be applied chronically to be effective. When in a canine model VNS treatment was terminated after 6 months CHF severity returned to baseline (Hamann et al., 2013).

The first-in-man study included patients with heart failure with reduced ejection fraction (HFrEF) (left ventricle ejection fraction-LVEF <35%) and New York Heart Association (NYHA) class II-III (Schwartz et al., 2008). This study by Schwartz et al. demonstrated improvement of left ventricle end-systolic volume (LVESV), NYHA classification, and quality of life (QoL). Based on those encouraging results, De Ferrari et al. conducted a pilot study in a similar cohort of 32 patients, implanted with the CardioFit system stimulator on the right vagal nerve. The study showed improvement in LVEF, cardiac volume, and 6-minute walking test at 6 and 12 months of follow-up; however, 3 out 32 patients died during the study. The mortality events were judged to be not related to the investigational device but rather attributed to the severity of CHF (De Ferrari et al., 2011).

The other three major trials of direct VNS in patients with HFrEF have yielded mixed results (Gold et al., 2016; Zannad et al., 2015; Nearing et al., 2021; Sharma et al., 2021). In the INOVATE-HF trial 707 patients were randomized to low frequency (1-2 Hz) right VNS in addition to guidelines medical therapy (GMT) or GMT alone. The NECTAR-HF was a randomized controlled trial that compared GMT to VNS at a delivery frequency of 20 Hz in 96 patients with HFrEF. Both these studies failed to meet their clinical endpoints and to demonstrate improvement in mortality, LV systolic dimensions, or LVEF, but they did show improvement in QoL and NYHA classification. Whereas the ANTHEM-HF was a single-arm study that investigated right or left cervical VNS in 60 patients with HFrEF. Stimulation was applied at a frequency of 10 Hz for 24 months, then the frequency was changed to 5 Hz to evaluate tolerance and ability to achieve autonomic engagement. The lower frequency allowed stimulation with a higher amplitude, which was tolerable and likely increased neuronal activation. The study showed improvement in LVEF, left ventricle end-systolic diameter, HRV, and also in QoL, NYHA class, and exercise capacity at 12-36 and 42 months of follow-up of continued autonomic regulation therapy (ART).

Notably, the methodologies used for VNS in these three studies differed considerably in their neurologic targets, technology platforms, and the mode and delivery of VNS for ART. Is also noteworthy that, unlike CardioFit, INOVATE-HF and NECTAR-HF, the ANTHEM-HF study used stimulation intensity and frequency near the natural frequency of vagal fibers activation (Anand et al., 2020; Jewett, 1964; Ardell et al., 2017). In order to confirm these data, an open-label, randomized, controlled pivotal study (ANTHEM-HFrEF) is currently underway to further evaluate ART in patients with advanced CHF [NCT03425422].

Ultimately, it's important to point out that one of the major drawbacks of cervical vagus stimulation is the invasive nature of this treatment with inherent surgical complications and poor patient tolerance. Furthermore, considering the invasive nature of this type of treatment, it has only been studied in patients with advanced CHF, where remodeling and fibrosis are already present, thus reducing the chances that a simple restore of the sympathovagal balance may at this stage modify the progression of the disease. Peripheral and non-invasive VNS, on the contrary, would allow intervention in the early stages of CHF, before the development of the structural alterations that characterize the progression of the disease. Therefore, the low-level tragus stimulation (LLTS) makes its way as a technique of VNS that can modulate the ANS stimulating the auricular branch of the vagus nerve. LLTS has shown promising data in animal models, but human studies of LLTS on CHF patients are limited. In a prospective, randomized, double-blind, 2 × 2 cross-over study, 1 h right LLTS (frequency 20 Hz) acutely ameliorated left ventricular function and favorably altered HRV frequency domain components in patients with diastolic dysfunction and heart failure preserved ejection fraction (HFpEF) (Tran et al., 2019).

In summary, the optimal parameters for LLTS remain undetermined and all studies highlight the importance of optimizing stimulation parameters. Large-scale long-term clinical trials on the effects of invasive VNS in patients with CHF have reported discordant results and failed to demonstrate efficacy on hard endpoints such as mortality. The reason would seem to be related to the critical conditions of the patients who are implanted. As a matter of fact, VNS improved cardiac function but did not reveal any reversive effects on structural cardiac remodeling, already present in subjects at the enrollment. Given the promising results of LLTS, intervention in the early stages of the disease with non-invasive stimulation approaches such as tVNS could lead to a significant change in mortality rates. Further studies with long-term evaluations and larger sample sizes are needed to confirm this hypothesis.

3.2. Hypertension

Chronic hypertension (HTN) affects more than 1 billion people worldwide and is associated with an increased risk of cardiovascular disease, lower quality of life and shorter life expectancy. Despite decades of continuous research, effective treatment of HTN remains challenging. Suboptimal compliance to medications plays a central role in treatment failure, with a large percentage of patients who struggle to adhere to prescribed medications (Michel and Egan Brent, 2019). These, combined with the significant share of patients with pharmaco-resistant hypertension calls for the need for different approaches to aid drug treatment.

The idea of treating HTN with neuromodulation reaches as back as the 1960s when clinical studies conducted in patients with resistant hypertension (RHT) showed lowering of blood pressure (BP) during stimulation with electrodes wrapped around the carotid sinus nerve. However, the technology available could not achieve reliable sustained baroceptor activation without causing significant adverse effects (Lohmeier et al., 2005; Lohmeier and Hall, 2020; Ng et al., 2016). This device-based approach was left behind by the mid-1970s as a result of technical issues and the beginning of an era of antihypertensive drug development. It wasn't until recently that new encouraging data from animal models brought back the interest in neuromodulation. Several studies not only confirmed the capability of baroceptor activation of lowering hypertensive subject BP, but also the possibility of reducing vascular shear stress, aortic stiffening, hypertension-induced endothelial dysfunction, and long-term survival in salt-sensitive hypertensive rats (Annoni et al., 2015; Tolkacheva, 2019; Chapleau et al., 2016).

The use of modern devices and improved surgical techniques allowed new clinical trials that showed promising results in long-term usage but failed to meet the criteria for short-term safety (Bisognano et al., 2011). Whereas, smaller, less invasive, more durable systems were developed and showed impressive results in one single arm, open-label trial (Hoppe et al., 2012), but still require validation as multicenter randomized control trials are still ongoing both in the USA and in Europe [NCT02880618, NCT02876042, NCT02627196].

Surgically related complications, long-term risk of tissue damage, and overall cost are the main constraining factors in the application of

device-based therapy for hypertension. Proving the efficacy of non-invasive VNS could avoid these factors and represent a new resource in the treatment of resistant HTN.

As of today, only one study focused on directly targeting BP using VNS was published (Silva et al., 2019). The study by Silva et al. included 30 hypertensive subjects, half of whom received a 20 min stimulation with a pulse width of 120 ms, and a frequency of 25 Hz, showing a slight reduction in BP with an improved LF/HF ratio in HRV analysis when compared to the control group (Silva et al., 2019). While data on the direct cardiovascular effect of tVNS on BP in hypertensive individuals are still scanty, on the other hand neuromodulation could provide other benefits for these patients. As a matter of fact, impaired autonomic function was found to be associated with increased all-cause mortality, especially in patients with hypertension or a history of cardiovascular disease (Gerritsen et al., 2001). Moreover, since a significant percentage of patients suffering from RHT are older than 55, it is useful to reiterate the importance of tVNS as a preventive treatment in this cohort of subjects, especially in individuals with greater baseline sympathetic prevalence, as seen in the aforementioned study by Bretherton et al. (2019). Finally, aiming at maximizing treatment effect, Sclocco et al. evaluated the effect of respiratory-gated auricular vagal afferent nerve stimulation (RAVANS), where the electrical stimuli are delivered during the exhalation phase of respiration, thus mimicking the breathing induced modulation of cardiac vagal activity. Preliminary data from their ongoing single-arm trial found that a medium intensity RAVANS stimulation increased the cardiovagal tone and reduced the sympathetic tone during a paced breathing task in hypertensive individuals (Sclocco et al., 2017). A later study from the same group tested RAVANS at different frequencies (0-2-10-25-50-100 Hz) reporting a significant increase in the cardiac parasympathetic modulation, assessed by the HF spectral index, during the RAVANS-100 Hz session when compared to sham stimulation with other frequencies not yielding significant results (Staley et al., 2020).

Thus, tVNS could represent a safe and cheap alternative when approaching patients with RHT, primarily targeting autonomic imbalance to reduce the cardiovascular effects of hypertension. Data on the direct effect of tVNS on BP in hypertensive patients remain scarce and will need further investigations. One of the major limits that hinders the replicability of available research is, once again, the variety of the stimulation patterns employed, with different times of exposure, frequencies, and intensities used among studies. Standardized, patient-tailored patterns of stimulation could yield great benefit in the development of new treatment strategies for hypertensive subjects.

Data from large, randomized trials are emerging for Invasive baroceptor activation and VNS techniques further proving the effectiveness of neuromodulation in the treatment of RHT and its complications.

3.3. Atrial fibrillation

Atrial fibrillation (AF) is the most frequent cardiac arrhythmia, affecting 1-2% of the population in Europe (Ceornodolea et al., 2017) with an age-dependent distribution (Lippi et al., 2021). AF is associated with significant cardiovascular morbidity and is a well-established leading risk factor for ischemic stroke (Jelena et al., 2020), determining an independent 1.5 to 2.0-fold higher long-term mortality risk (Ball et al., 2013). Consequently, the global burden of AF is showing an increasing trend in terms of hospitalizations and healthcare expenditure (Ball et al., 2013). Current pharmacological treatments for AF have major limitations, including limited efficacy especially in preventing recurrent AF (Noheria et al., 2008). Whereas, the non-pharmacological therapy (ablation) is improving, but the procedure is not entirely risk-free and only a limited number of patients are eligible (Haegeli and Calkins, 2014) and consistent risk of arrhythmia recurrence (Ganesan et al., 2013).

In order to find a treatment without side effects and limitations in its application, the research has focused on the role of the autonomic nervous system in the initiation and maintenance of AF. An unbalanced sympathovagal control over the ganglionated plexi (GP) can result in mismatched hyperactivity of the intrinsic cardiac autonomic nervous system. Under increased GP activity, the cholinergic activity can determine a further shortening of the effective refractory period in the myocardial sleeves of the pulmonary vein, while increased sympathetic activity may enhance the Ca2+ transient and early after depolarization instauration, all substrates for the AF initiation (Stavros et al., 2015a). Furthermore, since AF causes electrophysiological changes, acute atrial electrical remodeling occurs and an acute autonomic remodeling may ensue provoking a vicious cycle in which each perpetuates the other (Yu et al., 2012).

The aforementioned human studies on the effects of tVNS on cardiac autonomic control together with the antiarrhythmic evidence of invasive VNS in pre-clinical studies have paved the way for the application of non-invasive VNS for the treatment of AF in human studies (Salavatian et al., 2016; Li et al., 2009; Lu et al., 2016). Moreover, in 2013, Yu et al. demonstrated that tVNS applied at the right tragus and set at 80% below the voltages required to slow sinus rate was capable of inhibiting AF inducibility in a canine model, reversing the proarrhythmic effect of rapid atrial pacing (Yu et al., 2013). The same group evaluated the antiarrhythmic effect of acute tVNS applied at the right tragus in a first-in-human randomized sham-controlled study (Stavros et al., 2015b). 40 patients with paroxysmal AF referred for catheter ablation were randomized to 1 h of either real tVNS or sham tVNS and AF was induced under general anesthesia by burst atrial pacing before and after the neurostimulation. The stimulation was performed at 20 Hz and 50% below the voltages required to slow the sinus rate. Evidence was provided that tVNS acutely suppresses AF by prolonging the atrial effective refractory period and AF cycle length. Furthermore, in a second randomized clinical trial (TREAT AF), Stavrakis et al. evaluated the chronic effect of tVNS on AF burden in patients with paroxysmal AF (Stavrakis et al., 2020). Active tVNS at the tragus performed at 20 Hz and 1 mA below the discomfort threshold for 1 h daily over 6 months determined an 85% reduction of AF burden and an 83% reduction in total duration of AF compared to the sham stimulation. However, the tVNS treatment did not reduce the single AF duration. Another recent study, conducted by a different research group, tested the effects of a 3-day protocol of 1 h daily tVNS at 20 Hz and 0.13 mA (Trobec et al., 2020). 13 patients affected by paroxysmal AF were randomized to receive real or sham stimulation applied bilaterally at the cavum conchae. The AF burden analysis showed a favorable trend in the real tVNS group; however, the results did not reach statistical significance probably due to the short duration of treatment and registration. Finally, Andreas et al. assessed the protective effect of tVNS applied at the triangular fossa of the ear in preventing post-operative atrial fibrillation (POAF) (Martin et al., 2019). Forty-two participants were randomized to receive either the active tVNS, at 1 Hz and 1 mA, or sham stimulation for 2 weeks after cardiac surgery. The number of patients who suffered from POAF was significantly lower in the active group than in the sham group, no effect on the duration of POAF or during the day of onset was observed.

All these results suggest that the beneficial effect of tVNS is performed through the prevention of AF initiation rather than the interruption of the arrhythmic events. The local antiarrhythmic activity seems to be a consequence of the tVNS-mediated restoration of the tonic vagal inhibition over the hyperactivity of the intrinsic cardiac nervous system, ultimately resulting in an electrical and autonomic remodeling phenomenon (Yu et al., 2012; Salavatian et al., 2016; Stavros et al., 2015b; Stavrakis et al., 2020; Chen et al., 2015). An anti-inflammatory effect of tVNS was also observed in 2 of the above-mentioned studies: a reduction of tumour necrosis factor alpha (TNF-α) systemic levels was shown after both acute and chronic tVNS treatment (Stavros et al., 2015b; Stavrakis et al., 2020) and a reduction of c-reactive protein (CRP) levels was shown after acute tVNS treatment (Stavros et al., 2015b).

Further investigations are needed to optimize the tVNS parameters in

the treatment of AF, with great attention to the best ear area to stimulate to achieve an effective stimulation and the highest patient comfort. Moreover, the minimum duration of stimulation necessary to produce stable long-term effects is yet to be defined.

4. Conclusion

The imbalance of the ANS, namely an increased sympathetic predominance is a well-established risk factor for the onset and poor prognosis of cardiovascular diseases, as shown through HRV studies. tVNS, aimed at restoring the sympathovagal balance, is emerging as an innovative non-invasive and side-effect-free approach in the treatment of cardiovascular diseases such as arrhythmia, heart failure, and hypertension.

From the evidence cited above, the efficacy of tVNS would seem to be ascribable to a neuromodulation process mediated by the direct stimulation of vagal auricular afferents fibers innervating the ear. Compared to invasive VNS, this type of treatment would thus allow earlier and safer application in patients at the initial stage of cardiovascular diseases, avoiding the perioperative risks and the onset of irreversible remodeling of the cardiovascular system.

Further studies are needed to investigate optimal stimulation parameters, long-term effects and possible couplings with biofeedback loops such as respiratory activity.

Declaration of competing interest

The authors declare no conflict of interest.

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Cardiovascular responses to low-level transcutaneous vagus nerve stimulation

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ABSTRACT

Aims: The aim was to determine cardiovascular responses to an arbitrary protocol of transcutaneous low-level vagus nerve electrical stimulation (tVNS).

Methods: Study was performed in 15 male volunteers, mean age 23 years. Data were collected during two sessions – sham stimulation (no stimulation) and stimulation. Each session included one-hour resting phase followed by 15-min autonomic nervous system testing phase (Valsalva, deep breathing, wet-cold face tests), all in supine position. The right tragus stimulation parameters were: 20 Hz, constant current at sensation threshold, 1 ms rectangular pulse width. The ECG, noninvasive arterial blood pressure and thoracic impedance cardiography measurements were recorded and analyzed continuously with the Task Force® Monitor (CNSystems Medizintechnik GmbH, Graz, Ver. 2.2.10.0). *t*-Test for paired samples, paired Wilcoxon signed-rank, and one-way ANOVA for repeated measurements were carried out. $P < 0.05$ was considered significant.

Results: We demonstrated significant reductions of left ventricular contractility and output parameters, a trend for heart rate reduction, and resulting beneficial reduction of left ventricular work load. However, significant increases of blood pressure and total peripheral resistance were recognized, possibly as a reflex response.

Conclusion: It seems that our tVNS protocol has a potential for cardiac autonomic modulation. This gives us opportunity to advance our stimulation parameters with participant-specific adjustments. Further studies are however needed to prove the therapeutic potential of such approach in different patient groups.

1. Introduction

It is believed that transcutaneous vagus nerve electrical stimulation (tVNS), targeting the auricular branch (ABVN) or cervical branch of the vagus nerve (Yap et al., 2020), should produce therapeutic effects similar to direct vagus nerve stimulation and would overcome its limitations like invasiveness and higher costs (Johnson and Wilson, 2018). Indeed, the therapeutic potential of tVNS has already been documented in patients with depression, epilepsy, migraine as well as atrial fibrillation (AF) (Stavrakis et al., 2015, 2020). As most of the referenced methods differ in the parameters and protocols applied, there is currently no firm evidence on the optimal stimulation parameters that would provide the greatest therapeutic effects for a specific condition (Yap et al., 2020). The standardization of tVNS protocol is therefore of great importance to reach its full potential as a non-invasive and clinically relevant therapy. For the optimal therapeutic vagus nerve stimulation goal, the concept of balanced autonomic cardiovascular response has been suggested (Fukuda et al., 2015; Ardell et al., 2016, 2017). Consequently, optimal stimulation modalities may require participant-specific adjustments in a closed-loop setup, where stimulation parameters are set online, based on individual discomfort tresholds and recorded central and peripheral autonomic responses (Yap et al., 2020). For assessment of central responses, magnetoencephalographic imaging may be the most appropriate, since it has higher spatial resolution than electroencephalogram and higher temporal resolution than functional magnetic resonance imaging (Baillet, 2017). For assessment of peripheral cardiovascular responses, heart rate variability (HRV) (Malik and Camm, 1993) and microneurography (muscular sympathetic neural activity, MSNA) (Vallbo, 2018) may be applied in addition to heart rate (HR) and arterial blood pressure measurements (BP). However, HRV reflects indirect changes of parasympathetic activity at the sinus node level only. The MSNA records the sympathetic efferent

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activity directed toward peripheral blood vessels and is more appropriate for research purposes than for clinical setting. Surprisingly, changes of sympathetic efferent activity recorded with MSNA were neither associated with expected BP nor with expected cardiac output changes (Vallbo, 2018). To determine which parameters may be of additional benefit in assessing the cardiovascular autonomic responses, we performed continuous measurements with impedance cardiography, noninvasive arterial blood pressure (BP), and electrocardiography (ECG) during low-level tVNS in a group of young healthy men.

2. Methods

In this open label pilot study, 15 healthy male medical students aged 23 years (range 20–25), who signed written informed consent to participate in the study, were enrolled from December 2014 until May 2015. The study was conducted at Laboratory for Autonomic Neurology, Department of Neurology, University Medical Center Ljubljana (Fig. 1), and was approved by the State's Ethics Committee (107/07/14).

Testing was performed in non-sedated state, during single visit between 7 and 9 AM, in a quiet surrounding with air temperatures between 20 and 22 °C. A light meal was allowed 2 h before the visit. Examinees were asked to avoid caffeine and nicotine for 12 h and to void their bladder on the morning of the study. They were lied down to supine relaxed position and all measuring devices were connected and calibrated. A 20-min habituation phase followed. Data were collected during two sessions. Each session comprised of 60-min resting phase followed by the autonomic nervous system testing (ANST) phase that lasted for additional 15 min. During the first session a sham stimulation (no stimulation) was performed and during the second session a real stimulation was performed. A 15 min pause was allowed between the sessions for toilet use and stretching. The ANST included: a) Valsalva test, repeated 3-times with forced 20-second expiration against 40 mmHg pressure, b) deep breathing test with controlled respiration 6-times/min, repeated 2-times, and c) single wet-cold face test (~0 °C).

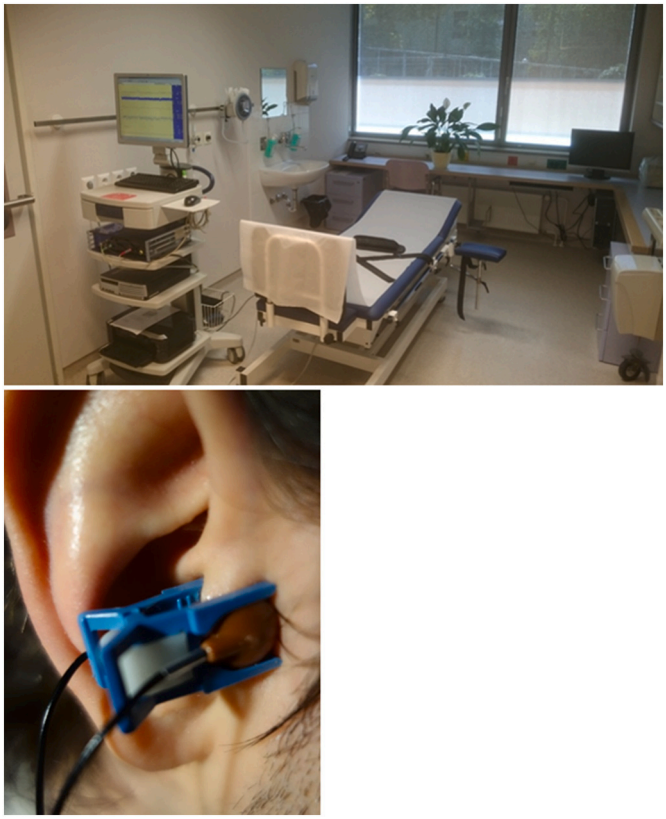

Fig. 1. Laboratory for Autonomic Neurology with equipment for continuous ECG, noninvasive arterial blood pressure, and noninvasive impedance cardiography recordings (see text for details). Two silver stimulation electrodes were positioned with plastic clip on the tragus of the right ear (below).

The tVNS protocol was adjusted according to Stavrakis et al. (2015). Stimulation was applied with transcutaneous electrical nerve stimulator (TENS, Gorenje, Slovenia) to the skin inside the right tragus, using two clip mounted silver electrodes (diameter 5 mm) (Fig. 1). A constant current stimulation was performed with 1 ms monophasic rectangular waveforms with frequency 20 Hz. The current was adjusted individually to the level of being barely perceptible and was typically less than 150 μA. ECG, noninvasive BP and thoracic impedance cardiography measurements were recorded continuously with the Task Force® hemodynamic Monitor (CNSystems Medizintechnik GmbH, Graz, Ver. 2.2.10.0, customer ID: 20040034) (Fig. 1). Standard 6-lead extremity ECG was used for monitoring and recording of HR (or RR intervals). Continuous BP measurements, using the vascular unloading method from the third and fourth finger of the left hand, were automatically corrected to oscilometric brachial BP values obtained from the right arm (error ± 5 mmHg). The cardiac function parameters were continuously measured with the impedance cardiography (Kubicek et al., 1966). These biosignals were recorded in 16-bit resolution with a maximal sampling frequency of 1000 Hz.

The parameters of left ventricular (LV) contractility (acceleration index – ACI [$100/s^2$]), LV output (stroke index – SI [ml/m^2]), LV work index (LVWI [$kg\ m/m^2$]), and total peripheral resistance index (TPRI [$dyne\ s/cm^5\ m^2$]) were analyzed off-line (Fortin et al., 1998). The time-domain analysis was performed for: HR [beats/min], RR intervals [ms], systolic BP (sBP), diastolic BP (dBP), and mean BP (mBP), all in [mmHg]. The ratio of maximal HR to minimal HR (HRmax/HRmin quotient) was a marker of Valsalva, respiratory arrhythmia and trigeminal nerve-sympathovagal responses. The spontaneous activity of the baroreceptors was determined by the sequence method using ΔRRI/ΔsBP slope [ms/mmHg]. The power frequency-domain spectra of heart dynamics were analyzed by the fast Fourier transform of instantaneous RR intervals as HR variability (HRV) power [ms^2] and of corresponding diastolic BP amplitudes as dBP variability (dBPV) power [$mmHg^2$]. Power spectral analysis was calculated for: very low frequency spectrum (VLF-HRV: 0–0.04 Hz), low frequency spectrum (LF-HRV: 0.04–0.15 Hz), and high frequency spectrum (HF-HRV: 0.15–0.4 Hz). For BP, the low frequency power spectrum LF-dBPV was analyzed only. All parameters were analyzed for each of six 10-minute resting phase recordings and ANST of the sham and stimulation sessions.

For the integral responses with predominant sympathetic modulation were considered increases of: ACI, SI, LVWI, TPRI, LF-dBPV, HR and BP. Responses with predominant parasympathetic modulation were considered decreases of: ACI, SI, LVWI, TPRI, HR and BP, and an increase of HF-HRV. For the marker of sympatho-vagal balance was considered: LF-dBP/HF-HRV quotient. The discomfort of testing was assessed after each session with a 10-point gradation questionnaire. We evaluated: stimulation perception, discomfort of lying still, fear of the procedure, coldness perception, and discomfort due to cuff inflations.

All datasets were gathered, stored and analyzed on PC HP Compaq using CNSystems software (Ver. 2.2.10.0). The Anderson-Darling normality test was performed before statistical analysis. Two-tailed *t*-test for paired samples, paired Wilcoxon signed-rank test for HRV and BPV data and one-way ANOVA for repeated measurements were carried out using IBM SPSS software (version 21.0). Value $p < 0.05$ was considered significant.

3. Results

The obtained results (mean ± SD) are listed in Table 1, with further graphical presentations for each examinee in separate Figs. 2 and 3. Comparing baseline (no stimulation) with one-hour low-lewel tVNS, indices of LV contractility, LV output, and LV work, significantly

Table 1
Aggregate data and statistical differences between no-stimulation and stimulation measurements. Data are presented as: mean ± SD.

	No-stimulation $n = 15$	Stimulation n = 15	p value[a]
Heart rate (HR) (beats/min)	63 ± 9.9	59 ± 8	*0.066*
Arterial blood pressure (mmHg)			
Systolic	116 ± 9.0	120 ± 8.4	**0.007**
Diastolic (dBP)	70 ± 6.3	74 ± 5.6	**0.0001**
Mean	86 ± 6.8	90 ± 5.8	**0.0052**
Acceleration index (100/s^2)	98.5 ± 22.23	86.7 ± 18.14	**0.0004**
Stroke index (ml/m^2)	60.0 ± 9.8	55.7 ± 9.1	**0.0027**
LV work index (kgm/m^2)	4.3 ± 1.68	3.9 ± 1.09	**0.029**
Total peripheral resistance index (dyne s/cm^5 m^2)	1876 ± 524	2225 ± 537	**0.0002**
Valsalva test (HR max/HR min)			
1	1.79 ± 0.40	1.92 ± 0.38	0.18
2	1.83 ± 0.32	1.96 ± 0.46	0.32
3	1.77 ± 0.34	1.78 ± 0.28	0.94
HR min ($n = 30$)	50 ± 6.04	49 ± 5.55	*0.11*
Deep breathing test			
HR max/HR min			
1	1.59 ± 0.22	1.52 ± 0.18	0.15
2	1.55 ± 0.26	1.50 ± 0.18	0.54
Baroreceptor sensitivity slope (ms/mmHg)			
1	35.4 ± 13.09	37.0 ± 14.34	0.63
2	33.6 ± 13.16	34.38 ± 13.06	0.72
Wet-cold face test			
HR max/HR min	6.5 ± 4.25	6.5 ± 3.14	1.0
Power frequency spectra (ms^2)			
LF/HF-HRV	1.33 ± 0.79	1.22 ± 0.65	0.55
LF-dBPV/HF-HRV	1.16 ± 0.61	1.05 ± 0.56	0.39
LF-dBPV	3.6 ± 2.81	5.5 ± 3.66	*0.08*

LV – left ventricle; HRV – heart rate variability; dBPV – diastolic arterial blood pressure variability; LF – low frequency spectrum; HF – high frequency spectrum.
[a] Calculated by paired *t*-test.

decreased (Table 1, Fig. 2). A trend in the direction of slower HR is seen (Table 1, Fig. 2). However, BP and total peripheral resistance, significantly increased (Table 1, Fig. 3). The HRV, dBPV, LF-dBP/HF-HRV, BRS and ANST parameters did not differ significantly between baseline and stimulation sessions (Table 1). Overall discomfort index in the group increased from 1.8 out of 10 points (no stimulation) to 3.6 (stimulation). There were no significant differences in any of the tested parameters between six 10-min recordings inside the baseline session and inside the stimulation session.

4. Discussion

The main result of this study is a significant decrease of LV contractility and LV output parameters caused by an arbitrary protocol of low-level right tragus tVNS. This response, in concert with a trend for slower HR, resulted in significant reduction of LV workload. Such result may be considered a beneficial parasympathetic neuromodulatory response. The tVNS protocol we used in this study (adjusted from Stavrakis et al. (2015)) therefore seems to have a potential for clinical application. In theory, this result might be an indirect efferent response transmitted through the medullary parasympathetic projections. The evoked sympathetic modulation response, demonstrated through significant increases in BP and peripheral resistance parameters, might be a secondary baroreceptor mediated reflex response. The divergent coactivation of the efferent parasympathetic and sympathetic systems might be another possibility. However, discomfort related or an afferent-driven withdrawal of background parasympathetic drive with divergent sympathetic overflow to peripheral vasculature, but not to sinus node or ventricles, may not be excluded. This response is supposed to be non-beneficial.

In favour of the low-level tVNS as feasible option for cardiac autonomic modulation are some data as follows. 1) There is sensitivity of the intrinsic cardiac nervous system to low-level vagus nerve stimulation. The functional threshold for activation of vagal afferent fibers was lower than that for activation of efferent fibers (Ardell et al., 2015). 2) The ABVN is a general A-group beta and delta sensory fiber and is one of the few branches to contain no motor fibers in man (Safi et al., 2016). The cardiac responses seen with ABVN stimulation are mediated through central vagal projections that involve the nucleus tractus solitarii (NTS) before projecting to other regions of the brain and brain stem (Tekdemir et al., 1998; Frangos et al., 2015). The direct efferent parasympathetic activity from tVNS used in this study is therefore unlikely. 3) NTS efferents activate other brainstem structures like nuclei ambigui and brainstem reticular formation, which then deliver processed signals to the heart surface bilaterally via the cervical vagus and thoracic sympathetic nerves. As such, tVNS may potentially exert more balanced cardiac autonomic effects than direct VNS (Armour, 2008; Ardell et al., 2016). In theory, divergent and cooperative sympathetic and parasympathetic responses may be possible (Shen et al., 2011; Hadaya and Ardell, 2020), explaining partially the opposite autonomic cardiac and BP responses in this study. Efferent parasympathetic fibers modulate several cardiac indexes, including chronotropy, dromotropy, inotropy, and lusitropy with direct connections to nodal and conduction tissues, atrial and ventricular myocytes and also indirectly via the ganglionated network of intrinsic cardiac nervous system (Armour, 2008; Ardell et al., 2017). This may be cardiac neurophysiological background for the reduction of LV contractility, LV output, and LV workload in this study. 4) In a dog model, VNS-evoked changes in cardiac function reflected the dynamic interplay between direct activation of efferents against afferently mediated withdrawal of central parasympathetic drive. Therefore, the potential exists for low-level sympathoexcitation as a result of activation by vagal afferents (Ardell et al., 2015). This mechanism might also contribute to the sympathetic modulation of BP demonstrated in the present study.

In contrast are results from Clancy et al. (2014) who studied responses to left auricular tragus stimulation in man (30 Hz, 0.2 ms pulse duration, 15 min active phase, at sensory threshold, sham was no stimulation). They recorded an increase of HRV (drop of LF/HF ratio) and reduction of sympathetic nerve outflow by microneurography (MSNA). They concluded that tVNS would be desirable in conditions characterized by enhanced sympathetic nerve activity, such as heart failure. However, the study group was heterogenous regarding age and gender, stimulation protocol was different from ours, and they did not record LV function parameters. Anyway, they demonstrated a significant increase in BP despite reduced MSNA. It was attributed to constriction and edema in the finger due to Finometer method of BP measurement. This would be less likely since stimulation lasted 15 min only. In addition, it was found that in subjects with high sympathetic efferent activity documented with MSNA an unexpectedly low cardiac output was found and vice versa (Vallbo, 2018). The mechanisms controlling this reverse relationship between the amount of efferent sympathetic activity recorded with MSNA and cardiac output remain to be clarified.

The main limitation of our study is nonrandomized sequence of stimulation applications. Sham stimulation (no stimulation) was always the starting session and stimulation was the second one, introducing the risk of potential time-related outcomes inside our enduring protocol. Although mild, the discomfort level was twice as high at the end of the stimulation session than it was after the sham stimulation. Typically, the sensation of discomfort has a growing character over time. However, all our recordings demonstrate completely stable values throughout each session that is pointing against significant time-related interferences. Nevertheless, the procedure related discomfort could not be completely excluded since painful sensation can undoubtly elicit sympathetic excitation. Next limitation is a cumbersome testing protocol on a single day visit. Two visits with randomly allocated stimulation sessions would be better option. However, a busy laboratory time schedule prevented

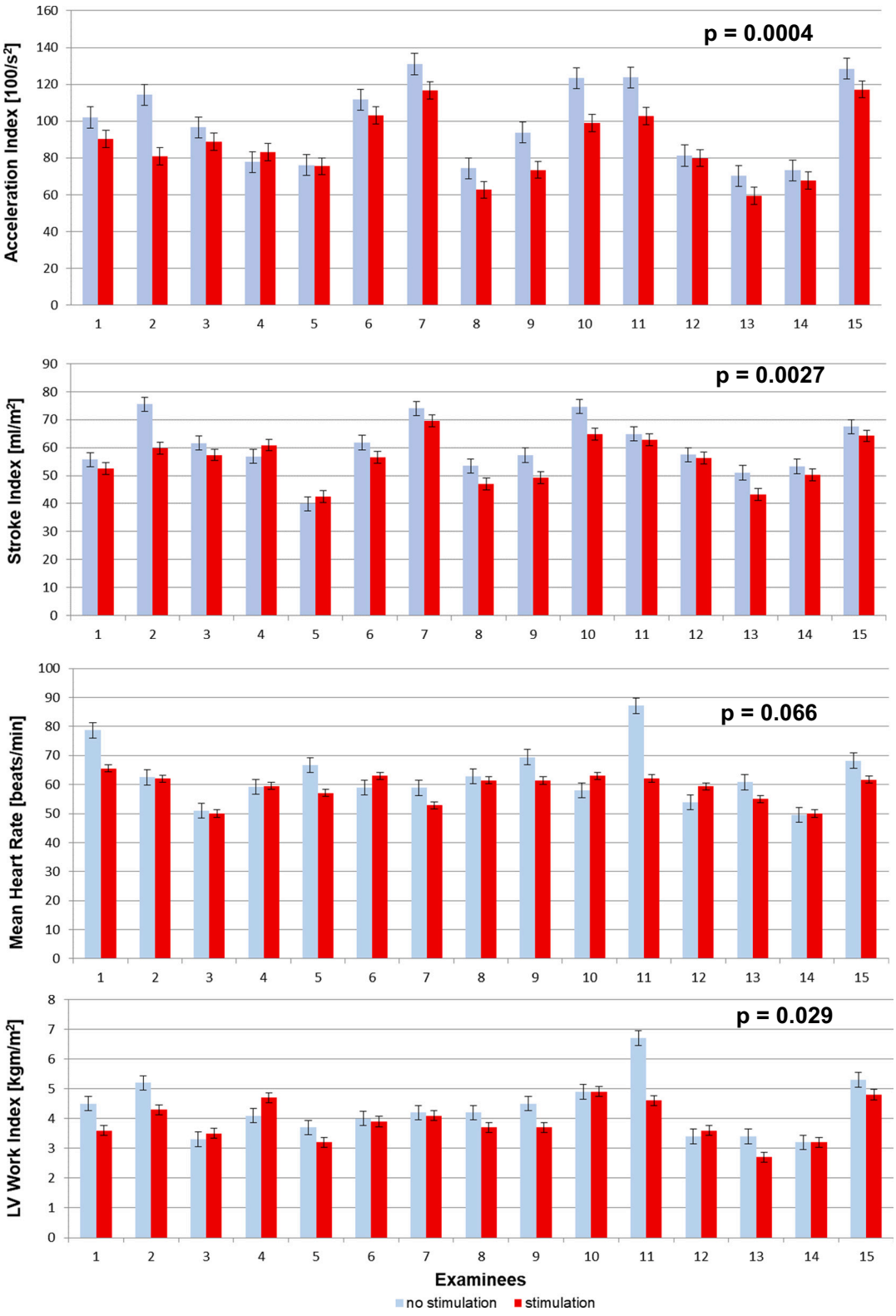

Fig. 2. Obtained results (mean ± SD) for each examinee during one-hour, low-level, transcutaneous vagus nerve stimulation. We demonstrated significant reductions of: acceleration index, stroke index, left ventricular (LV) work index, and a trend toward slower heart rate.

such implementation. Next limitation is a small sample size. However, the homogeneity of the sample and the paired sample approach assured a reasonable power for the concept verifying results. In addition, we presented all significant data for each examinee separately (see Figs. 2, 3). Another limitation of our study and others is that we tested healthy young male participants. The extent of the tVNS effects that might be observed in female, some patient groups, sedentary or older populations which characteristically have different or reduced parasympathetic modulation capacity therefore seems likely to be underestimated.

In conclusion, it seems that our adjusted arbitrary protocol of tVNS

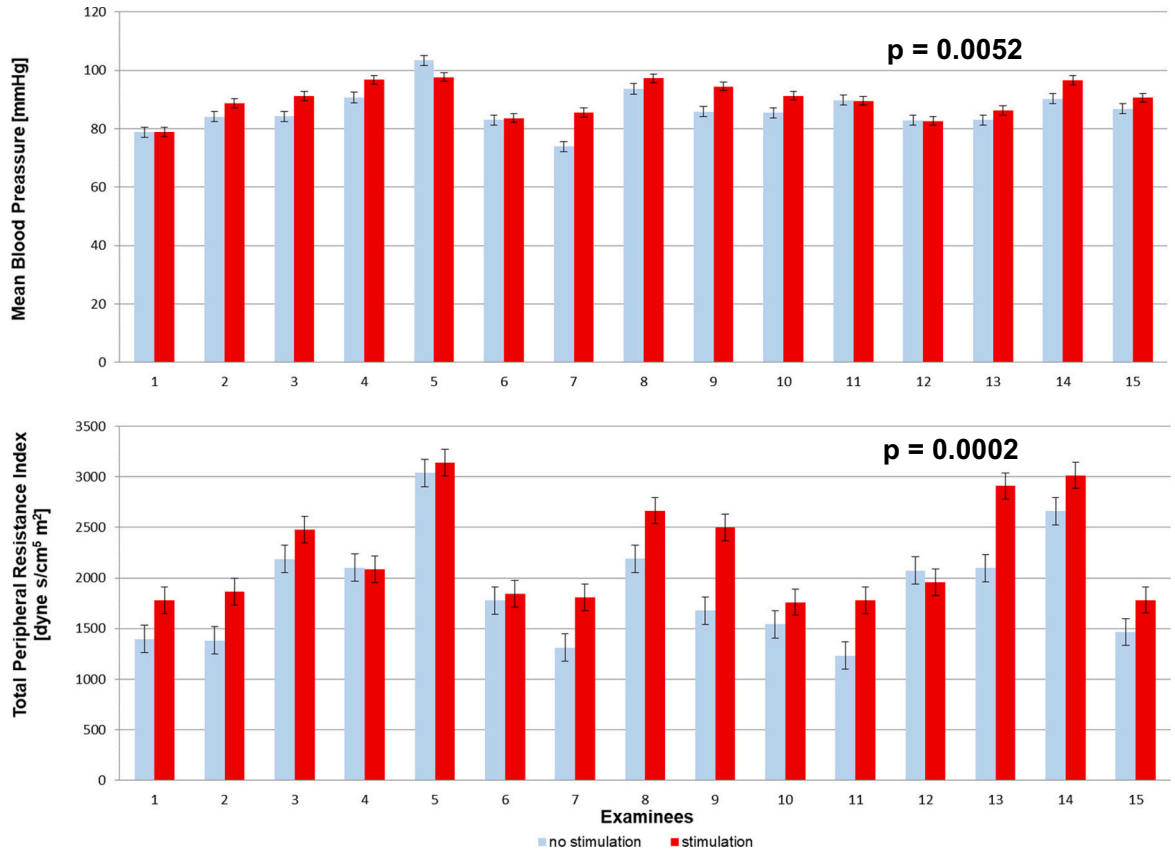

Fig. 3. Mean arterial blood pressure (mean ± SD) and total peripheral resistance index significantly increased during stimulation, possibly as a reflex response.

has a potential for cardiac autonomic modulation, since we demonstrated significant reductions of LV contractility, LV output, and LV workload parameters assessed non-invasively with impedance cardiography. This gives us opportunity to advance our stimulation parameters with participant-specific adjustments. Further studies are however needed to prove the therapeutic potential of such tVNS approach in different patient groups.

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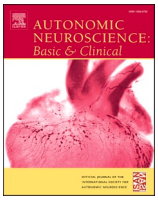

Review

Technical aspects and future approaches in transcutaneous vagus nerve stimulation (tVNS)

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ABSTRACT

With the emergence of transcutaneous vagus nerve stimulation (tVNS) as a therapy option for a multitude of clinical indications, the development and improvement of the stimulators becomes an increasingly important point of focus. This paper aims to discuss electrotechnical and software-based improvements to the state-of-the-art stimulators, in order to reduce the experienced side effects of the subjects as well as to increase the efficacy of the stimulation. It was found that side effects such as erythema and pain at the stimulation site are caused by electrolysis at the site of stimulation, which can be reduced by maintaining a voltage below the decomposition voltage. This can be achieved by using electroactive materials and rare-earth fractal metal coatings on the electrode, or by stimulating at the chronaxie with a biphasic rectangular waveform and an in-built short circuit to avoid an after-potential. It is furthermore discussed how the currently most promising stimulation site, the cymba conchae, can technically be stimulated in a feasible and tolerable way for the subject. Utilizing the subjects individual pain threshold is also demonstrated as a good indicator for optimal stimulation, as stimulation just below the pain threshold activates Aα and Aβ-fibers, while being unable to polarize the smaller diameter Aδ and C-fibers responsible for pain signaling. Finally, an outlook to individualized tVNS therapy is given, by using evolution algorithms that utilize device and subject data to optimize stimulation parameters.

1. Introduction

Transcutaneous vagal nerve stimulation (tVNS) is a relatively young technology which is quickly gaining more and more acceptance from physicians and patients alike. Although there are many tVNS devices currently on the market, many technical aspects and problems have not yet been solved. This paper aims to discuss a variety of technical aspects that may play an important role for current and future developments of tVNS devices.

Firstly, the design of the stimulation output stage is discussed, which plays an important role in potentially alleviating the more common side effects of tVNS such as itching, redness or swelling at the stimulation site. Secondly, a personalized software-based approach to tVNS is discussed, which aims to analyze stimulation parameters and subject data through a mobile application. Using this approach in conjunction with evolution algorithms, a software that can optimize the collected stimulation data, will likely be a key next step in improving tVNS in the next device generation.

1.1. Electrostimulation of nerves

Luigi Galvani was the first to describe the effects of electrical neuromuscular stimulation in frogs in 1780 (Bresadola, 1998), a discovery that led to the first electrical nerve stimulator by Perthes in 1912 (Goerig and Agarwal, 2000). Electrostimulation is currently one of the most advanced therapeutic tools in modern medicine and finds application in pacing the heart, the spine, the cochlea as well as in various other applications (Reilly and Diamant, 2011).

However, each application requires a specialized adaptation of electrostimulation, as the excitability of the cells, the anatomy of the surrounding area, the electrode placement and shape as well as the duration of the stimulation vary between each type of application and subject. Consequently, the special needs related to the application of electrostimulation via tVNS must be explored more closely.

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1.2. The electrode-skin interface

The basic, but not trivial, problem of electrostimulation is the issue of how to get the electrical stimulus into the subject and how to avoid or minimize discomfort or pain. In transcutaneous electrostimulation the current must overcome the barrier between the human skin and the applied metallic electrode (Morrison, 1980). Electrons transport the electrical current within the stimulator and its electrodes towards the skin surface. Yet, within the human body, different ions transport the electrical current, i.e. the electron current must be converted into an ionic current (Morrison, 1980). In an ionic current the electrical impulse is carried by cations (H^+, K^+, Na^+) and anions (OH^-, Cl^-). This transfer process from electron to ion current takes place at the so-called Helmholtz double layer (Fig. 1): The ions on the skin surface are solvated in water and thus form a hydrate cover causing their size to increase and their movement to slow. This hydrate cover prevents the ions from getting close enough to the electrode to assure the exchange of electrons and causes the formation of the electron double layer: one layer consists of electrons at the surface of the electrode (symbolized as "–" in Fig. 1), a second layer consists of ions in the electrolyte surrounding these ions.

How does the electron current generated by the stimulator cause an electrical stimulus, i.e. an ion current within the subject? There are two different ways for the current to transfer at a double layer interface: reversibly and irreversibly, depending on the voltage used. Their distinction is an important technical aspect in understanding and reducing the associated side effects of tVNS.

1.2.1. The capacitive way - reversible current induction

From an electrotechnical point of view, a double layer consists of two layers of opposite charges which are separated by an insulator. In the case of the subject's skin the insulator is a thin layer of water. The two layers of opposite charges and the insulating water form a capacitor (originally known as a condenser or condensator), the so-called Helmholtz- or double layer-capacitor C_{dl}. At low voltages as many ions as possible are attracted to the interface, the area where the liquid electrolyte contacts the surface of the stimulator's electrode. As soon as there is no more space for additional ions in the other layer, the current will stop. Thus, a larger surface area, i.e., a larger electrode, provides a larger capacity of the capacitor and allows for more current to be transported from the stimulator into the subject. The capacitive way is therefore ideally suited for alternating currents, as the capacitor must be permanently charged and discharged to maintain a constant current. It thus allows for a reversible induction of current into the subject.

1.2.2. The Faradaic way – irreversible current induction

Electrons can also overcome the hydrate cover barrier by using the so-called tunnel mechanism, a quantum mechanical effect facilitated by large electric potentials that are applied to the interface. The current-voltage relationship for a typical aqueous electrolyte (Fig. 2) shows

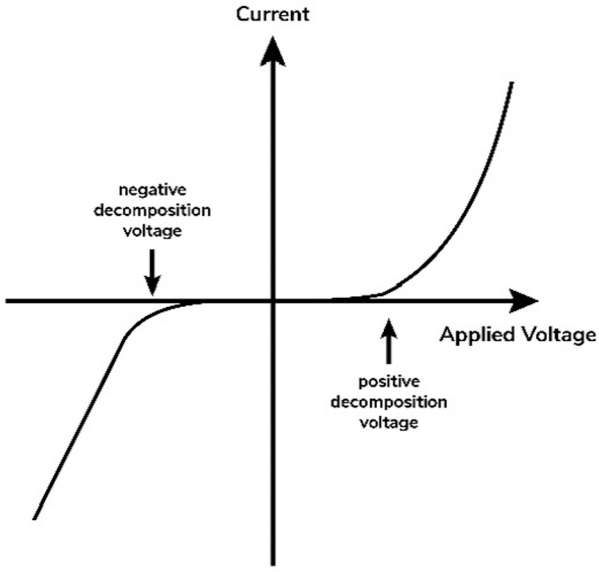

Fig. 2. An illustration of the decomposition voltage showing the start of the tunnel mechanism.

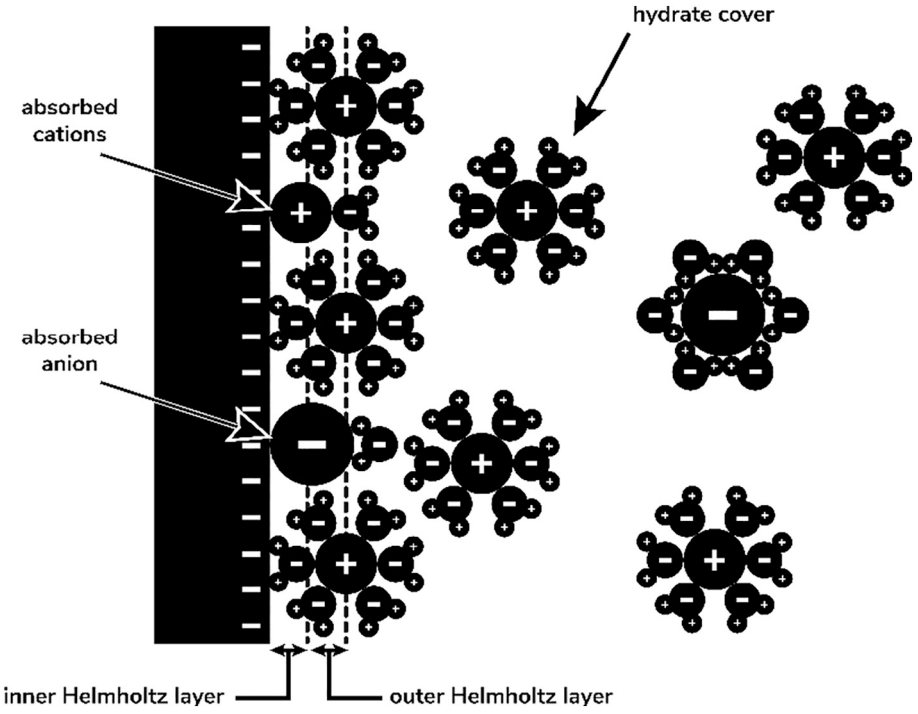

Fig. 1. The Helmholtz double layer forming around the electrode leading to a transfer in current.

that there is a certain threshold, the decomposition voltage, above which the current flow increases significantly and the tunneling process starts. This decomposition voltage is different for all ions as it varies due to the ions' difference in size and physical parameters. Provided the voltage applied by the electrical stimulator is higher than the decomposition voltages of the various ions on the subject's skin, the tunnel mechanism is initiated, i.e., electrons are enabled to tunnel between the metal electrode and the adjacent ions; this "flux" of electrons induces electrolysis in the aqueous sweat solution on the skins surface. The sweat on the skins surface mainly consists of aqueous NaCl. The electrolysis induced by the tunneling electrons causes the reduction of H^+ (from H_2O) to hydrogen gas, H_2, and the oxidation of Cl^- (from NaCl) to chlorine gas, Cl_2. As a result, the current is induced irreversibly due to the formation of the electrolysis products.

1.2.3. Technical stimulation objective

As shown, the use of a stimulation voltage above the decomposition voltage causes electrolysis of human sweat at the skin-electrode interface and leads to a change in pH of the skin. This causes many known side effects of tVNS, such as skin irritation, itching, pain, or erythema at the stimulation site. To avoid any of these side-effects, the output voltage of the stimulator must be reduced below the threshold of the decomposition voltage beyond which the tunneling mechanism starts. Current should therefore only be induced using the capacitive reversible way in order to reduce the subjects side effects.

1.3. How to reduce voltage below the decomposition voltage

There are several proposed ways how to keep the output voltage of the stimulator below the decomposition threshold and thus avoid or reduce unpleasant side-effects of tVNS. Lowering the output voltage by means of increasing capacitance, stimulating at the chronaxie and with the correct pulse shape will be discussed as follows.

1.3.1. Increasing the capacitance lowers the applied voltage (larger surface – less pain)

The decomposition voltage can be lowered by using electrodes with large double layer capacitance (Bolz et al., 1995). Since capacitance is defined as $C = Q/V$, where C is the capacitance, Q the electric charge and V the voltage, any increase in capacitance is associated with a decrease in the applied voltage. The capacitance depends on the capacitor's geometry and the type of dielectric material used, as demonstrated by the formula $C = k * \varepsilon_0 * (A/d)$, where k is the dielectric constant, ε_0 the permittivity of free space, A the area of the capacitor plates and d the distance between the capacitor plates. Consequently, an increase in the plate area of the capacitor increases its capacitance, which in turn yields a lower output voltage. In practice, this increase in plate area can be achieved with surface coatings, such as fractal coatings, on the stimulating electrodes (Bolz et al., 1995). As a result, the electrode's surface area increases and the output voltage reaching the skin decreases. This means that the risk of exceeding the decomposition voltage and thus causing electrolysis is reduced, leading to a reduction in side-effects.

In addition to increasing the electrode surface, the capacitance of an electrode can also be increased by using electroactive materials as a coating or as the base metal of the electrode. Electroactive materials can provide a high charge capacity and form a reversible redox system on their surface. This leads to an electroactive material with a higher capacitance and much lower impedance than other more common metal electrodes (Bolz et al., 1995). The most common example of an electroactive material is the well-known silver – silver chloride electrode, that is commonly used for short term applications in ECG recordings. For long-term applications iridium oxide is more stable and less cytotoxic. Although it is quite expensive, fractal iridium coatings are the state of the art in implantable pacing electrodes and other stimulation devices. The combination of fractal surface structure (largest active surface area) and iridium (electroactivity with a redox system on the surface that provides additional charge exchange capability) allow for a lower output voltage which in turn reduces the risk of exceeding the decomposition voltage and of experiencing any of the electrolysis induced unpleasant side effects of stimulation.

1.3.2. The chronaxie-rheobase relationship and the decomposition voltage

To successfully stimulate nerve cells with an electric pulse, a minimum depolarization threshold needs to be reached to excite the cell, i.e., a minimum voltage or current is needed. In order to reach this threshold, there are two variables to be considered: the stimulus duration and the stimulus strength. They are inversely proportional, with a larger stimulus duration allowing for a lower stimulus strength and vice versa (Fig. 3) (Irnich, 1980).

To decrease the stimulation voltage, the stimulus duration needs to be increased. The minimum stimulus strength required to reach the depolarization threshold is defined as the rheobase. The rheobase depends on the electrode geometry (smaller electrodes result in smaller rheobases), the subject's anatomy and the shape of the electrical pulse. However, stimulation at the rheobase theoretically requires an infinite stimulus duration which makes stimulation at rheobase intensity highly impractical. Furthermore, as most of the electrical current is dissipated through the surrounding tissue, the longer the pulse width the more energy is lost to the surroundings.

Therefore, an electrotechnical compromise must be reached that assures that the stimulation duration is long enough to guarantee a stimulation voltage below the decomposition voltage, yet still as low as possible in order to be feasible. The compromise to this problem is the use of the chronaxie, which is defined as twice the stimulus strength of the rheobase. Stimulation at double the rheobase intensity, with the respective stimulation duration, i.e. the chronaxie, balances the need for a low current (that avoids or minimizes unpleasant side-effects due to the tunnel mechanisms and subsequent electrolysis occurring above the decomposition threshold) and the attempt to minimize the amount of energy increasingly lost during stimulation to surrounding areas that comes with longer stimulus duration. The chronaxie depends on the diameter of nerve fibers and is estimated to be around 126 μs for larger fibers and around 225 μs for the smaller fibers (Tasaki, 2014).

1.3.3. The pulse shape

From an electrotechnical view, a stimulation pulse is created by simply using a battery with an on/off switch. Switching the device on or off then creates a pulse. However, while the pulse width can be adapted by changing the time between switching, the amplitude of the stimulation cannot. To be able to change the amplitude of the stimulation, a small capacitor is used that is charged by the battery until the desired voltage is achieved, which is then discharged. This is still the current state of the art in implantable pacemakers (Webster, 1995).

It is furthermore of importance to consider the variation of different

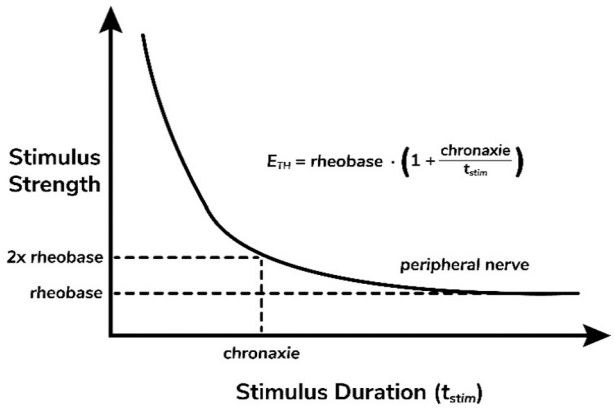

Fig. 3. An illustration of the chronaxie-rheobase relationship.

skin impedances when discussing the pulse shape used, as this directly affects the induced current. While skin impedance is already different in each person due to individual skin thickness and different amounts of sweat glands, it can also vary due to age or underlying causes such as diabetes. Due to this range of impedances the constant voltage approach leads to different output currents according to Ohm's law. This poses significant problems as different currents can be induced in people with different skin impedances, making medical comparisons impossible.

Instead of the constant voltage approach, it might be a better, though more expensive, approach to use a constant current output device with a closed loop regulator: the output current is measured, and the output voltage is immediately adapted to keep the current constant. Thus, the output pulse is rectangular and automatically adapted to any individual impedance differences in different types of skin.

However, the use of monophasic constant voltage or constant current pulses leads to a residual charge remaining on the Helmholtz capacitor of the electrode, called an after-potential. The remaining charge could cause long-term pH changes and electrochemical effects due to absorption of ions without a hydrate cover. Furthermore, having a residual charge makes it unfavorable to measure any sort of bio-signals with the stimulating electrode. To avoid such residual charges and subsequent skin irritation, the ideal waveform would be a biphasic rectangular waveform that discharges the interfaces after each pulse, with an automatic short circuit built in after each pulse in order to discharge the electrodes completely.

1.4. Achieving effective stimulation

So far, various ways of lowering the output voltage below the decomposition voltage have been outlined for trancutaneous nerve stimulation in general. However, to achieve transcutaneous stimulation of the vagus nerve, further aspects must be utilized to achieve effective stimulation. The correct level of current output and the site of stimulation are both essential to ensure effective stimulation and will be discussed in the following.

1.4.1. Current output

The tVNS threshold current to be applied in a subject is an individual parameter that depends on the subject's ear anatomy, skin thickness, humidity, and a variety of other factors (Farmer et al., 2021). If the current is too low it cannot be effective, if it is too high it will cause pain and other negative side effects. Thus, the optimal current must be identified in order to ensure both efficacy and safety for the subject.

Table 1 summarizes the classification of somatic and autonomic nerve fibers according to Erlanger and Gasser. Nerve fibers with a larger diameter can be depolarized with a lower stimulus intensity, as a larger nerve will allow more current to flow through and thus depolarization is achieved earlier. The auricular branch of the vagus nerve (ABVN) contains on average 385 myelinated nerve fibers on its left and 363 on its right, with approximately 20% (64 and 78 axons respectively) Aβ fibers that have a diameter of more than 7 μm (Safi et al., 2016). It is important to mention that this study used embalmed subjects with varying medical histories, which could have an impact on the results found.

Table 1
Nerve classification according to Erlanger and Gasser (Gasser, 1941).

Nerve fiber type	Myelinated	Diameter (μm)	Speed (m/s)	Function
Aα	Yes (thick)	10–20	60–120	Proprioceptors; somatic motor
Aβ	Yes	7–12	30–75	Mechanoreceptors of skin touch, pressure, vibration sense
Aχ	Yes	4–8	20–40	Efferent motor fibers to muscle spindles
Aδ	Yes (thin)	2–5	10–30	Sensory fibers mediating, cold perception, and pain
B	Yes (thin)	1–3	3–20	Preganglionic autonomic fibers
C	No	0,5-1,5	0,5–2	Sensory fibers mediating pain, warmth, and heat perception. Postganglionic autonomic fibers

Since pain signaling fibers are smaller diameter Aδ or C-fibers, the right current level for tVNS can be found by utilizing the subjects individual pain threshold. The small diameter Aδ and C-fibers require significantly higher stimulus intensities for depolarization than the thickly myelinated, larger diameter Aα or Aβ fibers. To avoid unpleasant or even painful sensations during tVNS, the stimulus intensity needed to still activate Aα or Aβ-fibers can be found by raising the output intensity of the stimulator to a level where the subjective pain threshold is reached, and then reducing the stimulus intensity just below the individual pain threshold. This approach ensures successful stimulation of the thickly myelinated nerve fibers but no longer stimulates the small diameter pain mediating fibers (Kaniusas et al., 2019). However, the mechanism of this approach is still not fully understood, and more research is needed to clarify the specific mode of action of tVNS.

1.4.2. Site of stimulation

Currently, various tVNS devices are commercially available. All of them claim to stimulate the subject's vagus nerves at different locations, most frequently at the ear. In order to maximize the efficacy of this treatment the different sites should be evaluated.

Historically the first devices were implantable solutions that stimulated the cervical nerve. Thus, the first generation of tVNS devices used the same location at the neck. The disadvantage of this approach is that efferent as well as afferent fibers are stimulated, although only the afferent fibers cause the therapeutic benefit. In addition, these devices often also stimulate the surrounding muscles causing undesirable muscle contractions.

The next generation of tVNS devices used brackets of electrodes that were attached to the ear lobe or the tragus of the human ear. This approach assured easy attachment, as well as electrode contact with low impedance and enabled the subject to integrate the therapy into their daily life. A disadvantage of the approach was the high pressure of the electrode brackets onto the ear causing discomfort, skin irritation, swelling and inflammation.

Furthermore, it has been shown that the ear lobe does not contain any vagal nerve fibers and that the tragus contains rather few vagus nerves. Instead, the highest fiber density of the auricular branch of the vagus nerve is located at the cymba conchae (Peuker and Filler, 2002). This was furthermore confirmed by fMRI studies, showing that stimulation of the cymba conchae activated the nucleus solitary tract (NTS) and the locus coeruleus (LC) (Yakunina et al., 2017). These results indicate that stimulation at the cymba conchae is likely the current best stimulation site for tVNS. The NEMOS® device (by Cerbomed, Erlangen, Germany) was the first tVNS device utilizing the cymba conchae (Yap et al., 2020). The electrode applicator of this device consists of a silicone ring placed between the tragus and the antitragus and uses a plastic spacer to position the electrode into the cymba conchae. While this electrode applicator is useful in directly targeting the cymba conchae, it brings with it two disadvantages: it provides rather poor horizontal fixation to the stimulating electrode, and it partially blocks the external ear canal. Recently, a headphone-like applicator has improved the positioning and fixation of the stimulating electrode at the cymba conchae for many subjects (Fig. 4). However, due to its *one-size-fits-most* design, there are as of now subjects for whom no suitable electrode applicator has been found, for instance for children. A future solution to this problem may be found using permanently applied or implantable electrodes.

Fig. 4. A headphone-like electrode design that allows stimulation at the cymba conchae. Courtesy of tVNS Technologies GmbH.

2. Future outlook for next generation tVNS devices

All the currently available tVNS devices provide similar options to each subject: The subjects receive a stimulator that provides the same, fixed set of stimulation parameters such as the frequency, amplitude, pulse width and pulse cycle. In general, the user only has control over the stimulation intensity or duration of applying tVNS. In an attempt to simplify the development and approval of tVNS devices, this "standard" approach does not consider any individual differences between subjects regarding anatomical and neurological aspects. However, it seems important for improved efficacy and compliance to implement the above mentioned electrotechnical aspects of tVNS in the next generation of devices. Moreover, future tVNS devices should assure a more personalized, patient-oriented approach towards vagal stimulation. For example, software might be developed that utilizes application-based data collection or back-end evolution algorithms to find optimized treatment routines for the individual subject.

2.1. Application based data collection

A first step towards a more personal tVNS treatment could use application-based data collection gathered from the individual subjects. The data might comprise both technical data from the device itself, as well as personal data regarding the subject's wellbeing. Theoretically the subject could learn on their own which stimulation time or intensity works best for them, however in practice it seems hard to notice trends when the improvements in a subject's wellbeing are incremental, non-linear and subject improvement typically sets in after several months of stimulation. A better approach to solving this problem might be a smartphone application that automatically logs the stimulation time, duration, and intensity, and allows the user to input digitizable information on their status improvement or deterioration and overall quality of life in form of a standardized questionnaire. The application could collect and analyze historic data and learn from weekly input how the applied technical parameters affect the subject's wellbeing. It could then either recommend the subject how to optimize their individualized treatment routine or automatically modify treatment parameters.

Furthermore, this approach could in theory allow for the utilization of linked smart-devices currently on the market to collect physiological data from the subject, such as heart rate, sleep quality and activity. This data could be incorporated into evaluating the subject's treatment routine in conjunction with data collected from the tVNS application.

2.2. Evolution algorithms

A more futuristic outlook for future generations of tVNS devices would be the use of evolutionary algorithms (Rechenberg, 1973). These types of algorithms are typically used in mathematical problems to optimize the solution to a given problem by comparing the results of incrementally changed inputs, when a perfect solution cannot be calculated (Rudolph, 2000). In practice this approach means that by incrementally changing a subset of parameters while controlling their effects, an optimal solution to the problem may be found. This evolutionary approach may also be applied to tVNS devices.

The technical implementation of this evolutionary algorithm could be to use a cloud service behind the smartphone application. That cloud service could either analyze the data of one subject locally or of an entire cohort of device users. By incrementally changing the device parameters experienced by the device users and correlating these to their respective wellbeing, a more optimal and personalized treatment routine is determined. This approach would also allow for a differential treatment of different clinical indications (e.g. through varying questionaries or collected data for each indication) and optimize existing treatment routines. It is furthermore important to mention the necessity of a large enough cohort of participants to directly establish a causal link between the effectiveness of the parameter set and the subject's wellbeing.

However, this approach would most likely lead to regulatory difficulties as it would require that device parameters can change by themselves, as well as subject data being stored on external servers. Furthermore, if the data is analyzed for all subjects collectively, this would lead to a subset of device users acting as the control group which would also cause regulatory scrutiny. It would therefore be essential that the tVNS device can only modify a small range of parameters that have been pre-approved and have shown efficacy. Moreover, subjects must consent to such device-initiated modification of parameters before the algorithm can be implemented. Despite unresolved technical and regulatory obstacles, this approach currently seems to be the most viable option to personalize treatment with tVNS and to better adjust therapy to so far uncontrollable variables, such as skin impedance or nerve fiber density.

3. Summary

The future development of tVNS will be based on technical improvements. On the one hand the stimulation itself will be improved by optimized electrodes (i.e., fractally iridium coated electrodes) and improved output wave forms like biphasic constant current pulses. On the other hand, personalization will further improve the therapy. This can be achieved by careful data collection of technical as well as personal data and an automatic evaluation of trends and therapeutic results. The integration of evolution algorithms may further improve the tVNS technology.

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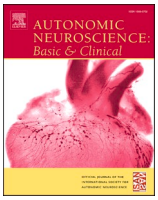

Review

t-VNS to treat disorders of behaviour in Prader-Willi Syndrome and in people with other neurodevelopmental conditions

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ABSTRACT

This paper proposes that tVNS has the potential to be a new treatment for some of the behaviour difficulties that may affect people with intellectual disabilities and/or autism, particularly those people born with specific neurodevelopmental syndromes. Behaviours, such as emotional outbursts, physical aggression, and self-injury are a relative common occurrence in these groups and have a significant impact on wellbeing and quality of life for the individuals and their families. Such behaviours have generally been understood through the lens of learning theory, the likelihood of their occurrence being shaped and reinforced by the responses of others. However, when vagus nerve stimulation has been used to treat epilepsy improvements in cognition, behaviour, and general wellbeing have been noted suggesting that with these behaviours other causal mechanisms are also important. More recently incidental findings from a proof of concept study where vagus nerve stimulation was given, using an implanted device, to people with the genetically determined neurodevelopmental disorder, Prader-Willi Syndrome (PWS), findings of benefit supported the above view. A second study, this time using tVNS, reported a similar result. In this paper we review the evidence for the use of tVNS for behavioural problems, consider the challenges when conducting trials in this population, and reflect on what the preliminary observations in people with PWS tell us about the possible mechanisms that underpin such behaviours.

1. Introduction

This paper explores a potentially important new use for vagus nerve stimulation for people with intellectual disabilities, which is as an intervention for what is generally referred to as 'challenging behaviour'. These are behaviours, such as aggression or self-injury that commonly manifest in people with an intellectual disability and impact severely on their quality of life and that of their families. Vagus nerve stimulation is approved for, and has been extensively used, in treatment-resistant epilepsy, a condition that is particularly common in people with intellectual disabilities (ID), with the prevalence of epilepsy increasing the greater the severity of the disability (Robertson et al., 2015). 'Intellectual disabilities' is the generic term for a group of people who have in common: evidence of global developmental delay in early childhood; a delay in or a failure to acquire living, educational and/or social skills appropriate for their age; and evidence on testing of an intellectual impairment, usually taken to be an IQ of less than 70 on a standardised and professionally applied intellectual assessment. Whilst people with ID have these traits in common, as a group they are heterogeneous in terms of their developmental profiles; in the degree of functional, social and/or intellectual impairments; in the cause of their impairments and disabilities; and in the presence or absence of other developmental, physical or mental health co-morbidities. Whilst there is much still to be established about the different causes of intellectual disabilities, there has been an increasing number of specific syndromes identified associated with the presence of intellectual disabilities. These are usually of genetic origin. Given the presence of delayed or atypical brain development, these syndromes are generally referred to as 'neurodevelopmental disorders or conditions'. Down syndrome is the most prevalent of the specific neurodevelopmental syndromes affecting around 1:1000 births. Others include Prader-Willi, fragile-X, Smith Magenis, Williams Syndrome and many more. The term, neurodevelopmental disorder/condition is also used to include people who meet criteria for autism spectrum conditions (ASC).

Whilst people with these neurodevelopmental syndromes usually have in common the presence of intellectual disabilities, it is clear that people with different syndromes differ in their propensity for specific behaviour problems or mental ill-health. The behavioural, cognitive and

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mental health characteristics of a syndrome have become known as the neuropsychiatric or behavioural phenotype of the syndrome (see Holland, 2020 for review). In this paper the impact of VNS on challenging behaviours in people with one specific condition, Prader-Willi syndrome (PWS), is described and we consider these findings within the broader context of intellectual disabilities and autism, where evidence for the benefits of VNS has yet to be systematically determined. We argue that if the present evidence suggesting benefit from VNS is supported by findings from larger and more systematic trials, VNS will provide an important and safe new treatment for what are often serious and intractable problems. In addition, these observations would also provide important and novel insights into underlying mechanisms that may underpin challenging behaviours.

2. Prader-Willi Syndrome: a genetically determined neurodevelopmental syndrome

PWS should generally be diagnosed in the neonatal period as the infant with PWS is extremely floppy (hypotonic) at birth, is unable to suckle normally and often requires tube feeding, and boys usually have undescended testes. This early phenotype evolves over time with delay in the acquisition of developmental milestones and eventually the onset of a marked tendency to over-eat (hyperphagia), which can result in life-threatening obesity if the diet is not carefully managed. Without hormone supplementation the phenotypic effects of relative growth and sex hormone deficiencies become apparent with short stature and sexual immaturity, respectively (Cassidy et al., 2012). In childhood and throughout adult life the hyperphagia remains and also, usually during childhood, an increased propensity to emotional outbursts, repetitive and ritualistic behaviours, and severe skin picking become apparent, and their occurrence are recognised as characteristic of the syndrome (Holland et al., 2003). For those with one particular genetic form of PWS (referred to as a chromosome 15 maternal uniparental disomy) there is also an increased risk for developing serious mental illness in late childhood and early adult life (Soni et al., 2008).

The genetics are complex. PWS results from the absence of expression of specific genes located on chromosome 15 at the q11–13 locus. These specific gene(s) are imprinted on the maternal chromosome 15 (not expressed), only the copies inherited from the father being expressed. PWS arises when there is the presence of one of three possible genetic abnormalities, which result in the absence of the paternal copy of the relevant genes and the subsequent complete absence of expression of these genes (Angulo et al., 2015). The combination of many of the characteristic features of the PWS phenotype, such as the hormone deficiencies and the hyperphagia, indicate that there is an abnormality of hypothalamic functioning (Tauber and Hoybye, 2021). Impairments in the development and functioning of the hypothalamus and its afferent and efferent projections may also account for some of the neuropsychiatric aspects of PWS, and much of the PWS phenotype can be conceptualised as an impairment of normal homeostatic regulation.

3. VNS to treat hyperphagia in people with PWS

The first study published on VNS involving people with PWS investigated the use of an implanted device to determine whether it impacted on the hyperphagia. This proof of concept trial was informed by previous observational and neuroimaging studies of eating behaviour in people with PWS that indicated there was a failure of satiation (Hinton et al., 2006) and also that food may have a greater reward value (Holsen et al., 2009). These studies suggested that there was an insensitivity of the pathways, as opposed to the presence of a complete absence of activity, and therefore the regulatory system may be modifiable. The clinical picture was one of leptin insensitivity as a result of impaired functioning of satiety pathways in the hypothalamus and in the resultant activation of the cortex. Conscious experiences of hunger only diminish and feelings of fullness only increase after excessive amounts of calories have been eaten (McAllister et al., 2011 for review).

The justifications for this first trial of VNS in people with PWS were twofold. The first was theoretical, based on the above observations and the known role of the vagus nerve in the feedback loop between the gut and the hypothalamus, which activates during food intake, and eventually leads to a cessation of eating. It was reasoned that over-driving the system through stimulation of the vagus nerve might overcome this insensitivity. Secondly, there was evidence, which was largely anecdotal, of a significant reduction in weight when VNS was used for treating epilepsy or depression. A search of PubMed and Web of Knowledge^{SM} undertaken at that time, with the terms ‘vagus nerve stimulation’ and ‘eating’, ‘appetite’ or ‘weight’, identifying eight relevant studies. Five referred to people undergoing VNS for intractable epilepsy; two were studies of people undergoing VNS for treatment-resistant depression; and one was to assess the effects of VNS on otherwise healthy obese participants. However, the largely incidental findings reported were contradictory and it was often not possible to discern participants' initial weights thereby complicating the conclusions of these reports. In the one study reporting VNS use in obese individuals free from epilepsy or depression, considerable benefit was indicated for some, but not others (Roslin and Kurian, 2003). Another study described acute reductions in cravings for sweet foods with active VNS in obese participants (Bodenlos et al., 2007). However, the methodology and conclusions from this study have been challenged (Gibson and Mohiyeddini, 2008).

For the full details of this first trial of VNS in people with PWS see the published paper (Manning et al., 2016). Three adult participants with PWS agreed to have a Cyperonics model 102 generator implanted. The studies used a repeated measures longitudinal design with baseline, active stimulation and reduced stimulation phases prior to stopping stimulation. At the start of the stimulation phase the following stimulus parameters, which are approved for treating epilepsy, were used: 0.25 mA output current, 30 Hz frequency and 500 μs pulses, with a 30 s on and 5 min time off cycle. The output current was increased by 0.25 mA steps to a maximum of 1.5 mA. The primary outcome measures were weight and body composition and a measure of food consumption over 1 h. However, contrary to our prediction, no impact on weight or eating behaviour was observed (see Fig. 1).

As can be seen in Fig. 1, one participant's weight increased over the period of stimulation, and in the other two, their weight either remained stable or decreased marginally (for one participant the weight was 2.9% below his baseline weight at the end of the study). Whilst with two of the participants the carers reported some improvements in their eating behaviour, there was no reduction in food intake when exposed to 1 h's free access to food under experimental conditions. It was concluded that VNS, used for at least six months with stimulation parameters similar to those used for epilepsy, did not improve hyperphagia. However, some months into the stimulation phase, the families of two participants and the two participants themselves reported marked improvements in behaviour with reductions in the severity and frequency of emotional outbursts to the extent that both of these participants asked that the device remain activated after the end of the study. As this study was investigating hyperphagia, no other behaviours, no baseline measures of these other behaviours had been collected, and therefore a direct comparison across different conditions was not possible. One participant did not report any benefits but she had not had significant behaviour problems on entry into the trial. Ten years on, the two participants who benefitted from VNS continue to have stimulation and both have had a battery replacement. In the case of one of the participants, their previous problematic behaviours returned before they realised that the battery was flat. Since replacement his behaviours have again improved.

These observations not only raised the possibility of a new treatment for challenging behaviour, at least in people with PWS, but also provided some insight into the likely biological mechanisms that might underpin such behaviour, suggesting that impaired emotional regulation in response to real or perceived threat as mediated through the autonomic

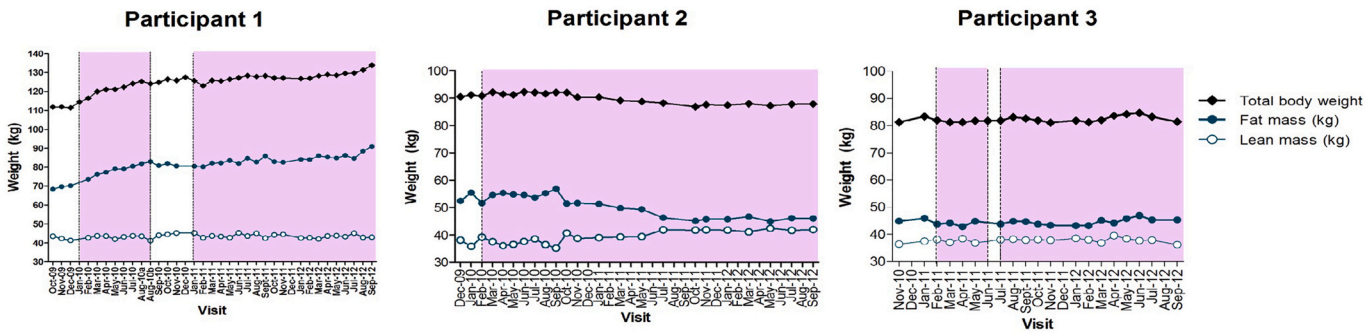

Fig. 1. Showing weight over time in the three participants receiving stimulation of the vagus nerve from an implanted device.

nervous system, was the primary deficit much in line with the polyvagal theory (see Mulkey and du Plessis, 2019 for review). Whilst such behaviours may still be reinforced and shaped over time, this finding suggested that this was not the main explanation for the occurrence of such behaviours. In preparation for a proposed new trial of VNS, this time focusing on the treatment of challenging behaviour in people with PWS, we undertook a review of the literature to determine the nature and significance of any existing evidence in the wider population of people with neurodevelopmental conditions, including people with autism with and without ID.

4. VNS, temper outbursts and other behaviours in people with ID

A broad search of the PubMed and Web of Knowledge[SM] databases was again undertaken using specific search terms covering vagus/vagal nerve stimulation and a variety of outcomes (see below). In total 169 articles and conference abstracts were identified concerning possible influences of VNS on quality of life and cognitive, social, behavioural and psychiatric functioning, including case studies and studies that were questionnaire-based or were using experimental paradigms. For example, there were reports that when VNS was combined with behaviour therapy for people with ASC there were additional improvements in behaviour and general quality of life (Engineer et al., 2017; Jin and Kong, 2017; Galli et al., 2003). Memory and social functioning has also been found to improve in people receiving VNS for epilepsy (Sun et al., 2017; Marwan et al., 2008), as has cognitive functioning in general (Schachter, 2004). These benefits appear to be independent of the effects of VNS on seizure control. However, some of the findings are contradictory, with findings suggesting that VNS does not lead to improvements in these areas of functioning or that any improvements observed are in fact dependent on the level of seizure control achieved. In addition, conclusions are further complicated by the use of anticonvulsant medication and the fact that the quality of life measures used often included items directly referring to seizure control. Similar problems arise in conclusions drawn from the literature regarding VNS use in depression, as global functioning when in a depressed state would be expected to improve as depressive symptoms were alleviated. However, we consider the evidence overall is sufficient to justify more systematic investigations of the use of VNS in people with neurodevelopmental syndromes to treat challenging behaviour. As described below findings from the second trial of VNS in people with PWS, this time using tVNS, supported this conclusion.

5. tVNS and temper outbursts in PWS

The second trial of vagus nerve stimulation involving people with PWS was prompted by the unexpected and serendipitous observations from the trial described above. Since the first PWS trial, tVNS had become more established and for this reason this non-invasive intervention for this second proof of concept study was considered more appropriate. As set out in the paper, the aim was to recruit a minimum of six people with PWS who had histories of at least two emotional outbursts a week on average (Manning et al., 2019). A repeat measures longitudinal design was again used with baseline phase of four months, active stimulation over 12 months, reduced stimulation phase (stimulation time 2 h a day) over four months. The primary outcome measures were changes in the mean number of daily outbursts between baseline and in each successive three months of the 12-month stimulation phase. The secondary outcome measures were changes in scores on the Challenging Behaviour Inventory. The stimulation protocol was that approved for treating epilepsy: four hours stimulation a day using the Nemos® tVNS device with an electrode placed in the cymba concha region of the left external ear. Stimulation intensity was increased by 0.1 mA steps, from 0.1 mA up to a maximum of 5 mA, using a pulse width of 250 μs at 25 Hz, until the participant reported a just detectable tingling sensation. tVNS was worn but switched off during baseline. As shown in Fig. 2 below from Manning et al., 2019, five participants completed all phases. Four of these five showed significant improvements in the outcome measures over successive three month periods during the 12 months of stimulation. In all four cases their behaviour deteriorated after a few weeks following a 50% reduction in daily wearing time. All requested to increase the wearing time back to 4 h daily. Since the end of the study three of the four participants have continued with daily tVNS with on-going benefit. The one participant who stopped has requested an implanted device and this is due to be implanted soon.

In addition to the quantitative data, semi-structured interviews of participants and informants were undertaken at the baseline and towards the end of the active stimulation phase. This qualitative data indicated that participants were better able to regulate their behaviour in response to some minor events so that it was possible for them to engage in other strategies to help them remain calm. In addition, they appeared more able to switch attention when required to do so, possibly due improvements in cognition and reduced stress. These early observations made when using tVNS indicated the need for further systematic study, both to definitively establish effectiveness and to identify potential biomarkers and mechanisms that both predict and mediate any beneficial effects.

6. Discussion

Importantly, the small studies described above with participants with PWS provided evidence for improvements in global functioning with VNS in individuals whose behavioural symptoms were not secondary to depression or seizures. This gave additional support to the view that the wider benefits of VNS observed when used to treat seizures or depression in other populations may be independent from any anti-seizure or antidepressant effects. VNS influences activity throughout the brain (Wang et al., 2021), and, consequently, has the potential to modulate a range of neural and cognitive processes. We propose that the observed favourable effects on emotional outbursts as seen in people

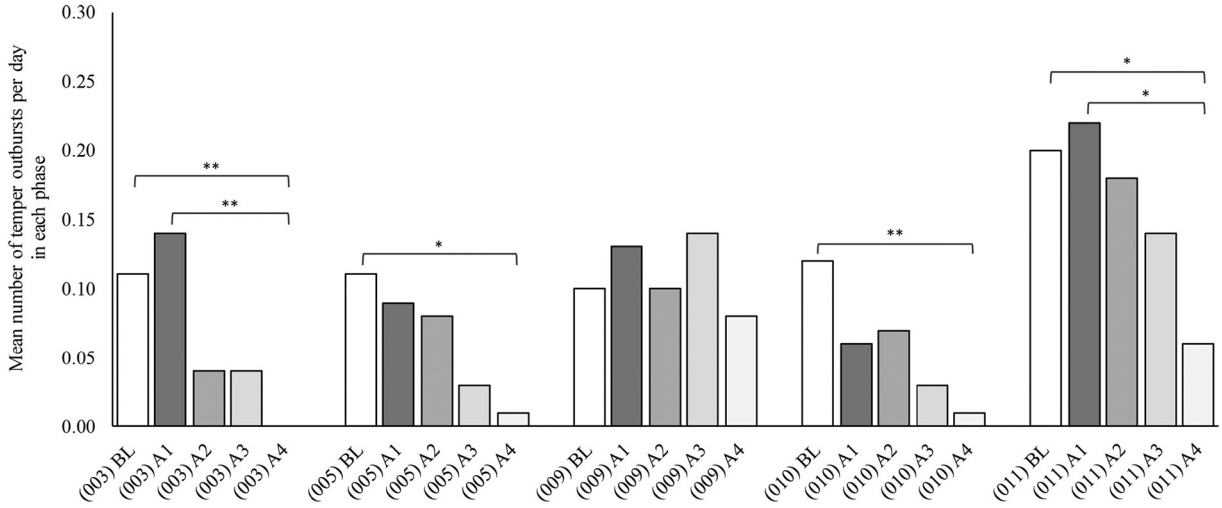

Fig. 2. From Manning et al., 2019. Mean number of temper outbursts per day in each phase for each participant. BL, baseline phase; A1, first three months of active phase; A2, second three months of active phase; A3, third three months of active phase; A4, fourth three months of active phase. Pairwise comparisons Dunn test. BL compared to A4: 003 ($p = .009$); 005 ($p = .021$); 010 (p = .009); 011 ($p = .045$). A1 compared to A4: 003 ($p = .003$); 011 ($p = .034$).

with PWS may be consequent upon the modification of afferent and efferent vagal projections on specific neural networks and the functioning of the autonomic nervous system in line with the polyvagal theory (see Porges, 2007). This may occur through the rebalancing of aberrant neural communications between systems involved in motivation and/or emotion and those associated with cognitive control and higher order processes that have been implicated in both aberrant satiety and problem behaviours (Eugenijus et al., 2019). Particularly with respect to PWS, the possible role of VNS in enhancing interoceptive awareness is of interest (Paciorek and Skora, 2020). PWS is characterised by insensitivity to satiety signals from the gut, thus contributing to hyperphagia, as well as a high pain threshold. However, the two studies reported would suggest that there is no direct impact of VNS on eating behaviour in contrast to the marked and direct effect on behaviour.

Experience from the trial of tVNS in people with PWS, however, identified some likely significant challenges to undertaking studies in this population of people with ID, including those with specific neurodevelopmental syndromes and/or autism spectrum conditions. First, tVNS requires that participants in any trial wear the device for the requisite period of time each day. Since the participants in a trial aimed at treating outbursts are necessarily selected on the basis of having such behaviours, maintaining compliance may be problematic. In the PWS tVNS trial there were early dropouts from the trial for this reason.

Secondly, in both PWS trials evidence of significant benefit was only apparent after some months of treatment, thus when using tVNS compliance has to be maintained over months. This length of time before there is an effect raises questions as to whether the tVNS or some other process is leading to this positive response, such as unintentional changes in support.

Thirdly, whilst there is still uncertainty about the use of medication to treat problem behaviour other treatments are being evaluated. In a case series of 14 people with PWS given sertraline it was reported that 13 improved significantly after 6 months of treatment (Deest et al., 2021). Medication has the disadvantage of possible side-effects but the advantage of a quicker response time. In any future trial a direct comparison between tVNS and medication may be indicated.

Finally, undertaking gold standard placebo controlled trials of VNS, whether from implanted devices or using t-VNS, raises the problem of the placebo condition. Sham stimulation is problematic as in both forms of VNS participants are aware of receiving actual stimulation. Furthermore, if a positive response to treatment may only become apparent after some months, there is concern about the ethics of allocating participants to a sham stimulation arm for a long period of time. These issues are compounded further when the condition in question is a rare disorder and recruitment may therefore be a challenge. Given such challenges, we propose that first, single cases or small case series from several sites, using a structured repeat measures single case or small group design with data collected at baseline and throughout the period of stimulation, should be undertaken as a means of building the evidence base. This is now more feasible, given that transcutaneous devices exist, one of which is already approved in Europe for PWS. These small studies should use a common approach to outcome measures and stimulus parameters. Secondly, clinicians and product engineers should work together to develop both psychological strategies to enhance compliance and design solutions that might, for example, enable the device to be worn during sleep. Thirdly, discussions with regulatory agencies need to take place to explore the trial solutions that would be acceptable to such agencies but at the same time would be feasible. Fourthly, if the developing evidence is convincing, if then required, a formal multi-site trial with sham and stimulation arms may be both possible and justifiable to deliver a definitive outcome through, for example, programming the devices to switch off after a few minutes when in the sham arm. Finally, our own experience in the second trial of participants with PWS was that those who may have gained the most benefit from VNS were the ones most difficult to engage. If sufficient evidence is established for the benefits of tVNS on behaviour, it would be clinically justified to recommend an implanted device for those whose behaviour prevented the use of tVNS. There are clearly important issues about consent that would need to be considered, particularly for those people with more disabling neurodevelopmental syndromes. However, the limited evidence so far, certainly for people with PWS, indicates that the benefits of treatment have been life changing and it is worth the effort to resolve these issues.

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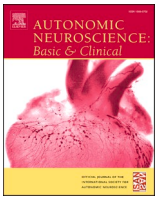

Functional anatomy of the vagus system – Emphasis on the somato-visceral interface

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ABSTRACT

Due to its pivotal role in autonomic networks, the vagus attracts continuous interest from both basic scientists and clinicians. In particular, recent advances in vagus nerve stimulation strategies and their application to pathological conditions beyond epilepsy provide a good opportunity to recall basic features of vagal peripheral and central anatomy. In addition to the "classical" vagal brainstem nuclei, i.e., dorsal motor nucleus, nucleus ambiguus and nucleus tractus solitarii, the spinal trigeminal and paratrigeminal nuclei come into play as targets of vagal afferents. On the other hand, the nucleus of the solitary tract receives and integrates not only visceral but also somatic afferents. Thus, the vagus system participates significantly in what may be defined as "somato-visceral interface".

1. Introduction

The vagus (the "wandering" nerve) is a major cranial nerve connecting the brain with neck, thoracic, abdominal and eventually also some pelvic organs. Although often introduced as the paradigmatic "parasympathetic" nerve, its visceral efferent component (and only this is referred to as parasympathetic) represents just one and not even the largest portion of its fibers. The vagus contains also branchiomotor (formerly called special visceral efferent) neurons and a quantitatively even more impressive afferent fiber contingent. It is this afferent component which is targeted by current methods of invasive (through electrodes wrapped around the cervical vagus) and non-invasive transcutaneous auricular (electrode on cymba conchae) or cervical (stimulation device over anterolateral neck skin) vagus nerve stimulation (VNS) for intractable epilepsy, depression and cluster headache (see Butt et al., 2020 for review; Silberstein et al., 2016). This review aims to briefly summarize the topography, peripheral innervation territories including the targets of preganglionic neurons and sensory structures as well as the brainstem nuclei of the vagus with only a short account of their major central connections. Special attention will be given to vagal innervation of the external ear and the upper aerodigestive tract, organs developmentally derived from foregut, pharyngeal arches, pouches and clefts where the somatic and visceral domains merge.

2. Vagus nerve: topography, branches, innervation territories, fiber spectrum

The rootlets of the vagus exit the medulla oblongata in the retro-olivary sulcus together with the root of the glossopharyngeal nerve and the cranial root of the accessory nerve. In rodents, the medullary exit and entrance of efferent and afferent vagal axons are separated into a ventral and dorsal root, respectively, a division which is not so evident in human. After piercing the dura, they leave the skull through the neural part (pars nervosa) of the jugular foramen, where the jugular or superior ganglion is located. The dural "sleeve" enveloping the vagal rootlets continues into the capsule of the jugular and nodose ganglia as well as the epi- and perineurium of the nerve, thereby significantly increasing its diameter. Immediately below the base of the skull, the large spindle-shaped nodose or inferior ganglion is formed. The vagus nerve continues its course through the neck located between the internal and, more caudally, common carotid artery and the internal jugular vein. On average, the nerve is found at the level of the laryngeal prominence at 3.5 cm lateral to the midline and 3.5 cm deep to the skin, with some variability (Hammer et al., 2018). Both blood vessels and

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vagus nerve, together with the ansa cervicalis profunda, are enveloped by the carotid sheath, in human a rather firm fascial structure which is anchored to the surrounding connective tissue (Fig. 1).

Average thickness of the human cervical vagus was variably described in post mortem studies between 2 mm (Pelot et al., 2020; Stakenborg et al., 2020) and 4.5 mm (Hammer et al., 2018), decreasing with age, but without sex or side differences. However, a study using high-resolution ultrasound revealed a larger cross-sectional area of the right versus left cervical vagus (Pelz et al., 2018). Five to seven fascicles were counted in the human cervical vagus (Hammer et al., 2018; Pelot et al., 2020; Stakenborg et al., 2020), while only one and as much as 46 fascicles were found in the mouse and pig cervical vagus, respectively (Pelot et al., 2020; Stakenborg et al., 2020).

2.1. Cranial and cervical branches

The first branch given off is the small ramus auricularis (auricular branch of the vagus nerve, ABVN), also called Arnold's nerve (Fig. 2; Testut, 1922; for review see Butt et al., 2020). Leaving from the jugular ganglion, it enters the mastoid canaliculus, engaging in anastomotic connections with branches from the facial and glossopharyngeal nerves within the petrosal bone and innervates parts of the external ear; a branch of the ABVN reaches the dura of the posterior cranial fossa. Dissection studies on embalmed specimens provided a fairly detailed map of the innervation territories of the ABVN and other auricular nerves (greater auricular, auriculotemporal, lesser occipital) in human showing some overlap but also an autonomous ABVN area in the cymba conchae (Peuker and Filler, 2002).

At the level of the nodose ganglion, a branch is given off to the pharynx. At the distal pole of the nodose ganglion, the superior laryngeal nerve splits off, coursing medial to the carotid artery and, before piercing the thyrohyoid membrane, issues its external branch to the cricothyroid muscle and branches to the cervical esophagus and the cricopharyngeal muscle, the latter branches being endangered during thyroid surgery (Prades et al., 2009; Uludag et al., 2017; Fig. 2). It is also this area where connecting branches with the cervical sympathetic trunk, eventually providing some sympathetic postganglionics to the vagus (Yang et al., 1999; Verlinden et al., 2016), and with the hypoglossal nerve, leading afferent fibers from the tongue to their cell bodies in the jugular ganglion (Neuhuber and Mysicka, 1980 and references therein), are regularly observed.

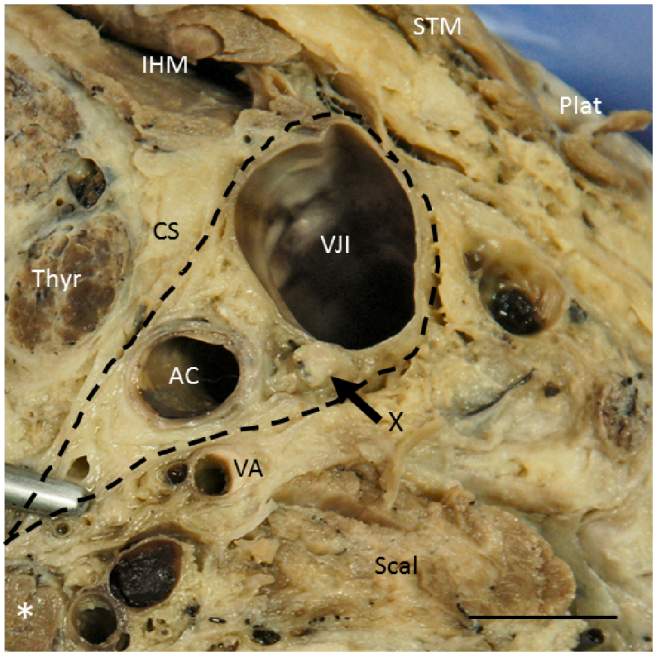

Fig. 1. Transverse section through the antero-lateral quadrant of the neck of an embalmed body at level C7. The vagus nerve (X, arrow) is enwrapped together with the common carotid artery (AC) and the internal jugular vein (VJI) by the carotid sheath (CS, dashed). IHM, infrahyal muscles; Plat, Platysma; Scal, scalenus anterior muscle; STM, sternocleidomastoid muscle; Thyr, thyroid gland; VA, vertebral artery; asterisk, prevertebral muscle. Body donated to the Institute of Anatomy and Cell Biology, University of Erlangen-Nürnberg, for didactic, research and postgraduate training purposes. Bar 1 cm.

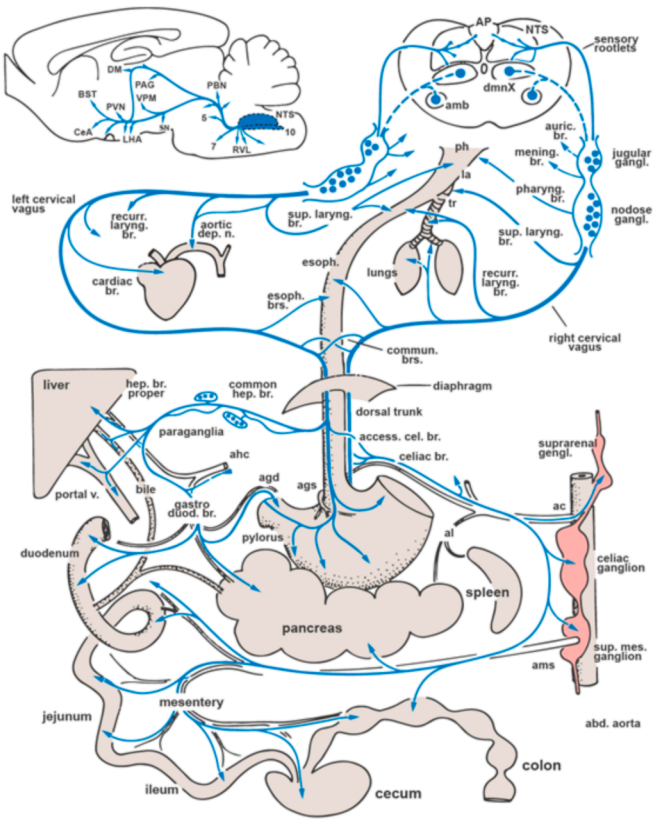

Fig. 2. Schematic overview of the distribution of vagus nerve's branches. Inset on top left summarizes the central distribution of vagal afferent information through the NTS. Abbreviations for periphery: ac, celiac artery; agd, right gastric artery; agd, left gastric artery; ahc, common hepatic artery; al, splenic artery; ams, superior mesenteric artery, la, larynx; ph, pharynx; tr, trachea. Abbreviations for brain areas: amb, nucleus ambiguus; AP, area postrema; BST, bed nucleus of stria terminalis; DM, dorsomedial thalamic nucleus; CeA, central nucleus of amygdala; LHA, lateral hypothalamic area; NTS, nucleus tractus solitarii; PAG, periaqueductal gray; PVN, paraventricular hypothalamic nucleus; PBN, parabrachial nuclei; RVL, rostroventrolateral medulla; SN, substantia nigra; VPM, ventroposteromedial thalamic nucleus; 5, trigeminal motor nucleus; 7, facial nucleus; 10/dmnX, dorsal vagal motor nucleus. Modified from Berthoud and Neuhuber, 2000, Auton. Neurosci. 85, 1-17.

Branching of the vagus within the carotid sheath at mid-cervical levels, the typical site where stimulating electrodes for invasive VNS are implanted, was observed in almost one-third of bodies in a post mortem dissection study, either uni- or bilaterally (Hammer et al., 2015). At low cervical levels, a superior cardiac branch was regularly observed (Kawashima, 2005).

2.2. Thoracic branches

The vagus enters the mediastinum between the subclavian vein and artery, on the right side giving off the recurrent laryngeal nerve around the artery. On the left, it abuts upon the anterolateral aspect of the aortic arch where the left recurrent laryngeal nerve winds around dorsally, bound by the ligamentum arteriosum (Botalli's ligament). Inferior

cardiac branches are given off by both recurrent nerves and thoracic cardiac branches split off distal to the recurrent nerve's origin (Kawashima, 2005). The recurrent laryngeal nerves travel in a groove between trachea and esophagus, giving off branches to both organs, and eventually enter the larynx from below as the inferior laryngeal nerves (Fig. 2). Because of their close relationship to the inferior thyroid artery, they are vulnerable during thyroid surgery.

In its mediastinal course, the vagus crosses the principal bronchi dorsally, providing branches to the pulmonary plexus and follows the esophagus, embedded in its adventitia. Here, the right and left vagus form a plexus suggestive of fiber exchange between both sides (Fig. 2). Tracing and functional studies in rat indicated that this occurs for efferent and afferent fibers to different though small extents (Fox and Powley, 1985; Norgren and Smith, 1988; Horn and Friedman, 2005).

2.3. Abdominal branches

Both left and right vagi enter the abdominal cavity as the anterior and posterior, respectively, trunk, together with the esophagus through its hiatus in the diaphragm. In some species, e.g., the ferret, the posterior trunk contained -twice as many axons than the anterior trunk (Asala and Bower, 1986) which was, however, not observed in the rat (Prechtl and Powley, 1990). The vagal trunks divide into two gastric and two celiac branches, each one from the anterior and posterior trunk, and one common hepatic branch from the anterior trunk (Fig. 2; Powley et al., 1983). However, terminology does not strictly correlate with innervation territories of the respective branches. In particular, the hepatic branch supplies, besides liver, biliary tract and portal vein, also the pancreas and duodenum which makes the interpretation of functional studies on the effects of “hepatic branch vagotomy” difficult (Berthoud and Neuhuber, 2019). The celiac branches serve primarily as a conduit for vagal efferent and afferent axons to the prevertebral plexus and from there along the branches of mesenteric arteries to the small and large intestines (Wang and Powley, 2007). However, anterograde tracing in rat demonstrated also presumed synaptic vagal preganglionic terminals on sympathetic postganglionic neurons of prevertebral ganglia (Berthoud and Powley, 1993).

The vagal efferent and afferent innervation extends well into the distal colon (equivalent to the descending colon in human) as demonstrated with anterograde tracing in rat (Berthoud et al., 1991; Wang and Powley, 2000). Anterograde tracing in female rats indicated vagal afferent innervation even of the uterus (Collins et al., 1999); it is unknown if there is also an efferent vagal influence on pelvic organs. There is no indication for vagal innervation of kidney, adrenal gland, lymphatic organs and adipose tissue (Cano et al., 2004; Berthoud et al., 2006; Giordano et al., 2006). Although it is possible that kindney, adrenal gland, adipose tissue and spleen are influenced by the vagus through a relay in prevertebral ganglia (Berthoud and Powley, 1993), this is controversially debated in particular for the spleen (Cano et al., 2001; Bratton et al., 2012; Kressel et al., 2020). Earlier data on vagal input to these organs rely on retrograde tracing notorious for diffusion artifacts (Fox and Powley, 1989).

2.4. Fiber spectrum

About 50% of axons in the cervical vagus in species of different body sizes (mouse, pig, human) are myelinated (Stakenborg et al., 2020). However, in this light microscopic study the proportion of unmyelinated fibers was underestimated as the neurofilament immunostaining demonstrated Remak bundles rather than individual unmyelinated axons. Remak units in the vagus nerve harbor on average two to four unmyelinated axons as demonstrated by electron microscopy (cat cervical vagus: Mei et al., 1980; ferret cervical vagus: Asala and Bower, 1986; rat abdominal vagus: Prechtl and Powley, 1990). Thus, a myelinated to unmyelinated axon ratio of one to five was calculated for the cat cervical vagus (Mei et al., 1980). Assuming a similar ratio also for the human cervical vagus, 20,000 myelinated axons (Stakenborg et al., 2020) were accompanied by some 100,000 unmyelinated axons. In human, about one half to two thirds of large myelinated fibers are motor axons of the recurrent laryngeal nerve, the others presumed afferent Aβ axons most likely from low threshold pulmonary mechanosensors; the numbers of thick myelinated axons in the thoracic vagus equals the number of this class of axons in the cervical vagus after subtracting the recurrent nerve's number (Safi et al., 2016). These axons are presumably activated by current methods of invasive VNS (Evans et al., 2004). As there are very few or almost no muscle spindles in laryngeal muscles (Paulsen, 1958; Loucks et al., 2005) and thus almost no Aγ axons to be expected, small and medium sized myelinated axons in the cervical vagus are also most likely afferents or preganglionic cardioinhibitory efferents (see Section 3.1.2). In contrast, more than 90% of axons in the subdiaphragmatic vagus are unmyelinated (99.5% in rat: Prechtl and Powley, 1990; 94% in human: Stakenborg et al., 2020, see caveat above), encompassing both preganglionic efferents and visceral afferents. The nature of the few myelinated fibers is unknown.

In the context of VNS strategies, knowledge of the anatomy of the vagus nerve's non-neuronal components, which were hitherto poorly studied, is also important. A recent study found that the perineurium of vagal axon fascicles is thicker as compared to other peripheral nerves (Pelot et al., 2020). This has to be taken into account when defining stimulation parameters.

Blood supply to the cervical vagus is provided by branches of vertebral, inferior thyroid and other neighbouring arteries. The number of subepineurial arteries correlated positively with fascicle numbers in human (Hammer et al., 2018).

3. Efferent neurons

3.1. Preganglionic parasympathetic neurons

3.1.1. Dorsal motor nucleus

Most of the vagal preganglionic parasympathetic neurons are located in the dorsal motor nucleus (DMX), a flat sheeth of medium-sized neurons sandwiched between the nucleus of the solitary tract (NTS) dorsally and the hypoglossal nucleus ventrally (Fig. 3a). It extends from the level of the pyramidal decussation throughout the entire medulla oblongata. Together with the medial nucleus of the solitary tract, it bulges the caudal floor of the fourth ventricle forming the vagal trigone (trigonum nervi vagi); rostrally, it decreases in size and is displaced, together with the solitary tract and nucleus, ventro-laterally by the medial and inferior vestibular nuclei which increased in size in the dorsal medulla. The majority of DMX neurons are cholinergic preganglionics targeting the enteric nervous system from the esophagus down to the distal colon, including pancreatic ganglia (Fig. 3b; Berthoud and Powley, 1991; Berthoud et al., 1991). Surprisingly, DMX neurons project only to a negligible extent to the area of the rat liver pedicle (Berthoud et al., 1992), and the absence of cholinergic markers in intrahepatic nerve fibers, at least in rodents, argues against a significant parasympathetic cholinergic innervation of the liver (Arvidsson et al., 1997). DMX neurons project also to cardiac ganglia (retrograde tracing data in rhesus monkey: Hopkins and Armour, 1998; anterograde tracing data in rat: Cheng et al., 1999). However, it is unclear if they exert a negative chronotropic or rather a negative inotropic effect (Gourine et al., 2016). Preganglionic neurons of the rat DMX are viscerotopically arranged in longitudinal columns medio-laterally (Fox and Powley, 1985), which combines with a rostro-caudal somatotopy of visceroafferent terminals in the NTS (Altschuler et al., 1989) forming a lattice for reflex connections across organ borders (Powley et al., 1992). It is unknown how the somatotopy of the rat DMX relates to the subnuclei of the human DMX (Huang et al., 1993).

Besides cholinergic preganglionic neurons, the DMX harbours some subdiaphragmatically projecting neurons which display catecholaminergic (Yang et al., 1999; Tsukamoto et al., 2005) and nitrergic (Krowicki

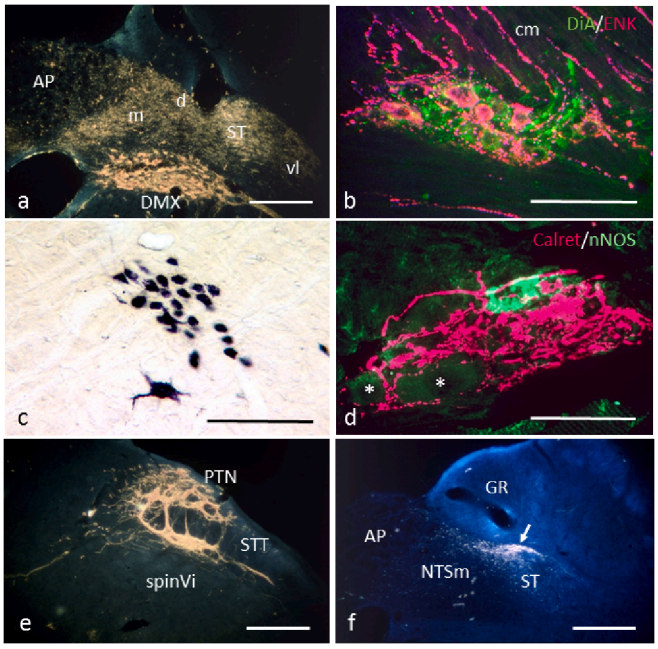

Fig. 3. a. Retrogradely labeled neurons in the dorsal motor nucleus (DMX) and transganglionically labeled afferent terminals in the nucleus tractus solitarii (NTS) and area postrema (AP) upon horseradish peroxidase (HRP) application to the rat cervical vagus. d, m, vl, dorsal, medial, ventrolateral subnuclei of the NTS; ST, solitary tract. **b.** Preganglionic axons anterogradely labeled with DiA from the DMX (green) project to a myenteric ganglion in the rat pyloric antrum. Enkephalinergic myenteric neurons (ENK, red) project to the circular muscle layer (cm) Extended focus confocal image. **c.** Compact formation of the nucleus ambiguus labeled by HRP application to the rat cervical vagus. The large multipolar neuron belongs to the rostral semicompact ambiguus formation. **d.** Intraganglionic laminar endings (IGLEs, calretinin-positive, red) enwrapping a myenteric ganglion in the rat esophagus. A nitric oxide synthase (nNOS) positive myenteric neuron is labeled green, another two unlabeled neurons are indicated by asterisks. Extended focus confocal image. **e.** Dense afferent terminal labeling in the paratrigeminal (PTN) and interpolar spinal trigeminal nucleus (spinVi) upon wheat germ agglutinin-HRP injection into the rat jugular-nodose ganglion complex. STT, spinal trigeminal tract. **f.** Transganglionically HRP labeled terminals of rat aortic branch afferents concentrate in the dorsal NTS subnucleus (arrow). Note some terminal labeling also in dorsomedial subnucleus and area postrema. GR, nucleus gracilis. Hitherto unpublished micrographs from the authors' previous work. Bars in a, c, e, f 200 μm, in b 100 μm, in d 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al., 1997) markers. Furthermore, small neurons which do not project through the vagus nerve are considered interneurons or centrally projecting neurons (McLean and Hopkins, 1982; Jarvinen and Powley, 1999), some of them being GABAergic (Gao et al., 2009).

3.1.2. External formation of the nucleus ambiguus

In addition to the DMX, parasympathetic preganglionic neurons are also found in the external formation of the nucleus ambiguus (see Section 3.2). They target postganglionic neurons in subepicardial ganglia of the heart and in the tracheobronchial tree. The axons of the cardioinhibitory ambiguus neurons are small myelinated in contrast to those from cardiac DMX neurons which are almost all unmyelinated (Cheng et al., 1999; Cheng and Powley, 2000; Gourine et al., 2016). Depending on species, the proportions of cardiac DMX and AMB preganglionic neurons vary, with more than 80% located in the cat's and 60% in the monkey's ambiguus nucleus (Hopkins and Armour, 1998; Taylor et al., 1999 for review). Interestingly, all vagal preganglionic neurons of the shark's ambiguus homologue in the ventrolateral medulla innervate the heart with myelinated axons and account for 45% of cardiac preganglionic neurons in this phylogenetically old species, the other 55% residing in the DMX (Taylor et al., 2014). Thus, myelinated cardioinhibitory neurons are phylogenetically highly conserved and not a new trait specific of mammals as proposed by the polyvagal theory (Porges, 2001).

Both right and left ambiguus and dorsal motor nuclei, respectively, project to all cardiac ganglia as shown with anterograde tracing in rat (Cheng et al., 1999; Cheng and Powley, 2000). However, axons from the right DMX terminate slightly more often in ganglia close to the sinoatrial node whereas axons from left ambiguus neurons are slightly biased to ganglia in the vicinity of the atrioventricular node. It is unknown if this lateralization applies also to human and may explain the clinical notion that stimulation of the right vagus elicits bradycardia more readily (see Hammer et al., 2018 for references).

3.2. Branchiomotor neurons

Branchiomotor neurons projecting their axons mainly through the vagus but also the glossopharyngeal nerve and the cranial root of the accessory nerve are musculotopically grouped in the ambiguus nuclear complex (AMB) which extends ventrolaterally throughout the length of the medulla. The seminal study of Bieger and Hopkins (1987) demonstrated a rostro-caudal arrangement with the compact formation (AMBc, Fig. 3c) innervating striated muscle of the esophagus, the semicompact formation (AMBsc) the pharynx and the loose formation (AMBl) the intrinsic muscles of the larynx. The stylopharyngeus muscle, innervated by the glossopharyngeal nerve is represented in the most rostral portion of the AMB. The external formation (AMBext) with its loosely scattered preganglionic neurons lies largely ventral to the branchiomotor clusters. Different packing densities of motoneurons are also observed in the human AMB, although the rostro-caudal arrangement may be modified by a dorso-ventral gradient, the compact formation being located dorsal and the semicompact and loose formations ventral (Schwarzacher et al., 2011).

Remarkably, AMBc neurons target not only motor endplates in the striated esophageal muscle, but issue collaterals also to myenteric neurons which are known to co-innervate this unusual muscle fiber type (Neuhuber et al., 1994; Powley et al., 2013b). This preganglionic feature of branchiomotor AMBc neurons is one of the several ambiguities of the nucleus ambiguus.

Branchiomotor and preganglionic neurons of the AMB intermingle extensively with neurons of the ventral respiratory group complicating a cytoarchitectonic definition of the AMB (Ellenberger and Feldman, 1990; Schwarzacher et al., 2011).

3.3. Neurons displaying catecholaminergic markers

The cervical and abdominal vagi contain fibers displaying catecholaminergic markers (tyrosine hydroxylase/TH and dopamine β-hydroxylase/DBH) (rat: Yang et al., 1999; human: Seki et al., 2014; Verlinden et al., 2016;). Some of them may be authentic sympathetic postganglionic "hitchhiking" fibers originating in the superior cervical ganglion (Yang et al., 1999; Verlinden et al., 2016). They are considered a possible source of unintended side effects of VNS. Although the functions of these fibers are unknown, a reasonable target is epineurial blood vessels of the vagus nerve itself (Fig. 4; Hammer et al., 2018). The proportions of "true" sympathetic postganglionic fibers was however considered minimal (Yang et al., 1999). As immunostaining for TH was used (Seki et al., 2014; Verlinden et al., 2016) and only a fraction of fibers co-stained for DBH (Verlinden et al., 2016), many of these "catecholaminergic" axons may be nodose primary afferents, since the nodose ganglion of rats contains numerous TH positive neurons which innervate the esophagus and stomach without being authentic catecholaminergic (Kummer et al., 1993). Another source is presumed dopaminergic neurons in the rat dorsal motor nucleus (Yang et al., 1999; Tsukamoto et al., 2005). Thus, it seems not very likely that cervical or

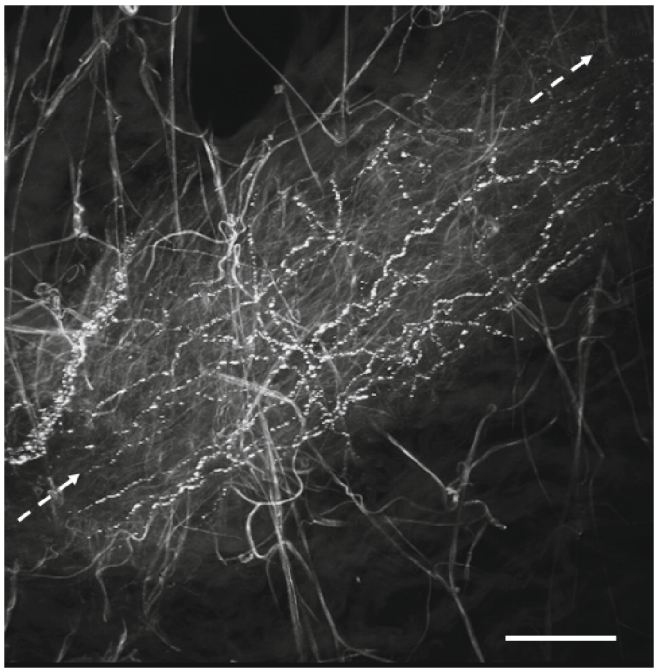

Fig. 4. A small artery in the epineurium of the human cervical vagus (longitudinal axis of the vessel indicated by dashed arrows) enmeshed by a plexus of varicose TH positive sympathetic nerve fibers. Smooth-contoured connective tissue fibers display autofluorescence. Wholemount preparation; extended focus image of 13 stacked single optical sections, z-step 0.5 μm. Body donated to the Institute of Anatomy and Cell Biology, University of Erlangen-Nürnberg, for didactic, research and postgraduate training purposes. Bar 100 μm.

abdominal VNS produces sympathetic side effects.

4. Afferent neurons

Primary afferent axons account for about 70% of fibers in the rat subdiaphragmatic vagus (Prechtl and Powley, 1990). The values for the cervical vagus were estimated between 50 and 70% in cat (Mei et al., 1980) and 80% in ferret (Asala and Bower, 1986). Pseudounipolar vagal primary afferent neurons reside in the jugular and nodose ganglia (Fig. 2). Neurons of the jugular ganglion originate from the cranial neural crest whereas nodose neurons are derived from an epibranchial placode (Baker and Bronner-Fraser, 2001). This differential origin is reflected by differences in peptide content, equipment with purinergic and growth factor receptors as well as transcriptomic differences. Thus, nodose ganglion neurons are typically non-peptidergic, express P2X2 and P2X3 receptors as well as TrkB, the high-affinity receptor for brain-derived neurotrophic factor (BDNF). Jugular ganglion neurons often contain peptides such as CGRP and express TrkA, the high affinity receptor for nerve growth factor (NGF) while another non-peptidergic subpopulation expresses TrkB (Wank and Neuhuber, 2001; Nassenstein et al., 2010). Likewise, transcriptomic analysis revealed significantly different molecular profiles, the jugular ganglion neurons being similar to the also neural crest derived dorsal root ganglion cells but completely different from placode derived nodose neurons (Kupari et al., 2019). Neurons expressing the vanilloid receptor TRPV1 were found in both jugular and nodose ganglia (Kim et al., 2020). These molecular differences most likely determine the functional properties of nodose and jugular neurons. Thus, neurons with a typical nociceptive phenotype are more commonly, though not exclusively found in the jugular rather than in the nodose ganglion (Kupari et al., 2019). Nevertheless, the esophagus is supplied with both crest- and placode-derived vagal C-fiber nociceptors (Surdenikova et al., 2012; Yu et al., 2014). TRPV1 expression correlates also with projections of vagal afferents to medial and dorsal (TRPV1+ afferents) and ventrolateral (TRPV1- afferents) areas of the nucleus tractus solitarii (Kim et al., 2020) as well as to sensitivity for certain inflammatory mediators (Zanos et al., 2018).

4.1. Visceral afferents

Vagal afferents from neck, thoracic and abdomino-pelvic viscera have their cell bodies in jugular and nodose ganglia. Afferents from the upper aerodigestive tract are represented in both ganglia, while afferents from subdiaphragmatic organs are almost confined to the nodose ganglion. Afferents from thoracic organs are both myelinated and unmyelinated, whereas afferents from subdiaphragmatic organs are almost exclusively unmyelinated.

4.1.1. Afferents from thoracic organs

Vagal pulmonary and cardiovascular afferents play vital roles in cardiorespiratory reflexes and coordination. Most prominent are thick myelinated pulmonary slowly and rapidly adapting mechanoreceptors (SARs and RARs, respectively) originating in bronchial smooth muscle and neuroepithelial bodies (NEBs) (Adriaensen et al., 2006; Chang et al., 2015; Brouns et al., 2021), some of which express Piezo2 (Nonomura et al., 2017). NEBs together with their associated vagal afferents may represent complex chemo- and mechanoreceptors. It is still debated whether NEBs or smooth muscle associated airway receptors (SMARs) represent the SARs which mediate the Hering-Breuer reflex (Brouns et al., 2021). Small myelinated and unmyelinated peptidergic and non-peptidergic pulmonary afferents are equally important as irritant receptors mediating the cough reflex, reacting to inflammatory stimuli or slowing breathing upon inhalation of toxins (Canning et al., 2004; Nassenstein et al., 2010; Krasteva et al., 2011; Chang et al., 2015; Driessen, 2019).

Aortic baroreceptor afferents are also partly myelinated (Krauhs, 1979) and utilize Piezo2 as transduction channel (Min et al., 2019). Nodose ganglion afferents from atria establish complex terminal structures around small intensely fluorescent cells of cardiac ganglia, in the myocardium including conduction fibers and in the endocardium (Cheng et al., 1997).

Among the small myelinated population are also mechanosensitive intraganglionic laminar endings (IGLEs) afferents from the esophagus involved in deglutition control (Raab and Neuhuber, 2007; Neuhuber and Bieger, 2013).

4.1.2. Gastrointestinal afferents

Vagal afferent structures in the gastrointestinal tract can be grouped into muscular and mucosal sensors. IGLEs which wrap around myenteric ganglia sandwiched between outer and inner layers of the tunica muscularis function as low-threshold mechanosensors (Fig. 3d; Neuhuber, 1987; Berthoud and Powley, 1992; Zagorodnyuk and Brookes, 2000; Zagorodnyuk et al., 2001). They extend throughout the vagal innervation territory from the esophagus to the distal colon (Wang and Powley, 2000) and mediate essential satiety signals (Bai et al., 2019). IGLEs form synaptic contacts with enteric neurons, express the vesicular glutamate transporters 1 and 2 (VGLUT1 and 2) as well as purine receptors P2X2/3, muscarinic and CGRP receptors (Hübsch et al., 2013; Horling et al., 2014; Raab and Neuhuber, 2007 for review); the functional significance of these features remains still to be elucidated. The other muscular sensors are the so-called intramuscular arrays (IMAs), found mainly in the outer muscle layer of the stomach and in sphincters (Berthoud and Powley, 1992; Kressel et al., 1994; Powley et al., 2013a, 2014). They are intricately related to interstitial cells of Cajal, a relation which is poorly understood (Powley et al., 2008). Vagal afferents in the mucosa display various distinct patterns which may relate to specific funtions (Berthoud et al., 1995b; Powley et al., 2011). In particular, close relationships to enteroendocrine cells (Berthoud and Patterson, 1996a; Kaelberer et al., 2018) and mucosal mast cells (Williams et al., 1997) are supposedly of

functional relevance.

Anatomical and functional studies indicate some degree of lateralization of gastrointestinal vagal afferents. The ventral and dorsal halves of the rat stomach and duodenum are supplied by IGLEs connected to cell bodies in the left and right nodose ganglia, respectively; this lateralization vanishes analwards (Wang and Powley, 2000). Using optogenetic manipulation of nodose ganglion neurons in mice, gut induced reward was found to be mediated through right but not left vagal intestinal afferents (Han et al., 2018). This suggests side-specific channeling of some vagal afferents to the limbic forebrain.

In the rat pancreas, vagal afferents concentrate in islets (Neuhuber, 1989), most likely monitoring endocrine activity (Iwasaki et al., 2013; Makhmutova et al., 2021). Vagal afferents of the proper hepatic branch innervate the portal vein and biliary ducts. Within the rat liver, they distribute to periportal fields but are absent from the hepatic lobules (Berthoud et al., 1992).

4.1.3. *Afferents from pelvic organs*

Both retrograde and anterograde tracing studies in rat indicated vagal afferent supply of the uterus (Ortega-Villalobos et al., 1990; Collins et al., 1999). There is also evidence that patients with complete spinal cord lesion are able to sense cervico-vaginal stimulation (Komisaruk et al., 2004). In the intact organism, vagal and spinal afferents from uterus and vagina are interacting in the nucleus tractus solitarii (Hubscher and Berkley, 1995). Retrograde tracing in rat demonstrated vagal afferents also from the urinary bladder, confirmation by anterograde tracing from the nodose ganglion pending (Herrity et al., 2014).

4.1.4. *Afferents from vagal paraganglia*

All along their course, vagal branches contain paraganglia, most of them supplied by vagal afferent terminals (rat: Powley et al., 1983; Kummer and Neuhuber, 1989; Dahlqvist et al., 1994; Berthoud et al., 1995a; Berthoud and Patterson, 1996b; human: Plenat et al., 1988). A chemosensory function for pO2 monitoring was proposed analogous to carotid and aortic bodies. There are also indications for vagal paraganglia as sensors for inflammatory mediators (Goehler et al., 1999).

4.2. *Somatic afferents*

The external ear and the upper aerodigestive tract, i.e., soft palate, pharynx, larynx and partly also trachea forming the transition zone from the “somatic” oral and nasal cavities innervated by trigeminal afferents to the “visceral” organs, i.e., lower airways and gastrointestinal tract, are innervated to different extents by neural crest-derived jugular and placode-derived nodose sensory neurons. While the cell bodies of afferents in the vagal auricular branch (ABVN) were found only in the jugular ganglion, afferents from the other organs are distributed to both jugular and nodose ganglia and also to the petrosal ganglion of the glossopharyngeal nerve which in rat fuses with the jugular-nodose ganglionic complex (Altschuler et al., 1989). As to the sensory qualities mediated by these vagal “somatic” afferents, there is a shift from awareness of touch, temperature and pain to feelings of irritation and the urge to cough, depending on the distance from the dental ridge where a stimulus is applied.

4.3. *Central termination sites of vagal afferents*

The major central termination area of vagal afferents is the nucleus tractus solitarii (NTS) in the dorsal medulla (Figs. 3a, 5). However, as detailed below in Section 6.1, vagal afferents from organs in the somato-visceral transition zone terminate also in the spinal trigeminal and paratrigeminal nuclei (Fig. 3e; Nomura and Mizuno, 1984; Altschuler et al., 1989; Driessen, 2019). The NTS is a complex of several subnuclei organized in medial, dorsal and ventrolateral groups similar across species (rat: Altschuler et al., 1989; Herbert et al., 1990; cat: Loewy and Burton, 1978; human: McRitchie and Törk, 1993). Besides second order

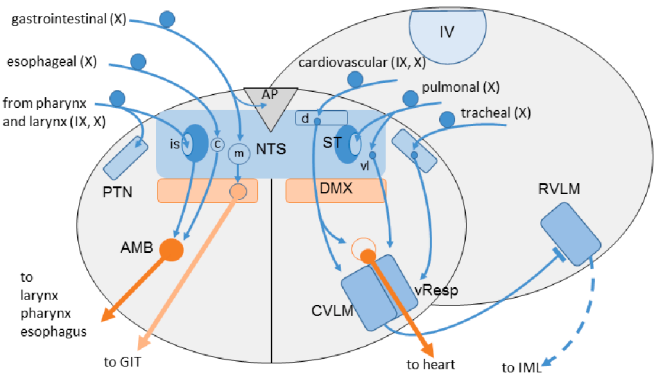

Fig. 5. Schematic simplified summary of vagal (X) and glossopharyngeal (IX) afferent projections to the medulla oblongata and their major reflex connections to the dorsal motor nucleus (DMX), ambiguus nucleus (AMB; left: branchiomotor division; right: parasympathetic neurons of external formation) and the ventrolateral medulla. Note afferents from pharynx, larynx and trachea to the paratrigeminal nucleus (PTN). Arrows indicate excitatory (glutamatergic) connections; the bar on rostral ventrolateral medulla (RVLM) symbolizes the inhibitory GABAergic projection from caudal ventrolateral medulla (CVLM) to glutamatergic presympathetic neurons in the rostral ventrolateral medulla (RVLM). AP, area postrema; c, d, is, m, vl, central, dorsal, interstitial, medial, ventrolateral subnuclei of NTS; DMX, dorsal motor nucleus of vagus; IML, sympathetic intermediolateral nucleus; IV, fourth ventricle; vResp, ventral respiratory group; ST, solitary tract.

neurons relaying the glutamatergic primary afferents, the NTS contains excitatory glutamatergic and inhibitory GABA/glycinergic interneurons (Saha et al., 1999) and catecholaminergic neurons of the A2 and C2 groups. A number of hormones and peptides, e.g., oxytocin, ghrelin, CCK and neurokinins as well as nitric oxide modulate transmission in the NTS through their respective receptors (Colin et al., 2002; Atkinson et al., 2003; Boscan and Paton, 2005; Peters et al., 2008; Wan et al., 2008; Cui et al., 2011). In the NTS, most vagal afferents terminate in more than one subnucleus (Figs. 3a, 5). There is some overlap of afferents from different organs already at the anatomical level which is even more pronounced when second-order NTS neurons activated by different specific stimuli are mapped (Paton and Kasparov, 2000). Nevertheless, gastrointestinal afferents dominate medial, gelatinous and commissural (Norgren and Smith, 1988; Altschuler et al., 1989), cardiovascular afferents dorsal (Fig. 3f; Ciriello and Calaresu, 1981) and afferents from the respiratory tract ventral, ventrolateral, intermediate, interstitial and commissural (Kalia and Richter, 1985; Altschuler et al., 1989; Hayakawa et al., 2001) subnuclei. The termination of e.g. single identified pulmonary SAR afferents in several subnuclei and their rostro-caudal extension over the entire NTS favor their access to functionally diverse NTS modules (Kalia and Richter, 1985). This principle may apply to some extent also to other vagal afferents. Afferents from the rat esophagus concentrate in the central subnucleus which in turn projects to AMBc, thus closing a deglutition reflex arc (Altschuler et al., 1989; Cunningham and Sawchenko, 1989). Likewise, pharyngeal afferents in rat are relayed by the interstitial NTS subnucleus to the AMBsc (Broussard et al., 1998).

It is conceivable that nociceptive neurons of largely jugular origin terminate preferentially in trigeminal and paratrigeminal nuclei whereas non-nociceptive neurons of largely nodose origin terminate preferentially in the NTS. However, this difference is certainly not clear-cut as for example crest-derived and placode-derived putative nociceptive afferents from the cervical esophagus expressing the TRPV1 receptor (Surdenikova et al., 2012) appear to regularly terminate in the interstitial nucleus of the NTS whereas additional afferent terminal labeling in the paratrigeminal nucleus was inconsistent (Wank and Neuhuber, 2001).

There is also a significant vagal afferent projection to the area

postrema from intestines and thoracic organs (Fig. 3a; Norgren and Smith, 1988).

5. Central connections of vagal nuclei

The NTS connects reciprocally to respiratory and vasomotor centers in the caudal and rostral ventrolateral medulla, communicating information from bronchopulmonary, baro- and chemosensors vital for respiratory and cardiovascular control (Blessing and Benarroch, 2012). In the ambiguus nuclear complex, both branchiomotor and cardioinhibitory preganglionic neurons are reflexly activated by secondary NTS neurons mediating deglutition and baroreceptor reflexes, respectively (Blessing and Benarroch, 2012; Neuhuber and Bieger, 2013). The medial NTS subnuclei are intricately connected to the DMX and the area postrema, thus forming the dorsal vagal complex, the medullary center of gastrointestinal regulation (Travagli and Anselmi, 2016). The different NTS subnuclei are engaged in complex reciprocal connections with the parabrachial nuclei and the Kölliker-Fuse nucleus (Herbert et al., 1990) and project to the locus coeruleus and periaqueductal gray (Van Bockstaele et al., 1999; Carrive and Morgan, 2012). Reciprocal connections link the NTS also to the paraventricular and posterior hypothalamus and the central nucleus of the amygdala. Most of these connections originate from separate neuron populations rather than collateralizing neurons (Hermes et al., 2006). Thus, being much more than a simple relay station, the NTS second-order and interneuronal network integrates and channels the signals from different peripheral sensors in highly specific ways to medullary, pontine, mesencephalic and prosencephalic autonomic centers for appropriate reflex and behavioural responses (Figs. 2, 5).

6. Somato-visceral interface

Breathing, chewing, swallowing, speaking and singing require coordination of several muscles in the head, neck and trunk which is secured by afferent feedback via cranial nerves V, VII, IX and X as well as cervical spinal nerves. The territories supplied by these nerves comprise somatic (face, oral and nasal cavities, neck muscles and skin, diaphragm) and visceral (pharynx and esophagus, larynx, tracheobronchial tree and lung) domains. On the level of medullary and spinal nuclei, it is thus not surprising to find mutual terminations of vagal, trigeminal and cervical primary afferents in their "private" as well as "alien" nuclei, i.e., the NTS, trigeminal sensory nuclei and the cervical dorsal horn (Altschuler et al., 1989; Neuhuber and Zenker, 1989; Marfurt and Rajchert, 1991; Driessen, 2019). Thus, these nuclei are the central representatives of the somato-visceral interface on the afferent side. At the motor level, there is a sophisticated pontomedullary interneuronal premotor network in the parvocellular reticular formation which integrates trigeminal propriopceptive afferents and coordinates trigeminal, facial, glossopharyngeal, vagal and hypoglossal motor nuclei as well as neck muscle motoneurons (Dessem and Luo, 1999; Zhang et al., 2012). In turn, vagal and glossopharyngeal afferents from pharynx and larynx are relayed via premotor neurons in the NTS to trigeminal motor neurons (Oka et al., 2013). The ventral respiratory group projects to phrenic and intercostal nerve motoneurons as well as to cranial nerve motor nuclei conferring respiratory rhythmicity to their activity patterns (Ellenberger and Feldman, 1990). The nucleus retroambiguus harbours another premotor neuron pool which integrates signals from vagal afferents and periaqueductal gray and projects to laryngeal motoneurons of AMBl (Subramanian et al., 2018).

6.1. Vagal afferents to non-vagal nuclei

As mentioned above, afferents of vagal branches innervating the external ear and upper aerodigestive tract terminate in the spinal trigeminal nucleus (medullary dorsal horn) and in the cervical dorsal horn, where they converge with trigeminal and cervical spinal afferents (Nomura and Mizuno, 1984; Sessle et al., 1986; Altschuler et al., 1989). A particularly interesting structure is the paratrigeminal nucleus (PTN), groups of neurons interspersed between the fiber bundles of the spinal trigeminal tract (Figs. 3e, 5, 6). It receives input largely from jugular sensory neurons innervating soft palate, pharynx, larynx (Altschuler et al., 1989; Hayakawa et al., 2001) and trachea (McGovern et al., 2015) as well from the trigeminal area (Marfurt and Rajchert, 1991) and cervical cutaneous nerves (Neuhuber and Zenker, 1989) and projects to the NTS, ventrolateral medulla, parabrachial nuclei and thalamus (Saxon and Hopkins, 1998; Driessen et al., 2018). Besides a role in nociception, the PTN is also involved in respiratory reflexes (Driessen, 2019). Although not explicitly mentioned by Nomura and Mizuno in their landmark ABVN tracing study (Nomura and Mizuno, 1984), a closer look on their figures reveals ABVN afferent terminals also in the PTN.

6.2. Non-vagal afferents to the nucleus tractus solitarii

On the other hand, the NTS receives primary trigeminal afferents, in particular from intraoral structures and the mandibular area (Jacquin et al., 1982; Takemura et al., 1987; Marfurt and Rajchert, 1991; Hayakawa et al., 2001). There are also projections from cervical cutaneous and auricular nerve afferents preferentially to dorsal and lateral areas of the NTS (Neuhuber and Zenker, 1989; for auricular nerve afferent projections see Section 6.3.2 below). Secondary afferents to the NTS originate in the spinal cord, spinal trigeminal and paratrigeminal nuclei (Menétrey and Basbaum, 1987; Gamboa-Esteves et al., 2001; Ma et al., 2005). These pathways provide the NTS also with nociceptive input from somatic domains which can modulate visceral reflexes, e.g., the baroreceptorreflex (Boscan et al., 2002). In addition, there is even a pathway for proprioceptive afferents to the NTS. The intermediate nucleus, a small structure wedged between the lateral edges of the hypoglossal and vagal dorsal motor nuclei, receives neck muscle afferents

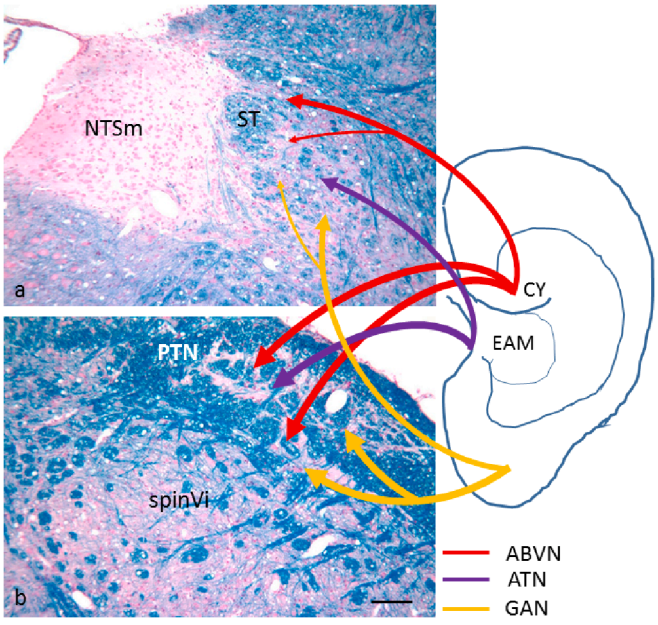

Fig. 6. Schematic summary of brainstem projections of auricular nerve afferents as demonstrated by transganglionic tracing studies. All three nerves (ABVN, auricular branch of the vagus; ATN, auriculotemporal nerve; GAN, greater auricular nerve) project to spinal trigeminal, paratrigeminal and solitary tract nuclei. In the NTS, afferents terminate preferentially in dorsal and lateral subnuclei. CY, cymba conchae; EAM, external auditory meatus; NTSm, medial subnucleus of the nucleus tractus solitarii; PTN, paratrigeminal nucleus; spinVi, subnucleus interpolaris of the spinal trigeminal nucleus; ST, solitary tract. Micrographs are from rat dorso-medial (a) and dorso-lateral (b) medulla, Klüver-Barrera stain. Bar 100 μm.

(Neuhuber and Zenker, 1989; Edwards et al., 2015) and projects to the NTS (Edwards et al., 2007). Forelimb muscle afferents were shown to inhibit baroreceptor signals in the NTS via GABAergic interneurons (Potts et al., 2003).

6.3. Auricular nerves

6.3.1. Peripheral distribution

The auricle and the external auditory meatus (EAM) develop from derivatives of the first and second pharyngeal arches, although the contribution of each of them is debated (Anthwal and Thompson, 2016). The ectoderm-derived epidermal lining of the EAM continues covering the outer surface of the tympanic membrane while its inner aspect is covered by a squamous epithelium derived from the endoderm of the first pharyngeal pouch (Thompson and Tucker, 2013). Thus, somatic and visceral domains are in touch back-to-back. This development is reflected by a complex sensory innervation provided by trigeminal, facial, glossopharyngeal, vagal and cervical spinal nerves, besides sympathetic and parasympathetic periarterial fibers (Cakmak et al., 2018). Although there is consensus that the lateral aspect of the human auricle, which is the preferred site of transcutaneous auricular VNS, is supplied by the trigeminal auriculotemporal nerve, auricular branch of the vagus nerve (ABVN) and the greater auricular nerve from the plexus cervicalis, the exact innervation territories are debated and there is some overlap (Fig. 6; Peuker and Filler, 2002; see discussion in Butt et al., 2020). Nevertheless, the cymba conchae is said to be exclusively supplied by the ABVN. However, intrapetrosal anastomoses of the ABVN with facial and glossopharyngeal branches (Testut, 1922; Butt et al., 2020) suggest that non-vagal fibers intermingle with authentic vagal afferents in the terminal arborizations of the ABVN even in the cymba. The medial aspect of the auricle receives fibers from the lesser occipital nerve, the ABVN and the greater auricular nerve (Peuker and Filler, 2002). Tracing studies on the dog's pinna, where a rostral, middle and caudal auricular nerve can be identified, indicated also a contribution by the facial nerve because tracer application resulted in retrogradely labeled neurons in the geniculate ganglion in addition to trigeminal, jugular and superior cervical spinal ganglia. More than one of these sensory ganglia contained labeled neurons upon tracer application to one particular nerve indicating anastomotic connections (Chien et al., 1996).

6.3.2. Central termination of auricular nerve afferents

In the quest to elucidate mechanisms of transcutaneous auricular VNS, central afferent terminations of auricular nerves are of great interest. Afferent terminals of all nerves were detected in the spinal trigeminal and partly also principal trigeminal nuclei, paratrigeminal nucleus, cuneate nucleus and upper cervical dorsal horn. Remarkably, all nerves in question send projections also to the NTS, in particular to its lateral, interstitial and dorsal but rarely to its medial subnuclei (Fig. 6; rat auriculotemporal nerve: Jacquin et al., 1982; cat ABVN: Nomura and Mizuno, 1984; rabbit greater auricular nerve: Liu and Hu, 1988; dog intrinsic pinna nerves: Chien et al., 1996; rat ABVN: He et al., 2013). In this respect, the ABVN is not more “vagal” than the other auricular nerves. Afferent projections to the (ventro-)lateral and interstitial NTS subnuclei are noteworthy as these subnuclei are the main termination site of myelinated vagal respiratory afferents (Kalia and Richter, 1985) which are the most likely target of invasive cervical VNS (Evans et al., 2004).

The density of terminals of all auricular nerve afferents in the NTS is rather low. This may be due to the transganglionic tracing technique which probably labels only a fraction of afferent terminals. However, all auricular nerve afferents terminate even more densely in spinal trigeminal and paratrigeminal nuclei which may relay this input to the NTS (Fig. 6; Saxon and Hopkins, 1998; Menétrey and Basbaum, 1987). Thus, fMRI studies of auricular VNS stimulation in human found BOLD activity in both NTS and spinal trigeminal nucleus (Frangos et al., 2015). Rostral areas in which BOLD signals were recorded upon auricular VNS, e.g., parabrachial nuclei (PBN), locus coeruleus, periaqueductal gray and limbic subcortical and cortical structures (Frangos et al., 2015; Yakunina et al., 2017) may be activated via conjoined projections from trigeminal and paratrigeminal nuclei as well as from the NTS, because both NTS and trigeminal/paratrigeminal nuclei project to PBN (Herbert et al., 1990; Feil and Herbert, 1995), which are an important relay to the more rostral areas (Krukoff et al., 1992; Luppi et al., 1995). Thus, it would be surprising if this afferent neuronal chain can be activated specifically by stimulation of the ABVN only unless one proposes that its afferents were relayed preferentially over those of the other auricular nerves in a kind of “labeled vagus line”. Why stimulation with innocuous intensities outside the proposed specific ABVN territory in the cymba conchae, e.g., tragus or ear lobe, is reportedly less effective in activating all these nuclei (Frangos et al., 2015; Yakunina et al., 2017) is not easily explained. Earlobe stimulation resulted in BOLD activity signals in cuneate and spinal trigeminal nuclei but not in NTS or more rostral sites (Frangos et al., 2015), although greater auricular nerve afferents project to the NTS (Liu and Hu, 1988). On the other hand, cymba stimulation did not activate the cuneate nucleus (Frangos et al., 2015), although ABVN afferents project heavily to this site at least in cat (Nomura and Mizuno, 1984). One may speculate that low threshold ABVN afferents are preferentially channeled by the intricate interneuronal network of the NTS to rostral brainstem and forebrain sites where the beneficial effects are generated. A more parsimonious explanation may be found in different sensory innervation densities of cymba conchae and earlobe, respectively. Although a recent study mapped the distribution of perivascular autonomic nerves in the human auricle (Cakmak et al., 2018), estimates of sensory Aβ fibers which are the likely target of transcutaneous auricular VNS, are available for the cymba but not for the earlobe (Dabiri et al., 2020).

6.4. Transcutaneous cervical vagus stimulation

The question of what is exactly stimulated by transcutaneous VNS is even more urgent in case of transcutaneous cervical VNS using a handheld device (e.g., Silberstein et al., 2016; Frangos and Komisaruk, 2017) which may stimulate low-threshold afferents. However, there are several sheets of tissue between the skin and the cervical vagus, which abound of low threshold sensors. Immediately below the low-threshold cutaneous sensors, the platysma is also densely supplied with low-threshold sensors, i.e., muscle spindles (May et al., 2018). Within the carotid sheath, the ansa cervicalis profunda containing bundles of low threshold proprioceptive axons from infrahyoid muscles lies superficially to the carotid artery, internal jugular vein and vagus nerve. It may require a sophisticated tuning of stimulus parameters in order to specifically activate the 200 to 400 (on average) thick myelinated afferents in the cervical vagus (Safi et al., 2016).

7. Conclusion

Innervating a large portion of our body and contributing significantly to homeostasis, the vagus represents an attractive target for therapeutic attempts to influence a variety of pathological conditions. The accessibility of its cervical and auricular branches encouraged the development of non-invasive vagus stimulation strategies. However, anastomoses of the ABVN with facial and glossopharyngeal nerves, the suspicion that transcutaneous cervical VNS may affect afferents other than vagal, the mutual distribution of all auricular nerve afferents to both “vagal” and “trigeminal” termination sites and the likely involvement of both NTS and trigeminal nuclei in mediating the stimulation effects question the appropriateness of the term “vagus nerve stimulation” for transcutaneous techniques.

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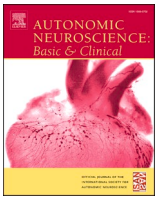

Electrical vagus nerve stimulation as a prophylaxis for SIRS and postoperative ileus

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ABSTRACT

Abdominal surgery results in an activation of immune cells of the bowel wall and a consecutive cytokine and nitric oxide (NO) release leading to an inflammation of the muscularis externa and a bowel paralysis, the so-called postoperative ileus (POI).

In addition to the local inflammation, major surgical trauma can also lead to a variable pronounced systemic inflammation up to its maximum variant, the systemic inflammatory response syndrome (SIRS), with hypotension, capillary leak and a breakdown of the intestinal barrier function followed by multi-organ dysfunction (MODS). Until now, neither for SIRS nor for POI, a prophylaxis or an evidence-based treatment exists.

Since the pioneering work from Kevin Tracey and his group in the late 90s characterizing the role of the vagus nerve in inflammation and describing the cholinergic anti-inflammatory pathway (CAIP) for the first time, substantial efforts have been made in the research field of neuro-immune interactions. Today, the anti-inflammatory potential of vagus nerve stimulation is moving more and more into focus resulting in new therapeutic approaches. This review focuses on the role of the CAIP in the development of SIRS and POI. Furthermore, new therapeutic options like transcutaneous vagus nerve stimulation are highlighted.

1. Background

1.1. The cholinergic anti-inflammatory pathway

Twenty years ago, the group of Kevin Tracey demonstrated, that a cervical transection of the vagus nerve (VN) in rats followed by intravenous lipopolysaccharide (LPS) administration resulted in a pronounced liberation of proinflammatory cytokines and a higher mortality compared to LPS application with an intact VN (Borovikova et al., 2000). On the contrary, electrical stimulation of the peripheral end of the transected VN before LPS application resulted in a significant reduction of cytokine release and diminished shock symptoms during endotoxemia. It was particularly interesting, that the electrical stimulation of VN improved survival after LPS challenge compared to rats with an intact, but unstimulated VN. As a consequence of these results, the cholinergic anti-inflammatory pathway (CAIP) was described for the first time.

In the next years, the mechanism of the CAIP was further investigated (Fig. 1): When afferent vagal fibres are stimulated by proinflammatory stimuli (cytokines, damage and pathogen-associated molecular patterns (DAMPs/PAMPs)), the information is sent to the vagal nuclei in the brain stem, where it is interconnected with efferent vagus nerve fibres, resulting in action potentials traveling down to several effector organs. Two important effector organs are the gastrointestinal (GI) tract and the spleen, harbouring the largest set of immune cells within the body. Both are targeted organs of the CAIP and macrophages expressing the alpha7 nicotinic acetylcholine receptor (nAChRa7) (Baez-Pagan et al., 2015) are the distinct target-cell population of efferent CAIP signaling. In the spleen, sympathetic neurons of the splenic nerve become activated on the celiac plexus level by the vagus nerve. This signaling activates $ChAT^{+}$ T-Cells in the spleen that release ACh that finally acts on resident macrophages via the nAChRa7 to dampen inflammation (Rosas-Ballina et al., 2009). The mechanism in the intestine seems to be different and does not require the interconnection of the sympathetic and parasympathetic signals in the cervical ganglion as shown by Costes et al. in a model of postoperative ileus (POI) (Costes et al., 2014). Interestingly, although ACh is released by vagal efferents in the intestine, it does not directly act on macrophages but

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first acts on nNOS, VIP and ChAT+ enteric neurons which lay in close proximity to resident macrophages (Matteoli et al., 2014; Cailotto et al., 2014). This indirect mechanism leads to immune modulation of macrophages. Nevertheless, independently of the cellular source, ACh, either neuronal- or immune cell-derived, activates nAChRa7, which inhibits proinflammatory NF-κB and p38MAP-Kinase-pathways and activates anti-inflammatory JAK2/STAT3 as well as IRAK-M pathways (Maldifassi et al., 2014; de Jonge et al., 2005). This leads to a reduced release of proinflammatory (TNF-alpha, IL-1beta, IL-6, IL-8) cytokines, a reduced expression of CD14 and toll like receptor (TLR) 4 and an increase of anti-inflammatory cytokines (IL-10) liberation (Hamano et al., 2006; Bernik et al., 2002; Huston et al., 2007).

1.2. Immune modulation via vagus nerve stimulation

These animal studies led to the idea, that the surgical implantation of a stimulation electrode beside the cervical VN could be a promising treatment for inflammatory diseases. As a consequence, it was demonstrated in several studies that electrical stimulation of the VN ameliorated inflammation and improved the outcome of diseases in animal models of ischaemia-reperfusion injury, haemorrhagic shock, pancreatitis and experimental colitis (Van Der Zanden et al., 2009). Based on these results, the first pilot trials in humans were conducted: Koopman and co-workers observed a significant decrease in serum TNF-alpha levels as well as lesser symptoms in patients with rheumatoid arthritis after surgical implantation of an electrode and stimulation of the cervical VN (Koopman et al., 2016). In another pilot trial, patients with Crohn's disease were treated with invasive (by surgical exploration and placement of a stimulation electrode beside the cervical neurovascular bundle) VNS (iVNS) resulting in a reduction of Crohn's Activity Index (Bonaz et al., 2016). However, until now no randomized controlled trials could demonstrate a convincing and permanent effect of iVNS in chronic inflammatory diseases. The explanation for this phenomenon could be the smouldering chronic inflammation caused by an autoimmune disease, which cannot be cured by VNS. Furthermore, for iVNS a surgical procedure with exposure of the VN is mandatory to implant the stimulation electrode baring the risk of wound infection or injury of surrounding structures. Additionally, a prophylactic stimulation before the beginning of an inflammatory stimulus, which could be the ideal indication considering the results of animal models, is impossible due to permanent inflammation in chronic disease (Hong et al., 2019).

Based on the potential side effects of iVNS due to invasiveness of stimulation, the technical and scientific progress in the last years led to the development of medical devices for transcutaneous stimulation of the VN (tVNS). The tVNS is mainly based on the fact, that the Ramus auricularis nervi vagi (RANV), a branch of the NV, sensitively innervates the skin of the concha auricularis at the outer ear (Fig. 2). This branch can be stimulated transcutaneously with electrical impulses without the potential risks of the invasive procedures. The afferent fibres of the RANV project into the nucleus tractus solitarii (NTS) in the brain stem, which is the starting point for the activation of a complex cerebral network, involving efferent fibres of the VN via the dorsal motor nucleus and which largely corresponds to that of iVNS (Badran et al., 2018). As an alternative to auricular stimulation of the RANV, VNS with consecutive CAIP activation can also be performed at the cervical level, directly stimulating efferent vagal fibres (McIntire et al., 2021). Several devices are commercially available for both, auricular (e.g., the tVNS®L device by tVNS TECHNOLOGIES (Fig. 2), the PARASYM™ device by PARASYM Ltd., the tVNS Stimulator device by VagusNet) and cervical VNS (e.g., the gammaCore™ device by electroCore™).

Several trials investigating the effects of tVNS in treatment of migraine, cluster headache, epilepsy or atrial fibrillation show auspicious results without severe side effects (Wu et al., 2020; Zhang et al., 2021; Lainez and Jensen, 2015; Stavrakis et al., 2015). These devices facilitate an activation of the CAIP at any time independently of an invasive, surgical procedure and without severe side effects (Sperling et al., 2010). The device would be a promising tool to activate the CAIP as a prophylaxis before the inflammatory cascade has started. Two interesting indications could be iatrogenic complications associated with surgical procedures. One is POI, an inflammation-based postoperative motility disorder that involves activation of resident nerve-associated macrophages and the enteric nervous system (Wehner et al., 2007; Schneider et al., 2021).

Another one is a systemic inflammatory response syndrome (SIRS). The idea would be to start tVNS before surgery and continue it until the end or for several days after the operation to activate the CAIP to avoid postoperative complications.

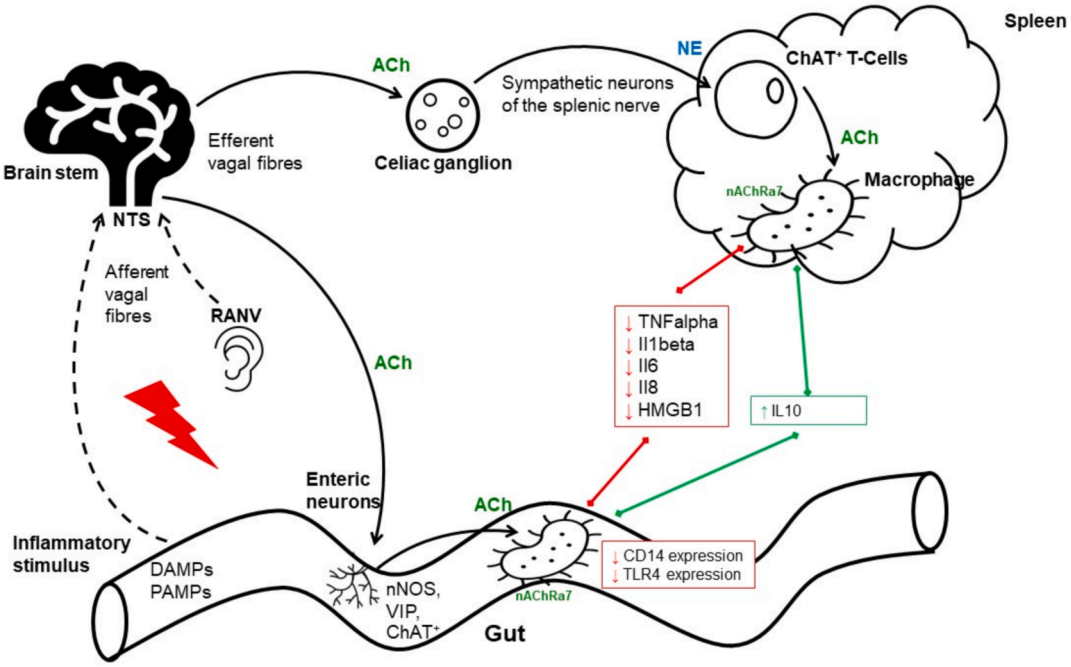

Fig. 1. Schematic overview of the CAIP.

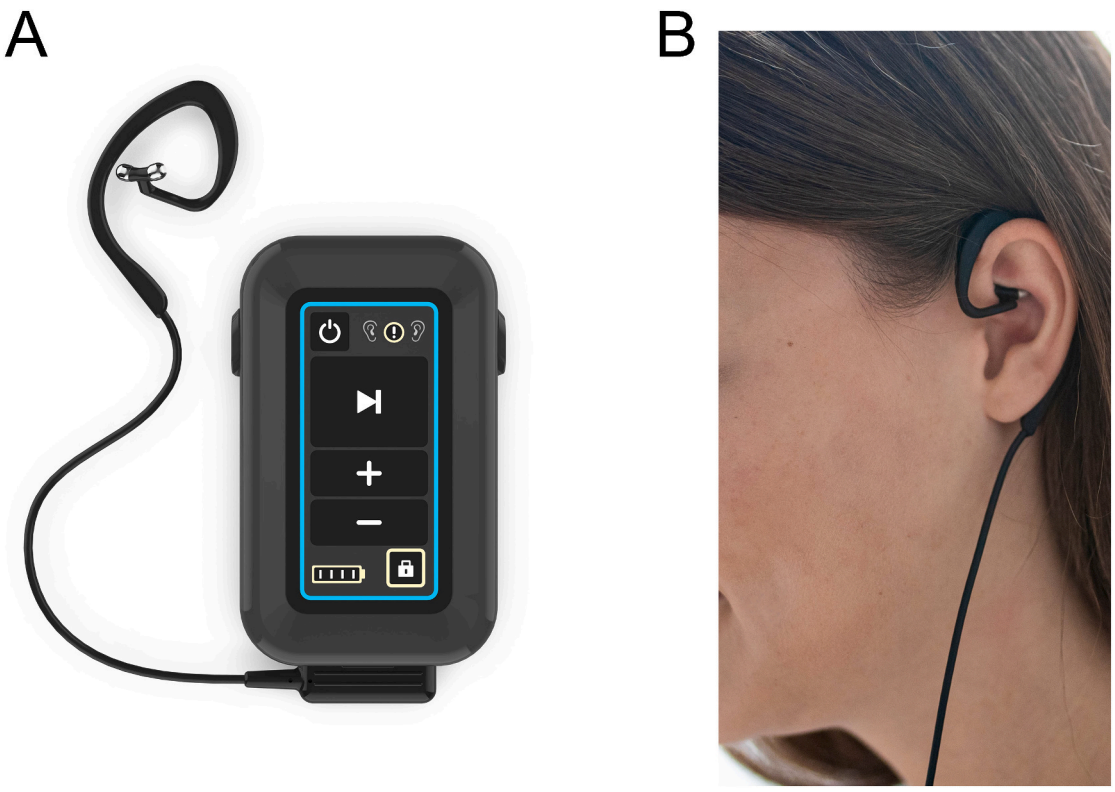

Fig. 2. A, Medical device for transcutaneous VNS via RANV purchased by tVNS TECHNOLOGIES GmbH. B, in-person application of the device.

2. Postoperative Ileus

2.1. Symptoms and clinical impact of Postoperative Ileus

POI is a common complication after abdominal surgery with an occurrence between 10 and 40% depending on the surgical procedure. Symptoms vary from bowel distension with mild abdominal cramps for one or two days to nausea and vomiting, abdominal cramps, absence of gas and stool passage and intolerance to food for more than one week. Due to the gastrointestinal paralysis, an increased gastro-oesophageal reflux with a high risk of aspiration, stasis of intestinal content with bacterial overgrowth, breakdown of the intestinal barrier and bacterial translocation could be observed, potentially leading to abdominal sepsis with distant organ failure. Occurrence of postoperative complications and prolonged hospitalization results in an increase of costs by up to 70% (Mao et al., 2019).

2.2. Activation of the CAIP as a prophylaxis for POI

Pathophysiology of POI consists of an interaction of inflammation, neural reflexes, neuro-humoral pathways and pharmacologic effects (Sommer et al., 2021). However, resident macrophages of the muscularis externa (ME) as well as enteric neuronal cells play a crucial role in development and maintenance of POI via cytokine and nitric oxide release (Vilz et al., 2014; Wehner et al., 2007; Schneider et al., 2021). Interestingly, the ME macrophages were shown to be inactivated by intrinsically release of ACh from vagal efferents via nAChRa7 (Boeckxstaens and de Jonge, 2009). As vagal innervation counter-regulates the inflammatory extend, VNS had attracted considerable attention in prophylaxis and treatment of POI. More than fifteen years ago, de Jonge and co-workers could demonstrate in a rodent model, that cervical exposure of the VN followed by perioperative electrical VNS prevented surgical induced inflammation of the ME and ameliorates POI via STAT3 activation in macrophages of the ME (de Jonge et al., 2005). As this approach requires an additional surgical procedure, a search for alternative options is mandatory. Stakenborg and co-workers published interesting data of a pilot study about infradiaphragmal electrical VNS during human laparoscopic colorectal surgery. Although the procedure seemed to be safe and feasible, a reduction of IL-6 and IL-8 release in LPS-stimulated whole blood could only be observed on postoperative day (POD) 1 (Stakenborg et al., 2017). Beside the small sample size, the pilot trial has one major issue: Due to the surgical exposure of the infradiaphragmal VN, a prophylactic stimulation before POI-inducing surgery is impossible. Hong and co-workers followed a different way in order to activate the CAIP: Within a rodent model a non-invasive tVNS was tested. First, the group could demonstrate, that tVNS via the RANV led to an activation of the efferent arm of the CAIP. Furthermore, tVNS diminishes inflammation of the ME after surgery and prevents POI (Hong et al., 2019). Based on these results, a pilot study in humans was performed investigating the effects of tVNS via RANV on smooth muscle activity of the stomach during abdominal surgery. Furthermore, serum gastrin levels as a surrogate marker for VN activation were analysed. Hong and co-workers could demonstrate that tVNS led to significant changes of action potential frequency and amplitude. Additionally, gastrin levels were significantly elevated 3 h after tVNS compared to levels before tVNS. At the beginning of 2021, Chapman and co-workers published data from the first parallel group, randomized-controlled early development study with 40 patients and could show a shorter time to first flatus and a faster tolerance of solid food after tVNS. However, in this trial, the efferent branches of the cervical VN are stimulated transcutaneously, not the RANV. Nevertheless, these results are interesting, whereby a power calculation and a sample size calculation before starting the trial was lacking (Chapman et al., 2021).

Despite these promising results, a well-designed randomized controlled clinical trial with an adequate sample size is still needed to generate evidence for the use of tVNS as a prophylaxis of POI after abdominal surgery.

3. Systemic inflammatory response syndrome (SIRS)

3.1. Clinical impact and pathophysiology of systemic inflammatory response syndrome (SIRS)

A well-known problem after major surgery or trauma is the development of the so-called systemic inflammatory response syndrome (SIRS). SIRS is mainly characterized by tachycardia, hypotension, tachypnea, leucocytosis or leucopenia as well as an increase or decrease in body temperature. These symptoms result from the excessive release of proinflammatory mediators (HMGB-1, TNF-alpha, IL-1beta, IL-6, IL-8) and can lead to multi-organ failure (MOF) and fatal outcome (Fry, 2012). Highly relevant for the further prognosis of patients during SIRS is the involvement of the GI tract: Hypotension with reduced bowel perfusion as well as the cytokine storm lead to a diminished peristaltic activity, the so-called septic ileus. As a consequence, bacterial overgrowth of intestinal content occurs followed by a breakdown of intestinal barrier function due to ischemia. This results in bacterial translocation and abdominal sepsis. Therefore, dysfunction of the gut is a major prognostic factor during SIRS, Deitch and co-workers coined the sentence "the gut is the motor of critical illness" (Deitch et al., 2006). However, until now, there is no existing prophylaxis or goal-directed treatment for SIRS, the therapy is symptomatic.

The incidence of SIRS after operations is up to 60% (Dieleman et al., 2017), but severity and duration of SIRS cannot be predicted. It depends for example on the type and invasiveness as well as the duration of the surgical procedure, intraoperative blood loss etc.

3.2. Vagus nerve stimulation as a prophylaxis for SIRS development

After the pioneering work of Tracey and co-workers describing the CAIP and demonstrating the benefits of iVNS in a SIRS-inducing endotoxemia model, other studies concerning VNS in SIRS rodent models followed: Zhou and co-workers demonstrated in a mouse SIRS model that iVNS reduced cytokine release and attenuates tight junction disruption resulting in less bacterial translocation (Zhou et al., 2013). Huston and co-workers as well as Hong and co-workers could demonstrate equal effects for tVNS in rodent SIRS models: Both groups demonstrated a reduced cytokine liberation and a reduced mortality for mice treated with tVNS before SIRS induction (Huston et al., 2007; Hong et al., 2019).

Furthermore, there is growing evidence about a relationship between vagal tone and the status of the immune system in humans. For example, a reduced heart rate variability as an indicator of a reduced vagal tone is associated with an increase in IL-6 and CRP serum levels in healthy subjects (Haarala et al., 2011). Additionally, an increased morbidity and mortality following sepsis or cardiac surgery, which implies a high risk for SIRS development, could be observed in patients with decreased vagal activity (Huston and Tracey, 2011).

Despite the convincing data from animal models and evidence from human observations about vagal tone and immune response, there are no recruiting or completed clinical trials (search on www.clinical trials.gov on May 25th 2021) for VNS as SIRS prophylaxis. Especially the use of non-invasive tVNS could be an interesting indication without the apprehension of severe side effects. However, this could be challenging due to the unpredictable incidence and severity of SIRS resulting in a trial with a large sample size and high expenses. To investigate tVNS as a potential SIRS prophylaxis, well-designed randomized controlled trials with adequate case calculations, a high incidence of SIRS and a comparable extent of symptoms to allow for a comparison between intervention and control group are needed.

4. Potential challenges in the design of a VNS trial and outlook

Preclinical data from animal models as well as human pilot studies indicate the potential of tVNS as a prophylaxis in acute inflammatory diseases. However, it remains challenging to design clinical trials to investigate the benefits of VNS. One key challenge when designing a tVNS clinical trial is the high interindividual variability in reaction to stimulation and the absence of a viable biomarker to measure responsiveness. Most often, stimulation parameters are set with regard to the individual stimulus threshold, when a "tingling sensation" at the outer ear becomes painful. Stimulation is exerted below this threshold. This system remains rather unstandardized and might result in unreproducible data, several biomarkers are currently under investigation (Keute et al., 2021; Vosseler et al., 2020) Moreover, in blinded clinical trials the problem of sham-stimulation arises. If no stimulus is exerted, participants will notice no somatosensory stimulus leading to a potential unblinding. On the other hand, if a stimulus is exerted at another site, for instance the tragus or the outer earlobe, different afferent fibres of the VN might stimulated with unpredictable neuronal effects (Borges et al., 2021; Vosseler et al., 2020). These operational problems need to be considered in the de facto design of VNS clinical trials.

In our opinion, all strength should be used to launch a multicentre trial to investigate the effects of tVNS in POI, which should be achievable with an adequate funding. Concerning POI, different surrogate markers including solid food tolerance, time to first defecation, time to first flatus or combinations are used to define its severity and duration. To our knowledge, there is no parameter or biomarker to objectively measure POI, impeding research in its therapy. Because of this, although usually treated with prokinetic substances, these measures still lack evidence in recent meta-analyses. We recently demonstrated the safety of the SmartPill®, a capsule sized 26x13mm, developed for analysis of bowel motility. Using the device, we were able to acquire first objectifiable data on POI in patients undergoing abdominal surgery, making the device a superior tool to investigate POI (van Beekum et al., 2021). Despite current clinical practice of prokinetic medication as therapy of POI, tVNS might hold major advantages because of its prophylactic nature, considering results of animal models and first human clinical trials, making research concerning tVNS as POI prophylaxis a worthwhile goal.

Concerning SIRS prophylaxis, the situation however is even more complex due to the unpredictable incidence and individual severity of the inflammatory extent. Therefore, a trial using a human SIRS model with a standardized induction of inflammation and a reproducible inflammatory answer to the stimulus is urgently needed to investigate possible therapeutic effects of tVNS. If a decrease of SIRS symptoms and cytokine liberation is observed in such a standardized model, a clinical trial examining tVNS before surgery is within reach.

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Review

Current challenges in reliably targeting the noradrenergic locus coeruleus using transcutaneous auricular vagus nerve stimulation (taVNS)

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ABSTRACT

Transcutaneous auricular vagus nerve stimulation (taVNS), as a non-invasive brain stimulation technique may influence the locus coeruleus-norepinephrine system (LC-NE system) via modulation of the Vagus Nerve (VN) which projects to the LC. Few human studies exist examining the effects of taVNS on the LC-NE system and studies to date assessing the ability of taVNS to target the LC yield heterogeneous results. The aim of this review is to present an overview of the current challenges in assessing effects of taVNS on LC function and how translational approaches spanning animal and human research can help in this regard. A particular emphasis of the review discusses how the effects of taVNS may be influenced by changes in structure and function of the LC-NE system across the human lifespan and in disease.

1. Introduction

The *locus coeruleus* (LC) in the brainstem is one of our main sources of *noradrenaline* (also referred to as norepinephrine, NE) in the brain. It exhibits particular vulnerability in a wide range of neurological and clinical conditions that pose an increasing economic and societal burden. Changes to the LC-NE system in such conditions include an *increase* in NE modulation, e.g., in chronic pain (Llorca-Torralba et al., 2016), stress and anxiety (Berridge and Waterhouse, 2003; Bremner et al., 1996), but also a *decrease* in NE production and degeneration of NE-producing cells in the LC, e.g., in depression (Bernard et al., 2011), post-traumatic stress disorder (Berridge and Waterhouse, 2003; Pietrzak et al., 2013) and aging (Mather and Harley, 2016). Moreover, for the two most prominent neurodegenerative diseases, Parkinson disease (PD) and Alzheimer disease (AD), LC abnormalities can be observed before typical pathologies in substantia nigra (SN) and transentorhinal/entorhinal cortex respectively, occur (Braak et al., 2011; Braak et al., 2003). A number of these neurodegenerative and psychiatric diseases are currently being treated or investigated to be treated with pharmacological interventions that also target the noradrenergic system (e.g., sNRIs - selective norepinephrine reuptake inhibitors). However, pharmacological interventions are accompanied with the downside of a lack of anatomical specificity and thus increase the possibility of generating side effects that can have a negative impact on quality of life. Studies in rodents were able to show how to increase LC firing associated with NE release in the hippocampus and cortical target areas over the course of minutes to hours using an invasive vagus nerve stimulation (iVNS) approach (Follesa et al., 2007; Hulsey et al., 2017; Hulsey et al., 2019; Manta et al., 2009). IVNS is used in humans as an adjunctive therapy to treat refractory epilepsy (see Englot et al., 2011 for meta-analysis & Panebianco et al., 2016 for review) as well as depression (see Farmer et al., 2020 for review).

A promising technique to circumvent the caveats of pharmacological or invasive stimulation in humans is transcutaneous auricular vagus nerve stimulation (taVNS) applied mainly to the cymba conchae or, in some studies, to the tragus of the external ear (cf. Fig. 1). Peuker and Filler (2002) showed in an anatomical study that the cymba conchae was innervated solely by the Auricular Branch of the Vagus Nerve (ABVN),

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whereby the tragus was also innervated by the Great Auricular Nerve and the Auricolutemporal Nerve. The ABVN, together with the remaining nerve fibre bundles of the vagus nerve, reaches the brainstem at the nucleus tractus solitarius (NTS) which has prominent projections to the LC-NE system (cf. Fig. 1), (Butt et al., 2020; Ruffoli et al., 2011). Stimulation at these auricular sites has been shown to activate structures along the vagal afferent pathway in humans (Badran et al., 2018; Yakunina et al., 2017). Therefore taVNS holds great promise as a more anatomically precise and potentially rehabilitating NE therapeutic compared to pharmacological interventions (Collins et al., 2021; Hulsey et al., 2017; Mridha et al., 2021; Sharon et al., 2021). Moreover, it may also offer the possibility for more varied interventions as stimulation interventions are able to modulate local neuronal activity in a particular frequency and for an explicit duration and can thus attempt to mimic naturally occurring firing patterns of the stimulated brain structure (Polanía et al., 2018). Despite these promising properties, current studies using taVNS as a substitute for pharmacological interventions in depression are plagued by their lack of reliability (Martin and Martín-Sánchez, 2012). To improve the reliability of taVNS interventions, the link between taVNS and the LC-NE system in humans needs to be better understood. This review summarizes the main challenges in this endeavour (see also Fig. 1 for an overview of the main challenges). We draw attention to the still limited understanding of the mechanisms of actions of taVNS and control of mediating factors in humans. Furthermore, we outline how a translational approach might help to understand how interindividual differences in the integrity of the brain, and in particular the LC, might alter taVNS effects.

2. Current outcome measurements of taVNS

The vast majority of taVNS intervention studies in humans lack appropriately validated physiological as well as cognitive outcome measures to monitor temporal and spatial specificity of intervention effects on the LC-NE system. The most commonly used taVNS outcome

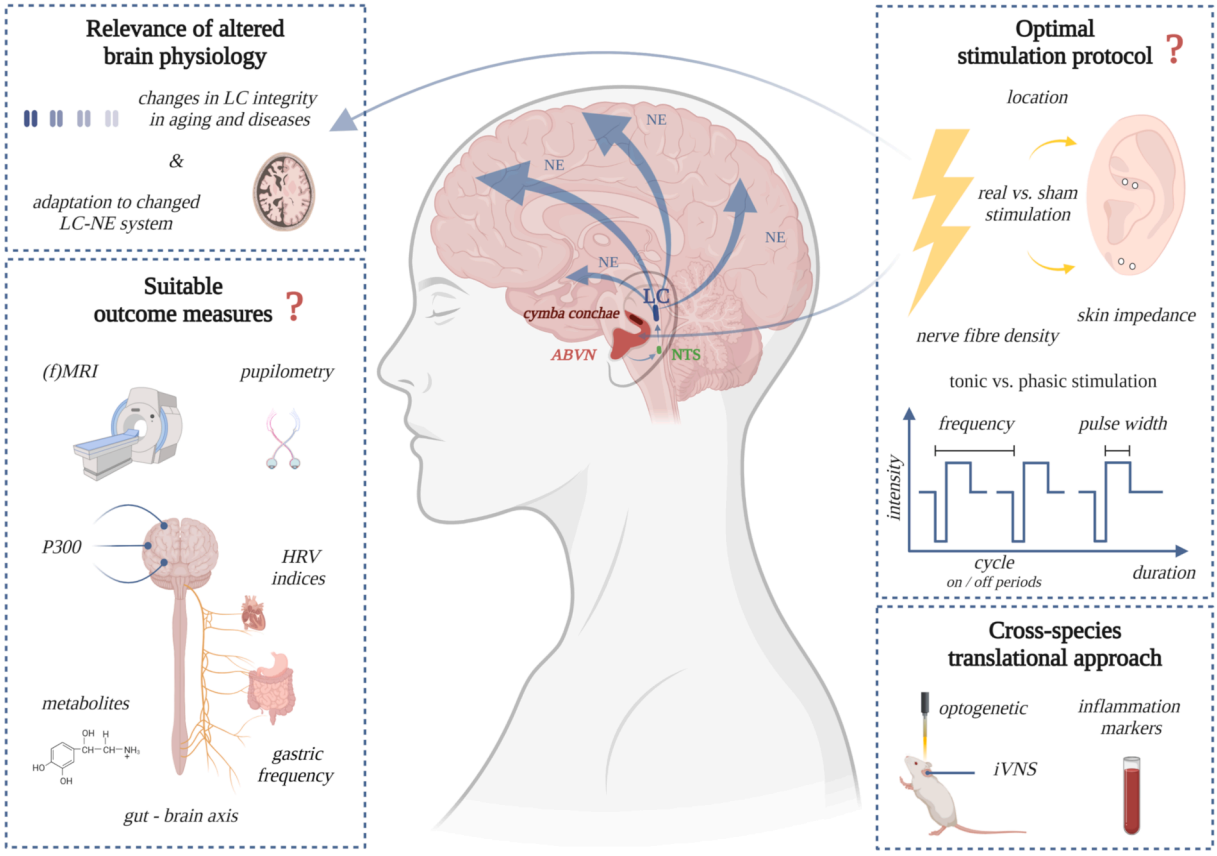

Fig. 1. Challenges in scrutinizing the link between taVNS and the LC-NE System.
Center: TaVNS is applied to regions in the left ear innervated by the ABVN. The stimulation is relayed via the ABVN to the NTS which projects to the LC from where NE is released into various projection areas. Top right: Currently, taVNS in humans is characterized by heterogeneous stimulation protocols. Phasic as well as tonic mono- or biphasic stimulation approaches are based on different stimulation parameters (intensity, frequency, pulse width, cycle, duration), which are tested on different stimulation locations. Most commonly used for real stimulation is cymba conchae and for sham stimulation earlobes (top right: white circles = 2× anode/cathode), which is currently under debate. Interindividual differences in nerve fibre density as well as lack of proper skin cleaning might be a cause for heterogeneous results from previous studies. Bottom right: Cross-species translational approaches can be used to investigate new applications for taVNS in humans and to improve current stimulation methods. Animal research using i/taVNS or optogenetic stimulation can help to improve our understanding of how VNS affects the LC-NE system and how its effects depend on changes in the LC-NE system (see also top left). Bottom left: Suitable outcome measures for taVNS are needed to study taVNS effects in an optimal manner. As of now only indirect measures of LC or noradrenergic function are available such as fMRI, pupillary changes, HRV indices (RMSSD, pNN50), gastric-frequency, P300 and potential NE metabolites. Of these, fMRI offers the most direct way to visualize LC-NE activity. Top left: Alterations in brain physiology, such as the integrity of the LC and adaptation to an altered LC-NE system might account for heterogeneous outcomes and need to be considered especially in clinical populations to adjust the stimulation parameters accordingly. Blue columns indicate the bilateral structure of the LC and the decrease in saturation symbolizes a decline of LC integrity.
Abbreviations: ABVN Auricular Branch of the Vagus Nerve, ERP Event related potential, fMRI functional magnetic resonance imaging, HRV Heart Rate Variability, iVNS invasive Vagus Nerve Stimulation, LC locus coeruleus, LC-NE system locus coeruleus norepinephrinergic system, NE Norepinephrine, NTS nucleus tractus solitarius, taVNS transcutaneous auricular vagus nerve stimulation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

measures are indirect measures of LC activity (cf. Fig. 1) such as heart rate variability (HRV) indices, pupil dilation, or the P300 event related potential (ERP) which have been discussed in their respective usefulness to indicate LC-NE activation in recent reviews (Burger et al., 2020; Farmer et al., 2020).

Briefly, HRV is a collective term for several indices derived from electrocardiography, whereby the Root Mean Square of Successive Differences (RMSSD) and percentage of consecutive normal sinus RR intervals spaced more than 50 ms apart (pNN50), are thought to reflect vagal activity (see (Burger et al., 2020) for review). However, the extent to which HRV actually reflects vagal nerve engagement is difficult to determine due to the differences in stimulation protocols and HRV indices assessed in previous studies (Burger et al., 2020). For this purpose, Wolf et al. (2021) have developed a Shiny web app that frequently incorporates new results into a Bayesian meta-analysis (termed 'living Bayesian meta-analysis') to investigate the extent to which HRV may be an indirect biomarker for taVNS. Likewise, it is important to critically investigate whether HRV is related to vagal activity at all, as a recent iVNS animal study showed that tonic vagal activity during respiration does not correlate with HRV metrics (Marmerstein et al., 2021).

Pupil dilation can be an easy-to-acquire proxy measure for LC firing. Anatomically, LC projections inhibit the Edinger-Westphal nucleus resulting in a relaxation of the iris sphincter muscle that can be measurable as a change in pupil dilation (Hall and Chilcott, 2018; Samuels and Szabadi, 2008a, 2008b). Functionally, stimulating the LC in monkeys has been shown to result in dilated pupils (Joshi et al., 2016). Still, the link between LC activity and pupil dilation is not exclusive. Other structures such as the hypothalamus or superior colliculus also target the Edinger-Westphal nucleus (Mathôt, 2018), and stimulating in the superior colliculus, for instance, also resulted in increased pupil dilations (Joshi et al., 2016). It is also important to note that not only noradrenergic but also cholinergic axons are involved in dilating the pupil (Reimer et al., 2016).

The P300 ERP, (short P3), occurs around 300 ms after the onset of behaviourally relevant or rare stimuli and especially the P3b subcomponent has been related to parietal noradrenergic pathways involved in decision making and memory (see (Polich, 2007) for a review). Nevertheless, event-related potentials are difficult to source-localize in the brain, especially when it is related to the brainstem structures, so there is currently no conclusive evidence how specifically LC activity and NE release is reflected in P300 ERPs (Farmer et al., 2020). Besides the P3, multiple studies investigated effects of vagus nerve stimulation on cortical oscillations. Results from three studies analysing power in different frequency ranges indicate that invasive and non-invasive VNS might be able to increase cortical arousal. This was observed as a decrease of power in lower frequencies (Bodin et al., 2015; Lewine et al., 2019; Sharon et al., 2021) and an increase of power in higher frequency ranges (Lewine et al., 2019). Of note, only the study from Sharon et al. (2021) used taVNS in a sample of 25 healthy adults. Bodin et al. (2015) used iVNS in 19 epilepsy patients and Lewine et al. (2019) used neck VNS in 8 healthy subjects. However, these results await replication in higher sampled studies. Other studies focused on oscillations related to different aspects of cortical processing. Keute et al. (2019a) observed increased frontal-midline theta power, related to executive function, in trials that elicited go/stop response conflicts during a cued go/no-go change task. In a different study, the authors observed a decrease in power in the theta range (4–8 Hz) over the course of the experiment, but this effect was observed after taVNS as well as after sham stimulation. Additionally, no taVNS effect on motor related beta oscillations or gamma oscillations related to early visual processing could be observed (Keute et al., 2021b). Thus, the use of different cortical oscillations as a proxy for LC-NE activation awaits further investigation in future studies.

Another possibility to evaluate the effect of taVNS would be to examine the concentration of NE and other neurotransmitters and their respective metabolites in blood and cerebrospinal fluid (CSF) following stimulation. The principal metabolite of NE is 3-methoxy-4-hydroxyphenylglycol (MHPG), which also serves as an indicator of noradrenergic activity (Elsworth et al., 1982; Kanda et al., 1991). In animal models, two weeks of iVNS increased the concentration of NE in prefrontal areas (Manta et al., 2013; Roosevelt et al., 2006). Neurochemical studies in humans, however, are sparse and until now limited to iVNS. Only one study directly assessed NE and MHPG in depressed patients ($N = 21$) implanted with iVNS (Carpenter et al., 2004). No effects on NE and MHPG concentrations were found in CSF taken from lumbar punctures, although an increase in homovanillic acid (HVA), a dopamine metabolite, was observed. However, all subjects were under constant pharmacological therapy, hence the authors could not determine the extent to which the psychotropic medication affected this increase in HVA (Carpenter et al., 2004). As of now more research is needed to determine how CSF metabolites may be used as an indirect biomarker of taVNS. Salivary Alpha-Amylase has also been considered as a proxy for LC-NE activation (e.g., Warren et al., 2019). However, the amount of studies using this proxy is low and their results are inconsistent with regards to how well they reflect an engagement of the LC (see Burger et al., 2020 & Farmer et al., 2020 for detailed reviews).

The VN does not only have a key role in the central nervous system (CNS) and in the autonomic nervous system (ANS), but also in the enteric nervous system (ENS) by signalling from gastrointestinal microbiota to the brain and vice versa (see (Cryan et al., 2019) for review) (see also Section 5). Previous work suggests that the VN may exert anti-inflammatory effects via hypothalamic-pituitary-adrenal (*vagal afferents*) or via cholinergic anti-inflammatory (*vagal efferents*) pathways (e.g., Bonaz et al., 2013, 2017; Tracey, 2002). Furthermore, LC-NE stimulation may also exert anti-inflammatory and neuroprotective effects by increasing the expression of neurotrophic substances such as brain-derived neurotrophic factor (BDNF) (Braun et al., 2014; Furmaga et al., 2012) and by attenuating the release of cytokines such as TNF alpha, IL-1b, IL-6, IL-18 (Borovikova et al., 2000; Meregnani et al., 2011; Subramanian et al., 2020). Of note, it is difficult to determine whether differences identified in blood or CSF reflect peripheral or central effects respectively (Molinuevo et al., 2018), so whilst fluid biomarkers may provide a more direct measure of NE levels compared to pupillometry or HRV, the origin of these effects cannot be exclusively determined.

Compared to Pupillometry, ERPs or HRV, *functional magnetic resonance imaging* (fMRI) can be a more direct tool to visualize LC responses. Although fMRI is not a direct measure of, for example, LC firing as it reflects changes in blood flow in the LC area which serves as a proxy of LC activation (e.g., Jacobs et al., 2020; Yi et al., 2021), it is arguably currently our best measure for visualizing LC activation in humans. FMRI studies in combination with taVNS in humans corroborate an involvement of the LC-NE system (see Table 1 for an overview of tVNS-fMRI studies), evident by taVNS-induced functional activation from NTS in LC-NE projection areas such as the amygdala and hippocampus (Sclocco et al., 2019; Yakunina et al., 2017). An overview and recommendations on how to proceed best during combined fMRI and taVNS studies (e.g., imaging resolution 1–2 mm voxel size) have recently been published (see section 'Functional Neuroimaging' (Farmer et al., 2020)). Furthermore, many current studies lack sufficient sample sizes, spatial resolution or postprocessing methods to reliably identify activation in the LC (Yi et al., 2021). Additionally, it should be noted whether the reported increased or decreased functional activation is based purely on real i/taVNS stimulation or on the comparison between real and sham stimulation, with the latter being preferred as a more optimal experimental control.

3. Insufficiently validated stimulation protocols

An important current challenge in evaluating taVNS in human research are the heterogeneous and often poorly-validated stimulation protocols (cf. Fig. 1 – 'Optimal stimulation protocol'). A recent consensus paper provides an overview of this issue (Farmer et al., 2020). The combination of different parameters such as stimulation intensity

Table 1
Studies reporting fMRI activation in LC and its projection areas following taVNS.

Scanner	Author/year	N	Design	Head coil	Structural parameters	fMRI sequence parameters	Smoothing	fMRI results
1.5 T								
	Dietrich et al., 2008	4 healthy male subjects	50 s ON vs 100 s OFF, 250 μs, 25 Hz, 4–8mA fMRI for 700 s - four alternating ON and OFF sequences were performed	Not reported	**MPRAGE** 176 sagittal slices **Thickness**: 1 mm **Matrix**: 256 × 256 **FOV**: 224 × 224 mm^2	**EPI** 36 axial slices **TR**: 110 ms **TE**: 60 ms **FA**: 90° **Thickness**: 3 mm **Matrix**: 64×64 pixel **FOV**: 224×224 mm^2	Not reported	**<u>Comparison left tragus vs earlobe stimulation</u>** *Activation* → left LC, thalamus, prefrontal cortex, posterior cingulated gyrus, insula →bilateral postcentral gyrus *Deactivations* → right nucleus accumbens, cerebella hemisphere
	Kraus et al., 2007	36 healthy subjects in 3 studies Study 1: N = 22 Study 2: N = 8 **Study 3: N = 6**	30 s ON vs 120 s OFF, 20 μs, 8 Hz, 4 mA in low condition/5 mA in high condition 130 blocks; 200 in case of alternating low-high stimulation - four alternating ON and OFF sequences were performed - stimulation during blocks 11–20, 41–50, 71–80, 101–110	Not reported	**MPRAGE** 160 sagittal slices **Thickness**: 1 mm **Matrix**: 256 × 256 **FOV**: 220 × 220 mm^2 In plane resolution: 0.98 × 0.98 mm^2	**EPI** 20 slices **TR** = 3000 ms **TE** = 60 ms **FA** = 90° **Thickness**: 4 mm **Matrix**: 128 × 128 **FOV**: 220 × 220 mm^2	Not reported	**<u>Comparison anterior wall vs. earlobe stimulation (N = 6)</u>** *Activation* → unspecific patterns *Deactivation* → paracentral lobe → right parahippocampal gyrus
	Kraus et al., 2013	16 healthy subjects 8 subjects per stimulation location	30 s ON vs 60 s OFF, 20 μs, 8 Hz, 32.6 V ± 13.4 V for taVNS, 30.0V ± 13.5V for sham 130 blocks - four alternating ON and OFF sequences were performed - stimulation during blocks 11–20, 41–50, 71–80, 101–110	Not reported	**MPRAGE** 160 sagittal slices **Thickness**: 1 mm **Matrix**: 256 × 256 **FOV**: 220 × 220 mm^2	**EPI** 20 slices **TR**: 3000 ms **TE**: 60 ms **FA**: 90° **Thickness**: 4 mm **Matrix**: 128 × 128 **FOV**: 220 × 220 mm^2	Not reported	**<u>Comparison anterior wall vs. earlobe stimulation (N = 8)</u>** *Activation* → left insula, medial frontal gyrus *Deactivation* → left parahippocampal gyrus → LC, solitary tract

(*continued on next page*)

Table 1 (*continued*)

Scanner	Author/year	N	Design	Head coil	Structural parameters	fMRI sequence parameters	Smoothing	fMRI results
					In plane resolution: 0.98 × 0.98 mm^2			
3T								
	Badran et al., 2018	17 healthy subjects	60 s ON vs 60 s OFF, 500 μs, 25 Hz, 3.14 mA ± 0.99 mA for taVNS, 2.43 ± 1.16 mA for sham two scanning sessions for 30 min - 6min each stimulation scan	32-channel	**MPRAGE** 208 slices **TR**:1900 ms **TE**: 2.26 ms **FA**: 9° **Voxel size**: 1 mm^3	**EPI** 47 slices **TR**: 2800 ms **TE**: 35 ms **FA**: 76° **Voxel size**: 3.0 mm^3	8mm FWHM Gaussian smoothing kernel	**Comparison tragus vs earlobe stimulation** *Increased activation* → right caudate → bilateral anterior cingulate, cerebellum → left prefrontal cortex → mid-cingulate
	Frangos et al., 2015	12 healthy subjects	Scan 1 as control 2 min rest – 7 min earlobe stimulation – 5 min rest Scan 2 as experimental 2min rest – 7 min left cymba conchae stimulation – 11 min rest 250 μs, 25 Hz, 0.43 ± 0.14 mA for taVNS, 0.58 ± 0.19 mA for sham	12-channel	**MPRAGE** 176 sagittal slices 1mm isotropic voxels **TR**: 1900 ms **TE**: 2.52 ms **FA**: 9° **Matrix**: 256 × 256 **FOV**: 256 × 256 mm^2 50% distance factor	**EPI** 33 axial slices 3mm isotropic voxels **TR**: 2000 ms **TE**: 30ms **FA**: 90° **Matrix**: 64 × 64 **FOV**: 192 × 192 mm^2 Interslice gap: 1.5 mm	5mm FWHM Gaussian smoothing kernel vs no spatial smoothing	**Comparison cymba conchae vs earlobe stimulation** Group brainstem analysis → activation of the ipsilateral NTS, STN, LC (contralateral), parabrachial area (contralateral) → bilateral activation in forebrain regions → bilateral deactivations in hypothalamus, hippocampal formation → spatial smoothing (5mm): activation throughout medulla, pons, midbrain, but not regional specific
	Garcia et al., 2017	16 migraine patients and 16 healthy controls	360 s stimulation duration, 14 s ON 20 s OFF, 450 μs, 30H z, 0.85 ± 1.07 mA – 1.22 ± 1.33 mA for tVNS, no stimulation during sham Two stimulation scan runs	12-Channel	**MPRAGE** 176 axial slices **TR**: 2530 ms **TE**: 1.64 ms **FA**: 7°	**T2* pulse sequence** 43 axial slices **TR**:2500 ms **TE**: 30 ms **FA**: 90° **Matrix**: 84×84	5mm FWHM Gaussian smoothing kernel	**Comparison cymba conchae vs cymba conchae (no current)** *Increased activation during eRAVANS* - NTS - anterior insula, mid-cingulate cortex *Post stimulation effects* - increased activation in nucleus raphe centralis, LC

(*continued on next page*)

Table 1 (*continued*)

Scanner	Author/year	N	Design	Head coil	Structural parameters	fMRI sequence parameters	Smoothing	fMRI results
			- 11 repetitions with air-puffs			**Thickness**: 2.62 mm gap: 0.5 mm		
					FOV: 256×256 mm^2 **Voxel size**: 1×1×1 mm^3	**FOV**: 220×220 mm^2 **Voxel size**: 2.62×2.62×3.12 mm^3		
	Peng et al., 2018	24 healthy subjects	30 s ON vs 60 s OFF,	Not reported	Not reported	**FSPGR** NEX = 1	6mm FWHM Gaussian	**<u>Comparison cymba conchae vs. earlobe stimulation (N = 16)</u>**
			250 μs, 20 Hz, between 4 and 8 mA			**TR**: 6.6ms **TE**: 2.8ms	smoothing kernel	*Activation*: → bilateral amygdala, prefrontal cortex → left caudate, posterior cingulum cortex, parahippocampal gyrus, putamen
			fMRI for 420 s - baseline for 60 s			**FA**: 60°		
			- four alternating stimulation ON and OFF sequences were performed			**Thickness**: 1mm **Matrix**: 256×256 **FOV**: 16cm/image		
	Sclocco et al., 2020	30 healthy subjects	Five 8.5-min duration fMRI scan runs	64 -channel	**MPRAGE** 176 axial slices	**EPI** multi-band factor 575 axial slices axial slices		**<u>Comparison cymba conchae vs. no current</u>**
			1× sham stimulation run 4× active RAVANS scans using different frequencies at 2 Hz (7.18 ± 0.95 mA),			2 mm isotropic voxel		*Greater activation for 100 Hz RAVANS vs. sham* - bilateral LC, dorsal and medial raphe nuclei, *Greater activation for 2 Hz RAVANS vs. sham* - right LC, dorsal raphe nuclei *Greater activation for 100 Hz RAVANS vs. Greater activation for 2 Hz vs.*
			10 Hz (6.46 ± 1.30 mA), 25 Hz (5.93 ± 1.21 mA), 100 Hz (5.57 ± 1.18 mA)		**TR**: 2530 ms **TE1/TE2/TE3/TE4:** 1.69/3.55/5.41/7.27 ms **FA**: 7°	**TR**: 1250 ms **TE**: 33 ms **FA**: 65 °		

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Table 1 (*continued*)

Scanner	Author/year	N	Design	Head coil	Structural parameters	fMRI sequence parameters	Smoothing	fMRI results
								10 Hz and 25 Hz - right LC, dorsal raphe nuclei a) *10 Hz RAVANS*: right LC b) *25 Hz RAVANS*: right LC, dorsal raphe nuclei *sig. correlation between 2 and 100 Hz* - right LC, dorsal raphe nuclei
			300 μs, 1.5 sec phasic bursts			**Thickness**: 2 mm		
					FOV: 256 × 256 mm^2	**FOV**: 220 × 220 mm^2		
	Yakunina et al., 2017	37 healthy subjects	30 s ON vs 60 s OFF,	32-Channel SENSE	**T1** coronal 3D	**EPI** 30 oblique coronal	8mm FWHM Gaussian	**Comparison cymba conchae vs. earlobe stimulation** *Activation* → unsmoothed data: bilateral LC and NTS
			500 μs, 25 Hz, 0.77 ± 0.42 mA	(Philips)		slices	smoothing kernel	
			at inner tragus, 0.81 ± 0.48 mA at ear canal, 0.91 ± 0.47 mA at cymba, and 0.81 ± 0.38 mA for sham		**TR**: 9.8 ms **TE**: 4.8 ms **FA**: 8 °	**TR**:2000 ms **TE**: 35 ms **FA**: 90°	vs no spatial smoothing	
			at cymba, and 0.81 ± 0.38 mA for sham		**Thickness**: 1.0 mm			
			- repeated for four times in a run - each subject eight 6-min fMRI runs with up to 90 s rest in between runs		**Matrix**: 256 × 256 × 195 **FOV**: 220 × 220 mm^2 **Voxel size**: 0.94 × 0.94 mm	**Matrix**: 80 × 80 **FOV**: 220 × 220 mm^2 **Voxel size**: 2.75 × 2.75 mm		
	Zhang et al., 2019	29 migraine patients	200 μs, 1 Hz, 1.5–3 mA	24-channel	**MPRAGE**	**EPI** 31 slices, 150 phases	6mm FWHM Gaussian smoothing kernel	**Comparison cymba conchae vs. tail of the helix stimulation** *Deactivation based on ROI analysis* →in the bilateral LC
			- each scan consisted of six 20 - s ON conditions separated by 20- or 30-s 'OFF' periods		**TR**: 1900 ms **TE**: 2.27 ms **FA**: 9 °	**TR**: 2000 ms **TE**: 30 ms		
			- 5 min real or sham taVNS fMRI scan - 8 min continuous real or sham taVNS without fMRI		**Thickness**: 1.0 mm **Matrix**: 256 × 256	**Thickness**: 3.5 mm **Matrix**: 64 × 64		

(*continued on next page*)

Table 1 (*continued*)

Scanner	Author/year	N	Design	Head coil	Structural parameters	fMRI sequence parameters	Smoothing	fMRI results
7T	Sclocco et al., 2019	16 healthy subjects	Four 8-min duration fMRI scan runs 1 s 'ON', 450 μs, 25 Hz, 1.6 ± 2.3 mA (*eRAVANS*) 1.7 ± 2.4 mA (*iRAVANS*) for taVNS, 1.4 ± 1.1 mA for sham - passive control scan - two active stimulation scans - one active control scan run	32-channel	**FOV:** 256 × 256 mm^2	**FOV:** 224 × 224 mm^2 **EPI** multi-band factor 2 38 coronal slices 1.2mm isotropic voxel size **TR:** 0.99 s **TE:** 23 ms **FA:** 58° **FOV:** 192 × 192 mm^2 band width: 1562 Hz pix^-1 echo spacing: 0.76 ms, R = 4 in-plane (GRAPPA)	smoothing kernel smoothing kernel	**Comparison cymba conchae vs. earlobe stimulation** *Greater activation for eRAVANS* - LC, dorsal and medial raphe nuclei

(mA), stimulation frequency (Hz), pulse width (μs) and duty cycle (stimulation on / stimulation off) provide a large parameter space from which researchers have to choose optimal stimulation protocols with for the most part unknown efficacy in humans. Moreover, many taVNS studies in humans use commercially available and certified devices with predefined stimulation parameters, e.g., a stimulation frequency of 25 Hz, pulse width between 200-300 μs and a duty cycle of 30 s on and 30 s off (Yap et al., 2020). Researchers are then only able to adjust the stimulation intensity to their individual needs (e.g., Bauer et al., 2016; Beste et al., 2016; Borges et al., 2019; Ferstl et al., 2021; Frangos et al., 2015; Warren et al., 2019). This already limits possible study designs, where often a more flexible manipulation of parameters is desirable. Indeed, 30 s stimulation with 25 Hz has been shown to increase LC firing and NE release in iVNS studies with rats (Dorr and Debonnel, 2006; Manta et al., 2009, 2013). Pulse width and the off-period in these studies however differed from the parameters pre-set in many taVNS devices. Using predefined stimulation parameters may simplify comparisons between human studies, however it is difficult to compare effects with animal studies where parameters often vary (Colzato and Beste, 2020).

Regarding the stimulation intensity, iVNS in rats has shown a dose-dependent relationship with higher intensities leading to increased LC firing and NE release. Driven activity in the LC increased monotonically with the tested stimulation intensities from 0.2 mA to 2.5 mA (Hulsey et al., 2017). However, a higher LC firing rate does not always appear to be beneficial. Animal studies on cortical plasticity using iVNS in rats suggest that the relationship between stimulation intensity and stimulation effects may not always increase monotonically. Plasticity was more pronounced at moderate intensities around 0.8 mA whilst at higher stimulation intensities (1.2–1.6 mA), iVNS disrupted cortical plasticity and behavioral benefits (Borland et al., 2016; Morrison et al., 2021; Souza et al., 2021). Currently, human studies which systematically investigate the effect of different stimulation intensities for taVNS are lacking. In human taVNS studies, two different approaches are used: (i) using a fixed stimulation intensity across all subjects and (ii) individual adjustment of intensity. In the second case, researchers can choose to stimulate below or above the individual perceptual threshold. Whilst the first approach assures uniform stimulation parameters across participants, the latter method gives the advantage of avoiding uncomfortable or even painful stimulation. Both options (fixed and individualized intensities) are viable given that the current intensity is high enough to activate myelinated A-fibres, which contribute a large part of the ABVN (Safi et al., 2016). From a theoretical point of view, it seems reasonable that stimulation intensities for taVNS should not fall below 0.75 mA to recruit A-fibres of the ABVN. Using computational models, Helmers and colleagues estimated stimulation intensities between 0.75 and 1.75 mA are sufficient to cause vagal activation with pulse widths between 200 and 500 μs. However, their model was restricted to the cervical VN and based on the histological examination of the VN from only one subject (Helmers et al., 2012). In practice, 'moderate', non-invasive stimulation intensities with regard to taVNS effect are likely to be higher, since skin impedance and properties of subcutaneous tissues affect the current flow (Keller and Kuhn, 2009). Inadequate skin cleaning and degreasing before stimulation can easily increase impedance at the skin level and thus may reduce the current that reaches the nerve fibres (Badran et al., 2019; Burger et al., 2020).

The most commonly applied ***frequency*** in human studies at present is 25 Hz (see Table 2 in (Farmer et al., 2020)), but conclusive evidence about the effectiveness of this frequency in humans is lacking. The effects of varying frequency (0, 7.5, 15, 30, 60, 120 Hz) keeping the other parameters constant, were shown with iVNS in rats (Hulsey et al., 2017). Specifically, they showed that higher stimulation frequencies lead to greater maximal discharge rates over a shorter duration. Varying the iVNS frequency thus influenced the timing but not the total amount of LC activity (Hulsey et al., 2017). A first more systematic approach in human studies based on perceptual thresholds was reported by Sclocco et al. (2020). They were able to show that perceptual ratings of

stimulation intensity did not differ between conditions when higher stimulation intensities were combined with lower frequencies (7.18 ± 0.95 mA (2 Hz) > 6.46 ± 1.30 mA (10 Hz) > 5.93 ± 1.21 mA (25 Hz) > 5.57 ± 1.18 mA (100 Hz)) and interestingly the perceptual rating did not differ between the conditions (Sclocco et al., 2020). Moreover, a wider cluster of fMRI activation in respiratory-gated taVNS (RAVANS) at 100 Hz was found in serotonergic (dorsal (DR) and median (MR) raphe nuclei) and noradrenergic (LC) nuclei, whilst lower 2 HZ RAVANS also lead to DR and right LC activation (Sclocco et al., 2020). These results also illuminate that high responders to 2 Hz RAVANS were also high responders to 100 HZ RAVANS and that due to the differentially perceived sensory stimulation the influence of sensory pathways on LC activations cannot be excluded (Sclocco et al., 2020). Based on these results, a high stimulation frequency (e.g. 25 Hz) should be tested in comparison to lower frequencies (e.g. 10, 15 Hz) in taVNS studies, keeping the other stimulation parameters constant, in order to be able to give conclusive evidence regarding the influence of stimulation frequency on the LC-NE system.

Besides stimulation intensity and frequency, the ***pulse width*** also affects iVNS efficacy in a dose-dependent manner. In rodent studies using iVNS, higher pulse width lead to increased LC firing rates (0, 30, 100, 500 μs (Hulsey et al., 2017)), pupil dilation (100, 200, 400 or 800 μs (Mridha et al., 2021)) and behavioral as well cortical arousal states (100, 500 or 800 μs (Collins et al., 2021)). In human taVNS studies, pulse width typically varies between 200 and 1000 μs (Redgrave et al., 2018) and needs to be further systematically investigated. Likewise, the relevance of changes in stimulus cycle requires further investigation in both animal and human research. A current trend towards investigating the effects of phasic (Sharon et al., 2021), event-related stimulation rather than tonic stimulation with particular stimulus cycles is interesting in this regard (summarized below in Section 3). Badran et al. (2019) were able to show that the perceptual threshold decreases with increased pulse width (real stimulation at tragus, $N = 15$), which suggests that parameter manipulations should be assessed in the context of manipulations of other parameters. However, at this point it is unclear how perceived intensity correlates with taVNS outcome measures.

Considering all stimulation parameters, it is evident that their optimal settings and interdependency is insufficiently studied in humans. The lack of studies systematically investigating stimulation parameters in humans is compounded by a frequent use of insufficiently validated outcome measures (see Section 1). Moreover, it is currently unclear to what extent perceptual ratings of stimulation intensity relate to stimulation effects on the VN, and result in additional LC engagement via sensory pathways. Another often neglected aspect, which Wolf et al. (2021) rigorously discussed based on neurobiological pathways, is the ***stimulation side*** of the ear, i.e., left vs. right (e.g., stronger HRV indices for right sided taVNS reported by De Couck et al. (2017)). In this regard, animal research suggests that the right nodose ganglia (NG) have better access to dopaminergic structures such as the SN (Han et al., 2018) and stimulation of the left ear in humans has a stronger effect on invigoration when food reward is involved (Neuser et al., 2020). These results suggest lateralisation effects and motivate further systematic studies in this respect. Currently, however, it seems likely that the stimulation side has no systematic impact on taVNS effects as measured based on HRV indices (Keute et al., 2021a) or mood changes (Ferstl et al., 2021).

One outstanding and non-trivial question remains, namely the systematic testing of the location for real vs. ***sham stimulation***. Electrical stimulation above the sensory threshold induces an easily recognizable somatosensory percept that could explain potential stimulation effects. Thus, a proper sham stimulation is necessary to assure that observed effects are based on LC stimulation and not merely on the somatosensory perception of the stimulation (Keute et al., 2018). Most study designs are based on the results of Peuker and Filler (2002) and even if Yakunina et al. (2017) already tested various locations for real stimulation using a taVNS-fMRI approach, sham stimulation locations in humans are not systematically tested yet. Typically, sham stimulation is applied to the left ear lobe (Burger et al., 2020; Butt et al., 2020) since it is considered to be relatively free of ABVN fibres (Peuker and Filler, 2002). However, this location has been challenged as an appropriate target for sham stimulation because of the inhomogeneous density of sympathetic nerves in the human ear (Borges et al., 2021; Cakmak, 2019; Rangon, 2018). Cakmak et al. (2018) recommend upper parts of the ear instead of the earlobe for sham stimulation, since they observed that perivascular, sympathetic neurotransmitters are denser in the upper rather than lower auricular areas adjacent to the cymba concha for real stimulation. A previous study with patients suffering from PD ($N = 14$) showed that stimulation of the anti-tragicus muscle zone located at the top of the ear lobe led to improved motor functions (Cakmak et al., 2017). Another proposed control method is to use real taVNS sites but without stimulation (Garcia et al., 2017) or with a drastically reduced stimulation frequency (e.g., 1 Hz) (Bauer et al., 2016). However, these approaches are rarely ever used and still need to be validated. Especially stimulation with 1 Hz at intensities above the perceptual threshold is easily recognized as different from and thus no longer indistinguishable from 'real' taVNS (Colzato and Beste, 2020). These results show that more research is needed to delineate proper targets for active sham stimulation. Stimulation protocols that differ in (subjective) intensity or stimulation patterns between real and sham control have to further consider placebo or expectancy-related confounds when comparing real and sham stimulation (Farmer et al., 2020). As of now, there is no sham stimulation that fulfils the criteria proposed by Butt et al. (2020), i.e., no innervation of ABNV fibres while being indistinguishable from taVNS.

4. Potential of phasic stimulation to illuminate the link between taVNS and LC-NE activation

LC neurons are thought to generally display two distinct firing modes with different discharge patterns and NE releasing properties (Aston-Jones and Cohen, 2005; Berridge and Waterhouse, 2003; Florin-Lechner et al., 1996): (i) a *tonic activity mode* (long-lasting, constant activity with 0.5–5 Hz) and (ii) a *phasic activity mode* (short bursts of activity with 10–20 Hz) (Aston-Jones and Bloom, 1981; Aston-Jones and Cohen, 2005; Clayton et al., 2004). A study in rats suggests that higher levels of NE release can be achieved by *phasic stimulation* compared to tonic stimulation (Florin-Lechner et al., 1996). Moreover, animal studies show that *phasic bursts* of NE release (through experimental interventions like electric foot shocks) support memory encoding by fostering LTPs (long term potentiation) in hippocampal projection areas (Luo et al., 2015) and are able to support inhibitory control in prefrontal areas by increasing the signal to noise ratio (Aston-Jones and Cohen, 2005; Berridge and Waterhouse, 2003). Pupil dilation has emerged as an increasingly used indirect measure of *phasic LC activity* (see Section 1) in human and animal studies (Gilzenrat et al., 2010; Murphy et al., 2014). Both, animal and human research has already shown that an increased pupil dilation is associated with *phasic LC activation,* although there is no exclusive link between LC firing and pupil dilation (Aston-Jones and Cohen, 2005; Eckstein et al., 2017; Joshi et al., 2016; Murphy et al., 2014; Samuels and Szabadi, 2008a).

Animal research has explored the influence of different stimulation parameters on the LC-NE system more systematically, in particular the effects of phasic stimulation. For instance a recent iVNS study in monkeys showed that phasic bursts of more than 30–50 Hz lead to stronger vagus evoked potentials compared with low frequency bursts of 5 Hz (Rembado et al., 2021). Hulsey et al. (2017) verified that short bursts of 0.5 s of iVNS drives phasic LC activity even at 0.2 mA and that increased VNS amplitude leads to increased LC firing. This relationship is consistent with recent findings by Mridha et al. (2021) who adjusted various stimulation parameters (amplitude, frequency, pulse width) in a study in mice and observed the strongest effects of VNS on pupil dilation, at 0.9 mA, 20 Hz and 800 μs with short bursts of 10 s (Mridha et al., 2021). Collins et al. (2021) confirmed a dose dependent effect of VNS on the LC-NE system, whereby a higher stimulation intensity and longer

stimulation duration (0.8 mA and 5 s instead of 0.5 s of short bursts) induced larger pupil dilation. Furthermore, Mridha et al. (2021) found that VNS stimulation intensity was correlated with the extent of cholinergic axon activation. This specific timing response of cortical activation due to VNS was also addressed by Collins et al. (2021), showing that after VNS onset, both NE and ACh cortical activation was observed, followed by whisking and locomotion approx. 1 s thereafter as well as pupil dilation about 1.5 s afterwards in awake as well as anesthetized rats. Additionally, Hulsey et al. (2019) also showed an involvement of the motor cortex during iVNS stimulation (0.8 mA, short bursts of 0.5 s). Effects of phasic iVNS on stimulus-specific plasticity were also observed in rat auditory cortex (see Section 5), where previously induced tinnitus pathology could be eliminated with a short burst of 0.5 s of iVNS at 0.8 mA (N. D. Engineer et al., 2011).

In *human research*, Sharon et al. (2021) were able to show a robust pupil dilation based on short bursts of 3.4 s taVNS (2.20 ± 0.24 mA). Similar short bursts of 4 s taVNS (2 mA) (Keute et al., 2021a) or 1 s taVNS (Sclocco et al., 2019) in humans, resulted in changes in HR and HRV indices (Keute et al., 2021a) as well as changed HRV indices during the exhalation phase of the respiratory cycle (eRAVANS) (Sclocco et al., 2019). Moreover, the LC activation observed by Sclocco et al. (2020) already reported in Section 2, was also based on short bursts, in this case 1.5 s taVNS. However, it should be noted that Keute et al. (2021a, 2021b) and Sclocco et al. (2020, 2019) did not choose an active sham control stimulation location (no current at all) in comparison to Sharon et al. (2021) (see Section 2 for sham-controlled designs). In summary, phasic stimulation approaches might be more useful than tonic approaches when investigating the direct effects of different stimulation parameters and can prove a useful tool for understanding how i/taVNS affects the LC-NE system. Studies with longer stimulation bursts found no immediate effects of taVNS, neither with respect to pupil dilation (e.g., 60 s of taVNS (Keute et al., 2019a, 2019b)) or HRV indices (e.g., 30 s of taVNS (Borges et al., 2019; De Couck et al., 2017), see (Burger et al., 2020) for review). Moreover, as the majority of findings reporting optimal stimulation parameters were from animal studies focusing on phasic or burst-like stimulations, phasic stimulation approaches may be more preferable for determining whether these optimal stimulation parameters translate to comparable taVNS stimulation effects in humans.

5. Potential factors influencing stimulation effects between individuals

Apart from open questions in the stimulation protocols and outcome measures, interindividual differences in ABVN properties and the status of the LC-NE system itself may influence taVNS effects (cf. Fig. 1 – 'Relevance of altered brain physiology'). This is mainly relevant when studying clinical subpopulations which are often the target for taVNS interventions. Regarding the *ABVN*, only one study so far, by Safi et al. (2016), counted the amount of myelinated nerve fibres in the ABVN and observed considerable variability between subjects (for review see (Yap et al., 2020)). It should be noted here, that the subjects had different histories of medical conditions, so healthy populations might show lower variability (Safi et al., 2016). Moreover, the density of nerve fibres of the cavum conchae (recess auricle), which is part of the ABVN, varies as well (Bermejo et al., 2017). This variability might already play an important role in explaining why some individuals benefit from taVNS whilst others do not (Butt et al., 2020). Moreover, many of the conditions where taVNS can be usefully applied will involve a decline or alteration in LC-NE function. For instance in AD and PD, alterations in LC function may occur before clinical symptoms manifest (Braak et al., 2003, 2011). For AD, post-mortem studies have shown that, although the number of NE neurons is reduced, certain NE metabolites were not. This was taken as evidence for some compensatory upregulation in NE production in reaction to the loss of LC-NE neurons by which the remaining LC neurons increase their firing rate (Herrmann et al., 2004). Furthermore, there is evidence that, at least in early stages of LC decline, increased adrenoreceptor density in hippocampus and amygdala might compensate for reduced LC-NE signalling (Andrés-Benito et al., 2017; Szot et al., 2006). This means that adaptive mechanisms in the brain aimed at compensating altered LC function may influence the effects of externally applied stimulation by, e.g., increasing the response of individual LC neurons or the sensitivity of target areas through increased receptor levels. Similarly, a post-mortem study examining the expression of signalling genes and growth factors revealed a decline in *LC function* in individuals suffering from depression (Bernard et al., 2011), which might underlie the use of sNRIs in the treatment of depression (Moret and Briley, 2011). A meta-analysis has shown that the effects of depression treatment, one of the main areas of i/taVNS application, were only apparent after controlling for depression severity, which revealed stronger effects in more severely affected individuals (Martin and Martín-Sánchez, 2012). Correspondingly, Ferstl et al. (2021) were able to show that lower baseline levels of positive mood in healthy subjects were associated with greater taVNS (30 s on/off stimulation cycle) induced improvements in motivation. At present, it is unknown whether clinical and cognitive assessments of disease severity are associated with greater LC-NE system decline. Nonetheless, existing studies suggest variability in taVNS effects are also observed in cognitively normal populations as well. Stimulation studies should thus focus more on taking into account interindividual differences in the integrity of the stimulated LC-NE system when interpreting taVNS effects to reduce unreliable and heterogeneous results. Reduced *LC integrity* has been observed in several clinical populations such as PD and AD as well as major depression (see (Liu et al., 2017) for an extensive review). Using this approach, interindividual differences in LC integrity can however also be observed in older healthy adults (Betts et al., 2017; Hämmerer et al., 2018; Liu et al., 2019). Interindividual differences in LC integrity in humans can be determined in terms of signal intensity using neuromelanin-sensitive MRI (Betts et al., 2019), a technique developed in 2006 (Sasaki et al., 2006). A combination of ultra-high-field MRI and histological analyses on post-mortem brain tissues confirmed that the localization of the neuromelanin contrast in MRI corresponds to NE-neurons in the LC (Keren et al., 2015). Related to this, advances in our understanding of the relevance of an altered functionality of the LC-NE system can motivate different interventional avenues with different types of stimulation approaches, which have been as of yet insufficiently explored. Specifically, high-frequency stimulation might carry potential for inhibiting overcompensated (overactive) LC neurons which are thought to contribute to chronic pain (Bernard et al., 2011) and aggressive behavior in conditions of declining LC-NE integrity possibly related to excessive LC activity (Liu et al., 2018). However, the interactions among brain areas when investigating different stimulation protocols will also have to be considered. For instance, high-frequency (100 Hz) optogenetic burst stimulation of basolateral amygdala neurons was recently reported to drive excitatory neurons in the medial prefrontal cortex into a blocked state with reduced activity (Klavir et al., 2017). In another rat model, it was shown that an overactivation of the LC - BLA pathway provoked pain and that blocking this pathway led to a reduction in pain-induced anxiety (Llorca-Torralba et al., 2019). Therefore, high-frequency stimulation might carry potential for inhibiting overcompensated (overactive) LC neurons which are thought to contribute to chronic pain. Similarly, aggressive behavior in conditions of declining LC-NE integrity is possibly related to excessive LC activity (Liu et al., 2018). Due to a long-standing lack of appropriate imaging measures for the LC-NE system and a still developing understanding of the role of the LC-NE system in higher cognitive functions (Sara and Bouret, 2012), current commercially available taVNS devices might not take full advantage of the therapeutic potential of taVNS interventions in humans.

6. Potential of translational cross-species approaches to illuminate the link between taVNS and LC-NE activation

Before a new therapy or treatment is applied to humans, they are usually tested in animal models. The potential of iVNS to treat epilepsy, for instance, was first demonstrated in canine models (Zabara, 1985, 1992) before it was investigated in the first human trials (Penry and Dean, 1990). Animal research is now helping us to further our knowledge about the functional mechanisms of iVNS and provides new hypotheses for potential applications of taVNS in humans (cf. Fig. 1 – 'Cross-species translational approach'). One example for this, among others, is the evolution from studies investigating NE-related plasticity in the primary auditory cortex (A1) of rats towards the development of potential, non-invasive tinnitus interventions in humans. Tinnitus, the perception of sounds without corresponding stimuli, is thought to be based largely on maladaptive A1 map reorganizations leading to an increased number of neurons responding to certain frequencies (Eggermont, 2015; Eggermont and Roberts, 2015; N. D. Engineer et al., 2011; Wu et al., 2016). In recent years, i/taVNS approaches were investigated as adjunctive treatment options for tinnitus due to its potentially neuromodulating effect (Stegeman et al., 2021). Early work investigating NE effects on auditory cortical plasticity used ionophoretic infusion of NE directly into rat auditory cortex paired with tone stimuli and observed frequency specific modulation in neuronal tuning curves (Manunta and Edeline, 2004). In a subsequent study, also in rats, direct, phasic stimulation of the LC paired with pure tones altered response characteristics of A1 neurons, corroborating the role of the LC-NE system for neural plasticity. Of note, frequency-specific increases in spike rates were observed already after 100 pairings, persisting up to 15 min after stimulation (Edeline et al., 2011). These observations were then used to generate hypotheses that iVNS in rats could yield similar results. Indeed, pairing pure tones with iVNS (performed 300 times a day for 20 days) increased the number of recording sites that preferably responded to the paired frequency compared with an unpaired control group (N. D. Engineer et al., 2011). Similar results were observed when speech sounds were used as stimuli (C. T. Engineer et al., 2015), which is in line with increased temporal flexibility of A1 neurons due to VNS-tone pairing (Shetake et al., 2012). These results generated new ideas to use iVNS and potentially taVNS to modulate cortical plasticity in a targeted manner in therapeutic settings to treat tinnitus. N. D. Engineer et al. (2011) used iVNS to reverse tinnitus in a rat model. Stimulation was applied as phasic bursts of 0.5 s, beginning 150 ms before tone onset and these pairings were repeated 300 times a day for 18 days. In rats receiving iVNS-tone pairings, behavioral correlates of tinnitus were eliminated after the therapy, whereas animals from the three control groups (iVNS without tones, tones without iVNS or no therapy) showed consistent impairments. Three weeks after the therapy, neural recordings from A1 revealed that pathological changes in the treated group but not in the control groups returned to normal levels (N. D. Engineer et al., 2011). Following these results, pilot studies in humans with implanted VNS electrodes emerged, using iVNS-tone pairing paradigms to treat tinnitus (De Ridder et al., 2014 ($N = 10$); Tyler et al., 2017 ($N = 30$); Vanneste et al., 2017 ($N = 18$)). Random tones were presented together with 0.5 s phasic (Tyler et al., 2017; Vanneste et al., 2017) or 30 s tonic (De Ridder et al., 2014) iVNS in order to reduce pathological, neuroplastic changes in auditory cortex regions. Subjects reported a reduction in subjective tinnitus symptoms (Tyler et al., 2017; Vanneste et al., 2017). Likewise, electrophysiological recordings performed before and after the therapy revealed that iVNS-tone pairing reduced gamma band activity (30–44 Hz) in left auditory cortex and phase coherence between auditory cortex and other brain areas associated with the tinnitus perception including the cingulate cortex (Vanneste et al., 2017). Hypersynchronous activity in the gamma band of the auditory cortex is an electrophysiological marker of tinnitus (Langguth et al., 2013; Weisz et al., 2007) while the cingulate cortex is associated with its affective components (e.g., distress) (Vanneste et al., 2010). In parallel, researchers aimed to establish taVNS as a non-invasive procedure to circumvent the invasiveness and high costs of iVNS. Lehtimäki et al. (2013) used a tailored sound therapy (ST) combined with continuously applied taVNS (25 Hz, 45–60 min) at the left tragus. Additional subjects ($N = 8$) were presented with pure tones centred at their tinnitus frequency while their brain activity was recorded via magnetencephalography (MEG) either during taVNS or no stimulation. The ST group not only showed decreases in subjective tinnitus symptoms after ST paired with taVNS but also a mood improvement while the MEG group showed a reduced amplitude of the N1m during taVNS (Lehtimäki et al., 2013). The N1m, the magnetic equivalent of the N1 ERP, reflects early auditory processing in A1 (Näätänen and Picton, 1987) and an increased N1m amplitude has been observed in many tinnitus patients, indicating hyperactivity in the A1 (Lehtimäki et al., 2013). A major drawback of this study, however, is that both results could have been obtained based on ST alone (e.g., Pantev et al., 2012). Hyvärinen et al. (2015) recorded brain activity via MEG while presenting tinnitus patients ($N = 7$) with tones matched to their individual tinnitus frequency either during continuous taVNS (25 Hz) on the left tragus or no stimulation. Additional control subjects ($N = 8$) without tinnitus were presented with 1 kHz tones and sham-stimulation at the left earlobe. They showed that taVNS in tinnitus patients' modulated tone evoked synchronicity in the beta- and gamma-band (Hyvärinen et al., 2015). Hypersynchronous activity in the auditory beta range has also been observed in patients suffering from tinnitus and auditory hallucinations (Vanneste et al., 2013).Yet, it is imperative to notify that these results have to be interpreted with caution as highlighted in detail by two recent reviews (Stegeman et al., 2021; Yakunina and Nam, 2021). Both, studies that used taVNS alone as well as studies that used taVNS in combination with ST, have severe methodological flaws. They lack sufficient sample sizes and appropriate blinding, are not designed as randomized controlled trials and results and methods are often reported with low quality (Stegeman et al., 2021). Furthermore, they rely on the assumption that cortical reorganization in the tonotopic map of A1 is a major cause for tinnitus, which is highly debated (Yakunina and Nam, 2021). With these caveats in mind, no clear statement for the effectiveness of taVNS in the treatment of tinnitus and more research using randomized controlled trials is needed (Stegeman et al., 2021; Yakunina and Nam, 2021). Nevertheless, animal research is still valuable to further delineate the exact cortical and neuronal causes of tinnitus and potential ways to reverse these. If properly conducted (i.e., sham controlled, blinded, sufficiently powered), human studies can then use non-invasive electrophysiological markers to improve the usefulness of taVNS in the remedy of tinnitus.

Apart from the auditory system, Hulsey et al. (2019) showed an involvement of the motor cortex during iVNS indicating stimulus-specific cortical plasticity of iVNS. Specifically, iVNS in rats paired with proximal forelimb movements increased the cortical representation of these movements (Hulsey et al., 2019). Similar, iVNS in rats paired with rehabilitative training after spinal cord injury improved forelimb strength compared to rehabilitative training without iVNS (Darrow et al., 2020). In line with the cortical plasticity potential of iVNS, Capone et al. (2017) were able to demonstrate that taVNS at the inner side of the tragus (2.0–4.5 mean mA, 20 Hz, 300 μs, 30 s every 5th min for 60 min) combined with robotic rehabilitation can improve arm functionality in patients with ischemic ($N = 5$) or haemorrhagic ($N = 2$) chronic stroke. However, this study also has a weakness in power, as only 7 subjects received real stimulation and 5 subjects (ischemic: $N = 3$) sham stimulation. As mentioned above, there is currently an increasing interest in understanding how taVNS might affect the gut-brain axis. Animal as well as human studies indicate an i/ta VNS induced reduction of food intake accompanied by weight loss and reduction of gastric frequency via vagal afferents (for review see (Farmer et al., 2020)). Additionally, Gil et al. (2009) were able to show not only that long-term iVNS with a low stimulation frequency (0.05 Hz), but also by applying a higher stimulation frequency (10 Hz, 10 ms, 200 mV, 12 h per day for 42 days)

(Gil et al., 2011) can lead to reduced food intake and body weight in rats on a high-fat diet. Similarly, at 1 Hz, stimulation of the afferent fibres was shown to reduce food intake in rats by influencing the response to stomach peristalsis within 100 days (Yao et al., 2018). Gil et al. (2011) further observed neuronal responses in NTS, decreased levels of leptin and increased levels of ghrelin after iVNS in rats on a high-fat diet. Both hormones are important because leptin contributes to inhibiting food intake and ghrelin to stimulating appetite (see (Klok et al., 2007) for review). An imbalance of this hormone release can promote obesity (Cryan et al., 2019), which is associated with health problems not only in animals but also in humans. Teckentrup et al. (2020) investigated the potential role of taVNS on gastric frequency in healthy adults ($N = 21$). Specifically, it was shown that afferent stimulation (25 Hz, 30 s on/off stimulation cycle) had an effect on metabolic efferents and resulted in reduced myoelectric frequency, but did not affect resting energy expenditure (Teckentrup et al., 2020). This effect might be driven by dopamine release in the brainstem (Teckentrup et al., 2020) which highlights once again that the VN is involved in the regulation of the activity of a variety of brain structures and internal organs. Yet, the extent to which taVNS can really contribute positively as an additional treatment option for obesity still needs to be investigated in more detail. In the future, translational approaches could try to establish iVNS and taVNS in the same animal models using outcome measures that have been shown to be indicative of LC-NE activity in both animals and humans. Different parameter combinations could then be systematically investigated in their respective effectiveness and compared between iVNS and taVNS. If based on this, an effect of similar magnitude can be shown in animals, one could show (i) to what extent the parameters for iVNS differ from taVNS in the animal itself and (ii) in comparison to common taVNS parameters used in humans. Thus, it would then also be possible to adapt the stimulation parameters used in animal research more specifically for taVNS in humans.

7. Summary and conclusion

A dysregulation of the LC-NE system characterizes a wide range of clinical and neurological conditions, including depression, chronic pain, post-traumatic stress disorder, neurodegenerative diseases, as well as cognitive decline in aging (Betts et al., 2019; Hämmerer et al., 2018; Liu et al., 2017). Compared to pharmacological therapies, taVNS has the potential for a more anatomically and functionally targeted intervention which can provide a valuable tool if properly validated. Here we reviewed the current challenges in evaluating the effectiveness of taVNS in reaching the LC-NE system in humans and outline experimental approaches that may help to overcome them. Challenges in assessing the effects of taVNS on the LC-NE system in humans (cf. Fig. 1) include most importantly difficulties in (i) identifying adequate biomarkers that index taVNS efficacy on the level of an engagement of the LC-NE system as well as (ii) identifying optimal stimulation protocols.

We outline how both of these shortcomings can be overcome by moving towards phasic i/taVNS protocols as well as investigating i/taVNS effects in cross-species translational approaches. In comparison to tonic stimulation interventions, phasic stimulations have the advantage of allowing for an immediate and repeated assessment of stimulation effects on the LC-NE system. Moreover, the ability to elicit LC firing and NE release by phasic or burst-like interventions has been well validated in animal studies that investigate NE and LC function using event-related interventions such as foot shocks or direct stimulation interventions to the LC (Chen and Sara, 2007). Indeed, phasic i/taVNS has been shown to modulate LC-NE activity in animal studies (Collins et al., 2021; Hulsey et al., 2017, 2019; Mridha et al., 2021) as well as human studies (Keute et al., 2019a; Sclocco et al., 2019, 2020; Sharon et al., 2021). In contrast, human taVNS studies using tonic stimulation (e.g. Borges et al., 2019; De Couck et al., 2017; Keute et al., 2019a, 2019b) have not always yielded reliable effects on the LC-NE system.

Secondly, cross-species translational research is of great importance to increase our understanding of how taVNS in humans affects the LC-NE system. Considerable knowledge regarding optimal stimulation parameters (e.g., Hulsey et al., 2017) or outcome measures (e.g., Collins et al., 2021; Mridha et al., 2021) has already been gained from iVNS in rodents. Such results can form the starting points for testing similar effects using taVNS in humans. TaVNS has emerged as a potential treatment option for tinnitus via modulating cortical plasticity in A1. However, results from human studies are few and inconclusive at best due to methodological shortcomings. Additional studies that are better controlled (e.g., sham controlled, randomized, balanced and properly blinded designs) with sufficient power are required to delineate how VNS findings in rodents can be translated to further understand how taVNS can be used as a reliable intervention for human tinnitus. Similarly, first approaches that show how i/taVNS can influence the rehabilitation of motor areas in the brain or regulate gastric frequency as well as weight loss and food intake await a more thorough validation in humans. In particular, fMRI offers great potential as an outcome measure as it provides the advantage that taVNS induced changes can be observed in the LC more directly with high spatial acuity compared to more peripheral outcome measures of LC activity such as pupillometry or HRV. Additional electrophysiological recordings with high temporal resolution such as EEG could then provide information about the timing of these effects. Not only the different stimulation sites (left vs. right ear) and locations (real vs. sham stimulation), but also stimulation parameters and their influence on the LC-NE system could be addressed using taVNS-fMRI. In addition, taVNS-fMRI might also help identify how the interaction of different stimulation parameters influences LC activation. At present, we do not know how the different stimulation parameters have to be combined in order to optimise taVNS effects on the LC-NE system.

Future stimulation devices used for basic research, individual at-home treatments as well as to study different clinical conditions should thus have the potential to let practitioners manipulate all stimulation parameters such as intensity, frequency, pulse width and duty cycle to adjust to their individual needs. Although taVNS can be assumed to stimulate VN and LC via the ABVN, the involvement of sensory pathways cannot be completely excluded due to for instance somatosensory reactions to the sensation of being stimulated. Studies assessing the painfulness or discomfort of taVNS applications should consider these as covariates when assessing interindividual differences. It remains to be systematically investigated to what extent an engagement of the LC via sensory stimulation effects, complicates the assessment of differences between real and sham stimulation.

A further reason for the heterogeneous results of taVNS interventions in humans, might be interindividual variability in the LC-NE system and the ABVN. The integrity of the LC is especially important when taVNS is considered as adjunctive treatment in clinical populations that may be affected by reduced NE modulation such as depression or neurodegenerative diseases. However, some evidence points towards an adaptation in the LC-NE system in response to a reduced NE supply in form of an upregulated NE release of the remaining LC neurons or an increase in NE receptors in target areas (Andrés-Benito et al., 2017; Herrmann et al., 2004; Szot et al., 2006). Such changes in the impact of NE release would then have to be taken into account when externally modulating NE release via taVNS. It is therefore important to add measures that allow to characterize interindividual differences in the LC-NE system in particular in the evaluation of taVNS in clinical populations. Measures such as neuromelanin-sensitive MRI sequences which help to assess the role of LC integrity can for instance prove relevance in this regard. Neuromelanin-sensitive MRI has already provided insight about the interindividual variability in LC integrity in healthy, older adults (Betts et al., 2017; Hämmerer et al., 2017; Liu et al., 2017). Additional measures that inform about interindividual differences in LC-NE function or responsiveness might then ultimately also inform the choice of individualized stimulation parameters. Stimulation parameters derived from studies with healthy subjects may prove less effective when applied

to patients with pathological changes in the LC-NE system. Establishing a tailored stimulation intervention, which takes into account interindividual differences in the reactivity of an altered LC-NE system as well as establishing suitable physiological and cognitive outcome measures for evaluating its success are therefore crucial to use the full potential taVNS offers as a valuable therapeutic approach in any of the above mentioned conditions characterized by a dysregulation of the LC-NE system.

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