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TITLE

Transcutaneous Vagus Nerve Stimulation (tVNS) in Stroke: The Evidence, Challenges and Future Directions

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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 [1]. A significant proportion of chronic stroke survivors are left with long term disability despite physiotherapy and rehabilitation [2]. These neurological deficits include weakness, sensory impairment, loss of coordination, spasticity, dysphasia, dysphagia, visual field dysfunction and cognitive impairment [3]. The wider socioeconomic impact of stroke includes both direct costs such as healthcare expenditure and indirect costs including lost economic productivity and carer burden [4]. 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 [5][6]. 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 [7]. 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 [8] and/or mechanical thrombectomy [9] 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 [10]. It is safe and well tolerated in research studies to date [11] and has been shown to activate similar vagal nerve projections and vagus nerve mediated pathways as invasive VNS [12][13]. 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 second trains of taVNS (pulse width 0.5ms, pulse frequency 20 Hz, pulse amplitude 0.5 mA) at the left cavum concha were delivered at 5 minute intervals for one hour starting 30 minutes after unilateral transient middle cerebral artery occlusion in adult male Wistar rats [14]. They found that taVNS in acute MCAO was associated with a 28% reduction in infarct volume and improvement in neurological outcome at 24 hours[14]. Interestingly, the neurological outcome did not improve at 3 hours 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 [15]. Whilst magnitude of reduction in infarct size was lower than that seen in invasive VNS [16], 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 [17], 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 [17,18] , here we detail neurobiological effects of tVNS in animal models of stroke. These include reduced systemic inflammation [19], increased M2 microglial polarisation [20], reduced spreading depolarisation [21], reduced blood-brain barrier breakdown [22], increased angiogenesis [23], and improved axon regeneration and reorganisation [24] (Figure 1).

2.2 Mechanisms

2.2.1. 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 [25][26]. 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 [27]. Further, the afferent vagus nerve transmission can increase cholinergic activity in the basal forebrain which could increase α7nAChR activation centrally [28].

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 [29]. Secondly, α7nAChR agonists have been shown to reduce cerebral oedema in a mouse model of intracerebral haemorrhage [30] . 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 [31]. In keeping with this, Li *et al* (2020) 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 [32].

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 [33] . 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 [34]. 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 [34]. Agonism of the α7nAChR has been associated with increased M2 polarisation suggesting a potential mechanism through which tVNS may promote anti-inflammatory effects [35] . 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 hours post MCAO [36]. Zhao *et al* (2020) further demonstrated that tcVNS in acute MCAO was associated with M2 polarisation, improved neurological outcomes and reduced infarct size [20]. 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) [19] and peroxisome proliferator- activated receptor gamma (PPAR-γ) [23] which are both associated with the M2 microglial phenotype [37].

2.2.2. 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 [38]. This occurs through several mechanisms including the activation of matrix-metalloproteinases (MMP) by pro-inflammatory cytokines such as TNF-α [38]. 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 hours [22]. IL-17A has also been demonstrated to increased BBB breakdown [39] therefore, the aforementioned inhibition of IL-17A production via tcVNS [20] may be an alternative mechanism through which tVNS reduces infarct size.

2.2.3. Excitotoxicity

Glutamate-mediated excitotoxicity is a sequelae of acute stroke and leads to secondary, post-ischaemic neuronal injury [40]. The effect of VNS on excitotoxicity in stroke is not well characterised. Invasive VNS has been shown to reduce ischaemia-induced glutamate release and increase hippocampal neuronal survival in a gerbil model of ischaemia [41]. Spreading depolarisations (SDs) occurs spontaneously after a stroke and are associated with dysregulated blood flow and altered metabolism that leads to infarct propagation [42]. Elevated glutamate is a proposed trigger for SDs [42]. Lindemann *et al* (2020) found that invasive cervical VNS and tcVNS reduced cortical SD frequency in the peri-infarct region [21]. 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.

2.2.4. 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 [14,36]. In contrast with this, longer durations of tVNS have been associated with increases in PPAR-γ [23], BDNF [32] and growth differentiation factor 11 (GDF-11) [43] – promoters of angiogenesis and neurogenesis.

PPAR-γ is a multifunctional nuclear transcription factor, expressed in neurons, endothelial cells and microglia, which has numerous neuroprotective and anti-inflammatory actions that make it a promising potential target in cerebral ischaemia [44]. 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 [23]. 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 [45].

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 [46]. 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 [23]. BDNF is a growth factor that has been shown to promote neurogenesis and regulate and maintain synaptic plasticity [47]. In keeping with this, sustained taVNS (up to 28 days following acute MCAO) has been shown to improve axon regeneration and re-organisation [32]. Similarly, taVNS increases GDF11 expression in the peri-infarct cortex [43]. 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 [48]. Therefore, it is feasible that taVNS may stimulate neurogenesis via a GDF11-dependent mechanism.

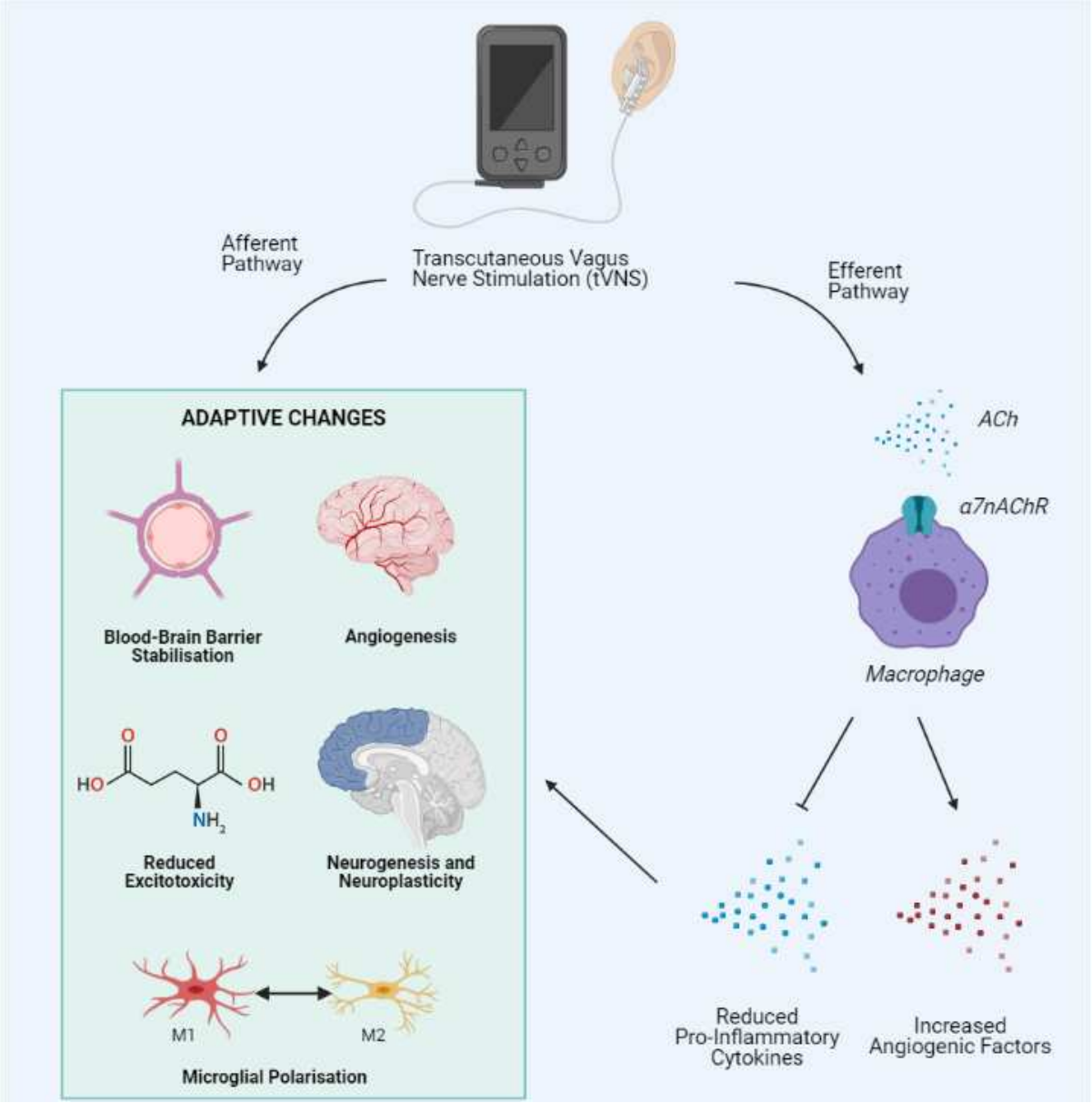

Figure 1. Effects of tVNS in Animal Models of Stroke

Author (Year)	Animal	tVNS Stimulation Parameters							Main Findings
		Site	Timing	Pulse Width	Frequency	Intensity	Interval	Duration	
Ay *et al* (2015) [14]	Adult Male Wistar Rats	Left cavum concha	30 mins post MCAO	0.5 ms	20 Hz	0.5 mA	30 sec trains every 5 mins	1 hour	No change in regional cerebral blood flow Significant reduction in infarct volume Significant reduction in Bederson scale at 24 hours (but not 3 hours) Bilateral c-Fos staining in the NTS and LC
Jiang *et al* (2016) [19]	Adult male SD Rats	Left cavum concha	30 mins post right MCAO	0.5ms	20 Hz	0.5 mA	30 sec trains every 5 mins	1 hour, 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 hours 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) [43]	Adult Male SD Rats	Left cavum concha	30 mins post MCAO	0.5 ms	20 Hz	0.5 mA	30 sec trains every 5 mins	1 hour, twice daily for	Transient decrease in blood pressure and heart rate during stimulation (not sustained).

								up to 7 days	No overall effect on regional cerebral blood flow. taVNS associated with higher mNSS score at 24 hours 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) [36]	Adult male spontaneously hypertensive rats	Right cervical vagus nerve	30 mins post right MCAO or 4 hours post right MCAO	1 ms	25 Hz	12 volts 350 ohm	2 min trains every 10 mins	1 hour	Reduced infarct volume, higher neurological scores and forelimb grip strength at Day 7 when treated at 30 mins post MCAO. Smaller infarct volume and improved neurological score if treated 4 hours post MCAO. Increased c-Fos positive cells in NTS. More HMGB1-positive cells at 24 hours. Fewer Iba-1-positive, TNF-α and CD68-positive cells at 24 hours.
Yang *et al* (2018) [22]	Adult male spontaneously hypertensive rats	Left cervical vagus nerve	30 mins post right MCAO	1 ms	25 Hz	15 volts	2 min trains every 10 mins	50 mins	Reduced infarct size on MRI Reduced BBB leakage in infarcted area at 24 hours 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) [20]	Male C57BL/6 mice	Right cervical vagus nerve	1 day pre right MCAO	1 ms	25 Hz	15 volts	2 min trains every 10 mins	1 hour	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* (2020) [23]	Adult male SD rats	Left cavum concha	30 mins post right MCAO	0.5 ms	20 Hz	0.5 mA	30 sec trains every 5 mins	1 hour, twice daily up to 28 days	taVNS increases PPAR-γ expression in the peri-infarct cortex at day 14 and 28. 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* (2020) [32]	Adult male SD rats	Left cavum concha	30 mins post right MCAO	0.5 ms	20 Hz	0.5 mA	30 sec trains every 5 mins	1 hour, 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) [21]	Adult Male Wistar Rats	Left cervical vagus nerve	30 mins post transient/ permanent left MCAO	1 ms	25 Hz	12 V	2 min train repeated once after 15 mins	15 mins	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.

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

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

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) [5] 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 hour 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 same group found that similar in-clinic therapy followed by self-delivered VNS paired with rehabilitation at home was feasible and effective [6][49]. 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 [50]. 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=0·0014). 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=0·0098) [50].. 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 [51]. 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 [52].

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 mins prior to robotic rehabilitation for 10 days in patients with chronic ischaemic or haemorrhagic stroke was safe and feasible [53]. 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 [51], 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 [5].

The effects of taVNS paired with concurrent upper limb rehabilitation were first demonstrated by Redgrave *et al* (2018) [54]. In that study, patients with ischaemic stroke more than 3 months prior (median time 1.16 years post-stroke) underwent three 60 minute 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 points 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 [55]. This is in keeping with a case report from Kilgard *et al* which showed that invasive VNS can improve sensory rehabilitation [56].

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 [57]. 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.

Authors	Type of Study	Population	N	Stimulation Parameters						Main Findings
				Site	Pulse Width	Frequency	Intensity	Interval	Duration	
Capone *et al* (2017) [53]	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 sec trains every 5 mins	60 mins 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* (2018) [54]	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) [55]*	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* [57] (2020)	RCT	Ischaemic stroke between 0.5 – 3 months prior	21	Left auricular vagus nerve	0.3 ms	20 Hz	Mean 1.66 mA	30 sec trains every 5 mins	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 *et al* (2020) [58]	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

Table 2. Clinical studies of tVNS in Stroke

*post-hoc analysis of Redgrave *et al* (2018)

Key
RCT – Randomised Controlled Trial
RTP – Repetitive Task Practice
UFM – Upper Limb Fugl – Meyer score
WMFT – Wolf Motor Function Test

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 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 [59]. One hundred and fifty patients with anterior circulation ischaemic stroke will be randomised to tcVNS delivered via Gammacore^{TM} 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 [22]. 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 25-35 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 multi-centre 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.

Study Name/ Registration Number	Location	Type of Study	Population	N	tVNS Parameters	Key Outcome Measures*	Estimated Completion Date
ACUTE STROKE							
NOVIS Trial NCT04050501 [59]	Netherlands	RCT	Acute anterior circulation ischaemic stroke (< 12 hours from onset)	150	tcVNS (gammaCore Sapphire™) 25 Hz, 0 – 24 Volts, 120 secs, every 15 mins for 3 hours 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 hours of onset or no ischaemia on FLAIR imaging)	60	tcVNS via gammaCore™ 7 x 2 min trains every 10 mins for 1 hour +/- repeated cycle after 3 hours	Safety Feasibility NIHSS at 24 hours Change in MRI infarct volume at 24 hours	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 0.3ms pulse width for 30 secs 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 x 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

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

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

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. Optimising 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 [60] 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 [61]. 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) [36], 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 [51]. Fifth, all the animal studies are performed in males; given there is evidence of sexual dimorphism in the mechanism of stroke-induced cell death [62] 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 [63]. 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 hours after ischaemia was still associated with a reduction in infarct size but that this protective effect was not present when delivered 5 hours post-infarct [36]. Given the presence of pre-hospital delays in stroke patients accessing urgent stroke services [61], 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 [59], 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 [64] .

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 [49]. 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) [36] found that the magnitude of reduction in infarct size was higher in their study of tcVNS compared to their study of taVNS [14], 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 [65] 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 [14], 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 [66]. 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 [67]. 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 [67]. 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 [67]. 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 [68]. 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 [69]. 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 [54]; 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 [68] and tVNS [70], an inverted U-shaped relationship between pulse frequency and cortical plasticity has also been reported [71]. 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 [49]. 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 [72]. There is limited evidence that any of these are associated with the degree or effectiveness of tVNS [72]. 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.

5.6. 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 [21]; 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 [73], 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 [74] 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.

5.7. 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 [55] and that invasive VNS paired with tactile training improved sensory dysfunction in a stroke survivor [56]. Given the fact that sensory dysfunction is common and a barrier to rehabilitation after stroke [75], 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 [76], depression [77] and migraine [78], 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) [79], the effect of tVNS on cardiac structure, electrophysiology and contractile function after stroke is a key future area of interest.

5.8. Practical Considerations

In acute stroke, there are several time sensitive processes that need to be coordinated amongst 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 [49], 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.

5.9 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 [80].

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 towards 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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CONFLICTS OF INTEREST

None

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