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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

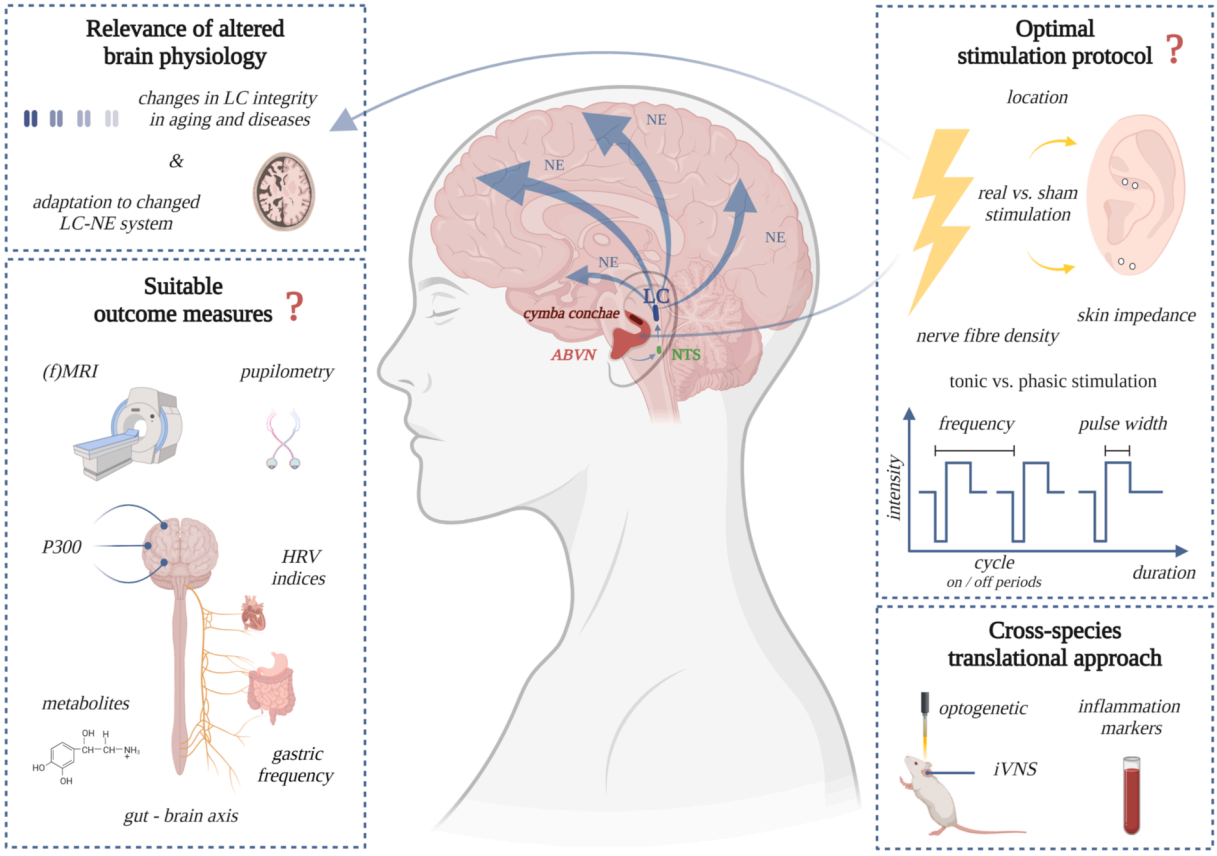

Fig. 1. Challenges in scrutinizing the link between taVNS and the LC-NE System.
Center: TaVNS is applied to regions in the left ear innervated by the ABVN. The stimulation is relayed via the ABVN to the NTS which projects to the LC from where NE is released into various projection areas. Top right: Currently, 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

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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

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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		

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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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