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Clinically-derived vagus nerve stimulation enhances cerebrospinal fluid penetrance

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ABSTRACT

Introduction: Vagus nerve stimulation (VNS) is an FDA-approved neuromodulatory treatment used in the clinic today for epilepsy, depression, and cluster headaches. Moreover, evidence in the literature has led to a growing list of possible clinical indications, with several small clinical trials applying VNS to treat conditions ranging from neurodegenerative diseases to arthritis, anxiety disorders, and obesity. Despite the growing list of therapeutic applications, the fundamental mechanisms by which VNS achieves its beneficial effects are poorly understood. In parallel, the glymphatic and meningeal lymphatic systems have recently been described as methods by which the brain maintains a healthy homeostasis and removes waste without a traditionally defined lymphatic system. In particular, the glymphatic system relates to the interchange of cerebrospinal fluid (CSF) and interstitial fluid (ISF) whose net effect is to wash through the brain parenchyma removing metabolic waste products and misfolded proteins.
Objective/Hypothesis: As VNS has well-documented effects on many of the pathways recently linked to the clearance systems of the brain, we hypothesized that VNS could increase CSF penetrance in the brain.
Methods: We injected a low molecular weight lysine-fixable fluorescent tracer (TxRed-3kD) into the CSF system of mice with a cervical vagus nerve cuff implant and measured the amount of CSF penetrance following an application of a clinically-derived VNS paradigm (30 Hz, 10% duty cycle).
Results: We found that the clinical VNS group showed a significant increase in CSF tracer penetrance as compared to the naïve control and sham groups.
Conclusion: (s): This study demonstrates that VNS therapeutic strategies already being applied in the clinic today may induce intended effects and/or unwanted side effects by altering CSF/ISF exchange in the brain. This may have broad ranging implications in the treatment of various CNS pathologies.

Introduction

Vagus nerve stimulation (VNS) is an FDA-approved treatment for refractory epilepsy, treatment resistant depression, cluster headaches, and migraine. Further, VNS has been proposed as a therapeutic option in an ever-expanding list of conditions including Alzheimer's Disease (AD), Bipolar Disorder, and obesity [1]. Despite this growing list of applications, the therapeutic mechanisms of VNS are not well understood, in part due to the vagus's highly complex and mixed nature. The vagus nerve carries a mix of efferent (~20%) and afferent (~80%) fibers providing parasympathetic innervation to, and providing sensory information from, the organs in the viscera [2,3]. Vagus sensory afferents mostly project to the nucleus of the solitary tract (NTS), and through NTS engage the major noradrenergic, cholinergic and serotonergic

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pathways of the brain [4,5]. Clinical investigations utilizing VNS indicate that it can exert significant cardiovascular and respiratory effects including bradycardia, dyspnea, and hypotension through both parasympathetic efferent and sensory afferent pathways [6–9].

In parallel, over the past several years the clearance systems of the brain have received increasing attention as a subject of research and potential target for therapeutic intervention in AD, Traumatic Brain Injury (TBI), and Epilepsy. Historically, the mechanisms by which the brain clears itself of extracellular waste and maintains homeostasis have remained poorly defined, as the brain does not contain the traditionally defined lymphatic vessels found in the periphery [10,11]. Recently, however, two novel systems for the clearance of misfolded proteins and cellular waste products from the brain, the glymphatic and meningeal lymphatic systems, have been described [12,13]. The glymphatic system, so-named due to its dependence on astroglial aquaporin-4(AQP4), describes the interchange of cerebrospinal fluid (CSF) and interstitial fluid (ISF) to clear the interstitial space [12]. These clearance pathways fit within a larger CNS clearance system that involves the production of CSF, its movement through the ventricles, subarachnoid space, and into the brain parenchyma wherein the CSF mixes with ISF collecting solutes from the extracellular milieu and finally draining the CSF/ISF/solute mixture through various routes (for a more thorough review see Ref. [11] and Supplemental Figs. 1 and 2). This collective system is believed to have particular relevance in the area of neurodegenerative diseases where the build-up of misfolded proteins is a significant contributor to disease progression [12,14]. In addition, this system becomes compromised in pathological states following TBI and AD progression [15,16]. Thus, it's been suggested that improvement or rescue of brain clearance function may be of potential therapeutic benefit.

While both the therapeutic mechanisms of VNS and the many factors affecting and regulating brain waste clearance are still being actively investigated, several of those described to date suggest that VNS may potentially be a neuromodulatory tool for influencing brain waste clearance. For example, the movement of CSF is linked to the cardiac cycle and the influx of CSF in the glymphatic model is hypothesized to be driven by cerebral arterial pulsatility or the movement of the arterial vessel wall over time [17,18]. Respiratory driven pulsations have also been linked to CSF pulsations and ultimately clearance [19]. And, the glymphatic system is sensitive to changes in noradrenergic signaling associated with sleep and arousal states [20]. As the literature is evolving rapidly the points of intersection between VNS and brain waste clearance is also constantly growing. Beyond cardiopulmonary effects these interactions potentially include VNS-induced intrinsic brain oscillations, modulation of perineural clearance efflux routes, effects on the blood-brain barrier, as well as direct and indirect effects on glial cell activity [21–26].

Given the growing clinical adoption of VNS, the impact of VNS on the clearance systems of the brain may be an important mediator of intended effects or unintended side-effects in existing patient populations. Here we measured brain-wide changes in the penetrance of a dye injected into the CSF in response to cervical VNS using clinically derived stimulation parameters to assess the potential of clinical VNS to modulate the clearance systems of the brain.

Methods

Animal use

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin-Madison. All animals were 8–14 week-old C57/BL6J male mice housed under 12 h light/dark cycles with ad libitum access to food and water. This study comprised of three animal groups including a naïve control (n = 12), sham VNS (n = 8), and clinically derived VNS (n = 8). Control mice underwent the same cisterna magna (CM) dye injection procedure and dye injection timeline but did not have a vagus nerve cuff implant. Sham VNS mice received the vagus nerve cuff implant, but the leads were left un-connected and no stimulation was applied.

Cuff electrode implantation procedure

Mice in the two VNS and sham groups were surgically implanted with a vagal cuff electrode prior to CM injection. Animals were induced at 5% isoflurane in O_2 and maintained at 1.75–2.5% isoflurane throughout the surgery. The clavicles, suprasternal fossa, and mid-line of the neck were marked prior to incision. Following mid-line incision, the skin, fatty tissue, and two submandibular glands were separated to reveal the sternohyoideus and sternocleidomastoid muscles. The sternocleidomastoid muscles were then separated from the sternohyoideus to expose the carotid sheath containing the vagus. While the internal jugular and carotid arteries were carefully separated, to reflect clinical practice no attempt was made to identify and separate the aortic depressor nerve (ADN). Bipolar cuff electrodes were made in-house using 500 μm inner-diameter silicone tubing (AM-Systems) and 38ga PTFE-coated platinum iridium wires (AM-Systems) with ≈1 mm interelectrode spacing. To maintain contact with the nerve during subsequent manipulations the ends of the cuff were secured with 9–0 suture (Ethicon). Following cuff implantation, the surgical area was closed with wires exiting through the skin.

Electrical stimulation parameters

Stimulation was provided by an RZ2 16-channel stimulator (Tucker-Davis Technologies). Stimulation parameters for the clinically derived group consisted of 200 μS cathodic-leading biphasic pulses with an amplitude of 800 μA delivered at 30 Hz and applied at a 10% duty cycle (30 s ON, 4.5 min OFF) over the course of 1 h. In clinical practice, the 10% duty cycle is implemented to minimize side-effects; the maximum tolerable amplitude is applied under the assumption that this will maximize engagement and enhance the beneficial effects of VNS [27]. However, optimization studies in rats showed an inverted U-function in the relationship between stimulation intensity and cortical plasticity with medium range amplitudes (400–800 μA) exhibiting optimal effects as compared to low (<400 μA) and high (>1.2 mA) amplitudes [28]. Thus, we selected 800 μA to reflect this maximally effective amplitude in rodents while remaining within the range of clinically applied parameters.

Nerve engagement was confirmed by monitoring of heart and respiratory rates using a pulse-oximeter with an attached thigh sensor (Starr Life Sciences). Pulse-oximeter output was plotted and visualized using MATLAB. Heart and respiratory rate were aligned with each stimulus event's onset/offset and plotted against time.

Cisterna magna (CM) dye-injection and VNS

Injection of a fluorescent tracer into the CSF system via the CM was performed as previously described [12,29]. Briefly, after cuff implantation, mice were placed into a head-holder with the head at a slight downward angle. A straight vertical incision was made caudal to the occiput bone and terminating at the atlas. The underlying fascia and muscle tissue were bluntly dissected exposing the CM. An injection port was created using a 30ga dental needle (Exel International) attached to PE-10 tubing (Instech Laboratories).

Using a motorized micro-manipulator (Siskiyou Corp.) the injection port was positioned just posterior to the cisterna magna at a 30-degree angle to the membrane and advanced until the tip was seen to puncture. To prevent leakage of CSF and dye the insertion site was sealed with cyanoacrylate glue. The injection port's tubing was then connected to a syringe pump (Kent Scientific) for dye injection.

The glymphatic system has specifically been shown to be sensitive to the type of anesthesia applied with isoflurane shown to diminish CSF penetrance while a combination of ketamine and xylazine is the most permissive [21]. Further, isoflurane inhibits synaptic transmission by suppressing neurotransmitter exocytosis into the synaptic cleft and is ill-suited to experiments involving electrical stimulation of the CNS [30,31]. Thus, following surgery we transferred animals to a combination of ketamine and dexmedetomidine (same class of anesthetic as xylazine). A delay period was implemented between the time isoflurane was turned off and VNS was initiated to allow the effects of isoflurane to dissipate (Fig. 1A). Following the delay period, the experimental groups received VNS for 30 min prior to injection of CSF tracer whereas the control and sham groups received no stimulation. A total of 10 μL of 0.5% TxRed-3kD dextran (Life Sciences) in artificial CSF was injected into the CM over a 5-min period and the tracer spread for a total of 30 min post-injection (Fig. 1A). Care was taken to keep the duration of the delay period across animals consistent. However, due to differences in complexity of the surgical procedures performed across the experimental groups, as well as the time taken to test vagus engagement, the delay period varied across animals. The variations in delay period between animals was not found to have any effect on CSF penetrance (Supplemental Fig. 3).

Ex vivo slice preparation and imaging

Thirty minutes post-injection animals were immediately perfused with saline followed by 4% paraformaldehyde (PFA). The brain was extracted and submerged in 4% PFA for an additional 24-h period before being transferred to phosphate-buffered saline. Brains were sliced on a vibratome (Leica Biosystems) into 100 μm thick coronal sections approximately spanning the region +1 to −2 mm relative to bregma corresponding with cortical regions largely perfused by the middle cerebral artery (MCA). Starting from the first slice, every other slice was mounted to glass slides with ProLong Gold containing DAPI (Life Sciences). Whole slice images were taken using a fluorescent stereoscope (Nikon SMZ18) and DS-Qi2 camera. All microscope and camera settings were kept constant across animals and experimental groups.

Data and statistical analysis

Images were analyzed in ImageJ with a thresholding approach as previously described [12]. Briefly, regions of interest (ROI's) encompassing the entire slice were generated by visualization of the DAPI-channel. The ROI's were then overlaid onto the TxRed-channel and the percentage of pixels within the ROI above a set

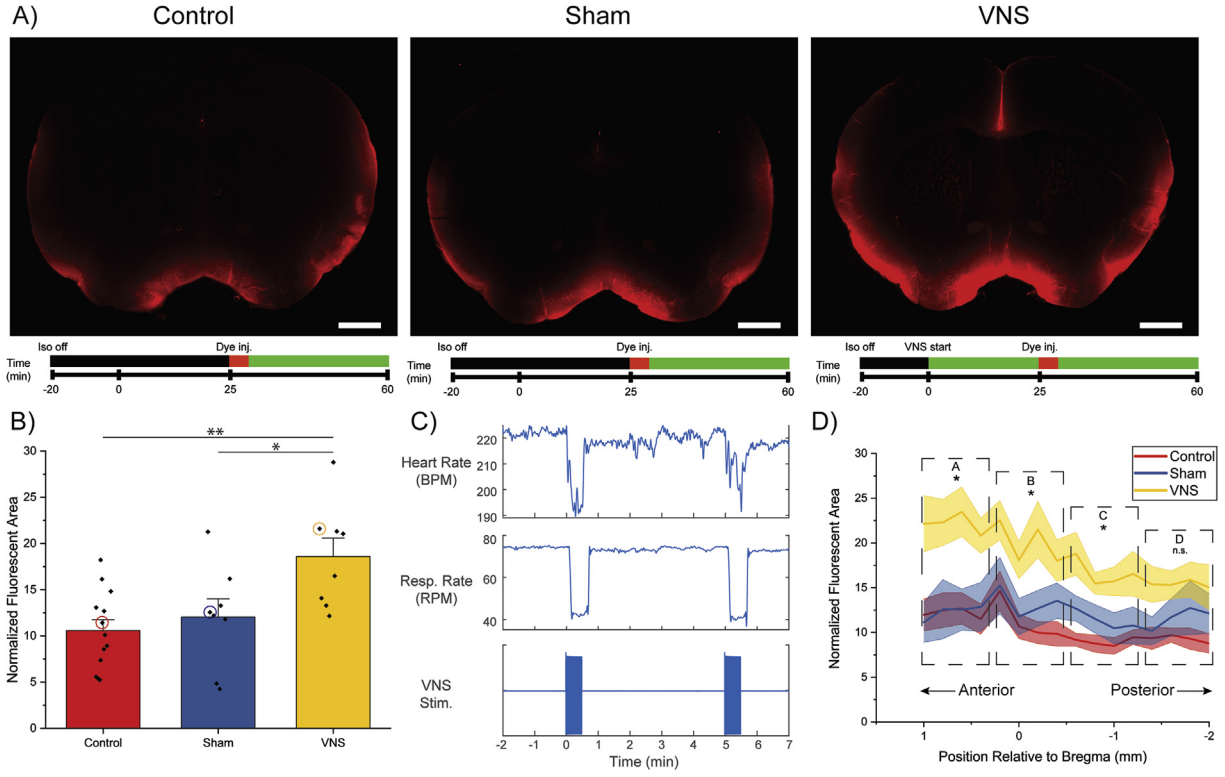

Fig. 1. *Vagus nerve stimulation increases CSF dye penetrance.* A) Representative images of whole slice images showing difference in CSF penetrance in response to VNS as compared to control and sham. Stimulation parameters were as follows: Biphasic, 30 Hz, amplitude = 800 μA, pulse width = 200 uSec with 30 s ON cycled every 5 min. The fluorescent portion of each slice was quantified as a function of the total slice area. A total of 12–16 slices from between the regions of 1 and -2 mm relative to bregma were assessed per animal in this way. Colored bar beneath shows the relative timeline for each group. In order to account for time of anesthesia a waiting period was incorporated into the control and sham groups. Scale bar = 1 mm. B) Average degree of CSF penetrance across groups, dots represent individual animals in each group. Colored circles correspond to the animals from which the slice images in (A) were taken. C) Representative traces of heart and respiratory rates in response to VNS. D) VNS increases CSF penetrance more in anterior regions of the brain than posterior. Positional slice-by-slice representation of the normalized fluorescent area at each position relative to bregma. Solid line represents the average normalized fluorescent area of all animals at that section per condition (shaded area = ±1 S E M). For analysis the data was subdivided into 4 equivalently sized bins which were individually analyzed. Linear mixed model analysis for repeated measures with Sidak correction formultiple comparisons. Error bars = ±1 S E M. Control n = 12, sham n = 8, 30 Hz periodic VNS n = 8, **p < 0.01, *p < 0.05.

threshold was calculated. The threshold was set by finding the mean pixel intensity of sections from unmanipulated animals ($n = 2$) to determine baseline autofluorescence and kept constant across all analyses. A total of 12–16 slices were analyzed per animal (Supplemental Fig. 4), as tissue sections that misfolded during the mounting procedure were excluded from analysis.

To assess global effects of VNS on CSF penetrance the fractional positive fluorescent area of all slices from each group was assessed using a linear mixed effects regression for repeated measures with each position treated as a repeated measure within a given animal, an AR(1) covariance structure between positions, and Sidak adjustment for multiple comparisons. This analysis, analogous to a spatially defined repeated measure ANOVA, allows for the incorporation of subjects with missing data resulting from the excluded mis-folded sections. For the anterior-posterior positional analysis, the same methods were used with slices organized into four equivalently sized non-overlapping bins spanning the entire region. Results were considered statistically significant with $p < 0.05$.

Results

Following implantation, we tested engagement of the vagus nerve by measuring the cardiorespiratory response to stimulation. Clinical stimulation parameters resulted in a range of changes to heart (HR) and respiratory rate (RR) for the duration of the stimulus followed by a rapid recovery once stimulation was stopped (Fig. 1C, Supplemental Fig. 5). The majority of animals exhibited decreases in HR and RR coinciding with the period of stimulation. Rebound tachycardia was common after the stimulation period in those animals, which has been noted in other studies of VNS [32,33]. Tachycardia during the period of stimulation was noted in at least one animal, which has also been reported in other VNS studies [34]. In total, stimulation locked effects on heart rate or respiration rate were observed in all animals, confirming target engagement.

Tracer injection to the CM occurred midway through stimulation and the degree of CSF penetrance was quantified by calculating the fractional area to which tracer had spread using previously established methods [12,29]. We found that the clinically derived VNS parameters significantly increased the degree of CSF dye penetrance (18.60% ± 1.98%) relative to naïve controls (10.58% ± 1.17%, $p = 0.004$) and sham VNS (12.05% ± 1.97%, $p = 0.049$) groups (Fig. 1A and B). Measurement of tracer penetrance at each slice position relative to bregma, rather than as an aggregate per animal, revealed that VNS increased CSF penetrance with increasing magnitude from posterior to anterior sections (Fig. 1D). Sections from each animal were combined into four equivalently sized positional bins and analyzed across experimental groups. VNS induced significant increases in CSF penetrance in the three anterior bins but not in the fourth, most posterior, bin. The more pronounced VNS-induced CSF penetrance in the anterior region may be due to closer proximity to the middle cerebral artery (MCA) and branching arterioles along which CSF travels in the glymphatic model [22]. Consistent with this observation, vagal connections to the sphenopalatine ganglion (SPG), which directly parasympathetically innervates the anterior circulation, are well-documented [35,36]. However, similar anterior/posterior distributions of an injected CSF tracer have been observed in other studies where VNS was not present. Consequently, anterior/posterior distribution of increased CSF penetration may simply be an artifact of the site of injection and relative flow direction of CSF [37,38].

Discussion

The brain's waste clearance systems are increasingly appreciated as a critical factor in the maintenance of healthy brain homeostasis and in the progression of neuropathological processes. In parallel, neuromodulation of the vagus nerve has been demonstrated to be clinically effective across a large and continuously growing number of indications. However, the mechanisms behind these beneficial outcomes remain poorly understood. Our results demonstrate for the first time that VNS using clinically derived parameters can alter penetrance of CSF into the brain parenchyma and therefore may provide a novel method for increasing CSF/ISF exchange and brain waste clearance.

The current model of the glymphatic system posits that CSF influx to the brain parenchyma is driven, in part, by cerebral hemodynamics and, more specifically, by the pulsatility of penetrating cerebral arterioles [17,38]. Thus, it is tempting to speculate that the effects of VNS on CSF penetrance observed here are a result of the intermittent VNS-induced swings to systemic cardiopulmonary parameters leading to rapid changes in cerebral hemodynamics through compensatory cerebral autoregulatory mechanisms. Although we measured VNS-induced changes to heart and respiratory rates to experimentally confirm vagus nerve engagement, the direction of these changes varied between periods during and immediately after blocks of stimulation (rebound tachycardia), and between animals during the period of stimulation (bradycardia vs tachycardia).

Attempts to isolate the VNS-induced increases in CSF penetrance to any one singular physiological outcome is problematic given the complexity of direct and indirect responses to VNS, and the myriad pathways through which the physiological consequences of VNS might impact the brain clearance systems. For example, although the cardiac effects of vagus are often presumed to be mediated through the activation of sensory afferent fibers leading from the baroreceptors, recent large animal and human data have indicated that in practice activating these afferent pathways at tolerable amplitudes is problematic [27,39–43]. Combined optogenetic/electrical stimulation studies with transections in rodent suggest that the majority – but not all - of the bradycardic effect of VNS is through activation of parasympathetic efferent fibers innervating the heart [9]. Isolating electrical activation to just the vagus nerve is also difficult, as the relative anatomy of the vagus with respect to nearby structures can be highly variable. Subject-to-subject differences include specific cross connections from the vagus to the carotid sinus nerve and sympathetic trunk, the presence/absence of the ADN within the cervical vagus trunk, variants where the sympathetic trunk fibers/fascicles are conjoined with the vagus trunk, and general differences in fascicular/fiber organization of the vagus may vary with respect to an epineural cuff electrode's placement [39,44–47]. The vagus also innervates multiple pathways/systems that may directly or indirectly impact any specific downstream cardiopulmonary outcome being measured (for example, the activation of the anti-inflammatory reflex pathway, Hering-Breuer reflex, or even vagal mediated innervation of the thyroid affecting circulating thyroid hormone may all indirectly or directly influence sympathetic/parasympathetic tone [48–51].

The VNS-induced effects on CSF penetrance may also be partially or completely independent of VNS systemic cardiopulmonary effects and instead result from more regionalized brain-specific VNS effects. The wide number of CNS targets connected to the vagus nerve and expanding number of factors affecting the brain's waste clearance systems certainly leaves open the possibility of an alternative mechanism. For example, transient changes in cerebral arterial blood flow due to VNS would not be expected to fully depend on systemic heart rate or breathing responses as they are mediated in part through cross connectivity to the facial nerve and subsequent parasympathetic innervation of the cerebral arteries [35,36,52,53]. VNS has also been reported to acutely decrease

intracranial pressure in pigs through an unknown mechanism that was determined to be independent of VNS-induced cardiovascular effects [54]. VNS can also alter the neurotransmitter and metabolite content of CSF possibly leading to unknown interactions with the astrocytic end-feet lining the paravascular space [12,55]. In rats, it was hypothesized that VNS-induced norepinephrine release from locus coeruleus reduced cortical edema following TBI [56]. The glymphatic system is also heavily influenced by brain noradrenergic signaling although reports indicate that inhibition of noradrenergic signaling leads to increased glymphatic flux [20,57]. Finally, classical models of CSF circulation indicate that some level of CSF drainage to peripheral lymphatics occurs through perineural spaces surrounding the cranial nerves as they exit the cranium and intraventricular tracers have been detected exiting along multiple cranial nerves including the vagus [37]. Consequently, a potential mechanism for the effects seen here may be VNS induced increases in metabolic demand and blood flow impacting the degree of efflux through the perineural space. VNS electrodes have previously been shown to alter the movement of fluorogold injected in the gut to locations within the brain [58].

Ultimately, there may not be one specific mechanism/pathway through which VNS and the brain waste clearance systems interact. Due to the inherent promiscuity of VNS there are likely multiple pathways which may be both synergistic and/or competing under specific circumstances as has been shown with VNS for the treatment of many conditions. This often results in an inverted u-curve with respect to current amplitude and specific physiological responses [34,59,60]. Further, the recent interest in the clearance systems of the brain has led to many new mechanisms that may impact clearance being identified and debated each year. As the literature is evolving rapidly the points of intersection between VNS and brain waste clearance is constantly growing. Beyond the effects already mentioned these interactions potentially include VNS-induced intrinsic brain oscillations, effects on the blood-brain barrier, and direct and indirect effects on glial cell activity function [21,25,26]. Thus, isolating each possible variable will likely involve a large and lengthy multi-disciplinary effort to both identify the mechanisms involved as well as optimize stimulation parameters for maximal beneficial effect.

It is important to note that in the present study all experiments were performed under anesthesia considering the proposed importance of arousal state on glymphatic function and the number of conflicting reports on the effect of anesthesia on CSF influx/efflux [20,21,61–63]. Recently, Hablitz et al. studied the effect of a range of anesthetic regimens on glymphatic function and found that CSF tracer penetrance was highest under ketamine/xylazine and significantly impaired under isoflurane [21]. Although surgeries in our experiments were performed under isoflurane, the remainder of our experiment was done under ketamine/dexmedetomidine anesthesia after allowing the isoflurane to wear off. Additionally, it has been suggested that the observed increases in glymphatic function under sleep and anesthetic states may be the result of tracer build-up within the CSF system as a result of significantly diminished outflow rather than increased throughput [63]; hence the glymphatic system model still remains a current topic of debate within the scientific community [64].

Regardless of isolated mechanism, our results demonstrate that VNS can alter the penetrance of CSF into the brain parenchyma under certain conditions, which has many potential implications outside of the clearance of misfolded proteins. Increasing the amount of CSF in the brain parenchyma presumably would increase the distance between neighboring electrically excitable cells. This would serve to decrease non-synaptic coupling between neurons, also known as ephaptic coupling. Ephaptic coupling has been demonstrated to strongly entrain action potentials within proximally located neurons and has been increasingly linked to epileptic pathologies [65–68]. Moreover, increasing CSF penetration into the parenchyma presumably alters extracellular concentrations of endogenous neurochemical transmitters and other molecules. Changes in tonic and/or phasic concentrations of neurochemical transmitters and other molecules that modulate synaptic transmission may have profound implications on circuit function by altering intrinsic excitability within the brain.

Here we applied only a limited set of stimulation parameters, yet multiple examples within neuromodulation demonstrate the importance of optimizing stimulation parameters to achieve the maximal desired effect and avoid unwanted side-effects. It is likely that the application of VNS to increase brain waste clearance would also benefit from such optimization and future studies should take into consideration testing of multiple frequencies, amplitudes, and duty cycles. For example, recent reports indicate that gamma (40 Hz) entrainment is effective at reducing amyloid deposition and improving cognitive performance in AD mouse models through an as yet unidentified mechanism [69,70]. Accordingly, we performed a preliminary assessment to determine if VNS delivered continuously at 40 Hz affected CSF penetrance. Unlike the clinically derived paradigm, there was no difference between the naïve controls, sham VNS, and the continuous 40 Hz VNS (12.77% ± 1.13%, $p = 0.930$) group (Supplemental Fig. 6). However, it should be noted the stimulation amplitudes applied to the continuous VNS group (100–300 μA) were not the same as the clinically derived VNS group. VNS in rodents at 40 or 80 Hz can cause a short cessation of breathing and in our initial attempts to stimulate continuously at 800 μA to match the 10% duty cycle stimulation, breathing would stop entirely for extended periods and without recovering to any regular breathing pattern [71]. Thus, we decreased the amplitude in this group to match to some extent the observed HR and breathing responses during intermittent stimulation. It is therefore possible the lowered amplitude was simply insufficient to activate the pathways needed to affect CSF penetrance, despite eliciting a cardiopulmonary response. Indeed, both the degree and cortical sites of VNS-induced alterations in cerebral blood flow are known to be amplitude dependent in humans [72]. Further, it is not clear whether the vagus nerve is able to maintain one-to-one entrainment with an electrical pulse train over a prolonged period, nor how central circuits connected to the vagus would respond and adapt to such continuous stimulation.

While we targeted the vagus nerve due to its widespread and growing list of clinical indications, it should be noted that other cranial nerve targets have recently been proposed as potential neuromodulation targets [73–75]. If the primary mechanism driving CSF influx into the brain is indeed cerebral arterial pulsation, then the facial nerve becomes a tempting target as it has been well-documented to regulate cerebral arterial dilation and blood flow [73]. Interestingly, transection of the facial nerve blocked the ability of VNS to induce cerebral bloodflow, indicating the effects of VNS may be due to crosstalk of the vagus and facial nerves since the facial nerve directly parasympathetically innervates the cerebral arteries [35,52]. Additionally, the trigeminal nerve has similar anatomical connections to key brain nuclei as the vagus and has gained an increased research focus as an alternative to VNS as, in humans, it can be more effectively stimulated by superficial, non-invasive, methods. Trigeminal nerve stimulation was also used to reduce cerebral edema, enhance cerebral bloodflow, and improve recovery following severe TBI in rats [74].

Conclusions

Our results demonstrate that VNS can be used to modulate one aspect of the brain's waste clearance systems. The emerging critical

role of these systems to maintaining healthy brain homeostasis highlights the need for future studies to both better define the stimulation parameter space and the mechanisms involved. Indeed, the discovery that VNS mimicking clinical use can increase the penetration of CSF into the brain has profound implications for the underlying mechanisms of VNS therapies and present a potential confound to studies intended to isolate their therapeutic mechanisms. As a previously unexplored facet of cranial nerve stimulation, the modulation of CSF penetration could also conceivably lead to unforeseen and unwanted consequences during chronic administration. As such, this phenomenon warrants scientifically rigorous study to isolate the fundamental mechanism causing increased CSF penetration in future studies.

Declaration of competing interest

The authors report no competing financial interest.

CRediT authorship contribution statement

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Appendix A. Supplementary data

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References

[1] Howland RH. Vagus nerve stimulation. Curr Behav Neurosci Reports 2014;1:64–73. https://doi.org/10.1007/s40473-014-0010-5.
[2] Ben-Menachem E, Revesz D, Simon BJ, Silberstein S. Surgically implanted and non-invasive vagus nerve stimulation: a review of efficacy, safety and tolerability. Eur J Neurol 2015;22:1260–8. https://doi.org/10.1111/ene.12629.
[3] Agostoni E, Chinnock JE, De Daly MB, Murray JG. Functional and histological studies of the vagus nerve and its branches to the heart, lungs and abdominal viscera in the cat. J Physiol 1957;135:182–205. https://doi.org/10.1113/jphysiol.1957.sp005703.
[4] Hulsey DR, Shedd CM, Sarker SF, Kilgard MP, Hays SA. Norepinephrine and serotonin are required for vagus nerve stimulation directed cortical plasticity. Exp Neurol 2019;320:112975. https://doi.org/10.1016/j.expneurol.2019.112975.
[5] Hulsey DR, Hays SA, Khodaparast N, Ruiz A, Das P, Rennaker RL, et al. Reorganization of motor cortex by vagus nerve stimulation requires cholinergic innervation. Brain Stimul 2016;9:174–81. https://doi.org/10.1016/j.brs.2015.12.007.
[6] Gierthmuehlen M, Plachta DTT. Effect of selective vagal nerve stimulation on blood pressure , heart rate and respiratory rate in rats under metoprolol medication. 2016. p. 79–87. https://doi.org/10.1038/hr.2015.122.
[7] Zaaimi B, Grebe R, Wallois F. Animal model of the short-term cardiorespiratory effects of intermittent vagus nerve stimulation. Auton Neurosci Basic Clin 2008;143:20–6. https://doi.org/10.1016/j.autneu.2008.07.002.
[8] Stauss HM. Differential hemodynamic and respiratory responses to right and left cervical vagal nerve stimulation in rats. Phys Rep 2017;5:1–10. https://doi.org/10.14814/phy2.13244.
[9] Rajendran PS, Challis RC, Fowlkes CC, Hanna P, Tompkins JD, Jordan MC, et al. Identification of peripheral neural circuits that regulate heart rate using optogenetic and viral vector strategies. Nat Commun 2019;10:1944. https://doi.org/10.1038/s41467-019-09770-1.
[10] Da Mesquita S, Fu Z, Kipnis J. The meningeal lymphatic system: a new player in neurophysiology. Neuron 2018;100:375–88. https://doi.org/10.1016/j.neuron.2018.09.022.
[11] Louveau A, Plog BA, Antila S, Alitalo K, Nedergaard M, Kipnis J. Understanding the functions and relationships of the glymphatic system and meningeal lymphatics. J Clin Invest 2017;127:3210–9. https://doi.org/10.1172/JCI90603.
[12] Iliff JJ, Wang M, Liao Y, Plogg BA, Peng W, Gundersen GA, et al. A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid β. Sci Transl Med 2012;4. https://doi.org/10.1126/scitranslmed.3003748.
[13] Louveau A, Smirnov I, Keyes TJ, Eccles JD, Rouhani SJ, Peske JD, et al. Structural and functional features of central nervous system lymphatic vessels. Nature 2015;523:337–41. https://doi.org/10.1038/nature14432.
[14] Tarasoff-Conway JM, Carare RO, Osorio RS, Glodzik L, Butler T, Fieremans E, et al. Clearance systems in the brain - implications for Alzheimer disease. Nat Rev Neurol 2015;11:457–70. https://doi.org/10.1038/nrneurol.2015.119.
[15] Iliff JJ, Chen MJ, Plog BA, Zeppenfeld DM, Soltero M, Yang L, et al. Impairment of glymphatic pathway function promotes tau pathology after traumatic brain injury. J Neurosci 2014;34:16180–93. https://doi.org/10.1523/jneurosci.3020-14.2014.
[16] Peng W, Achariyar TM, Li B, Liao Y, Mestre H, Hitomi E, et al. Suppression of glymphatic fluid transport in a mouse model of Alzheimer's disease. Neurobiol Dis 2016;93:215–25. https://doi.org/10.1016/j.nbd.2016.05.015.
[17] Iliff JJ, Wang M, Zeppenfeld DM, Venkataraman A, Plog BA, Liao Y, et al. Cerebral arterial pulsation drives paravascular CSF-interstitial fluid exchange in the murine brain. J Neurosci 2013;33:18190–9. https://doi.org/10.1523/JNEUROSCI.1592-13.2013.
[18] Stoodley MA, Brown SA, Brown CJ, Jones NR. Arterial pulsation-dependent perivascular cerebrospinal fluid flow into the central canal in the sheep spinal cord. J Neurosurg 1997;86:686–93. https://doi.org/10.3171/jns.1997.86.4.0686.
[19] Kiviniemi V, Wang X, Korhonen V, Keinänen T, Tuovinen T, Autio J, et al. Ultra-fast magnetic resonance encephalography of physiological brain activity – glymphatic pulsation mechanisms? J Cerebr Blood Flow Metabol 2016;36:1033–45. https://doi.org/10.1177/0271678X15622047.
[20] Xie L, Kang H, Xu Q, Chen MJ, Liao Y, Thiyagarajan M, et al. Sleep drives metabolite clearance from the adult brain. Science 2013;342:373–7. https://doi.org/10.1126/science.1241224. 80-.
[21] Hablitz LM, Vinitsky HS, Sun Q, Stæger FF, Sigurdsson B, Mortensen KN, et al. Increased glymphatic influx is correlated with high EEG delta power and low heart rate in mice under anesthesia. Sci Adv 2019;5:eaav5447. https://doi.org/10.1126/sciadv.aav5447.
[22] Iliff JJ, Wang M, Liao Y, Plogg BA, Peng W, Gundersen GA, et al. A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid. Sci Transl Med 2012;4. https://doi.org/10.1126/scitranslmed.3003748. 147ra111-147ra111.
[23] de Leon MJ, Li Y, Okamura N, Tsui WH, Saint-Louis LA, Glodzik L, et al. Cerebrospinal fluid clearance in alzheimer disease measured with dynamic PET. J Nucl Med 2017;58:1471–6. https://doi.org/10.2967/jnumed.116.187211.
[24] Lochhead JJ, Davis TP. Perivascular and perineural pathways involved in brain delivery and distribution of drugs after intranasal administration. Pharmaceutics 2019;11:598. https://doi.org/10.3390/pharmaceutics11110598.
[25] Yang Y, Yang LY, Orban L, Cuylear D, Thompson J, Simon B, et al. Non-invasive vagus nerve stimulation reduces blood-brain barrier disruption in a rat model of ischemic stroke. Brain Stimul 2018;11:689–98. https://doi.org/10.1016/j.brs.2018.01.034.
[26] Mercan D, Heneka MT. Norepinephrine as a modulator of microglial dynamics. Nat Neurosci 2019;22:1745–6. https://doi.org/10.1038/s41593-019-0526-9.
[27] Handforth A, DeGiorgio CM, Schachter SC, Uthman BM, Naritoku DK, Tecoma ES, et al. Vagus nerve stimulation therapy for partial-onset seizures: a randomized active-control trial. Neurology 1998;51:48–55. https://doi.org/10.1212/wnl.51.1.48.
[28] Borland MS, Vrana WA, Moreno NA, Fogarty EA, Buell EP, Sharma P, et al. Cortical map plasticity as a function of vagus nerve stimulation intensity. Brain Stimul 2016;9:117–23. https://doi.org/10.1016/j.brs.2015.08.018.

[29] Xavier ALR, Hauglund NL, von Holstein-Rathlou S, Li Q, Sanggaard S, Lou N, et al. Cannula implantation into the cisterna magna of rodents. JoVE 2018. https://doi.org/10.3791/57378.

[30] Wu X-S, Sun J-Y, Evers AS, Crowder M, Wu L-G. Isoflurane inhibits transmitter release and the presynaptic action potential. Anesthesiology 2004;100:663–70. https://doi.org/10.1097/00000542-200403000-00029.

[31] Baumgart JP, Zhou Z-Y, Hara M, Cook DC, Hoppa MB, Ryan TA, et al. Isoflurane inhibits synaptic vesicle exocytosis through reduced Ca 2+ influx, not Ca 2+ -exocytosis coupling. Proc Natl Acad Sci Unit States Am 2015;112:11959–64. https://doi.org/10.1073/pnas.1500525112.

[32] Thompson GW, Levett JM, Miller SM, Hill MRS, Meffert WG, Kolata RJ, et al. Bradycardia induced by intravascular versus direct stimulation of the vagus nerve. Ann Thorac Surg 1998;65:637–42. https://doi.org/10.1016/S0003-4975(97)01351-9.

[33] Frei MG, Osorio I. Left vagus nerve stimulation with the neurocybernetic prosthesis has complex effects on heart rate and on its variability in humans. Epilepsia 2001;42:1007–16. https://doi.org/10.1046/j.1528-1157.2001.0420081007.x.

[34] Ardell JL, Nier H, Hammer M, Southerland EM, Ardell CL, Beaumont E, et al. Defining the neural fulcrum for chronic vagus nerve stimulation: implications for integrated cardiac control. J Physiol 2017;595:6887–903. https://doi.org/10.1113/JP274678.

[35] Hara H, Zhang QJ, Kuroyanagi T, Kobayashi S. Parasympathetic cerebrovascular innervation: an anterograde tracing from the sphenopalatine ganglion in the rat. Neurosurgery 1993;32:822–7. https://doi.org/10.1227/00006123-199305000-00016. ; discussion 827.

[36] Agassandian K, Fazan VPS, Margaryan N, Dragon DN, Riley J, Talman WT. A novel central pathway links arterial baroreceptors and pontine parasympathetic neurons in cerebrovascular control. Cell Mol Neurobiol 2003;23:463–78. https://doi.org/10.1023/A:1025059710382.

[37] Ma Q, Ineichen BV, Detmar M, Proulx ST. Outflow of cerebrospinal fluid is predominantly through lymphatic vessels and is reduced in aged mice. Nat Commun 2017;8. https://doi.org/10.1038/s41467-017-01484-6.

[38] Mestre H, Tithof J, Du T, Song W, Peng W, Sweeney AM, et al. Flow of cerebrospinal fluid is driven by arterial pulsations and is reduced in hypertension. Nat Commun 2018;9:4878. https://doi.org/10.1038/s41467-018-07318-3.

[39] Yoo PB, Lubock NB, Hincapie JG, Ruble SB, Hamann JJ, Grill WM. High-resolution measurement of electrically-evoked vagus nerve activity in the anesthetized dog. J Neural Eng 2013;10. https://doi.org/10.1088/1741-2560/10/2/026003. 026003.

[40] Tosato M, Yoshida K, Toft E, Struijk JJ. Quasi-trapezoidal pulses to selectively block the activation of intrinsic laryngeal muscles during vagal nerve stimulation. J Neural Eng 2007;4:205–12. https://doi.org/10.1088/1741-2560/4/3/005.

[41] Tosato M, Yoshida K, Toft E, Nekrasas V, Struijk JJ. Closed-loop control of the heart rate by electrical stimulation of the vagus nerve. Med Biol Eng Comput 2006;44:161–9. https://doi.org/10.1007/s11517-006-0037-1.

[42] Castoro MA, Yoo PB, Hincapie JG, Hamann JJ, Ruble SB, Wolf PD, et al. Excitation properties of the right cervical vagus nerve in adult dogs. Exp Neurol 2011;227:62–8. https://doi.org/10.1016/j.expneurol.2010.09.011.

[43] De Ferrari GM, Stolen C, Tuinenburg AE, Wright DJ, Brugada J, Butter C, et al. Long-term vagal stimulation for heart failure: eighteen month results from the NEural Cardiac TherApy foR Heart Failure (NECTAR-HF) trial. Int J Cardiol 2017;244:229–34. https://doi.org/10.1016/j.ijcard.2017.06.036.

[44] Toorop RJ, Scheltinga MR, Moll FL, Bleys RL. Anatomy of the carotid sinus nerve and surgical implications in carotid sinus syndrome. J Vasc Surg 2009;50:177–82. https://doi.org/10.1016/j.jvs.2009.03.029.

[45] Schmidt E. Blood pressure response to aortic nerve stimulation in swine. Am J Physiol Content 1968;215:1488–92. https://doi.org/10.1152/ajplegacy.1968.215.6.1488.

[46] Nicolai EN, Settell ML, Knudsen BE, Mcconico AL, Gosink BA, Trevathan JK, et al. Sources of off-target effects of vagus nerve stimulation using the helical clinical lead in domestic pigs. BioRxiv 2020. 10.1101/2020.01.15.907246v1, https://doi.org/10.1101/2020.01.15.907246.

[47] Settell ML, Pelot NA, Knudsen BE, Dingle AM, McConico AL, Nicolai EN, et al. Functional vagotopy in the cervical vagus nerve of the domestic pig : Implications for the study of vagus nerve stimulation. Journal of Neural Engineering 2020. https://doi.org/10.1088/1741-2552/ab7ad4.

[48] Davidson NS, Goldner S, McCloskey DI. Respiratory modulation of baraareceptor and chemoreceptor reflexes affecting heart rate and cardiac vagal efferent nerve activity. J Physiol 1976;259:523–30. https://doi.org/10.1113/jphysiol.1976.sp011480.

[49] Ishii J, Shizume K, Okinaka S. Effect of stimulation of the vagus nerve on the thyroidal release of 131 I-labeled hormones. Endocrinology 1968;82:7–16. https://doi.org/10.1210/endo-82-1-7.

[50] Maschino ISE, Us TX, Guzman AW, Williams F, Amerson PC, Scott TL. (12) United States Patent 2010;2.

[51] Borovikova LV, Ivanova S, Zhang M, Yang H, Botchkina GI, Watkins LR, et al. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature 2000;405:458–62. https://doi.org/10.1038/35013070.

[52] Cobb S, Finesinger JE. Cerebral circulation. Arch Neurol Psychiatr 1932;28:1243. https://doi.org/10.1001/archneurpsyc.1932.02240060002001.

[53] Hardebo JE, Arbab M, Suzuki N, Svendgaard NA. Pathways of parasympathetic and sensory cerebrovascular nerves in monkeys. Stroke 1991;22:331–42. https://doi.org/10.1161/01.STR.22.3.331.

[54] Tubbs RS, Wellons JC, Blount JP, Oakes WJ. Left-sided vagus nerve stimulation decreases intracranial pressure without resultant bradycardia in the pig: a potential therapeutic modality for humans. Child's Nerv Syst 2004;20:309–12. https://doi.org/10.1007/s00381-004-0947-x.

[55] Ben-Menachem E, Hamberger A, Hedner T, Hammond EJ, Uthman BM, Slater J, et al. Effects of vagus nerve stimulation on amino acids and other metabolites in the CSF of patients with partial seizures. Epilepsy Res 1995;20:221–7.

[56] Clough RW, Neese SL, Sherill LK, Tan AA, Duke A, Roosevelt RW, et al. Cortical edema in moderate fluid percussion brain injury is attenuated by vagus nerve stimulation. Neuroscience 2007;147:286–93. https://doi.org/10.1016/j.neuroscience.2007.04.043.

[57] Benveniste H, Lee H, Ding F, Sun Q, Al-bizri E, Makaryus R, et al. Anesthesia with dexmedetomidine and low-dose isoflurane increases solute transport. Anesthesiology 2017;127:976–88. https://doi.org/10.1097/ALN.0000000000001888.

[58] Somann JP, Albors GO, Neihouser KV, Lu K-H, Liu Z, Ward MP, et al. Chronic cuffing of cervical vagus nerve inhibits efferent fiber integrity in rat model. J Neural Eng 2018;15. https://doi.org/10.1088/1741-2552/aaa039. 036018.

[59] Poon C-S. Organization of central pathways mediating the hering-breuer reflex and carotid chemoreflex. Adv Exp Med Biol 2004;551:95–100. https://doi.org/10.1007/0-387-27023-X_15.

[60] Hulsey DR, Riley JR, Loerwald KW, Rennaker RL, Kilgard MP, Hays SA. Parametric characterization of neural activity in the locus coeruleus in response to vagus nerve stimulation. Exp Neurol 2017;289:21–30. https://doi.org/10.1016/j.expneurol.2016.12.005.

[61] Benveniste H, Heerdt PM, Fontes M, Rothman DL, Volkow ND. Glymphatic system function in relation to anesthesia and sleep states. Anesth Analg 2019;128:747–58. https://doi.org/10.1213/ANE.0000000000004069.

[62] Gakuba C, Gaberel T, Goursaud S, Bourges J, Palma C D, Quenault A, et al. General anesthesia inhibits the activity of the "glymphatic system. Theranostics 2018;8:710–22. https://doi.org/10.7150/thno.19154.

[63] Ma Q, Ries M, Decker Y, Müller A, Riner C, Bücker A, et al. Rapid lymphatic efflux limits cerebrospinal fluid flow to the brain. Acta Neuropathol 2019;137:151–65. https://doi.org/10.1007/s00401-018-1916-x.

[64] Smith AJ, Yao X, Dix JA, Jin B-J, Verkman AS. Test of the "glymphatic" hypothesis demonstrates diffusive and aquaporin-4-independent solute transport in rodent brain parenchyma. Elife 2017;6:1–16. https://doi.org/10.7554/elife.27679.

[65] Traub RD, Dudek FE, Snow RW, Knowles WD. Computer simulations indicate that electrical field effects contribute to the shape of the epileptiform field potential. Neuroscience 1985;15:947–58. https://doi.org/10.1016/0306-4522(85)90245-3.

[66] Chiang CC, Shivacharan RS, Wei X, Gonzalez-Reyes LE, Durand DM. Slow periodic activity in the longitudinal hippocampal slice can self-propagate non-synaptically by a mechanism consistent with ephaptic coupling. J Physiol 2019;597:249–69. https://doi.org/10.1113/JP276904.

[67] Anastassiou CA, Koch C. Ephaptic coupling to endogenous electric field activity: why bother? Curr Opin Neurobiol 2015;31:95–103. https://doi.org/10.1016/j.conb.2014.09.002.

[68] Anastassiou CA, Perin R, Markram H, Koch C. Ephaptic coupling of cortical neurons. Nat Neurosci 2011;14:217–23. https://doi.org/10.1038/nn.2727.

[69] Martorell AJ, Paulson AL, Suk HJ, Abdurrob F, Drummond GT, Guan W, et al. Multi-sensory gamma stimulation ameliorates alzheimer's-associated pathology and improves cognition. Cell 2019;177:256–71. https://doi.org/10.1016/j.cell.2019.02.014. e22.

[70] Iaccarino HF, Singer AC, Martorell AJ, Rudenko A, Gao F, Gillingham TZ, et al. Gamma frequency entrainment attenuates amyloid load and modifies microglia. Nature 2016;540:230–5. https://doi.org/10.1038/nature20587.

[71] Siniaia MS, Young DL, Poon C-S. Habituation and desensitization of the Hering-Breuer reflex in rat. J Physiol 2000;523:479–91. https://doi.org/10.1111/j.1469-7793.2000.t01-1-00479.x.

[72] Henry TR, Bakay RAE, Votaw JR, Pennell PB, Epstein CM, Faber TL, et al. Brain blood flow alterations induced by therapeutic vagus nerve stimulation in partial epilepsy: I. Acute effects at high and low levels of stimulation. Epilepsia 1998;39:983–90. https://doi.org/10.1111/j.1528-1157.1998.tb01448.x.

[73] Borsody M, Sacristan E. Facial nerve stimulation as a future treatment for ischemic stroke. Brain Circ 2016;2:164. https://doi.org/10.4103/2394-8108.195281.

[74] Chiluwal A, Narayan RK, Chaung W, Mehan N, Wang P, Bouton CE, et al. Neuroprotective effects of trigeminal nerve stimulation in severe traumatic brain injury. Sci Rep 2017;7:1–13. https://doi.org/10.1038/s41598-017-07219-3.

[75] Wildenberg JC, Tyler ME, Danilov YP, Kaczmarek KA, Meyerand ME. Sustained cortical and subcortical neuromodulation induced by electrical tongue stimulation. Brain Imaging Behav 2010;4:199–211. https://doi.org/10.1007/s11682-010-9099-7.