NEUROMODULATORY PATHWAYS REQUIRED FOR TARGETED PLASTICITY THERAPY

by

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I dedicate this work to the boundless beauty of nature.

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TARGETED PLASTICITY THERAPY

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Targeted plasticity therapy (TPT) utilizes vagus nerve stimulation (VNS) paired with physical rehabilitation to direct plasticity and promote recovery. Pre-clinical trials in stroke, spinal cord injury, traumatic brain injury, and peripheral nerve injury models show improved functional recovery after VNS-pairing when compared to physical rehabilitation alone. Pairing VNS with motor movements in neurologically intact animals leads to expansion of task-specific cortical representations. Precise timing of VNS is required to drive plasticity and functional recovery. VNS engages pro-plasticity neuromodulators, but there is no direct evidence that they mediate VNS effects. Acute responses to VNS in key neuromodulatory centers are also unknown. This dissertation work aims to elucidate the neuromodulatory pathways required for VNS directed plasticity underlying TPT. A reliable preparation driving expansion of proximal forelimb representation in rats after one week of VNS pairing on a lever-press task is used in two experiments. Targeted neurotoxins selectively deplete cholinergic, noradrenergic, and serotonergic innervation of the cortex in experimental animals, testing the necessity of each key neuromodulatory pathway to VNS effects. Intracortical microstimulation reveal cortical

representations to compare across groups. The third experiment characterizes locus coeruleus (LC) responses to parametric variation of VNS. It uses acute VNS cuff implants and standard LC recording techniques to elucidate phasic response characteristics to a wide range of VNS intensity, pulse width, and frequency.

The results of this dissertation replicate previous findings that VNS drives robust plasticity in the motor cortex following VNS-movement pairings. Cholinergic, noradrenergic, and serotonergic depletion each block the effects of VNS. The cortical depletion of acetylcholine was complete, while noradrenergic and serotonergic lesions were confined to the experimental hemisphere. This result suggests that each neuromodulator system uniquely contributes to VNS-directed plasticity and TPT. Recordings from the LC reveal rapid phasic activity in response to VNS. Increases in intensity and pulse width monotonically increase LC activation. Alterations in stimulation frequency do not influence total driven activity, but allow for temporal shaping of the response. These results make substantial contributions to elucidating the mechanisms, resoundingly confirming the neuromodulatory basis for TPT and VNS-directed plasticity. They can help guide clinical considerations in terms of patient selection based on pharmacological profiles. Additionally, they contribute to efforts to optimize stimulation parameters by elucidating responses characteristics in a key neuromodulatory center.

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CHAPTER 1

INTRODUCTION

Neurological Injury: Motor Deficits and Recovery

Diverse neurological injuries effect motor function and often result in chronic deficits. Stroke is a leading cause of long-term disability in the United States, impacting almost 800,000 people each year[1]. Of these, 87% are ischemic, resulting from blockage of blood vessels leading to reduced blood flow to part of the brain causing cell death. The remaining 13% are hemorrhagic in nature, caused by ruptures in blood vessels releasing blood into the brain. Of survivors, 80% suffer some form of long-term motor disability[2]. There are an estimated 285,000 people currently living with spinal cord injuries (SCI) in the US, and about 17,500 new cases per year[3]. While there are a wide range of consequences to SCI, improvement in hand function has high impact and priority, as it severely impacts patient independence[4]. Treatment for long-term motor impairments in both injury types focus on physical rehabilitation therapy[5,6]. Subject retention and consistency drastically influences outcomes[7]. Improvements to therapy techniques and adjuvant therapies could improve speed and efficacy of rehabilitative therapy[8].

Widespread functional and structural plasticity of the motor system subserve motor skill learning and can support motor behavioral recovery[9,10] . Motor skill learning causes functional reorganization within the cortex[11]. Complex motor skill learning also drives plasticity in the striatum and cerebellum[12,13]. In addition to supra-spinal targets, circuitry within the spinal cord also changes in response to experience[14]. Plasticity across each of these modalities also occurs in humans[15–17]. Cortical representations have been extensively studied, likely due to the east of access for experimentation. Intracortical microstimulation of motor cortex results in observable

motor movements in primates and rodents[18,19]. Even limiting the scope of inquiry to cortical representations, the involvement of plasticity in recovery after injury is evident. Reorganization of representation in areas of motor cortex spared from ischemic stroke accompany functional recovery in adult primates and rats[20–23]. A further lesion to the functionally reorganized cortex abolishes motor recovery gains in rats[20].

Reorganization of neural circuitry after injury can also be maladaptive and harmful or even responsible for long term neurological disorders. Peripheral nerves often reinnervate following laceration[24]. Erroneous cross-reinnervation between sensory and motor axons produce suboptimal functional results, and inhibit full recovery[24]. Neonatal SCI have enhanced recovery over extended periods, but reinnervation and residual motor control are often under-developed due to compensatory activity[25]. Similarly, following stroke, compensatory mechanisms can become maladaptive, as spared-limb usage, ipsilateral projections, and inter-hemispheric balances compete and inhibit recovery of the damaged regions[26]. Preventing maladaptive reorganization and directing functional, adaptive plasticity could be key to promoting motor recovery after neurological injury. Further understanding plasticity throughout the central nervous system could lead to development of therapies and help boost the efficacy of functional recovery.

Neural Plasticity: Dynamic Conditions

Fully developed human nervous systems contain an estimated 100 billion neurons[27]. Refining the trillions of connections between them is prohibitively complex for purely genetic mechanisms[28]. Early in development rapid and extensive shifts in neural representations through competition, consolidation, and dynamic modulation shape future experience in critical periods[29]. Representations often stabilize, allowing a predictable, functional system to emerge[28]. Closing of

these critical periods does not signal the end of changing representations, as neural populations continue to shift in their response to sensory stimulation, and output organizations throughout the lifespan of an individual[30,31]. Manipulations to the adult system can prompt highly dynamic states to re-emerge[32]. Further insight into the mechanisms of plasticity of neural connections and closure of the critical periods can inform current approaches to enabling therapeutic neural plasticity.

Study of ocular dominance (OD) plasticity in the visual system of cats initiated a rich history of research in cortical plasticity. After regular development[33], most cells in the visual cortex respond to stimulation of both eyes. Monocular deprivation of visual input by suturing one eyelid shut alters cortical representations[34]. Later optical stimulation of the deprived eye results in little to no response in the cortex. However, monocular deprivation after the 12th week of development has no impact on OD[34]. During this critical period of development, as little as 3 days of deprivation leads to a sharp decline in cortical cells bilaterally driven. Extensive research has aimed to elucidate the mechanisms involved in the rapid shifts seen in the visual cortex.

Multiple neuromodulators mediate OD plasticity[35]. Destruction of noradrenergic and cholinergic innervations reduces the physiological response to monocular deprivation[36]. Pharmacological blockade of β adrenergic receptors suppresses OD shifts in a concentration dependent manner[37]. Similarly, destruction or pharmacological blockade of serotonergic signaling also prevents OD during the critical period[38]. The diverse neuromodulatory involvement in shifts of OD provide possible opportunities to induce plasticity beyond the usual critical period utilizing endogenous mechanisms. Indeed, microperfusion of norepinephrine into the visual cortex of adult cats restores OD plasticity beyond the critical period during development[39]. Similarly, chronic administration of a selective serotonin reuptake inhibitor restored plasticity in response to

monocular deprivation in rats[40]. Noradrenergic, serotonergic, and cholinergic neurotransmission facilitate systemic changes, and likely have specific cellular mechanisms that underscore their influence. Although many neuromodulators converge on similar downstream pathways, their synergy appears necessary to achieve plasticity.

Observing plasticity at a cellular and synaptic level provides insight into plasticity mechanisms that can explain these systems level phenomenon. Neurons communicate through release of neurochemicals, often directly adjacent to other neurons at a synapse[41]. The action of each neurochemical depends on receptors in the post-synaptic cell[42,43]. Each neurochemical has multiple receptors that vary from directly, rapidly acting ionotropic receptors, to often slower metabotropic receptors that influence activity through protein and kinase cascades[44]. Glutamate and gamma-aminobutyric acid (GABA) are the most common neurotransmitters, and cause excitation or inhibition of the post-synaptic cell through ionotropic receptors. Neuromodulators, including the previously discussed acetylcholine, norepinephrine, and serotonin, often act through metabotropic receptors, modulating activity caused by glutamate and GABA as opposed to directly influencing through excitation or inhibition. Complex circuitry within the cortex determines the systemic efficacy of each neurochemical.

Changes in synaptic strength are foundational to neural plasticity. *In vitro* slice electrophysiology in allows controlled study of synaptic dynamics[45]. Excitatory post-synaptic potentials (EPSPs) caused by release of glutamate at the synapse change based on post-synaptic receptor composition[42,46]. Long-term potentiation or depression (LTP or LTD) occur when alterations to synaptic strengths persist[47]. In a process known as spike-timing dependent plasticity (STDP), LTP strengthens synapses following sequential action potentials in pre and post-synaptic

cells, while LTD weakens the synapse if the post-synaptic cell is active preceding the pre-synaptic cell[48,49]. Mechanisms of STDP help explain complex organization and representations in large brain networks like primary sensory or motor cortices[50].

The abundance of synaptic activity *in vivo* make a theory that timing of pre and post-synaptic action potentials alone shape synaptic strengths implausible[51]. Multiple internal and external factors including firing rate and neuromodulation influence the efficacy, prevalence, and direction of changes in EPSP[52]. In visual cortex spike timing alone is insufficient to induce plasticity, but introduction of cholinergic and noradrenergic receptor agonists through bath application reinstates and facilitates both LTP and LTD[53,54]. Later studies found that specific phosphorylation of post-synaptic glutamate receptors mediate alterations to synaptic strength[55]. Neuromodulator receptors coupled to specific signaling cascades influence post-synaptic receptor composition following salient synaptic events, enabling the long-term changes[56]. This allows behavioral states associated with specific neuromodulators to also influence whether synapses are strengthen, weakened, or remain unchanged.

Changes throughout the developing nervous system influence the requirements for induction of plasticity. In the auditory cortex, multiple neuromodulatory and sensory inputs converge on a specific subpopulation of inhibitory inter-neurons[57]. They express 5-HT_{3A} and nACh receptors, fast acting ionotropic receptors for serotonin and acetylcholine respectively. Activation of these cells dis-inhibit sensory input to the cortex, allowing sensory inputs to shape cortical circuits. Many cholinergic receptors express later in development than nAChRs, downregulating the effects of acetylcholine release on these cells through competition, and closing the auditory critical-period plasticity. Small shifts in receptor distribution and neural circuitry impact the

requirements to induce plasticity. Driving systemic plasticity beyond the critical period may be possible through the same mechanisms, but would require specific neuromodulatory tone to engage the previously labile pathways.

New Pharmacology: Timing and Treatment Utilizing Vagus Nerve Stimulation

Pharmacological interventions facilitate pro-plasticity sates, but lack temporal specificity in delivery[40]. Rather than specifically promoting beneficial plasticity, traditional pharmacological agents could simply induce a labile state. Determinants of STDP operate on millisecond timescales, orders of magnitude faster than developed drug delivery systems[52]. With temporally specific neuromodulation LTP or LTD can be selectively enabled in mouse visual cortex *in vitro*[56]. Although presentation of exogenous agonists within 5 seconds is sufficient activate plasticity in a primed pathway, current pharmacological agents are not feasible for delivering event specific neuromodulation[56].

Activation of key neuromodulatory centers during stimulus specific neural activity enables targeted plasticity of cortical circuitry *in vivo.* Direct stimulation of the cholinergic nucleus basalis (NB) increases acetylcholine levels in the cortex, and facilitates stimulus-evoked responses[58–60]. Pairing NB stimulation with auditory tones causes reorganization of the auditory cortex, with increased representation of the paired tone[61]. Tones interleaved within 8 seconds of NB stimulation did not measurably affect cortical representations. Similarly, stimulation of the locues coeruleus (LC) increase levels of norepinephrine in the cortex[62], and pairing tones with electrical or optogenetic stimulation drives reorganization of cortical responses[63,64]. Pairing neuromodulatory stimulation with behaviors may be able to drive plasticity throughout the nervous system when

endogenous activity is insufficient. Promoting stimulus specific reorganization through precisely timed neuromodulation may help enhance recovery from neurological disorder.

Targeted plasticity therapy (TPT) aims to promote functional recovery by pairing vagus nerve stimulation (VNS) with rehabilitation to treat neurological disorders[65]. VNS paired with rehabilitation promotes enhanced forelimb function across a variety of rat models of neurological injury including ischemic and hemorrhagic stroke, SCI, traumatic brain injury, and peripheral nerve injury[66–70]. There are ongoing clinical trials to determine the efficacy of VNS pairing during rehabilitation as a treatment after stroke[71]. Further research of the mechanisms facilitating VNS mediated plasticity in the motor system could help guide clinical efforts.

The vagus nerve is the tenth cranial nerve and controls most communication between the viscera and the central nervous system. Sensory afferent fibers of the vagus project to the nucleus of the solitary tract (NTS)[72]. The LC receives di-synaptic excitation from the NTS, further projecting almost ubiquitously throughout the CNS[73]. Chronic VNS increases tonic activity of the LC and the serotoergic dorsal raphe nucleus (DRN), and acute VNS is known to alter norepinephrine levels in the cortex and hippocampus[74–76]. VNS pairing is thought to utilize these biological neuromodulatory pathways to target plasticity, but the direct involvement of any of these pathways is unknown[65].

Pairing VNS with movements or auditory tones drives plasticity in primary motor and auditory cortices respectively[77,78]. Distal and proximal forelimb representations can be differentiated in cortical representations using ICMS[18]. Pairing VNS with a lever pressing task emphasizing proximal forelimb use causes expansion of proximal representation within the motor cortex, whereas VNS paired with a wheel spin task emphasizing distal forelimb use causes

expansion of distal forelimb representations[78]. The plasticity enhancing effects of paired VNS are specific to the paired movement. Similarly, VNS paired with auditory tones results in expansion of cortical area responding to the paired tone, but not of tones interleaved 15 seconds apart from stimulation[77]. VNS pairing is thought to drive plasticity by coincident activation of cortical networks and delivery of pro-plasticity neuromodulators, enabling plasticity after otherwise inert activity.

While there is no direct evidence of neuromodulatory involvement in VNS directed plasticity, subjects in a clinical trial for treatment of tinnitus with paired VNS did not see beneficial effects if taking noradrenergic antagonists[79]. Determining neuromodulatory systems influencing VNS driven plasticity could help screen potential subjects in future studies. Chapters 2 and 4 of this dissertation aim to elucidate on which neuromodulatory pathways are required for VNS directed plasticity within the motor cortex. Targeted immunotoxins can create specific lesions to key neuromodulatory pathways[80,81]. Established techniques of ICMS and standard VNS pairing procedures produce reliable plasticity enhancement with only 5 days of pairing[78]. Combining these two techniques allows determination of the necessity of specific neuromodulatory pathway for VNS effects.

The timescale of acute neuromodulator activation by VNS is also unknown. The efficacy of VNS treatments rely on the timing in of stimulation[70,82]. VNS delivered 25 seconds after successful trials during rehabilitation does not enhance functional recovery, but concurrent or 2 second delayed stimulation promote significant recovery over rehabilitation alone[70]. Additionally VNS paired with auditory tones drives expansion of representation in the auditory cortex, but tone presentations interleaved within 15 seconds of paired tones do not have expanded representation[77].

The effects of chronic VNS on tonic activity levels have been studied, but the magnitude and duration of acute activity driven by VNS is unknown[74,75,83]. Chapter 4 of this dissertation focuses on the acute effects of VNS on LC activity. Characterizing phasic *in vivo* responses in a key neuromodulatory system could guide VNS delivery approaches, allowing optimization of key aspects of neuromodulatory delivery.

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CHAPTER 2

REORGANIZATION OF MOTOR CORTEX BY VAGUS NERVE STIMULATION REQUIRES CHOLINERGIC INNERVATION*

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Abstract

Background: Vagus nerve stimulation (VNS) paired with forelimb training drives robust, specific reorganization of movement representations in the motor cortex. The mechanisms that underlie VNS-dependent enhancement of map plasticity are largely unknown. The cholinergic nucleus basalis (NB) is a critical substrate in cortical plasticity, and several studies suggest that VNS activates cholinergic circuitry.

Objective: We examined whether the NB is required for VNS-dependent enhancement of map plasticity in the motor cortex.

Methods: Rats were trained to perform a lever pressing task and then received injections of the immunotoxin 192-IgG-saporin to selectively lesion cholinergic neurons of the NB. After lesion, rats underwent five days of motor training during which VNS was paired with successful trials. At the conclusion of behavioral training, intracortical microstimulation was used to document movement representations in motor cortex.

Results: VNS paired with forelimb training resulted in a substantial increase in the representation of proximal forelimb in rats with an intact NB compared to untrained controls. NB lesions prevent this VNS-dependent increase in proximal forelimb area and result in representations similar to untrained controls. Motor performance was similar between groups, suggesting that differences in forelimb function cannot account for the difference in proximal forelimb representation.

Conclusions: Together, these findings indicate that the NB is required for VNS-dependent enhancement of plasticity in the motor cortex and may provide insight into the mechanisms that underlie the benefits of VNS therapy.

Introduction

Neuromodulatory interventions have been extensively investigated as potential therapies to reverse maladaptive plasticity or boost limited plasticity to treat neurological disease. Recently, vagus nerve stimulation (VNS) has emerged as one such potential adjunctive intervention to enhance neuroplasticity [1]. Repeated presentation of auditory stimuli paired with short bursts of VNS drives long-lasting plasticity in auditory cortex [2-4]. Moreover, VNS paired with forelimb training drives robust, specific reorganization in motor cortex [5]. Based on this enhancement of plasticity, VNS has garnered attention as a method to support recovery in the context of neurological disease.

Recently, several studies have demonstrated that VNS paired with specific rehabilitative training regimens can provide therapeutic benefits in a variety of neurological disorders. VNS paired with specific tones reverses the neural and behavioral correlates of tinnitus in a rat model, and a pilot study indicates that VNS tone therapy promotes recovery in chronic tinnitus patients [2, 3, 6, 7]. Additionally, several studies have indicated that VNS paired with motor rehabilitation improves recovery in several mechanistically distinct models of brain injury. VNS paired with rehabilitative training enhances recovery of forelimb function after cortical ischemic stroke, subcortical intracerebral hemorrhage, and traumatic brain injury [8-12]. Based on these findings, physical rehabilitation with task-concurrent VNS is now under investigation in chronic stroke patients [13, 14]. A distinct implementation using long-duration VNS is already in use in over 60,000 patients for control of intractable epilepsy and treatment-resistant depression [15-18].

Despite the demonstrated and potential efficacy in a variety of neurological diseases, the mechanisms underlying VNS-dependent enhancement of neuroplasticity and recovery are largely

unknown. Previous studies have implicated the noradrenergic locus coeruleus (LC) and cholinergic nucleus basalis (NB) in the effects of VNS in the central nervous system [1, 19]. Electrical stimulation of the vagus nerve drives activity in both the LC and cholinergic basal forebrain [20-22]. VNS drives release of norepinephrine throughout the brain [23-26]. Lesions of the LC prevent the seizure-attenuating and antidepressant effects of VNS, indicating the importance of noradrenergic signaling in the effects of VNS [27, 28]. Acute antagonism of muscarinic acetylcholine receptors prevents VNS-dependent desynchronization of cortical EEG, suggesting that VNS exerts an effect on cortical processing by engaging cholinergic transmission [29]. Moreover, tones paired with either VNS or direct NB stimulation drive similar spectral and temporal features of plasticity in the auditory cortex [2, 3]. Both the LC and NB are key substrates in neural plasticity [30], and activation of these systems may underlie VNS-dependent enhancement of plasticity.

Acetylcholine and norepinephrine act both independently and synergistically to facilitate plasticity [31, 32], and it is not clear how the effects of VNS are mediated by these neuromodulatory systems. It is possible that NB activation is necessary for the plasticity enhancing effects of VNS. Alternatively, the noradrenergic or other neuromodulatory systems may substitute in the absence of NB activation. Here, we evaluate whether the NB is necessary for the plasticity-enhancing effects of VNS paired with motor training. A clear definition of the neuromodulatory systems engaged by VNS is needed to identify factors, such as drugs or disease states, that affect neuromodulatory transmission and may interfere with the benefits of VNS therapy.

Methods

Subjects

Twenty-six adult female Sprague-Dawley rats weighing on average 284 grams were used in this experiment. Rats were housed in a 12:12 hour reversed light cycle to increase daytime activity levels. Rats were food deprived during behavioral training, with body weights maintained above 85% to increase motivation for food pellet rewards. All handling, housing, behavioral training, and surgical procedures were approved by the University of Texas Institutional Animal Care and Use Committee.

Behavioral Procedure

Rats were trained on the bradykinesia assessment task, a quantitative, automated lever pressing task [33]. The behavioral chamber consisted of an acrylic cage with a slot located in the front right for access to a lever positioned 2.5 cm outside of the chamber (Fig. 2.1A). The lever was affixed to a potentiometer, which records the angle of the lever depression relative to horizontal. The lever was allowed to move 13° below horizontal, and lever depression exceeding 75 percent of the total range was considered a press. A spring provided 28 grams of resistance, returning the lever to its level resting angle. A controller board (Vulintus, Richardson, TX) sampled the potentiometer position at 100 Hz and relayed the information to custom MATLAB software that controlled the task and collected data.

During behavioral testing, a timer was initiated on the first press of the lever. If the lever was depressed a second time within 500 msec, the trial was recorded as a success and a reward pellet (45 mg dustless precision pellet, BioServ, Frenchtown, NJ) was delivered (Fig. 2.1B). A tone provided an auditory cue for successful tasks. If the lever was not pressed again or the second

press occurred more than 500 msec later, the trial was recorded as a failure and no reward or VNS was given (Fig. 2.1C). Training was conducted in stages, as previously described [33]. Behavioral training and testing was performed in two thirty minute sessions per day, five days a week, with at least two hours between daily training sessions. Rats continued behavioral training until they performed at least 100 successful trials during each training session. Once proficient, rats underwent 192-IgG-saporin or control injections and stimulating cuff implants. One week after lesion, rats returned for behavioral testing. After habituating to the stimulating cable, rats underwent 5 days of training with VNS paired with successful trials.

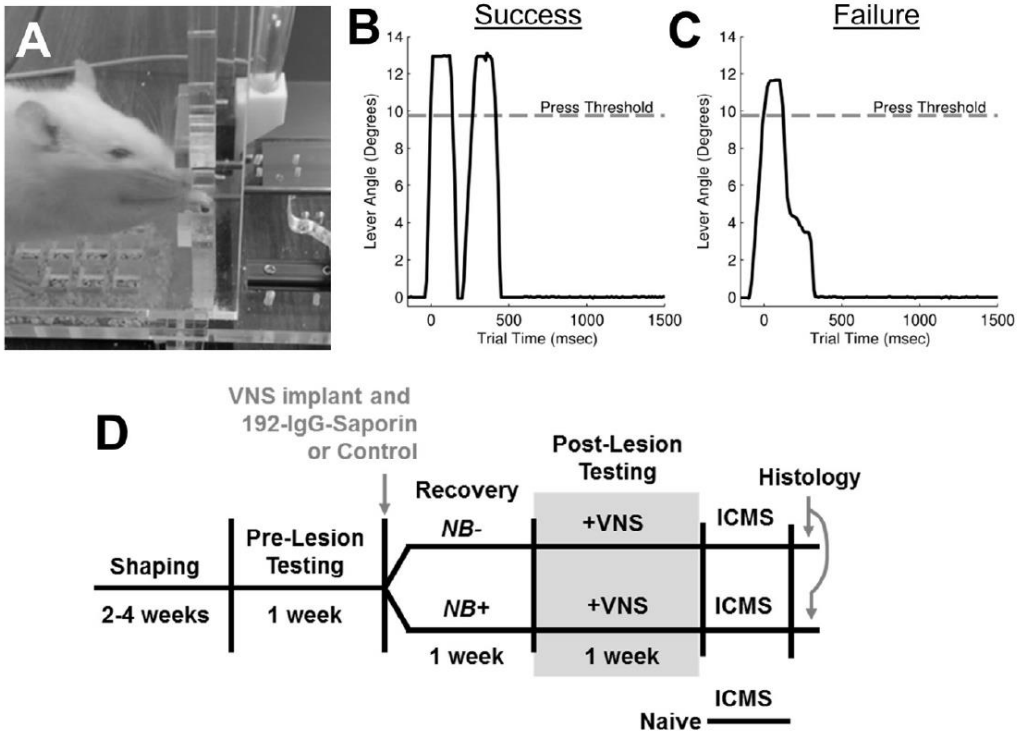

Figure 2.1 Experimental design
(A) Image of a rat performing the lever pressing task. (B, C) Representative data of lever pressing performance depicting a successful and unsuccessful trial. (D) Timeline of the experimental design.

Cortical Cholinergic Depletion

Cholinergic lesions were performed similar to previous reports [34-36]. Rats were anesthetized with ketamine hydrochloride (80 mg/kg, i.p.) and xylazine (10 mg/k, i.p.), and given supplemental doses as needed to maintain anesthesia levels. After placing the rat in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) burr holes were drilled over the nucleus basalis bilaterally. Rats received injections of either conjugated 192-IgG-saporin (Advanced Targeting Systems, San Diego, CA) to selectively lesion cholinergic neurons in the basal forebrain, or control injections of an untargeted antibody and saporin, which does not enter cells and induce cell death. Toxin or control peptide (0.375 mg/mL in saline) was injected through a pulled glass needle at 0.1 μL/min using a Nanoliter 2010 injector (World Precision Instruments, Sarasota, FL). Injections were made at the following sites (site 1&2: 0.3 μL, AP: -1.4, ML: +/- 2.5, DV: -8.0; sites 3&4: 0.2 μL, AP: -2.6, ML: +/- 4, DV: -7.0). A schematic of injection locations can be found in the supplementary data. The needle remained in place for 4-5 minutes after each injection to allow for diffusion and prevent backflow. Burr holes were sealed with bone wax.

Vagus Nerve Cuff Implant

Vagus nerve cuff implantations were performed as described previously [8-11]. Immediately following 192-IgG-saporin or control injections, four bone screws were manually drilled into the skull at points near the lamboid suture and over the cerebellum. A two-channel connector was attached to the cranial screws with acrylic. An incision and blunt dissection of the neck muscles exposed the left vagus nerve. The vagus nerve was isolated with blunt dissection and placed in a bipolar stimulating cuff electrode with platinum-iridium leads (~5 kΩ impedance). Cuff leads were tunneled subcutaneously and attached to the skull mounted connector and encapsulated

with acrylic. Neck and scalp incisions were sutured and treated with topical antibiotic ointment. Rats were provided with amoxicillin (5 mg) and carprofen (1 mg) tablets for 3 days following the surgeries and allowed to recover for one week before returning to behavioral training.

Vagus Nerve Stimulation Procedure

Upon returning to behavioral testing following surgery, rats were given 1-5 days to become habituated to the stimulating cable connected to the headcap while performing the task. Once rats consistently performed 200 successful trials per day while connected to the stimulator, VNS pairing commenced. VNS was delivered on successful trials in all rats, as previously described [9, 11]. VNS was delivered as a 500-ms train of 15 pulses at 30 Hz. Each biphasic pulse was 0.8 mA in amplitude and 100 μs in phase duration. These parameters are identical to previous studies [5, 8, 9, 11, 12]. Rats received VNS paired with behavioral training for 5 days before ICMS.

Intracortical Microstimulation Mapping

Within 24 hours of the final VNS paired training session rats underwent intracortical microstimulation (ICMS) of the left motor cortex to derive functional maps in cortex contralateral to the trained limb using standard procedures [5, 37-39]. An additional 8 rats that did not receive motor training underwent ICMS as naïve controls. Rats were anesthetized with ketamine hydrochloride (70 mg/kg, i.p.) and xylazine (5 mg/k, i.p.) and received supplementary doses as needed. To prevent swelling, a small incision was made in the cisterna magna. A craniotomy and duratomy exposed the left motor cortex, contralateral to the trained forelimb. A tungsten electrode (~0.7 MΩ impedance) was inserted following a grid with 500 μm spacing to a depth of 1.8 mm. Sequential electrode placements were made at least 1 mm apart where possible. Stimulation consisted of a 40 ms pulse train of ten 200 μs monophasic cathodal pulses delivered at 286 Hz.

Stimulation intensity was gradually increased from 20 μA to 200 μA until a movement was observed. If no movement was observed at 200 μA, responses were evaluated at 1.6 mm and 2.0 mm electrode depths to account for variability in cortical thickness. If no movement was observed at any depth at the maximal stimulation, the site was deemed nonresponsive. The borders of primary motor cortex were defined based on unresponsive sites and stopped at the posterior-lateral vibrissae area, which is known to overlap the somatosensory cortex [40].

Motor mapping procedures were conducted with two experimenters as previously described [5]. The first experimenter placed the electrode and recorded the data for each site. The second experimenter was blind to the treatment group and electrode position to avoid potential biasing. The second experimenter delivered stimulations and observed and classified movements. Movements were classified at the threshold current, but in some cases, slightly higher currents were used to disambiguate movements too small to be classified at threshold. Stimulation sites were randomly chosen and did not extend beyond established border (i.e., unresponsive) sites. Movements of the shoulder and elbow were classified as ‘‘proximal forelimb.’’ Movements of the wrist and digits were classified as ‘‘distal forelimb.’’ ‘‘Hindlimb’’ included any movement in the hindlimb of the rat. Neck, vibrissa, and jaw movements were classified as such. Cortical area was calculated by multiplying the number of sites eliciting a response by the area surrounding a site (0.25 mm^2). Complete borders were determined when possible, but some maps do not have complete borders. All maps were used for analysis, and raw ICMS maps from all subjects can be found in the online supplement.

Histology and Quantification of ACh Depletion

Following ICMS, rats were transcardially perfused with 250 mL of 0.02% heparin/100mM phosphate-buffered (PB) solution, followed by 450 mL of 4% paraformaldehyde/100 mM PB solution. Brains were removed and postfixed in 4% paraformaldehyde/100 mM PB solution, and then cryoprotected in a 30% sucrose/0.1 M PB solution. The full extent of the motor cortex was sectioned at a 40 μm thickness. Three sections from motor cortex contralateral to the trained limb were randomly selected and stained for acetylcholinesterase (AChE) activity using standard protocol [41]. In brief, free floating sections were washed in a Tris-Maleate buffer solution containing 6 mg/ml promethazine. After a series of washes in Tris-Maleate buffer, tissue was incubated in a solution containing 10 mM sodium citrate, 30 mM cupric sulfate, 5.0 mM potassium ferricyanide, and 0.5 mg/ml acetylcholine iodide. After washes in a Tris-HCl buffer, tissue was processed for DAB to intensify the labeling. Tissue was then mounted, dehydrated, and cover slipped.

Stained tissue was imaged using a NanoZoomer (Hamamatsu, Tokyo, Japan). Analysis of cortical cholinergic innervation was performed by counting AChE positive fibers crossing of a grid overlay, as described previously [42, 43]. A region of layer V of motor cortex from each section was randomly selected for analysis by an independent experimenter blind to the condition of each rat. A 6x6 grid (250 μm x 250 μm) was manually superimposed on the area using Adobe Photoshop CS4 (Fig. 2.2). All intersections between AChE stained fibers and a gridline were manually identified and counted by an experimenter using coded images. Four rats that received injections of 192-IgG-saporin failed to show greater than 90% depletion of cholinergic fibers and were excluded.

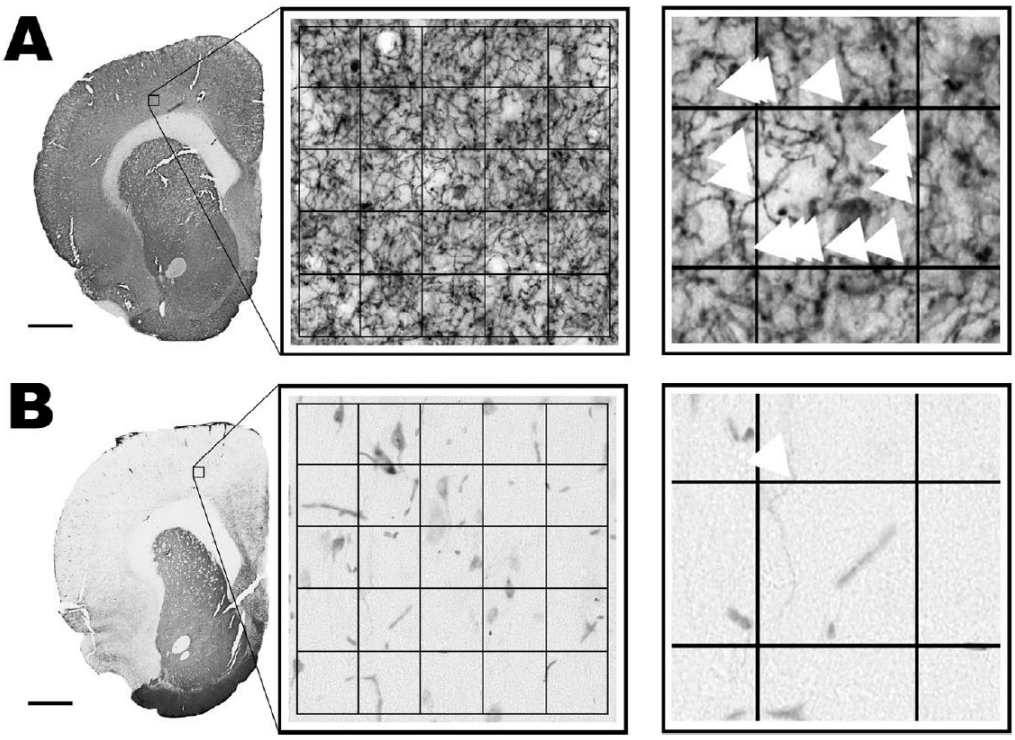

Figure 2.2 192-IgG-saporin lesions deplete cortical cholinergic innervation. Representative images of AChE fiber staining in layer V motor cortex of an NB+ control lesioned subject (A) and an NB- 192-IgG-saporin lesioned subject (B). The right-most panel shows a further magnification with white arrowheads marking fiber crossings. Calibrations are 1 mm for main image, and the spacing between gridlines is 50 μm for inset.

Statistics

All data are reported in the main text as mean ± SEM. All comparisons were planned in the experimental design *a priori*, and significant differences were determined using one-way ANOVA and t-tests where appropriate. Statistical tests for each comparison are noted in the text. Paired t-tests were used to compare performance before and after lesion, and unpaired t-tests were used to compare measures across groups. Alpha level was set at 0.05 for single comparisons, and Bonferroni-corrected to 0.017 for multiple comparisons where applicable. Error bars indicate SEM in all figures, and * denotes $p < 0.05$.

Results

192-IgG-Saporin lesions deplete cortical cholinergic innervation

Rats were trained to proficiency on an automated lever pressing task that required use of the proximal forelimb (Fig. 2.1) [33]. Once they reached proficiency, rats were randomly assigned to receive either an NB lesion (NB-) or control procedure (NB+). NB lesion subjects received bilateral NB injections of 192-IgG-saporin, a toxin that selectively lesions cortically-projecting cholinergic neurons while leaving other NB neurons intact [44, 45]. Depletion is complete by the seventh day post-injection [46]. Control subjects received bilateral injections of a saporin conjugated with a control antibody, which does not induce cell death. To confirm cholinergic lesion after 192-IgG-Saporin injections, AChE containing fibers were analyzed in motor cortex (Fig. 2.2 A&B). Fiber count analysis indicates that the 192-IgG-Saporin injections caused substantial cholinergic denervation. 192-IgG-saporin resulted in an 96.6 ± 1.0% reduction in cortical AChE fiber staining compared to control injections (Fig. 2.2 ; NB-, n = 8; NB+, n = 9).

Cholinergic depletion prevents VNS-dependent cortical plasticity

After injection, both the NB+ and NB- groups underwent five days of motor training during which successful trials were paired with a burst of VNS. On the day after the final session of VNS paired training, all subjects underwent ICMS mapping of motor cortex. Additionally, ICMS mapping was performed on a cohort of untrained subjects (Naïve, n = 8).

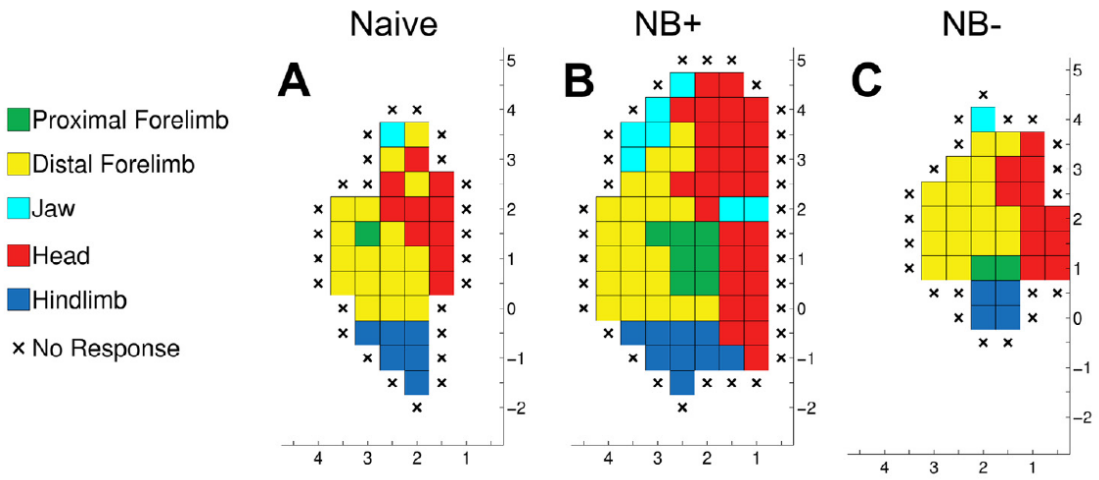

Figure 2.3 Representative ICMS maps.
(A) Example motor cortex map from an untrained control rat. (B) Example map depicting the substantial increase in proximal forelimb representation in rats with an intact NB that received VNS paired with motor training. (C) Example maps from rat with an NB lesion that received VNS paired with motor training. Note the similarity to the untrained control map. Each square represents a 0.25 mm^2 (0.5 x 0.5 mm) area. Electrode penetrations occurred in the middle of each square.

VNS paired with motor training in subjects with an intact NB increases proximal forelimb representation in motor cortex (Fig. 2.3). ANOVA on proximal forelimb representation revealed a significant effect (One-way ANOVA, $F[2,24] = 9.45$, $p = 9.39 \times 10^{-4}$). VNS paired with successful trials on the lever pressing task resulted in a 159% increase in proximal forelimb representation in cholinergically intact rats compared to untrained controls (Fig. 2.4; NB+: 2.02 ± 0.19 mm^2; Naïve: 0.78 ± 0.19 mm^2; unpaired t-test, $p = 2.21 \times 10^{-4}$). Depletion of cortical cholinergic projections substantially reduced VNS-dependent map expansion. Rats with cholinergic lesions exhibited a 54% smaller proximal forelimb representation compared to cholinergically intact rats (NB-: 0.94 ± 0.37 mm^2; unpaired t-test vs. NB+, $p = 0.012$) and similar to that observed in untrained controls

(unpaired t-test NB- vs. Naïve; p = 0.693). These results indicate cholinergic innervation is necessary for VNS-dependent map plasticity in motor cortex.

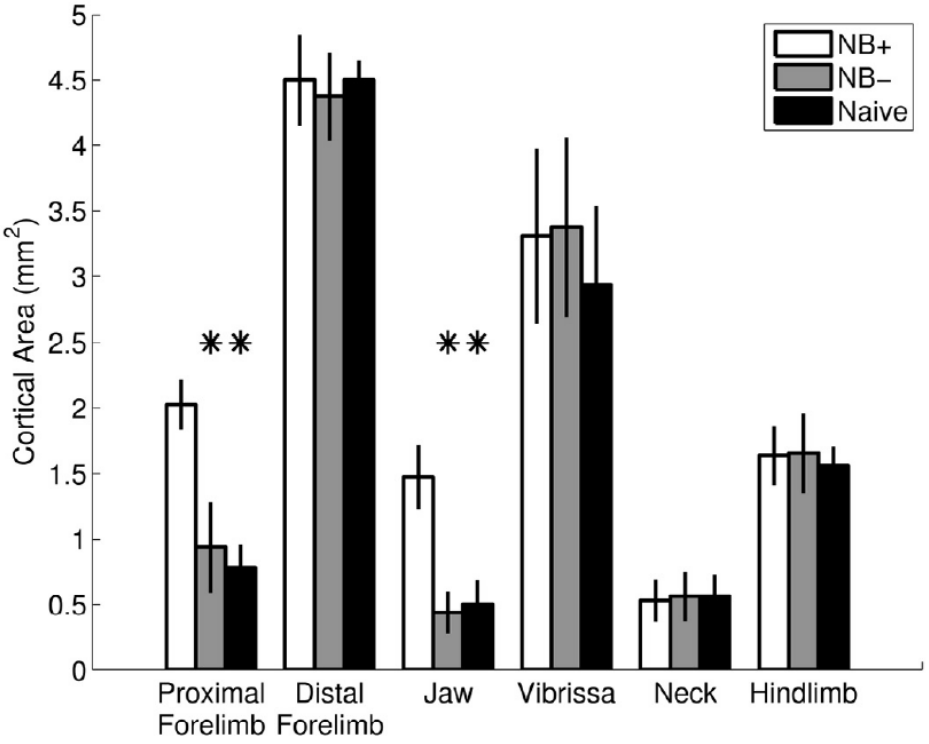

Figure 2.4 NB lesions prevent VNS-dependent motor cortex map reorganization. Total area of multiple movement representations in motor cortex. VNS paired with motor training in rats with an intact NB results in significantly greater proximal forelimb and jaw representations. NB lesions prevent VNS-dependent expansion of movement representations. Other movement representations are unchanged. * indicates $p < 0.05$ compared to NB+ group for each movement representation.

Jaw representation in the motor cortex was also altered by VNS paired with motor training. ANOVA on jaw representation revealed a significant effect of group (One-way ANOVA, $F[2,24] = 9.84$, $p = 7.56 \times 10^{-4}$). *Post hoc* comparison indicated that VNS paired with motor training drove a 234% expansion in jaw representation in the NB+ group compared to NB- group (Fig. 4; NB+: 1.47 ± 0.26 mm^2; NB-: 0.44 ± 0.17 mm^2; unpaired t-test, $p = 4.00 \times 10^{-3}$) and a 194% expansion

compared to untrained controls (Naïve: 0.50 ± 0.20 mm^2; unpaired t-test v. NB+, p = 7.50 x 10^{-3}). Jaw representation was similar in the NB- and Naïve groups (unpaired t-test NB- v. Naïve, p = 0.802), suggesting that the expansion of jaw map area is dependent on acetylcholine.

Pairing VNS with training on a lever pressing task did not alter of distal forelimb representations in any group (Fig. 2.4; One-way ANOVA, F[2,24] = 0.45, p = 0.646). There was also no difference in vibrissa, neck, or hindlimb representation between groups (One-way ANOVA, Vibrissa: F[2,24] = 1.55, p = 0.233; Neck: F[2,24] = 0.01, p = 0.986; Hindlimb: F[2,24] = 0.24, p = 0.790). The total area of motor cortex was not significantly different between groups (One-way ANOVA, F[2,24] = 2.52, p = 0.10). Consistent with previous reports, no differences in stimulation threshold were observed (One-way ANOVA, F[2,24] = 0.36, p = 0.70).

Depletion of cortical acetylcholine does not alter behavioral performance

Behavioral changes could potentially contribute to the observed differences in cortical representations. Prior to lesion or control surgery, there was no difference between groups in hit rate (Fig. 2. 5A; Pre; NB+: 67.7 ± 3.2%, NB-: 71.9 ± 3.7%; unpaired t-test, p = 0.39), number of trials per day (Fig. 2.5B; Pre; NB+: 398 ± 27 trials, NB-: 374 ± 44 trials; unpaired t-test, p = 0.645), or interpress interval (Fig. 2.5C; Pre; NB+: 404 ± 39 msec, NB-: 363 ± 41 msec; unpaired t-test, p = 0.49). Consistent with previous studies, NB lesions did not alter task performance group (Fig. 2.5; Post; Hit Rate: NB+: 64.4 ± 5.6%, paired t-test v. Pre, p = 0.53; NB-: 66.3 ± 5.6%, paired t-test v. Pre, p = 0.14; Trials per Day: NB+: 361 ± 34 trials, paired t-test v. Pre, p = 0.33; NB-: 341 ± 46 trials, paired t-test v. Pre, p = 0.48; Interpress Interval: NB+: 432 ± 54 msec, paired t-test v. Pre, p = 0.43; NB-: 409 ± 54 msec, paired t-test v. Pre, p = 0.24) [47]. No differences in performance were observed across groups after surgery (Post, NB+ v. NB-; unpaired t-test, Hit

Rate: p = 0.81; Trials per Day: p = 0.72; Interpress Interval: p = 0.77). Additionally, the number of stimulations received in each group was similar (Fig. 2.5D; NB+: 1084 ± 65; NB-: 1009 ± 96, unpaired t-test, p = 0.52). These results indicate that differences in task performance and amount of VNS cannot account for the observed differences in cortical representations.

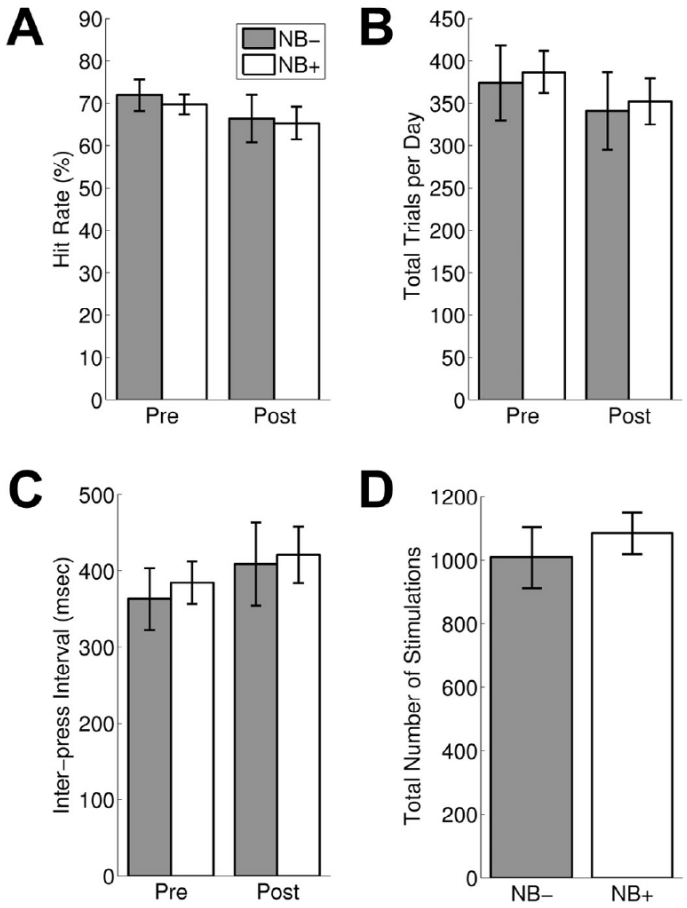

Figure 2.5 NB lesions do not change forelimb performance.
No differences in forelimb performance measures, including hit rate (A), total number of trials per day (B), or speed of lever presses (C), were observed between groups before or after NB lesion. Additionally, both groups received a similar number of VNS stimulations (D).

Discussion

Our previous study demonstrated that VNS paired with forelimb training enhanced map reorganization in the motor cortex [5]. The cholinergic nucleus basalis is a key substrate in training-dependent motor cortex map reorganization [36, 47, 48], and several lines of evidence suggest that VNS activates cholinergic circuitry in the basal forebrain [20, 29]. In this study, we examined whether the NB is required for the plasticity enhancing effects of VNS paired with motor training. VNS paired with motor training increased the proximal forelimb representation area in the motor cortex in rats with an intact NB. NB lesions prevent VNS-dependent expansion of proximal forelimb representation, demonstrating that the NB is required for the plasticity enhancing effects of VNS. No differences in forelimb performance were observed in either NB+ or NB- groups before or after lesion, excluding the possibility that differences in map representation may arise from differences in behavior. Together, the findings from this study demonstrate that the NB is required for VNS-dependent enhancement of plasticity in motor cortex.

VNS paired with training on a lever task in rats with an intact NB results in a larger representation of the proximal forelimb in the motor cortex, replicating the findings of a previous study [5]. Area corresponding to proximal forelimb is increased without altering the representation of the distal forelimb. This likely reflects the greater dependence on the proximal musculature compared to distal musculature to perform this lever pressing task [33]. Our previous study using a similar design demonstrated that lever training without VNS did not result in a measurable increase in proximal forelimb representation compared to untrained controls, indicating the importance of VNS paired with training to drive increased proximal representations [5]. The current study does not incorporate this lever-trained control group that did not receive VNS;

therefore, while unlikely, we cannot directly rule out the possibility that proximal forelimb expansion was a result of training alone. NB lesions prevented VNS-dependent expansion of proximal forelimb representations. The lesion method employed in this study using 192-IgG-saporin selectively lesions cortically-projecting cholinergic neurons and leaves surrounding neurons intact [44, 45], resulting in a specific depletion of cortical cholinergic innervation in the NB- group. This suggests that the cortical cholinergic innervation from the NB is required for VNS-dependent enhancement of plasticity, and that other neuromodulatory systems engaged by VNS cannot substitute for the loss of cholinergic input.

The proximal forelimb representations observed in this study are slightly smaller than those reported in some previous studies [49-51]. Differences in representational area observed across studies could arise from a variety of sources. The lower frequency stimulation train used to evoke movement during ICMS in the present study could in part account for disparity in proximal forelimb area. Additionally, differences in the amount of time between the beginning of behavioral training and ICMS and the specific behavioral training paradigm used may contribute. The proximal representations observed in this study are similar those reported in our previous study using the same design, suggesting the experimental conditions specified in this design yield consistent representations [5].

Map expansion in rats with an intact NB was not totally restricted to proximal forelimb area. We observed a significant increase in the representation of the jaw in rats with an intact NB that received VNS. As the timing between an event and VNS pairing is critical for map reorganization [2], the increase in jaw representation may arise from the fact that rats would receive VNS during a lever press and immediately afterwards (<2 seconds, on average) eat a reward pellet.

It is likely that the close temporal approximation of chewing the pellet and VNS was sufficient to enhance the representation of the jaw. The absence of an increase in jaw area in rats with NB lesions provides further support that cholinergic innervation is required for VNS-dependent map expansion.

The absence of differences in behavioral performance between the NB+ and NB- groups before and after VNS in the present study excludes the possibility that alterations in behavior arising from NB lesion or imbalanced experimental groups could account for differences in map plasticity. Lesion of the NB did not affect performance of the trained task, as demonstrated previously [34, 47]. Moreover, VNS paired with training did not enhance or impair task performance. Map reorganization despite an absence of change in motor performance is consistent with the notion that map plasticity may support learning but is unnecessary in the performance of a learned task [52-54]. It remains to be determined whether pairing VNS with motor training during early stages of acquisition of a motor task could speed learning. Future studies should evaluate the role of VNS-dependent enhancement of map plasticity in the context of known behavioral changes, such as improvement of motor recovery after brain injury driven by VNS paired with rehabilitative training [8-12].

The attenuation of map reorganization resulting from NB lesions observed in this study suggests that cortical cholinergic innervation is required for VNS-dependent enhancement of plasticity. However, these findings do not rule out the importance of other neuromodulatory systems in VNS-dependent plasticity. Several previous studies have indicated that the noradrenergic system is involved in the effects of VNS [23-28, 55]. Norepinephrine and acetylcholine often act synergistically to influence plasticity [32]; therefore, it is possible that both

the noradrenergic and cholinergic systems contribute to VNS-dependent plasticity. Delineation of the complex interaction of neuromodulatory pathways engaged by VNS may be required to understand the effect of VNS therapies.

The neuronal mechanisms engaged by VNS to promote plasticity are unknown. VNS and acetylcholine have been implicated in a variety of synaptic changes associated with cortical map reorganization [56]. LTP of connections in motor cortex is believed to underlie in part the increase in representational area resulting from training dependent plasticity [57, 58]. Several studies report that VNS enhances the induction of LTP [59-61]. Similarly, activation of muscarinic acetylcholine receptors facilitates induction of LTP [62, 63]. While these studies are restricted to hippocampus, it is possible that similar mechanisms are activated in the cortex in response to VNS. This convergent control of LTP may in part underlie VNS-dependent enhancement of map plasticity.

Based on the robust, specific enhancement of plasticity driven by VNS, a number of targeted plasticity therapies using VNS paired with rehabilitative training have been developed to support recovery after neurological injury and disease. VNS paired with rehabilitative regimens significantly improves recovery in animal models of chronic tinnitus, ischemic stroke, intracerebral hemorrhage, and traumatic brain injury [2, 8-12]. Moreover, pilot trials evaluating VNS therapies in patients have demonstrated reduced handicap in chronic tinnitus patients [64-66]. As these therapies are translated to the broader clinical population in larger trials, it is critical to identify conditions that interfere with the efficacy of VNS. The cholinergic system is affected by a number of common pharmaceuticals and pathologies. As such, cholinergic transmission will likely be at least partially compromised in many patients, which may consequently occlude the benefits of VNS therapy. Indeed, in a pilot study evaluating VNS therapy for chronic tinnitus, a

subset of patients were taking drugs that in part altered cholinergic and noradrenergic transmission. Patients on the drugs failed to improve, while those who were not on drugs demonstrated a significant reduction of tinnitus intensity and distress [66]. While more testing is required to provide a direct demonstration, these findings suggest that alterations of neuromodulatory transmission may occlude the effect of VNS therapy. The absence of VNS-dependent plasticity after NB lesion in this study suggests that further studies should evaluate whether cholinergic lesions prevent the benefits of VNS therapy in models of neurological disease. The delineation of the neuromodulatory pathways engaged by VNS therapy will provide insight into the mechanisms that underlie the benefits of VNS therapy and is critical to the successful translation of VNS therapy.

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CHAPTER 3

PARAMETRIC CHARACTERIZATION OF NEURAL ACTIVITY IN THE LOCUS COERULEUS IN RESPONSE TO VAGUS NERVE STIMULATION

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Parametric characterization of neural activity in the locus coeruleus in response to vagus nerve stimulation
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Abstract

Vagus nerve stimulation (VNS) has emerged as a therapy to treat a wide range of neurological disorders, including epilepsy, depression, stroke, and tinnitus. Activation of neurons in the locus coeruleus (LC) is believed to mediate many of the effects of VNS in the central nervous system. Despite the importance of the LC, there is a dearth of direct evidence characterizing neural activity in response to VNS. A detailed understanding of the brain activity evoked by VNS across a range of stimulation parameters may guide selection of stimulation regimens for therapeutic use. In this study, we recorded neural activity in the LC and the mesencephalic trigeminal nucleus (Me5) in response to VNS over a broad range of current amplitudes, pulse frequencies, train durations, inter-train intervals, and pulse widths. Brief 0.5 s trains of VNS drive rapid, phasic firing of LC neurons at 0.1 mA. Higher current intensities and longer pulse widths drive greater increases in LC firing rate. Varying the pulse frequency substantially affects the timing, but not the total amount, of phasic LC activity. VNS drives pulse-locked neural activity in the Me5 at current levels above 1.2 mA. These results provide insight into VNS-evoked phasic neural activity in multiple neural structures and may be useful in guiding the selection of VNS parameters to enhance clinical efficacy.

Introduction

More than 75,000 patients have received vagus nerve stimulation (VNS) therapy for the treatment of epilepsy and depression (Schlaepfer et al., 2008, Englot, Chang and Auguste., 2011, Berry et al., 2013, Ben-Menachem et al., 2015). Emerging studies provide evidence that VNS paired with rehabilitative training may be useful in the treatment of additional neurological disorders, including tinnitus and stroke (Dawson et al., 2016, De Ridder et al., 2014, Hays., 2016). Despite the widespread use of VNS, there is relatively little consensus on the optimal stimulation methods, perhaps owing to incomplete knowledge of the effects of VNS on structures throughout the brain. Therefore, a detailed understanding of the effects of VNS on neural activity in key structures may guide selection of stimulation parameters to maximize therapeutic benefits.

The noradrenergic locus coeruleus (LC) has been identified as a key mediator of VNS actions in the central nervous system. LC lesions block both the antiepileptic and antidepressant-like effects of VNS, demonstrating the requirement of noradrenergic engagement (Krahl et al., 1998, Grimonprez et al., 2015, Furmaga, Shah and Frazer., 2011). Moreover, 30 second trains of VNS increase firing rates of LC neurons over the course of minutes to hours (Groves, Bowman and Brown., 2005, Manta et al., 2009a, Manta et al., 2013, Dorr and Debonnel., 2006). Similar activation of brain structures, including the LC, is observed in human subjects minutes after delivery of VNS (Frangos, Ellrich and Komisaruk., 2015). Consistent with these actions on LC activity, VNS increases norepinephrine concentrations in the cortex and hippocampus on the order of minutes to hours (Hassert, Miyashita and Williams., 2004, Roosevelt et al., 2006, Follesa et al., 2007). Elevated norepinephrine is correlated with VNS-dependent seizure suppression, potentially linking LC activation to clinical efficacy (Raedt et al., 2011). Patients receiving VNS for epilepsy

control demonstrate cumulative benefits after several months of stimulation (DeGiorgio et al., 2000, Ching et al., 2013), providing support for the notion that VNS promotes long-lasting changes to suppress seizures.

In addition to these protracted effects, there is accumulating evidence that VNS rapidly activates structures in the central nervous systems in milliseconds to seconds. The vagus nerve innervates the nucleus tractus solitarius, which sends excitatory input to the LC via the nucleus paragigantocellularis, providing a pathway by which VNS could directly drive short latency spiking in the LC (Ruffoli et al., 2011). Indirect evidence from measures of cortical excitability suggests that VNS-dependent activation of neuromodulatory circuits rapidly influences cortical activity. Within 10 milliseconds of stimulation, VNS triggers scalp-recorded evoked potentials, reflecting ascending neural activation (Usami et al., 2013). Moreover, VNS modulates cortical synchrony via activation of the cholinergic system within 100 milliseconds of stimulation (Nichols et al., 2011). A recent study indicates that this rapid activation is required for VNS-dependent enhancement of plasticity (Engineer et al., 2011). Delivery of 0.5 s trains of VNS coincident with tones drives robust plasticity in auditory cortex that is specific to frequency of the paired tone. However, equivalent VNS delivered 15 s before or after tones fails to drive plasticity, indicating that VNS engenders rapid, phasic neural activation to support plasticity. Despite its potential importance in the functional consequences of VNS, little is known about the rapid action of VNS on neural activity in relevant brain structures. A detailed understanding of the rapid modulation of activity may lead to the development of optimized stimulation protocols that capitalize on these temporal patterns.

A clear understanding of stimulation intensity-dependent modulation of activity is also critical to maximizing the effects of VNS. Studies evaluating the memory- and plasticity-enhancing effects of VNS across a range of stimulation parameters report an inverted-U response, in which moderate intensity stimulation yields a greater effect than lower or higher intensities (Clark et al., 1998, Clark et al., 1999, Borland et al., 2016). One plausible explanation for the inverted-U response is VNS-dependent activation of a low threshold system that promotes plasticity and an overriding high threshold system that occludes plasticity. The majority of parameter optimization efforts have focused on driving greater activity in target structures, including the LC (Manta et al., 2009b). This has proven informative in the context of seizure suppression, in which stronger VNS paradigms appear to yield greater suppression (Ghani et al., 2015). However, the complex inverted-U effect of VNS on plasticity suggests that minimization of off-target responses may be equally useful and necessary to maximize therapeutic effects.

Materials and Methods

Subjects

The University of Texas at Dallas Institutional Animal Care and Use Committee approved all procedures. Female Sprague Dawley rats (Charles River), weighing 310 ± 11g, were used in all experiments. Rats were housed in a 12:12 hr reversed light cycle environment with ad libitum access to food and water.

Surgical Procedures

Rats were anesthetized with ketamine hydrochloride (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). Supplemental doses were administered as needed to maintain anesthesia throughout surgical procedures and data collection. Carprofen (5mg/kg, s.c.) was administered to reduce

inflammation. Body temperature was maintained throughout surgery and neural recording using a feedback-controlled electric warming pad (FHC, Bowdoin, ME). Subjects were implanted with a custom made platinum-iridium bipolar stimulating cuff electrode on the left cervical vagus nerve, as previously described (Engineer et al., 2011, Khodaparast et al., 2013, Khodaparast et al., 2014, Hays et al., 2014, Pruitt et al., 2016, Khodaparast et al., 2016, Hays et al., 2016, Hulsey et al., 2016). A transient drop in blood oxygen saturation in response to a short (~3 sec) VNS train was used to confirm that the cuff electrode was functional. Immediately after cuff implantation, subjects were positioned in a stereotaxic frame with bregma and lambda level. After exposing the surface of the skull, a hole was drilled centered at 1.1mm lateral and 3.6mm caudal to lambda and the underlying dura was carefully removed (George et al., 2013).

Electrophysiological Recordings in LC and Me5

Extracellular recording was performed with parylene coated tungsten microelectrodes (2-4 MΩ, FHC). Two electrodes (250 μm spacing) were lowered approximately 5.5-6.5 mm ventral from the dural surface at a 15 degree angle from the vertical axis until neural activity with appropriate response characteristics (described below) was observed. Neural signals were differentially amplified using an RA16PA preamplifier (Tucker-Davis Technologies, Alachua, FL) from a common reference and ground attached to the skin around the skull. Signals were digitized at 24.414 ks/s with 16-bit resolution using an RZ5 BioAmp processor (Tucker-Davis Technologies) and monitored online with Brainware. LC units were identified by a characteristic response to a hindpaw pinch of a phasic burst of spikes followed by inhibition, long duration positive-negative waveforms with a notch on the ascending phase, and spontaneous firing rate (Martins and Froemke., 2015) (Fig. 3.1). The Me5 nucleus is located lateral to the LC and was

identified by high frequency firing, accompanied by burst firing upon manipulation of the jaw (Linden., 1978) (Fig. 3.6A). Multi-unit recordings were made at sites identified as LC or Me5. Electrophysiological recording sweeps were 4.5 s in duration, and were initiated every 8 s (except as noted). One second of spontaneous activity was recorded prior to VNS presentation. A subset of recording sites contained readily identifiable single units. After complete stimulus set presentation at a site, the electrodes were advanced at least 100 μm and a new recording site was identified. In a subset of animals, electrolytic lesions were made at the final recording location to confirm electrode position (Fig 3.1D).

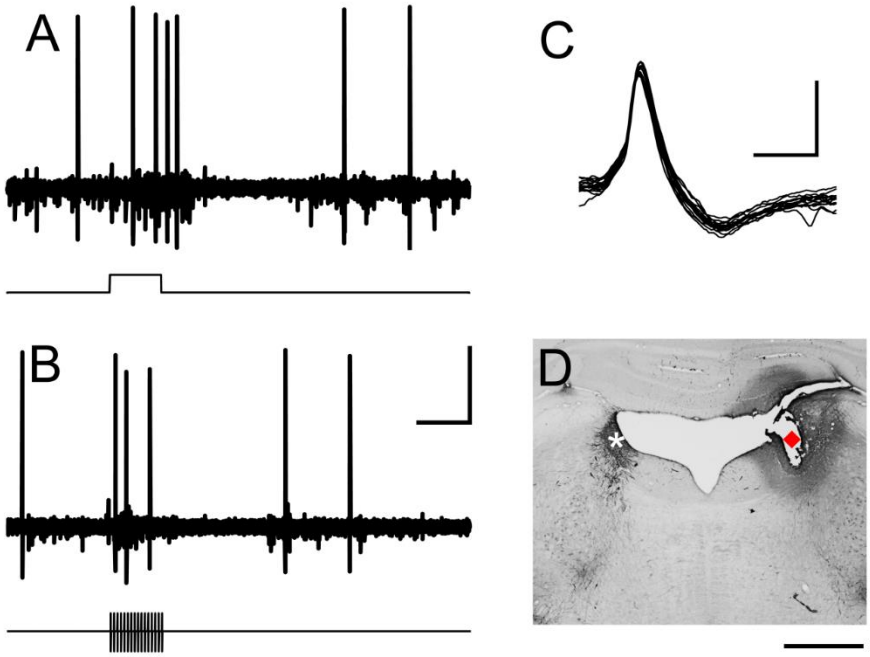

Figure 3.1 Identification of neurons in locus coeruleus.
(A) Recording sites in the LC were characterized by a brief increase in firing rate followed by a suppression in response to a hindpaw pinch (denoted by line below panel). (B) Brief trains of VNS elicited driven activity in LC neurons. (C) Characteristic wide spike shape was observed in well-isolated LC units. (D) Example histological verification of LC recording site. White * marks TH-positive neurons in LC contralateral to the recording site. Red diamond marks the electrolytic lesion location. Scale bar is 250 μV x 500 ms in panels A&B; 500 μV x 1 ms in C; 1 mm in D.

Vagus Nerve Stimulation

VNS was delivered through a constant current stimulus isolation unit (Model 2200, A-M Systems). The vagus nerve was stimulated at standard parameters of sixteen 0.8 mA 100 μs biphasic pulses at 30 Hz (Engineer et al., 2011, Porter et al., 2011, Borland et al., 2016, Hulsey et al., 2016). In addition, a broad stimulus set was delivered with varying pulse number per train (0 – 64 pulses), stimulation current intensity (0 – 2.5 mA), pulse frequency (0 – 120 Hz), and pulse width (0 – 500 μs) totaling 23 distinct stimulation parameters (Tables 3.1-3.3). Every VNS parameter was presented 20 times in a pseudorandom, interleaved order at each recording site. The main recording protocol lasted approximately 40 minutes at each site. At a subset of sites, standard parameter stimulation was delivered with 5 and 30 second inter-train intervals. Cuff voltage was monitored throughout data collection procedures.

Data Analysis and Statistical Methods

Data was processed using custom MATLAB software (MathWorks). Electrical stimulation artifact was removed from data by linearly interpolating between data points 0.2 ms before and 3 ms after each pulse in a VNS train. The neural signal was bi-directionally band pass filtered between 300-3000Hz. Spike activity for multiunit responses were automatically detected by positive crossings of a threshold initially set at 2.58 times greater than the standard deviation (99 percent confidence interval) of the signal for the entire stimulus set and adjusted as necessary to distinguish spiking activity. Spike data was sorted by VNS parameter and a mean peristimulus time histogram (PSTH) was generated with 50 ms bins. Firing rate in Hz was calculated by summing all spikes per 50 ms bin and dividing by bin time in seconds. Phasic excitatory responses were calculated as driven spikes (sum of spikes at a given parameter – sum spontaneous spikes)

Table 3.1 Range of Pulse Numbers Tested

Intensity (mA)	Pulse Width (μs)	Number of pulses	Frequency (Hz)
0	0	0	0
0.8	100	4	30
0.8	**100**	**16**	**30**
0.8	100	64	30

Table 3.2 Range of Current Intensities Tested

Intensity (mA)	Pulse Width (μs)	Number of pulses	Frequency (Hz)
0	0	0	0
0.1	100	16	30
0.2	100	16	30
0.4	100	16	30
0.8	**100**	**16**	**30**
1.2	100	16	30
1.6	100	16	30
2.5	100	16	30

Table 3.3 Range of Frequencies Tested

Intensity (mA)	Pulse Width (μs)	Number of pulses	Frequency (Hz)
0	0	0	0
0.8	100	16	7.5
0.8	100	16	15
0.8	**100**	**16**	**30**
0.8	100	16	60
0.8	100	16	120

Standard parameters are represented in bold text

Table 3.4 Range of Pulse Widths Tested

Intensity (mA)	Pulse Width (μs)	Number of pulses	Frequency (Hz)
0	0	0	0
0.2	100	16	30
0.2	500	16	30
0.4	100	16	30
0.4	500	16	30
0.8	30	16	30
0.8	**100**	**16**	**30**
0.8	500	16	30
1.6	30	16	30
1.6	60	16	30
1.6	100	16	30

Standard parameters are represented in bold text

Data Analysis and Statistical Methods

Data was processed using custom MATLAB software (MathWorks). Electrical stimulation artifact was removed from data by linearly interpolating between data points 0.2 ms before and 3 ms after each pulse in a VNS train. The neural signal was bi-directionally band pass filtered between 300-3000Hz. Spike activity for multiunit responses were automatically detected by positive crossings of a threshold initially set at 2.58 times greater than the standard deviation (99 percent confidence interval) of the signal for the entire stimulus set and adjusted as necessary to distinguish spiking activity. Spike data was sorted by VNS parameter and a mean peristimulus time histogram (PSTH) was generated with 50 ms bins. Firing rate in Hz was calculated by summing all spikes per 50 ms bin and dividing by bin time in seconds. Phasic excitatory responses were calculated as driven spikes (sum of spikes at a given parameter – sum spontaneous spikes)

from 1-750 ms after stimulation onset for all 500 ms VNS trains. An offset response was calculated from 751-1500ms as percent change from the spontaneous rate. For experiments evaluating the effects of varying VNS frequency, driven spikes were calculated based on positive response periods for each stimulus. Spike data during stimulation artifact cutout was interpolated to normalize cutout duration across frequencies. A cycle histogram of spiking activity after each pulse was created and vector strength calculations were used to quantify the degree of synchronization between VNS pulse timing and neural spiking activity (Shetake et al., 2011). For vector strength analysis, a value of 1 indicates perfect synchronization and 0 indicates no synchronization to VNS pulses. Repeated measures analysis of variance (ANOVA) followed by paired t-tests (Bonferroni corrected to an alpha of 0.007) were used where appropriate to determine significant differences.

Histology

Upon completion of daily recording, an electrolytic lesion was made by delivering current through one of the electrodes at the final recording site (1 mA current, 30 s). Immediately after electrolytic lesion, animals were transcardially perfused with phosphate buffered saline followed by 4% paraformaldehyde in phosphate-buffered saline (PBS). Brains were post-fixed for at least 24 hours and transferred to 20% sucrose solution for cryoprotection. The extent of the LC was sectioned with 40 μm sections. Sections were stained for tyrosine hydroxylase (TH) to identify the LC. In brief, sections were incubated in a permeabilization buffer of 0.3% triton in PBS, followed by a quenching solution of 0.3% H_2O_2 in methanol. After washing in PBS, sections were incubated in a blocking solution of 5% normal horse serum at room temperature for 1 hour. Tissue was then transferred to primary TH antibody (Cat # AB152, EMD Millipore) at a 1:1000 dilution and

incubated overnight at 4°C. The following day, sections were processed with horseradish peroxidase substrate (Vectastain ABC Elite, Vectorlabs) and stained with diaminobenzidine (ImmPACT DAB, Vectorlabs). Sections were then mounted on microscope slides dried, and cover slipped using DPX mounting medium (Cat #13512, EMS). TH staining and lesions were visualized using an Olympus BX51 microscope and imaged at 4x magnification using an Olympus DP71 acquisition system.

Results

VNS drives activity in LC neurons

LC neurons were identified according to stereotaxic location and well-validated electrophysiological characteristics: wide spike widths and consistent excitation-inhibition pattern in response to a hindpaw pinch (Martins and Froemke., 2015) (Fig. 3.1A & C). Electrode placement was histologically verified in a subset of animals (Fig. 3.1D). Electrophysiological recordings were made from 23 sites across 5 animals. A 0.5 s train of 0.8 mA, 100 μs biphasic pulses delivered at 30 Hz reliably evoked rapid, robust phasic neural activity in the LC (Fig. 3.1B & 3.2). Peak firing rate of the phasic response was increased approximately 450% over spontaneous firing rate. All LC recording sites (23 of 23) demonstrated significant increases in firing rate with these stimulation parameters. Longer train durations resulted in a linear increase in driven spikes (Table 3.1; Fig. 3.2C; Repeated measures ANOVA, $F[3,66] = 74.22$, $p < 0.0001$; 4 pulses: 14.5 ± 1.9 spikes, 16 pulses: 41.3 ± 4.2 spikes, 64 pulses: 131.2 ± 15.0 spikes, paired t-tests vs. spontaneous, all $p < 0.0001$). Similar increases in firing rate were observed for 5 and 30

second inter-train intervals (5 s ITI: 53.2 ± 5.8, 30 s ITI: 54.6 ± 9.4; Paired t-test, p = 0.69). These findings indicate that short trains of VNS drive rapid, phasic neural activity in the LC.

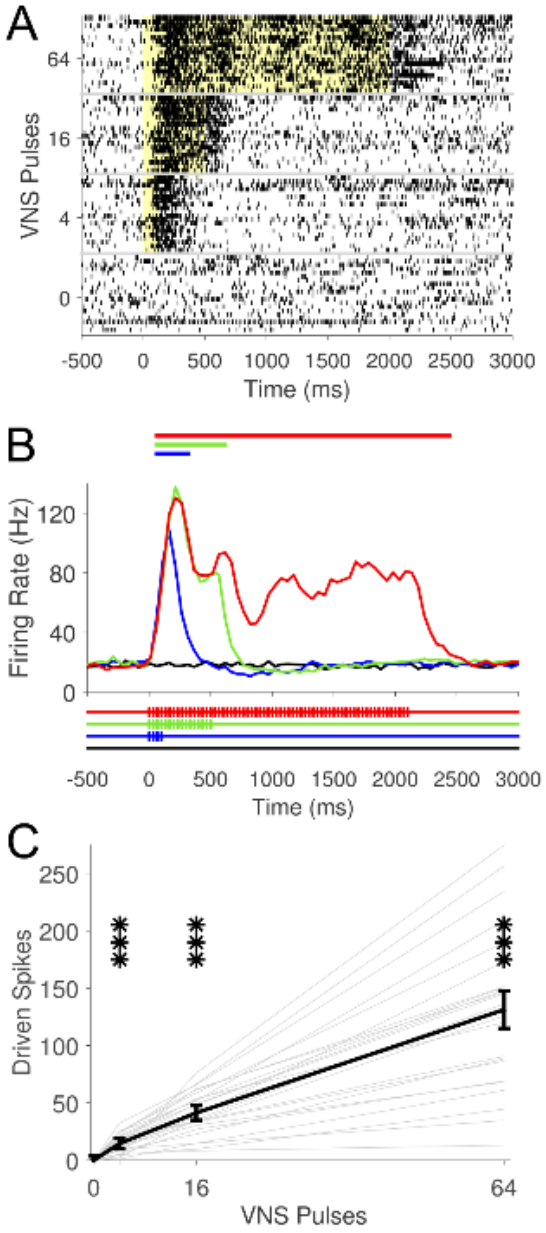

Figure 3.2 VNS drives rapid, phasic neural activity in LC.
(A) Example raster plot showing representative neural activity at one recording location in LC in response to 4, 16, and 64 pulse trains of VNS at 0.8 mA, 100 μs at 30 Hz. Yellow background denotes stimulation period. (B) Population PSTH of neural responses to 4 (blue), 16 (green), and

64 (red) pulses of VNS at 30 Hz. Colored lines above the PSTH represent significant positive driven response duration. VNS pulse timing is illustrated below PSTH. (C) Longer VNS train durations result in linear increases in number of driven spikes. *** $p < 0.001$; all statistical comparisons versus spontaneous rate.

Effects of stimulation intensity on LC neural activity

Stimulation intensity influences VNS-dependent norepinephrine release, neural plasticity, memory enhancement, and clinical seizure suppression, suggesting that neural activity in the LC activity is modulated by VNS intensity (Clark et al., 1995, Clark et al., 1999, Roosevelt et al., 2006, Zuo, Smith and Jensen., 2007a, Ghani et al., 2015, Borland et al., 2016). We examined LC firing rate across a range of VNS stimulation intensities from 0.1 to 2.5 mA, while holding other parameters constant (Fig 3.3A; Table 3.2). Firing rate was slightly, but significantly, increased at a stimulation intensity of 0.1 mA, consistent with recruitment of A and B fibers (Groves and Brown., 2005) (Fig. 3.3C&D; 0 mA: 13.7 ± 1.3; 0.1 mA: 20.8 ± 2.1; Paired t-test v. spontaneous, $p < 0.0001$). Driven activity increased monotonically across the range of stimulation intensities and was significantly increased at each tested intensity above 0.1 mA (Fig. 3.3D&E; Repeated measures ANOVA, $F[7,154] = 126.44$, $p < 0.0001$; Paired t-tests, 0 mA v. 0.2 - 2.5mA, all $p < 0.001$). Increasing stimulation intensity resulted in a shorter latency to the onset of significantly driven activity (Fig 3.3E). Well-isolated single units in a subset of electrophysiological recordings exhibited a similar monotonically increasing firing rate in response to greater stimulation intensities (Fig. 3.3A&B; Repeated measures ANOVA, $F[7,133] = 30.29$, $p < 0.0001$; Paired t-tests, 0 mA v. 0.4 - 2.5mA, all $p < 0.001$). An offset response (751 – 1500 ms) was observed that displayed a modest, non-monotonic change in firing rate with increasing current (Fig. 3.3E inset). Stimulation intensities from 0.2 – 0.8 mA resulted in a 20% suppression of neural activity

compared to spontaneous rate during the offset response, while stimulation intensities at 1.6 mA and 2.5 mA demonstrated a 30% increase in firing rate (Fig. 3.3F). These findings indicate LC neurons are engaged by VNS at low thresholds and that increasing stimulation intensities drive greater phasic neural activity.

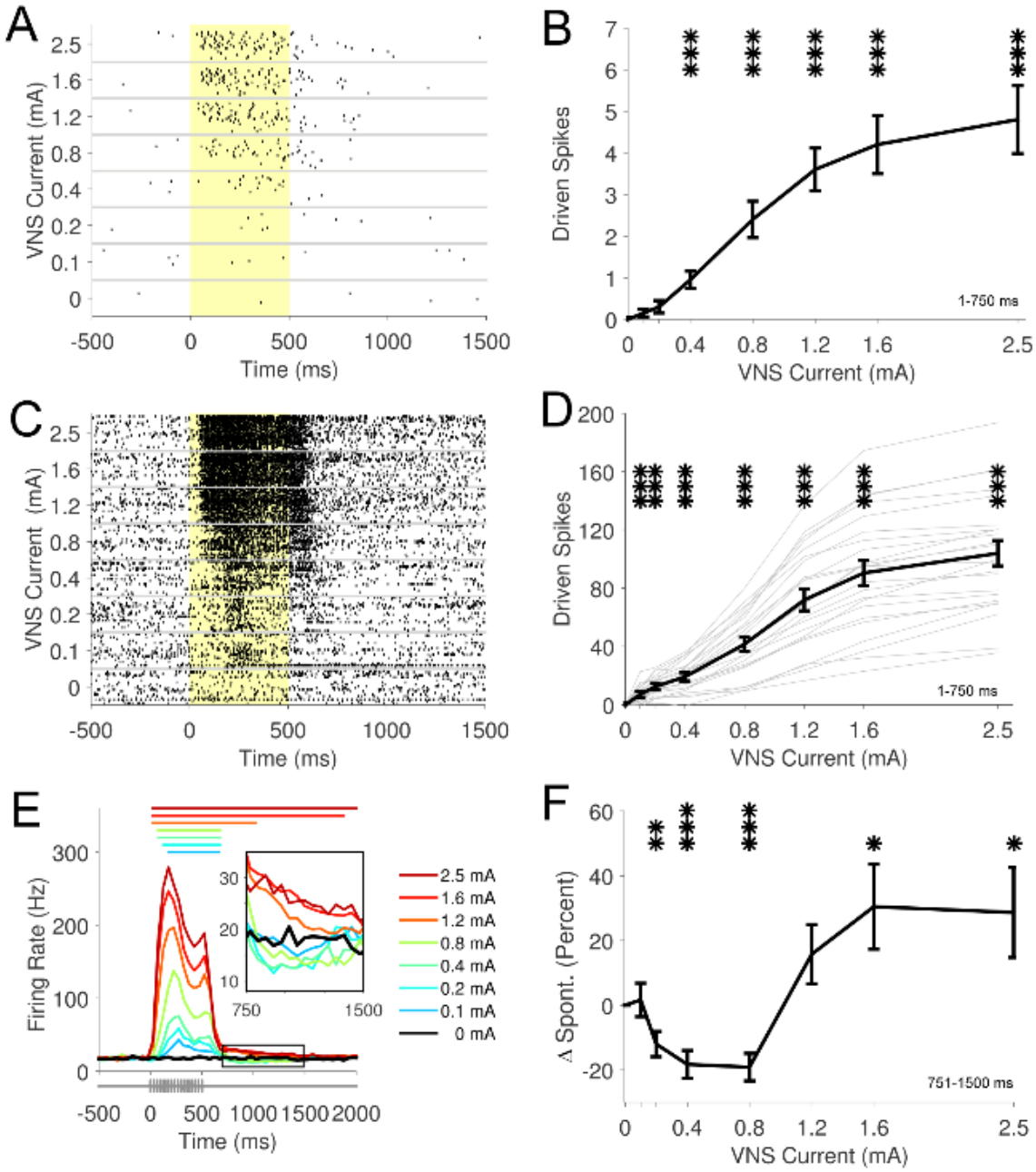

Figure 3.3 Increasing stimulation intensities drive greater phasic neural activity in LC. (A) Example neural activity from a well-isolated single unit across a range of current intensities. The yellow shaded region denotes stimulation period. (B) Average driven spikes for a single unit across 20 recording sweeps between 1-750 ms response period. (C) Example multiunit recording showing phasic driven activity (1-750 ms response period) across a range of current intensities. (D) Analysis of group data of the phasic driven response (1-750 ms response period) demonstrates significant increases in driven activity at 0.1 mA stimulation intensity. Stronger current intensities drive greater increases in firing rate. Bold black line represents group average across 23 sites. Thin gray lines represent data from individual sites. (E) PSTH illustrates monotonic increases in phasic response across stimulation intensities. Colored lines above the PSTH represents significant positive driven response duration. VNS pulse timing represented below. Inset highlights offset response from 751 – 1500 ms. (F) Offset responses (751 – 1500 ms) demonstrate a modest suppression of neural activity compared to spontaneous at intensities from 0.2 – 0.8 mA and an modest increase compared to spontaneous at 1.6 mA and 2.5 mA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; all statistical comparisons versus 0 mA (spontaneous rate).

Effects of pulse frequency on LC neural activity

We next sought to determine the effect of varying the frequency of pulses within a stimulation train on neural activity in the LC. Stimulation frequency was varied from 7.5 Hz to 120 Hz, while all other parameters, including number of pulses per train, were held constant (Fig. 3.4; Table 3.3). Firing rate was significantly increased at all frequencies tested (Fig. 3.4C; Repeated measures ANOVA, $F[5,110] = 73.47$, $p < 0.0001$; Paired t-tests, 0 Hz v. 7.5 – 120 Hz, all $p < 0.001$). The temporal profile of LC activity reveals that higher stimulation frequencies result in greater maximal discharge rates over a shorter duration (Fig. 3.4B). However, the total number of driven spikes in response to a VNS train was similar at most frequencies (Fig. 3.4C). A slight, but significant reduction in total driven spikes was observed at 120 Hz compared to 30 Hz (Paired t-test, 30 Hz v. 120 Hz, $p < 0.0001$). These results suggest that, for a fixed number of pulses, varying VNS frequency affects the timing, but not total amount of LC activity.

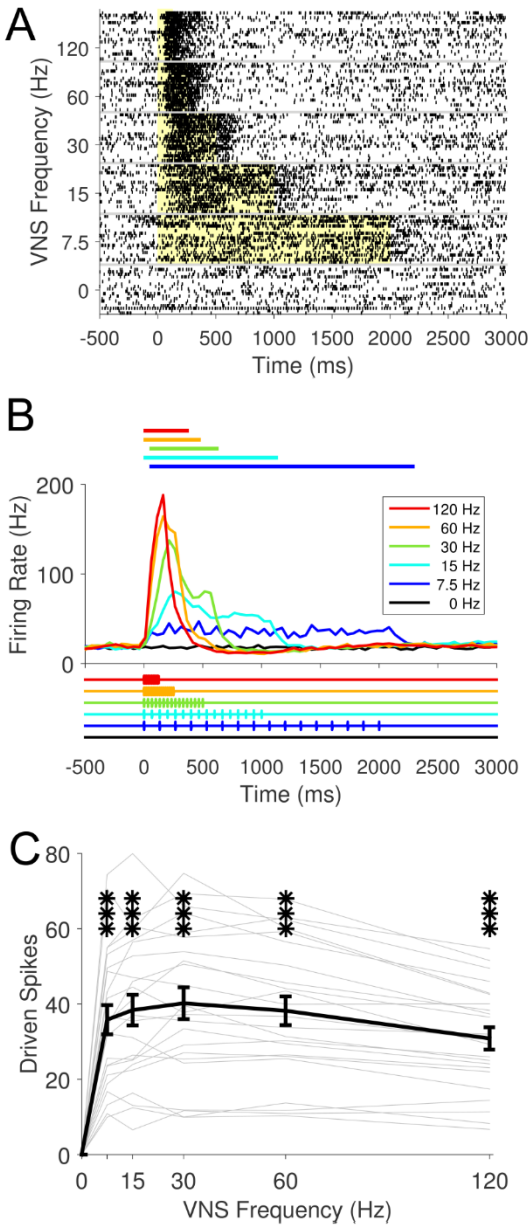

Figure 3.4 Frequency changes the timing, but not total amount of VNS-driven neural activity in LC.

(A) Example raster plot from a single recording site across a range of frequencies. The yellow shaded region denotes stimulation period. (B) PSTH of population data illustrates that the timing and maximal rate of driven activity is influenced by pulse frequency. Colored lines above the PSTH represents significant positive driven response duration. VNS pulse timing represented below. Note that the number of pulses was matched across conditions. Higher frequencies drive stronger, shorter neural activity for a fixed number of pulses. (C) At all frequencies tested, VNS drives significant increases in neural activity. *** $p < 0.001$; all statistical comparisons versus 0 Hz (spontaneous rate).

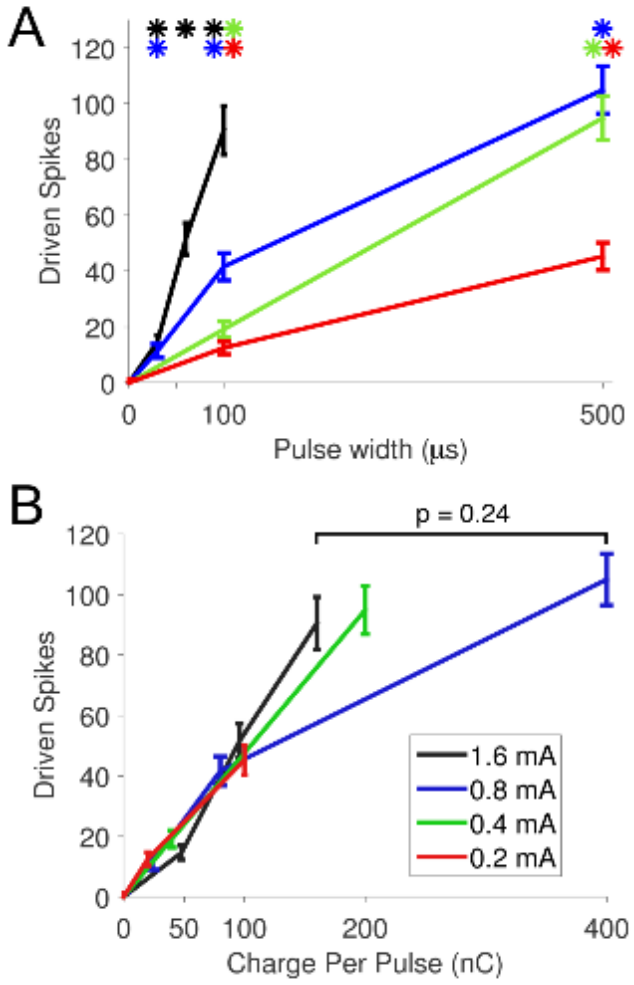

Figure 3.5 Increasing pulse widths drive greater neural activity in LC.
(A) At each current intensity, increasing pulse widths drive greater neural activity in LC neurons. (B) Driven spikes in the LC increase approximately linearly as a function of total charge per pulse (pulse width x current) up to 160 nC. After this point, additional charge results in diminishing increases in neural activity. Line colors in legend apply to both panels. * $p < 0.001$; all statistical comparisons in panel A versus 0 µs (spontaneous rate).

Effects of pulse width on LC neural activity

Pulse width influences tolerability and efficacy of VNS therapy; therefore, identification of parameters that maximize LC activity at minimal pulse widths may guide selection of clinical

parameters (Liporace et al., 2001, Heck, Helmers and DeGiorgio., 2002). Pulse width was varied from 30 μs to 500 μs at various current intensities while all other parameters were held constant (Table 3.4). At a stimulation intensity of 0.8 mA, pulse widths of 30 μs and greater resulted in significantly driven, monotonically increasing neural activity (Fig. 3.5A; Repeated measures ANOVA, $F[3,66] = 155.57$, $p < 0.0001$; Paired t-tests, 0 μs v. 30 – 500 μs, all $p < 0.0001$). Similarly, longer pulse widths drove significantly greater firing rates at all current intensities tested (Fig. 3.5A; Repeated measures ANOVA, 0.2 mA: $F[2,44] = 96.23$, $p < 0.0001$; Paired t-tests, 0 μs v. 100, 500 μs, all $p < 0.0001$; 0.4 mA: $F[2, 44] = 154.36$, $p < 0.0001$; Paired t-tests, 0 μs v. 100, 500 μs, all $p < 0.0001$; 1.6 mA: $F[3,66] = 107.78$, $p < 0.0001$; Paired t-tests, 0 μs v. 30, 60, and 100 μs, all $p < 0.0001$). Evaluation of driven activity as a function of total charge per pulse (pulse width x current intensity) allowed direct comparison of parameter sets with different pulse widths and intensities. Independent of current intensity and pulse width, LC activity increases approximately linearly up to an apparent plateau around 160 nC per pulse, after which greater charge delivery does not yield significantly increased LC activity (Fig. 3.5B; 160 nC vs. 400 nC; Paired t-test, $p = 0.24$).

Neural activity in the mesencephalic trigeminal nucleus (Me5) in response to varying VNS parameters

There is growing evidence that the beneficial effects of VNS are limited to moderate current levels (Clark et al., 1995, Clark et al., 1998, Clark et al., 1999, Zuo, Smith and Jensen., 2007b, Borland et al., 2016). It has been proposed that higher current levels may activate responses in other brain regions that limit the effective range of VNS. To test this hypothesis, we compared VNS-dependent activity in the LC to activity in the neighboring mesencephalic trigeminal nucleus

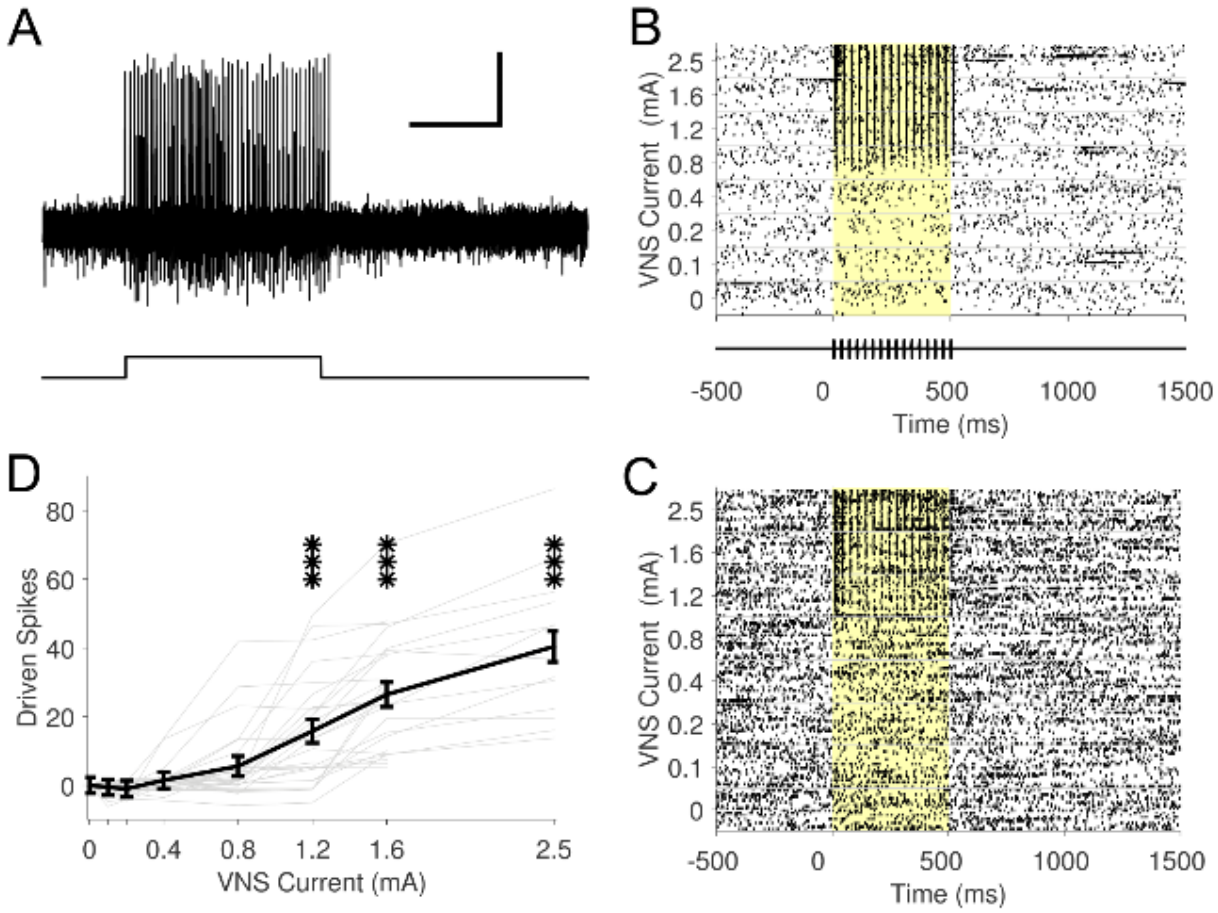

Figure 3.6 VNS drives pulse-locked activity in Me5 neurons.
(A) Example Me5 activity demonstrating characteristic increase in firing rate to movement of the jaw (denoted by line). (B & C) Raster plots of representative neural activity from two Me5 recording sites illustrate the strongly pulse-locked response to VNS. The yellow shaded region denotes stimulation period. Pulse timing represented below in (B). (D) Group data demonstrates a significant driven response in Me5 at 1.2 mA and above. Increasing current intensities drives stronger increases in firing rates. Bold black line represents group average across sites. Thin gray lines represent data from individual sites. *** $p < 0.001$; all statistical comparisons versus 0 mA (spontaneous rate).

(Me5). Me5 receives diverse sensory and proprioceptive input from many locations, including jaw musculature (Alvarado-Mallart et al., 1975, Jerge., 1963). We recorded multiunit neural activity in 26 sites in Me5 across 8 animals. Me5 neurons were identified by a strong response to changes in jaw position, as previously reported (Linden., 1978) (Fig. 3.6A). VNS resulted in short

latency driven activity in Me5 neurons after each individual pulse within a stimulation train, distinct from that observed in the LC (Fig. 3.6B&C). Vector strength at 1.6 mA was significantly stronger in the Me5 compared to LC, highlighting the pulse-locked activity pattern in Me5 neurons (Me5: 0.47 ± .04, LC: 0.13 ± .01; Unpaired t-test, $p < 0.001$). The threshold to drive neural activity was substantially higher than that observed for the LC, with only stimulation intensities at or above 1.2 mA yielding significant driven activity in Me5 neurons (Fig. 3.6D; One-Way ANOVA, $F[7,84] = 35.13$, $p < 0.0001$; Paired t-tests compared to 0 mA, $p < 0.0001$ for 1.2, 1.6, and 2.5 mA). Analysis of vector strength and threshold current intensity needed to evoke significantly driven activity illustrates the distinct VNS response characteristics observed in Me5 and LC neurons (Fig. 3.7). These findings demonstrate that Me5 neurons exhibit monotonic increases in phase-locked firing rate in response to VNS at stronger stimulation intensities than LC neurons.

Discussion

In this study, we assessed the response of LC neurons across a range of commonly used VNS parameters. Brief bursts of VNS drive rapid, phasic neural activity in the LC. Significantly driven phasic responses are observed at low (0.1 mA) stimulation intensities. Increasing the current intensity and pulse width drives greater neural activity. Varying the frequency of a fixed number of pulses affects the timing, but not the total amount of LC activity. The mesencephalic trigeminal nucleus, a brainstem nucleus nearby the LC that receives sensory input from laryngeal muscles, exhibits distinct pulse-locked neural activity in response to stronger stimulation intensities. Together, these findings provide insight into the neural responses to VNS in multiple brain regions and may be useful in selecting parameters to optimize VNS for clinical applications.

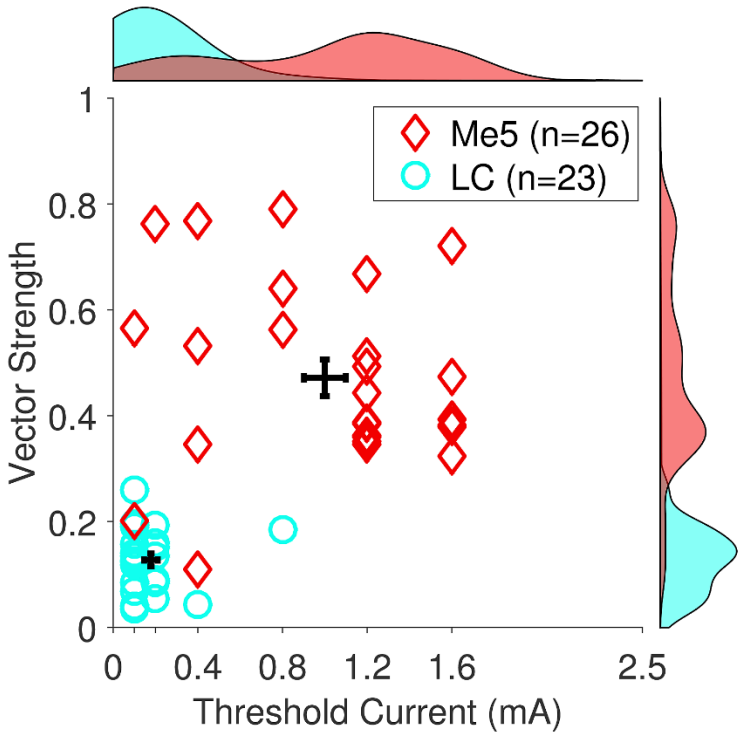

Figure 3.7 Comparison of LC and Me5 response to VNS at all recording sites. Evaluation of minimum stimulation needed to evoke driven activity and degree of pulse-locking highlights distinctive LC and Me5 response to VNS. LC neurons respond at significantly lower stimulation intensities compared to Me5 neurons. Vector strength at 1.6 mA is significantly greater in Me5 neurons, representative of the strongly pulse-locked responses to individual pulses within a VNS train. Group distributions are plotted on the top and right edge.

The ability of VNS to modulate neural activity in the LC corroborates previous studies which have examined this relationship over longer time scales (Groves, Bowman and Brown., 2005, Dorr and Debonnel., 2006). Here, we extend these findings and show that short bursts of VNS evoke rapid, phasic neural activity in the LC. This rapid recruitment of LC neurons likely mediates the memory- and plasticity-enhancing effects of VNS. We speculate that the short latency increase in LC activity drives a phasic release of norepinephrine which acts to facilitate plasticity

specific to ongoing experience (Hays., 2016). The role of the LC in plasticity is supported by evidence that antagonism of β-adrenergic receptors blocks VNS-dependent plasticity (Shen et al., 2012). Moreover, enhanced plasticity requires coincident (or closely-timed) presentation of VNS with stimuli, highlighting the importance of rapid activation of brain structures in the functional consequences of VNS (Engineer et al., 2011). Longer trains of VNS also facilitate plasticity, but considering the efficacy of short trains of VNS, it is likely that the initial rapid increase in firing rate mediates the majority of the effect (Zuo, Smith and Jensen., 2007b).

Stimulation intensities at 0.1 mA were sufficient to drive neural activity in the LC, suggesting that phasic activity is regulated, at least in part, by A- and B-fiber activation. Vagal C-fibers would not be expected to be activated at this low intensity (Woodbury and Woodbury., 1990). However, the increasing magnitude of LC activation suggests that C-fibers may also contribute at higher stimulation intensities. Given the role of norepinephrine levels in the reduction of seizures (Raedt et al., 2011), activation of the LC with low intensity stimulation supports the notion that C-fiber activation is unnecessary for the seizure suppressing effects of VNS (Krahl, Senanayake and Handforth., 2001). A notable limitation of the present study is the use of an anesthetized recording preparation. Application of α2-agonists for anesthesia, including xylazine used in combination with ketamine in this study, reduces spontaneous neural activity in the LC (Aghajanian and VanderMaelen., 1982, Aston-Jones et al., 1994, Berridge and Waterhouse., 2003). While excitability is reduced, the large magnitude of VNS-driven responses observed in LC neurons in this study suggests that similar, if not larger, increases in activity would be observed in the absence of anesthesia. Future efforts should examine VNS-dependent LC dynamics across a range of parameters in unanesthetized conditions.

Both greater current intensities and longer pulse widths increase firing rate in the LC. These findings are consistent with previous studies that indicate that stronger stimulation intensities yield greater increases in norepinephrine levels in cortical structures (Roosevelt et al., 2006). Improved seizure suppression is associated with higher levels of VNS-induced norepinephrine levels in an animal model of epilepsy, suggesting that greater LC activation may mediate the anti-epileptic effects of VNS (Raedt et al., 2011). Indeed, a meta-analysis examining the effect of VNS intensity on seizure suppression revealed that stronger stimulation parameters correlate with better clinical efficacy (Ghani et al., 2015). Increased activity in the LC in response to stronger VNS intensities likely represents the mechanistic link between increased stimulation current and better seizure suppression.

There is a dearth of direct evidence to define the optimal stimulation frequency for VNS. Our findings indicate that the timing, but not the total amount, of neural activity in the LC is influenced by frequency for a fixed number of pulses over the range tested. Higher frequencies elicit greater increases in LC firing rate over a shorter period of time. This modulation of spike rate over time by varying pulse frequency and train duration would, in principle, allow control of the temporal profile of norepinephrine levels. For instance, a short, high frequency train may drive a strong, transient release of norepinephrine, while a long, low frequency train would yield a smaller, more sustained increase in norepinephrine. These findings provide a rationale for more detailed investigation into the functional consequences of different stimulation frequencies.

Charge delivery influences tolerability of VNS in patients (Liporace et al., 2001, Heck, Helmers and DeGiorgio., 2002). Reductions in current intensity and pulse width reduce charge delivery and can be used to modify tolerability of the therapy and provide longer implantable pulse

generator battery life. However, higher current intensities and longer pulse widths increase charge delivery and are associated with greater clinical efficacy, pointing to a trade-off between minimizing side-effects and maximizing therapeutic benefit (Ghani et al., 2015, Heck, Helmers and DeGiorgio., 2002). The characterization of LC activation as a function of charge delivery in this study indicates that charge increases firing rate up to an apparent plateau at approximately 160 nC, after which additional charge yields substantially diminishing gains in LC activity. Additional studies are needed to fully characterize this relationship across a wider range of parameters, including potential interaction with train duration and pulse frequency.

In addition to the LC, the mesencephalic trigeminal nucleus exhibits driven neural activity in response to VNS. The pattern of activation in Me5 neurons is distinct from that observed in the LC, displaying strongly pulse-locked activation to each pulse within a VNS train. The Me5 receives sensory and proprioceptive input from external laryngeal muscles, including the digastricus and mylohyoideus (Alvarado-Mallart et al., 1975). VNS is known to drive activation of laryngeal muscles (Castoro et al., 2011). While we cannot make a direct assertion with the data from this study, we speculate that the phase-locked neural activity in Me5 reflects activation of proprioceptive neurons as a result of VNS-dependent contraction of laryngeal musculature. Increasing VNS current intensity drives greater EMG responses, consistent with the increased Me5 activity reported in our study (Castoro et al., 2011). The observation of VNS-driven activity in Me5 provides an intriguing link to voice alterations common in patients receiving VNS, as the same laryngeal muscles associated with speech production send proprioceptive input to the Me5 (Sokolowsky., 1943, Sataloff, Heman-Ackah and Hawkshaw., 2007, DeGiorgio et al., 2000). Because the threshold stimulation current that yields activation in Me5 was substantially higher

(1.2 mA) than that required to drive activity in the LC (0.1 mA), it may be possible to identify stimulation parameter sets that minimize Me5 activation to reduce adverse effects on voice while maintaining therapeutic efficacy.

Targeted plasticity therapies using short bursts of VNS paired with rehabilitative training regimens have emerged as potential treatments for a variety of neurological disorders (Hays., 2016). Preclinical studies demonstrate that VNS paired with rehabilitative training improves recovery in models of tinnitus, ischemic and hemorrhagic stroke, and traumatic brain injury (Engineer et al., 2011, Khodaparast et al., 2013, Khodaparast et al., 2014, Hays et al., 2014, Pruitt et al., 2016, Khodaparast et al., 2016, Hays et al., 2016). Moreover, clinical studies provide an initial indication of the clinical utility of VNS-based plasticity therapies for tinnitus and stroke patients (Dawson et al., 2016, De Ridder et al., 2014). Because of its clear link to plasticity and engagement by VNS, the LC represents a likely mediator of VNS-dependent enhancement of plasticity. Other neuromodulatory systems likely act synergistically to contribute, but VNS-driven phasic activation of LC reported here provide evidence that noradrenergic circuitry is activated at VNS parameters that effectively enhance plasticity (Seol et al., 2007, Shetake et al., 2011, Porter et al., 2011, He et al., 2015, Engineer et al., 2015, Hulsey et al., 2016).

Several studies evaluating the memory- and plasticity-enhancing effects of VNS have reported an inverted-U response, in which middle intensity stimulation yields greater effects than low or high stimulation intensities (Clark et al., 1995, Clark et al., 1999, Zuo, Smith and Jensen., 2007a, Borland et al., 2016). Given the potential role for the LC in VNS-dependent enhancement of plasticity, it was possible that neural activity in the LC would exhibit a similar inverted-U relationship, in which moderate stimulation intensities elicit maximal driven spikes. However, the

observed monotonically increasing phasic excitation of the LC with stimulation intensity does not support this conclusion, suggesting that firing rate in the LC itself does not mediate the inverted-U response. However, it is possible that presynaptic depletion or noradrenergic autoinhibition at strong stimulation intensities may limit norepinephrine release without directly suppressing neural activity in LC (Starke., 1981). Interestingly, the offset response to VNS (from 751 to 1500 ms) fits empirical data of the plasticity-enhancing effects of VNS (Borland et al., 2016). Many explanations could account for the inverted-U response. One likely model is a low-threshold system that drives positive effects and an overriding high-threshold system that drives negative effects (Hays., 2016). It is tempting to relate the LC as the positive system and Me5 as the negative system. Such a model would closely fit the experimental evidence of the inverted-U effect of VNS on plasticity, as stimulation parameters that drive maximal LC activity in the absence of Me5 activity (0.8 mA) yields the greatest enhancement of cortical plasticity (Borland et al., 2016). However, this model is unlikely to be complete, because while the pro-plasticity role of the LC is easily recognized, there is no clear evidence that would establish Me5 as the negative system to suppress the positive effects of the LC. However, it is conceivable that a different, yet-to-be-identified system with activation characteristics similar to that of the Me5 could interact with LC activation to account for the inverted-U response. Defining the inverted-U is of considerable clinical importance for VNS-based plasticity therapies, as more stimulation does not necessarily relate to greater efficacy.

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CHAPTER 4

SYNERGY OF MULTIPLE MONOAMINE PATHWAYS REQUIRED FOR TARGETED PLASTICITY IN MOTOR CORTEX.

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Abstract

Background: Vagus nerve stimulation (VNS) paired with forelimb training drives robust, specific reorganization of movement representations in the motor cortex. This effect is hypothesized to be mediated by VNS-dependent engagement of neuromodulatory networks and is known to require acetylcholine. VNS drives activity in the locus coeruleus (LC) and dorsal raphe nucleus (DRN), brain regions that produce neuromodulators important for plasticity, but it is not known whether these neuromodulatory networks are required for enhanced cortical plasticity.

Objective: We tested the hypothesis that cortical noradrenergic or serotonergic innervation are required for VNS-dependent enhancement of motor cortex plasticity.

Methods: Rats were trained on a lever pressing task. Once proficient, all rats received implantation of a stimulating cuff electrode on the vagus nerve and cortical injections of an immunotoxin to deplete either serotonergic or noradrenergic innervation or vehicle. One week post-implantation, rats resumed training and underwent five days of motor training with VNS or sham stimulation delivered during proximal forelimb movement. Intracortical microstimulation (ICMS) was performed in the motor cortex contralateral to the trained limb 24 hours after the final training session to examine movement representations.

Results: VNS paired with forelimb training resulted in a significant 180% increase in the representation of proximal forelimb compared to sham stimulation. Depletion of either cortical norepinephrine or serotonin prevented VNS-dependent increase in proximal forelimb area, and resulted in representations similar to untrained controls and sham stimulation. Motor performance was similar between groups, suggesting that differences in forelimb function cannot account for the difference in proximal forelimb representation.

Conclusions: These findings indicate that cortical noradrenergic and serotonergic innervation is required for VNS-dependent enhancement of plasticity in the motor cortex. This result provides further evidence engagement of diverse neuromodulatory networks by of VNS enhance plasticity.

Introduction

Targeted plasticity therapy (TPT) enhances recovery across a wide range of neurological disorders in rodents and humans[1–5]. TPT pairs brief bursts of vagus nerve stimulation (VNS) with sensory, motor, or cognitive events during rehabilitation to enhance plasticity and support lasting functional improvements[5,6]. In neurologically intact animals, pairing distinct distal or proximal forelimb movements with VNS drives expansion of paired movement representations in the motor cortex[7,8]. However, there is little direct evidence to indicate the neural mechanisms by which VNS facilitates plasticity.

Afferent projections of the vagus nerve innervate the nucleus of the solitary tract, and electrical stimulation engages multiple neuromodulator systems in the central nervous system[9,10]. Chronic stimulation leads to increased tonic activity in the locus coeruleus (LC) and dorsal raphe nucleus (DRN)[11]. Recent findings suggest VNS also rapidly engages the LC and cholinergic systems[12,13]. The cholinergic, noradrenergic, and serotonergic systems each are associated with facilitating cortical and synaptic plasticity[14–16]. Previous studies highlight the role of neuromodulatory networks in VNS effects[13,17,18]. Depletion of cholinergic innervation in the cortex blocks the robust enhancement of plasticity[8]. In the clinical setting, pharmacological norepinephrine and GABA antagonists appear to compromise TPT efficacy[2]. Given that VNS engages neuromodulatory networks associated with plasticity, we tested the hypothesis that cortical depletion of

norepinephrine and serotonin will prevent the plasticity-enhancing effects of VNS paired with motor movements.

Methods

Experimental Overview

All experiments were pre-registered on Open Science Framework (https://osf.io/xf3yj/), with planned outcomes, statistical tests, and experimental methods defined *a priori*. In brief, four groups were trained on a lever pressing task emphasizing proximal forelimb use. Once proficient, rats received a surgical implant of a cuff electrode around the vagus nerve and cortical injections of immunotoxins. Saporin conjugated to Anti-Dopamine-Beta-Hydroxylase (DBH-Sap) or Anti-serotonin transporter (SERT-Sap) antibodies were injected to deplete noradrenergic or serotonergic innervation respectively. Saporin conjugated to non-targeted pre-immune mouse IgG antibody (CTRL-Sap) was injected as a surgical control in two groups. Following recovery, rats received either VNS paired with proximal forelimb movements or equivalent behavioral testing without VNS. Within 24 hours of the final training session, motor representations were assessed with intracortical microstimulation by two experimenters blinded to group. An additional group of untrained animals received ICMS for comparison. Following ICMS, rats were transcardially perfused, and the brain was fixed for histological analysis of cortical NE and 5-HT innervation.

Subjects

66 adult female Sprague-Dawley rats weighing on average 287 grams were used in this experiment. Rats were housed in a 12:12 hour reverse light cycle. Rats were food deprived Monday-Friday during behavioral training to increase motivation for food rewards, and provided ad lib feeding on weekends. Supplemental food was provided as necessary to maintain a minimum

of 85% original body weight. All handling, housing, behavioral training, and surgical procedures were approved by the University of Texas Institutional Animal Care and Use Committee.

Behavioral Task

Rats were trained on with MotoTrak (Vulintus Inc, Richardson, TX) systems on the bradykinesia assessment task, a quantitative, automated lever pressing task (Fig. 4.1)[19]. The behavior chamber consisted of an acrylic cage with a slot for access to a lever automatically positioned -1 to 2 cm from the inside edge of the chamber. The slot was positioned next to a divider in the cage, forcing use of the right forelimb. Rewards pellets were delivered at the other side of the divider, forcing rats to travel between the two areas of the cage to receive rewards and re-set their position between successful trials. The lever was affixed to a potentiometer, which records the angle of the lever depression relative to horizontal. The lever was allowed to move 13° below horizontal. A spring provided 28 grams of resistance, returning the lever to its level resting angle. A controller board sampled the potentiometer position at 100 Hz and relayed the information to MotoTrak software that controlled the task and collected data.

Shaping of lever press behavior occurred across 5 stages. The first stage rewarded initial lever press with a sugar pellet (45 mg dustless precision pellet, BioServ, Frenchtown, NJ). The lever was positioned inside the cage, and the press threshold adaptively increased from 10 to 75 percent of the total range of motion, where it was kept for the remainder of training. A tone provided an auditory cue for successful presses. The second stage rewarded release of the lever to 50 percent of the press threshold. A second tone provided an auditory cue for successful releases. The third stage progressively moved the lever position from -1 to 2 cm from the edge of the cage where it was kept for the remained of training, rewarding on release. Beginning on the fourth stage, rats

were rewarded upon completing a second press after releasing an initial press. The time allow for a successful second press was adaptively reduced from 2000 to 500 ms following initial press. The final behavioral stage only rewarded double presses with under 500 ms inter press interval. After a trial there was a time-out period of 2.5 seconds before a new trial could be initiated. Behavior training was performed in two thirty minute sessions per day, five days a week, with at least two hours between daily training sessions. Proficiency at the task was defined as performing an average of 100 successful trials across 10 behavior session with a hit rate of at least 65 percent. 3 animals were excluded due to failure to learn the task.

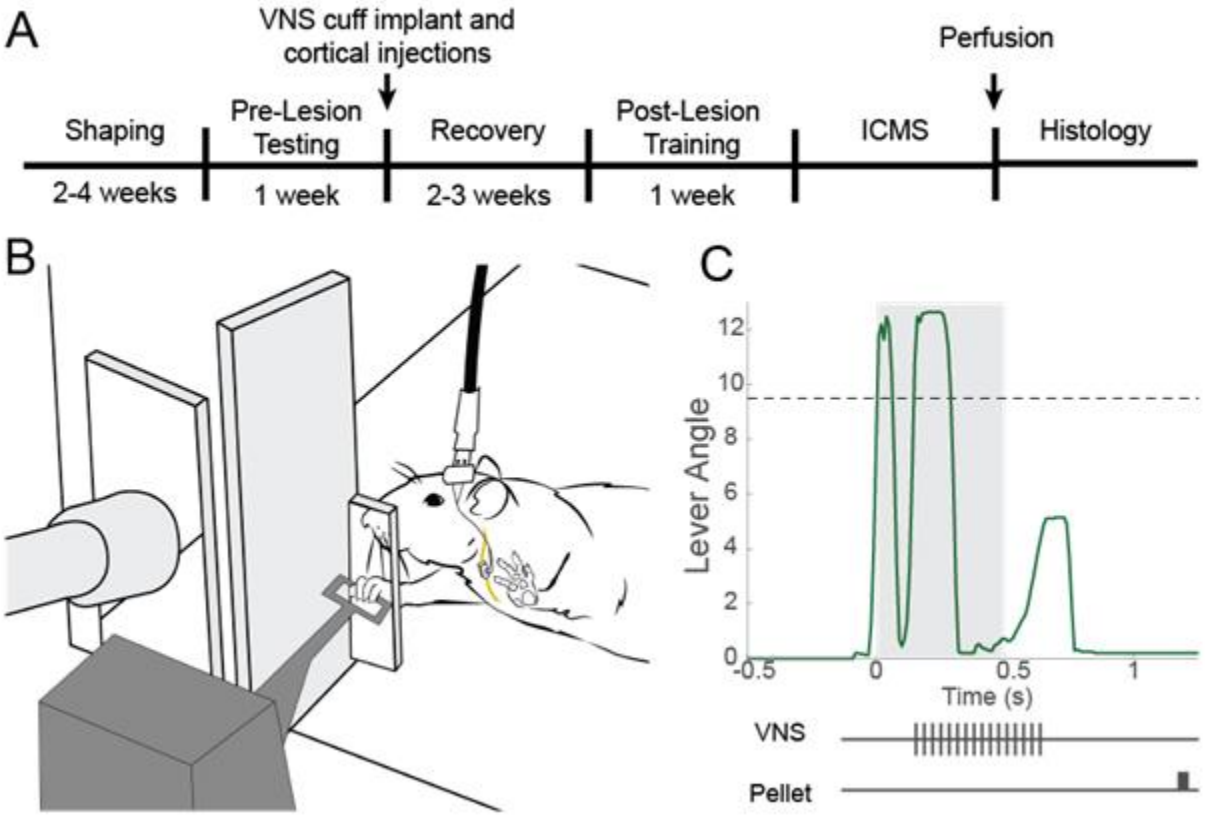

Figure 4.1 Experimental Design
(A) Timeline of the experimental design. (B) Schematic of a rat tethered to VNS stimulator performing lever press task. (C) Representative data from a successful tiral with timing of VNS train and pellet delivery

Cortical Noradrenergic and Serotonergic Depletion

Immunotoxins (Advanced Targeting Systems, San Diego, CA) were used to deplete noradrenergic or serotonergic innervation of the motor cortex. Rats were anesthetized with ketamine hydrochloride (80 mg/kg, i.p.) and xylazine (10mg/kg, i.p.), and given supplemental doses as necessary to maintain anesthesia. The head was stabilized in a stereotaxic frame (David Kopf Instruments, Tujunga, CA), and burr holes were drilled over the left motor cortex (contralateral to the trained forelimb). DBH-Sap, SERT-Sap, or CTRL-Sap (0.1 mg/ml) were stereotaxtically injected with neuros syringes (Hamilton, Reno, NV) at two stereotaxic coordinates typically in the caudal forelimb area (from bregma: 3mm lateral, 0 and 2 mm anterior; .25 μL, 0.1 μL/minute)[20,21]. The syringe remained in place for 4 minutes following each injection to allow for diffusion and to prevent backflow of the injection. Burr holes were sealed with Kwik-Cast (WPI, Sarasota, FL) silicone and acrylic.

Vagus Nerve Cuff Implantation

A vagus nerve cuff and headcap connector were implanted following cortical injections as in previous studies[3,8]. While in the stereotaxic frame, four bone screws were drilled into the parietal and supraoccipital bones. A two-channel connector was attached to the cranial screws with acrylic. Rats were transferred to a supine position, and an incision and blunt dissection of the neck muscles exposed the left vagus nerve. The vagus nerve was isolated and placed in a bipolar stimulating cuff electrode with platinum iridium leads (~5 kΩ impedance). Cuff leads were tunneled subcutaneously, attached to the skull mounted connector, and encapsulated with acrylic. Neck and scalp incisions were sutured and treated with topical antibiotic ointment. Rats were received an injection of buprenorphine (0.03 mg/kg, s.c) and provided carprofen and enroflaxcin

(1mg each) tables for 3 days following surgery and allowed to recover for one week before returning to behavioral training.

Vagus nerve stimulation procedure

Upon returning to behavior, rats were habituated to the stimulating cable connected to the headcap while performing the lever press task. Once rats consistently performed 200 successful trials per day while connected to the stimulator, VNS pairing commenced. 7 rats were excluded due to surgical complications and 2 for failure to regain proficiency at the behavioral task. VNS was delivered on successful trials using an isolated pulse stimulator (A-M Systems, Sequim, WA), triggered by the MotoTrak software on successful trials during the final behavioral training stage. The stimulation parameters are identical to previous studies[1,3,8]. VNS was delivered as a 500 ms train at 30Hz. Each biphasic pulse was 0.8mA in amplitude and 100 μs in phase duration. Rats received VNS paired with behavior training for 5 days before ICMS. Sham stimulation rats underwent the same habituation and training protocol, but did not receive stimulation. VNS voltages were monitored to evaluate cuff impedance. 9 rats were excluded due to inconsistent or high (>10V peak to peak) recoded stimulation voltages and 2 for mechanical failure of the head mounted connector. A drop in blood oxygen saturation follows successful VNS in rats. 3 further rats were excluded due to showing no drop in blood oxygen saturation after the completion of experimental procedures.

Intracortical Microstimulation

Within 24 hours of the final training session rats underwent intracortical microstimulation (ICMS) of the left motor cortex to derive functional maps contralateral to the trained limb using standard procedures[5,7,8]. An additional 8 rats that did not receive motor training underwent ICMS

as naïve controls. Rats were anesthetized with ketamine hydrochloride (75 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.) and received supplemental doses as necessary. To prevent cortical swelling, a small incision was made in the cisterna magna. A craniotomy and duratomy exposed the left motor cortex, contralateral to the trained forelimb. A tungsten electrode (0.1 – 1 MΩ, FHC Inc., Bowdin, MD) was inserted to a depth of 1.8 mm following a .5 mm grid (Fig. 4.3). Sequential electrode penetrations were made at least 1mm apart where possible. Stimulation consisted of a 40 ms train of twelve 200 μs monophasic cathodal pulses delivered at 300 Hz. Stimulation intensity was gradually increased from 20 to 200 μA until a movement was observed. If no movement was observed at maximal intensity, the site was deemed nonresponsive. Borders of the motor cortex were defined based on unresponsive sites and stopped at the posterior-lateral vibrissae area.

Motor mapping procedures were conducted blinded with two experimenters. The first experimenter placed the electrode and recorded data for each site. The second experimenter was blind to the experimental group and electrode position to avoid potential biasing. The second experimenter delivered stimulation and observed and classified motor movements at threshold. Stimulation sites were chosen at random and did not extend beyond established border sites. Movements were classified as "proximal forelimb" (shoulder and elbow), "distal forelimb" (wrist and digits), "hindlimb," and "head" (neck, vibrissa, and jaw). Cortical area was calculated by multiplying the number of sites eliciting a response by the area surrounding the site (0.25 mm^2). Complete borders were determined when possible, but some maps do not have lateral or posterior borders due to the median sinus or the headcap placement. The forelimb region was always mapped completely.

Histology and quantification

Immediately following ICMS, rats were transcardially perfused with 0.1 M phosphate buffered solution (PBS), followed by 4% paraformaldehyde in PBS (PFA). Brains were removed and post fixed in PFA, and then cryoprotected in 30% sucrose in PBS. The full extent of the motor cortex was sectioned at 40 μm thickness and collected in 240 μm series. Tissue was processed with standard immunohistochemistry protocol for DBH to determine cortical innervation of NE fibers. Protocol development for 5-HT staining is ongoing. In brief, free floating sections were washed in PBS, then placed in a blocking solution of 2% normal horse serum (NHS) and 0.5 % Triton X-100 for one hour. Following PBS washes, the tissue was placed in the blocking solution with added anti-DBH antibodies (EMD Millipore, Temecula, CA, 1:1000 dilution), and left overnight at 4° F. Tissue was then processed with ABC-HRP and DAB kits (Vector Laboratories, Burlingame, CA) following their standard protocol. After staining, tissue was mounted on glass slides and cover slipped.

The motor cortex was imaged using an VS120 (Olympus, Cenver Valley, PA) slide scanning system at 20x magnification. Cortical innervation was assessed by counting fiber crossings of a superimposed 5x5 grid with 50 μm spacing[8,22]. All fiber count analysis was performed on coded images by experimenters blind to experimental condition.

Statistics

All data are reported in the main text as mean ± SEM. All comparisons were planned in the experimental design *a priori*, and significant differences were determined using one-way ANOVA and t-tests where appropriate. Statistical tests for each comparison are noted in the text. Paired t-tests were used to compare performance before and after lesion, and unpaired t-tests were

used to compare measures across groups. Alpha level was set at 0.05 for single comparisons, and Bonferroni-corrected for multiple comparisons where applicable. Error bars indicate SEM in all figures, and * denotes $p < 0.05$.

Results

Cortical injections of anti-DBH or anti-SERT Saporin deplete innervation unilaterally

Tissue processing is ongoing. 5-HT tissue is awaiting development of a consistent staining protocol. DBH fiber counting is complete in 6 rats, and staining in 10 additional.

Injections of saporin-based immunotoxins in the motor cortex of rats unilaterally depletes cortical innervation of the targeted fiber type (Fig 4.2). There is no difference between

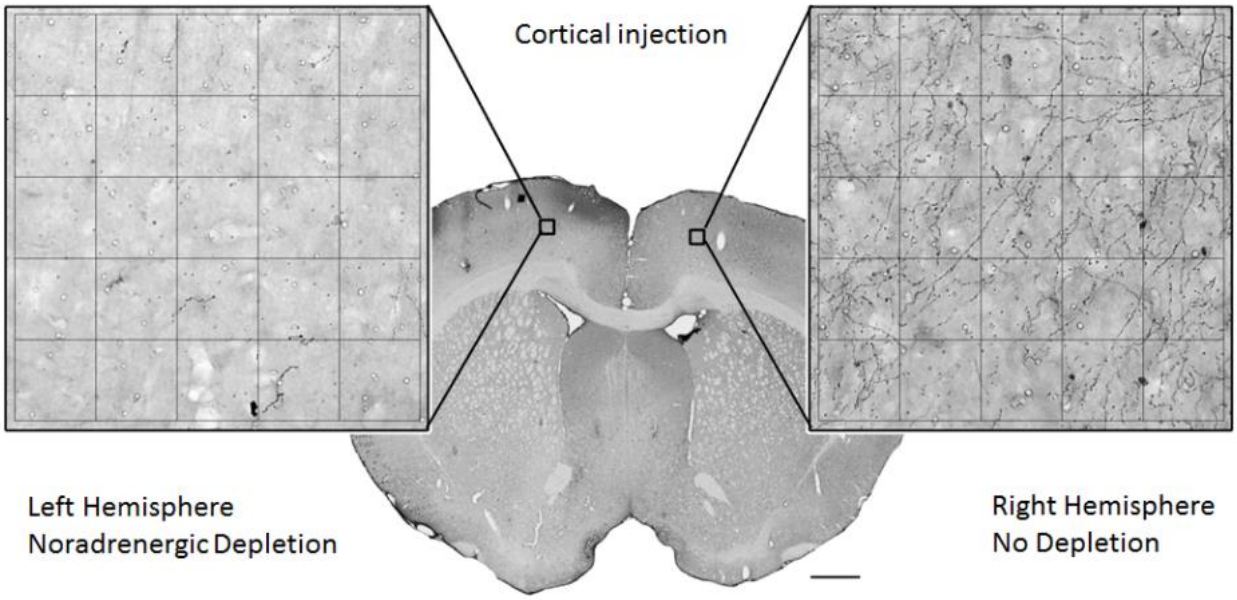

Figure 4.2 Saporin injections depleted targeted cell types
Representative staining in a Anti-DBH-Saproin injected rat. DBH-Sap effectively eliminates cortical axons containing DBH, a marker for noradrenergic fibers unilaterally. CTRL-Sap or saline injections do not significantly impact fiber counts.

average fiber crossings per section in the right, uninjected hemisphere, between toxin types (CRTL-Sap = 84 ± 8.3, DBH-SAP = 92 ± 5, unpaired t-test, p=0.38). DBH-Sap injections

significantly depleted cortical innervation by over 50% in all rats (Right = 92 ± 5.3, Left = 18 ± 10, unpaired t-test, $p<0.01$). There was no significant depletion of cortical innervation in CTRL-Sap injected animals (Right = 84 ± 8, Left = 68 ± 6, unpaired t-test, $p=0.18$). The significant depletion of cortical innervation is hemisphere and toxin specific, with complete innervation in the uninjected hemisphere of all rats.

Serotonergic or Noradrenergic depletion prevents VNS-dependent cortical plasticity

We hypothesized that VNS paired with proximal forelimb movement would result in larger proximal forelimb representation in motor cortex. Further, we hypothesized that serotonergic or noradrenergic depletion would prevent VNS effects. We measured proximal forelimb representation in four groups to test the hypothesis: Sham-control, VNS-control, VNS-DBH, and VNS-SERT.

VNS paired with motor training in subjects with intact neuromodulatory pathways increases proximal forelimb representation in the cortex (Fig. 4.3). ANOVA of proximal representation

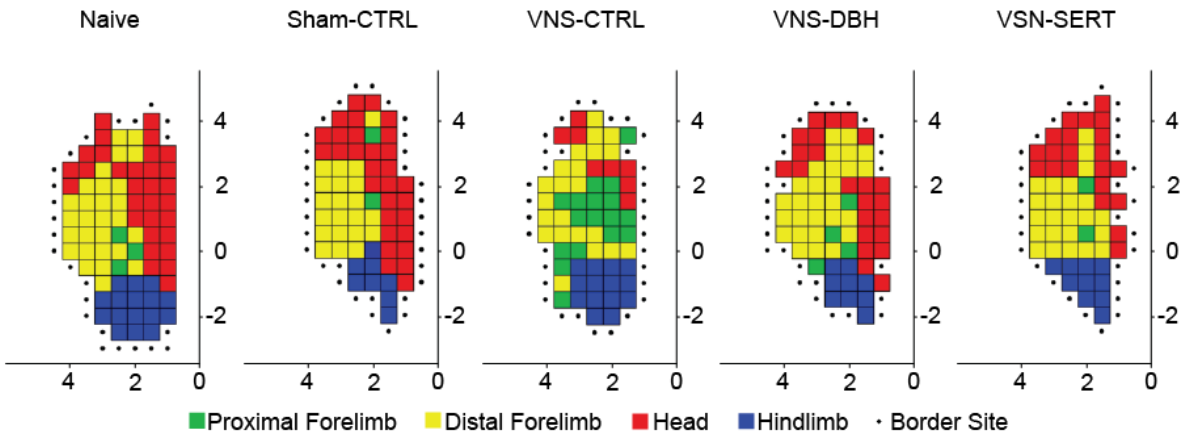

Figure 4.3 Representative Motor Maps
Experimental procedures did not alter overall map size or topography. Note the expanded proximal forelimb area in the VNS-CTRL map as compared to the similar representations in all other groups

revealed a significant effect between groups (one way ANOVA, $F[4,34] = 7.42$, $p<0.001$). Animals with control injections into motor cortex that received VNS paired with lever press and had a significant 180% increase in proximal forelimb representation compared to trained, unstimulated rats (Fig. 4.4, VNS-control = 2.78 ± 0.41, Sham-control = 0.96 ± 0.24, unpaired t-test, $p<0.01$). This finding replicates two previous studies in motor cortex[7,8], and corroborates numerous studies in the auditory system showing stimulus specific cortical plasticity in response to paired VNS[1,23–25].

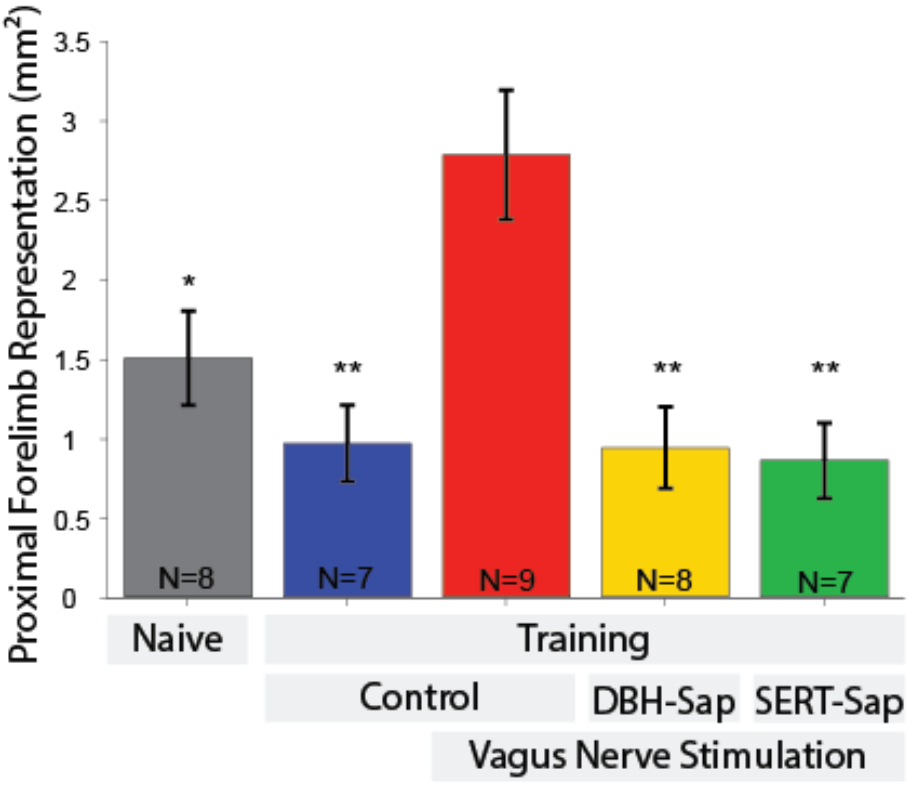

Figure 4.4 Proximal forelimb representations
VSN paired with forelimb training increases proximal forelimb representation in neurologically intact rats when compared to all other groups. Lesions of two distinct monoaminergic pathways prevent VNS-dependent motor cortex map reorganization. * $p<0.05$, ** $p < 0.005$

Depleting noradrenergic innervation with cortex immunotoxin injections prevented VNS mediated expansion of proximal forelimb representation. VNS-DBH rats had a significantly

smaller proximal forelimb representation than VNS-controls, and were comparable to Sham-control rats (Fig. 4.4, VNS-DBH = 0.93 ± 0.26, unpaired ttests, vs. VNS $p < 0.01$, vs sham $p = 0.94$). Similarly, depletion of serotonergic innervation prevented VNS mediated cortical plasticity. VNS-SERT rats had significantly smaller proximal forelimb representations than VNS-controls, and were comparable to sham-control and VNS-DBH rats (Fig. 4.4, SERT-VNS = 0.86 ± 0.24, unpaired ttests, vs. VNS $p < 0.01$, vs sham $p = 0.76$, vs DBH $p = 0.82$). Along with the previously published results on acetylcholine[8], this is the third type of specific neuromodulatory lesion to block cortical plasticity mediated by VNS pairing.

There are no differences in overall map size or any other movement representation between groups. ANOVA did not reveal differences between groups in representations of distal forelimb (one way ANOVA, $F[4,34] = 0.5$, $p = 0.74$), indicating a task-specific expansion. Additionally there are no differences in representations of head or hindlimb (one way ANOVA, $F[4,34] = 1.66$, $p = 0.18$, $F[4,34] = 1.74$, $p = 0.16$). Total motor representations in cortex were also not significantly different (one way ANOVA, $F[4,34] = 1.67$, $p = 0.18$). These results support the specificity of VNS-directed plasticity, as proximal forelimb movements were emphasized during task performance and VNS pairing.

Neuromodulatory depletion and VNS do not influence established behavior

Differences in duration, performance, or total VNS delivered during behavioral training may potentially account for observed changes in cortical representations. However, ANOVA showed no significant difference between groups in total days training or days training on double press

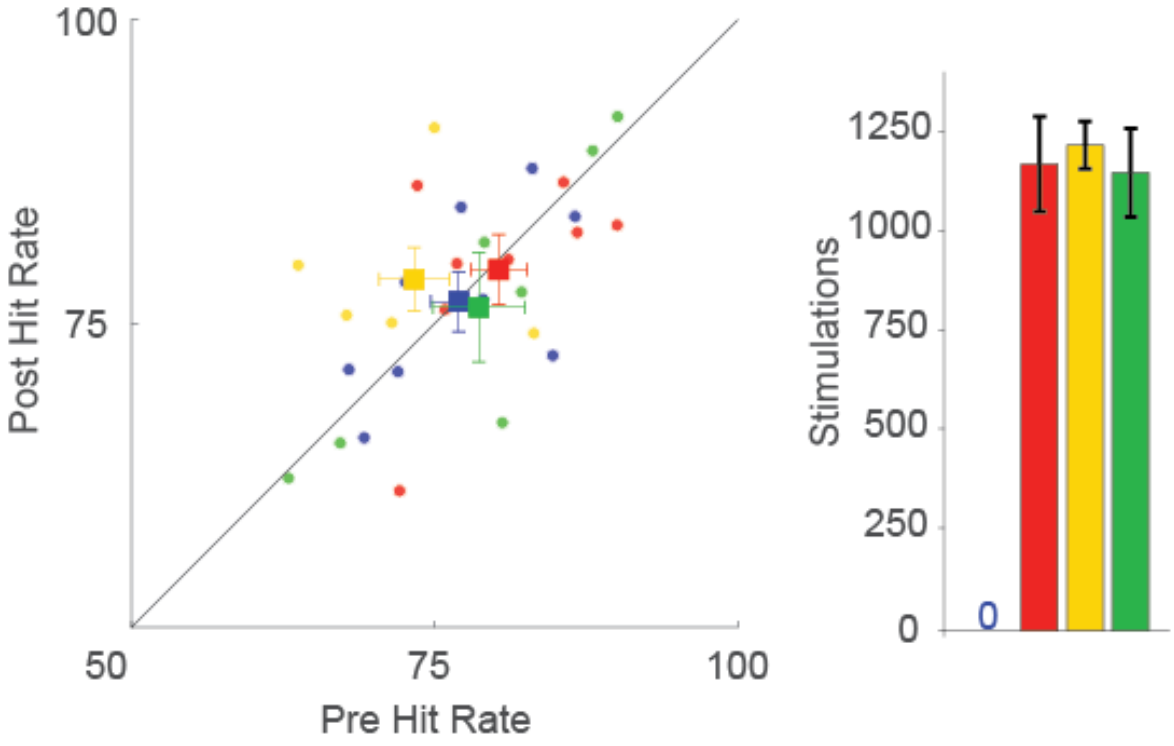

Figure 4.5 Monoamine lesions and VNS do not effect behavioral performance
There was no effect on hit rate of neuromodulatory lesions or VNS. Crucially, all groups received similar amounts of stimulation paired with proximal forelimb movements.

stages (one way ANOVA, F[3,27] = 1.01, p = 0.4; F[3,27] = 0.86, p = 0.47). Two way ANOVA revealed no significant main effect or interaction between experimental group or surgical procedures on hit rate (Fig. 4.5, two way ANOVA, Group, F[3,52] = 0.61, p = 0.61; Pre vs Post, F[1,52] = 0.05, p=0.81; interaction, F[3,52] = 0.52, p = 0.67) or inter-press-interval (two way ANOVA, Group, F[3,52] = 0.83, p = 0.48; Pre vs Post, F[1,52] = 0.07, p=0.79; interaction, F[3,52] = 0.23, p = 0.87). Additionally, no gross behavioral changes were observed after neuromodulatory lesions. Two way ANOVA revealed a significant main effect of surgical procedure on total number of trials, but no interaction and no main effect of experimental group (two way ANOVA, Group, F[3,52] = 1.08, p = 0.36; Pre vs Post, F[1,52] = 11.45, p < 0.01;

interaction, $F[3,52] = 0.51$, $p = 0.68$). This effect is likely due to stimulator cable connections encumbering upon speed of navigating between the lever and reward areas of the behavioral cage as rats performed the task, and is consistent across all groups (Fig. 4.1). Most importantly, similar amounts of VNS was delivered immediately following proximal forelimb movements to each of the appropriate groups (Fig. 4.5, one way ANOVA, $F[3,27] = 0.08$, $p = 0.97$). Together, these results exclude the possibility that that differences in task performance or amount of VNS could account for the observed differences in cortical representation between groups.

Discussion

Previous studies have established that pairing VNS with cognitive, sensory, or motor events drives robust, specific plasticity[1,7,8,26,27]. In this study, we provide the third independent replication that VNS paired with motor training drives expansion of cortical proximal forelimb representation in motor cortex[7,8]. These findings mirror previous studies in sensory systems, in which VNS paired with tones or complex sounds drives plasticity in auditory cortex[1]. Moreover, VNS paired with cognitive cues related to fear or drug-seeking behaviors modulates plasticity in key pathways between the prefrontal cortex and basolateral amygdala, highlighting the flexibility of paired VNS to drive stimulus-specific plasticity beyond cortical networks[26,27]. In each case, pairing of a stimulus with VNS drives specific, substantial reorganization in modality-specific circuits. The ability of VNS to direct plasticity in a wide variety neuronal networks may be subserved by engagement of common neuromodulatory pathways[6]. The present study supports this notion and provides direct insight into the neuromodulatory mechanisms required for VNS-dependent enhancement of plasticity.

Cholinergic innervation of the motor cortex is necessary for VNS effects on plasticity in motor cortex[8]. Other VNS effects rely on noradrenergic signaling, but the involvement of norepinephrine in plasticity effects of VNS had not previously been studied[11,18]. Additionally VNS is known to alter activity in the serotonergic DRN after chronic stimulation, but direct involvement of serotonergic signaling has not been studied in any effects of VNS[11]. In this study, we show that depletion of norepinephrine and serotonin each block the plasticity enhancing effects of VNS pairing in motor cortex. These findings do not constitute an exhaustive list of neuromodulators involved in directed plasticity, but provide evidence that norepinephrine and serotonin are critical for targeted plasticity using VNS. Identifying these neuromodulatory mechanisms opens new avenues to optimize and better understand VNS-based therapies.

Targeted plasticity requires precise pairing of VNS with activity in relevant circuits. VNS delayed 25 seconds from successful trials fails to enhance plasticity or facilitate motor recovery after spinal cord injury, while concurrent or 2 second delayed stimulation significantly improve performance[5]. VNS drives robust expansion of paired tone representation in auditory cortex, but tones interleaved 15 seconds apart from paired tones failed to drive expanded representation[1]. The rapid recruitment and phasic activation of LC by VNS, and the current finding that noradrenergic depletion prevents plasticity corroborate this theory[12]. Synaptic eligibility traces established by spike timing dependent plasticity (STDP) also support the importance of rapid noradrenergic signaling[16]. Eligibility traces of long-term potentiation (LTP) are activated by endogenous norepinephrine release, but decay within 10 seconds unless activated[16]. This provides a clear mechanistic link between TPT using VNS and rapid neuromodulator release.

Synergistic interactions of cholinergic, noradrenergic, and serotonergic systems appear vital to driving cortical plasticity with event paired VNS, as depletion of each individually blocks the robust effect seen in neurologically intact rats[8]. Individual neuromodulatory lesion yield mixed results influencing ocular dominance plasticity. In some cases, plasticity persists despite single system depletions, and only multiple combined lesions block plasticity[28,29]. Convergent downstream targets of neuromodulator receptors could provide redundancy, allowing compensation[14]. Alternatively, requiring unique contributions of multiple distinct neuromodulators could provide specificity necessary for a stable system[29]. Experiments utilizing VNS and neuromodulatory lesions are unique, in that VNS influences cortex activity through multiple neuromodulatory systems[11–13]. Despite the diverse pathways VNS engages, it could not compensate for noradrenergic or serotonergic denervation.

Depletion after cortical injections of DBH- or SERT-Sap is extensive, but hemisphere specific. The lack of variability in depletion precludes correlation analysis between extent of depletion and blockade of plasticity. Lower concentrations of toxins yield partial cortical lesions, while influencing behavior[21]. It is possible that graded depletion of neuromodulatory innervation would not block VNS mediated plasticity. The timing and duration of VNS training relative to lesion also may interact. Cholinergic depletion with 192-IgG-Saporin leads to changes in dopaminergic tone in cortex and the olfactory bulbs[30]. Receptor compositions also change in response to neuromodulatory depletions[31–33]. VNS pairing started within 3 weeks of saporin injections. Postponing delivery of VNS for a longer period could influence outcomes, as the nervous system fully adapts to a depleted state.

The time course for VNS mediated changes varies extensively. In some cases, VNS significantly influences behavior and plasticity states in as few as four stimulation pairings[27]. In motor cortex plasticity experiments 1000 pairings over 1 week drives reorganization, while in the auditory system 6000 pairings over 3 weeks are used to influence cortical representations[1,7,8]. VNS increase tonic firing of the serotonergic DRN only after two weeks of stimulation[9,11]. Intermediate or extended influences of VNS pairing are unknown. The time course of sensitization or habitation to VNS in neuromodulatory centers could influence the extent of beneficial effects of pairing. Priming the nervous system with unpaired VNS for two weeks prior to movement pairing may allow compensatory mechanisms to engage and facilitate plasticity despite lesions to other systems.

Following our previous study indicating the requirement of cholinergic innervation, this study adds functional noradrenergic and serotonergic pathways as requirements for targeted plasticity in motor cortex utilizing paired VNS[8]. Continued research into VNS induced activity in key neuromodulatory centers and effects of altered systems in other modalities is necessary. Regional differences even between areas of cortex influence neuromodulatory requirements for STDP[16]. Other cortical and subcortical targets for TPT may not require each of these pathways to be intact. Studies regarding sensitization or habituation to VNS in neuromodulatory nuclei could also inform and optimize approaches to stimulation scheduling in clinical settings.

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CHAPTER 5

CONCLUSIONS

Neuromodulation and Targeted Plasticity Therapy

Utilizing vagus nerve stimulation (VNS) to direct plasticity and promote recovery through targeted plasticity therapy (TPT) developed over the last decade[1–5]. Despite recent conception, safety and efficacy trials using TPT in human patients have been conducted to treat tinnitus and stroke[6,7]. In April of 2017 the US department of defense announced a new program funding research in targeted neuroplasticity training (TNT) utilizing peripheral nerve stimulation to support improved, accelerated training of military personnel. The growing interest and backing of TPT methodologies follows an incredibly diverse set of applications for treatment of neurological disorders. Applications grew across a spectrum of disorders from the auditory system in treating tinnitus, to cognitive disorders like post-traumatic stress disorder and addiction, to a variety of motor deficits including stroke and spinal cord injury[1,5,8–12]. The variety of applications of TPT is likely to continue growing.

Despite a growing list of applications, details of the mechanisms VNS and TPT utilize remained elusive. Synaptic and circuit plasticity has been studied extensively, and details of the wide influence of neuromodulators on the process continue to emerge[13–19]. VNS directs expansion of cortical representation toward paired stimuli in a similar fashion to stimulation of the basal forebrain or ventral tegmental area[1,15,18]. Stimulation of the locus coeruleus (LC) also drives auditory cortex plasticity[19,20]. These and other results lead to a clear neruomodulatory hypothesis for the basis of targeted plasticity utilizing VNS[3]. Pharmacological blockade or lesions of cholinergic and noradrenergic systems block other effects of VNS in the central

nervous system[21–23]. Although multiple veins of indirect evidence implicate noradrenergic and cholinergic signaling, the work in this dissertation is the first evidence published that neuromodulators directly mediate VNS enhanced plasticity.

Previous studies demonstrated VNS alters activity in the noradrenergic LC and increases norepinephrine levels across multiple brain regions[24–26]. The published research in chapter 3 of this dissertation further elucidates the effects of VNS on LC activity. Past publications focused on tonic changes in activity after chronic stimulation, and did not provide useful insight into phasic activation[24,26]. Timing of VNS is crucial to therapy outcomes[11,27]. The presented evidence of rapid, phasic activation of LC in response to VNS further supports the importance of pairing. It also allows clear predictions for mechanisms of VNS pairing related synaptic plasticity and spike timing dependent plasticity (STDP)[17].

Norepinephrine modulates a wide variety of neural plasticity effects. Depletion prevents ocular dominance plasticity in response to monocular deprivation, and local perfusion restores typical plasticity responses[28]. Electrical or optogenetic stimulation of LC paired with activation of primary auditory cortex drives changes in responses to tones[19]. On a synaptic level, norepinephrine can drive divergent responses based on transmitter availability and receptors activated[29]. α_1 and β-adrenergic receptor activation control long term depression (LTD) and potentiation (LTP) respectively. High norepinephrine concentrations facilitates activation of both pathways, resulting in typical STPD. Despite such extensive effects of norepinephrine, additional factors likely influence VNS effects.

VNS influences cortical plasticity, memory enhancement, and tonic activity of neuromodulatory nuclei with an inverted-u based on stimulation intensity[30–32]. Phasic activity in

the LC, however, monotonically increases with stimulation intensity. While immediate phasic activity monotonically increased in the LC, nonlinear responses to increased intensity were observed upon stimulation offset. Rising stimulation intensity at low to mild currents resulted in increased transient inhibition of tonic activity after VNS, similar to patterns following activation by noxious stimuli[33–35]. At stimulation intensities above 1 mA, LC activity did not dip below tonic levels, and instead slowly return to baseline activity levels. Increasing intensity above 1 mA prolonged activation, and delayed returns to baseline activity. Plasticity in the auditory cortex following VNS pairing with auditory tones varies based on stimulation intensity in an inverted-u, declining at intensities of 1.2 mA and above[32]. The LC offset response to VNS closely follows the same pattern. VNS may engage multiple other modulatory brain regions at varied stimulation intensities. The mesencephalic nucleus of the trigeminal nerve (Me5), anatomically adjacent to the LC, also responds to VNS with phasic activation. Me5 requires significantly high intensity VNS to drive activity when compared to LC. While Me5 is unlikely to influence the effects of VNS directed plasticity, what other brain regions VNS physically activates remains unknown.

Synergistic interactions of neuromodulators influence plasticity outcomes. In some *in vitro* preparations, neural activity alone does not drive spike timing dependent plasticity. Introduction of both cholinergic and noradrenergic agonists is required to restores the polarity of spike timing dependent plasticity (STDP)[36]. Bath application of β-adrenergic receptor agonist isoproterenol alone causes paired pulses to induce long term potentiation (LTP) regardless of timing, whereas cholinergic receptor agonist McN application alone causes all pairings to induce long term depression (LTD). A combination of the two pharmacological agents restores

normative STDP. Serotonin also facilitates LTD *in vitro*, and depletion *in vivo* prevents ocular dominance plasticity, similarly to norepinephrine and acetylcholine[17,28,37,38]. Even if VNS does not directly activate these key systems, depletion or intervention may block VNS mediated effects.

Chapters 2 and 4 of this dissertation examined the effects of VNS paired with motor movements after depletion of cholinergic, noradrenergic, or serotonergic innervation of the cortex. We hypothesized that cholinergic and noradrenergic depletion would prevent VNS-mediated effects on cortical movement representations. Each of the lesions prevented VNS-driven effects on cortical representations. The blockade of plasticity by serotonergic depletion was somewhat surprising. Although tonic changes in serotonin levels facilitate plasticity, and depletion prevents ocular dominance plasticity, cholinergic and noradrenergic agonism alone facilitate normal STDP[36,39,37]. It would be reasonable to hypothesize that those two systems alone could direct plasticity in the absence of serotonin, but all three systems are crucial to VNS-directed plasticity.

VNS drives rapid phasic activation of the noradrenergic system, but influence on serotonergic activity has been documented only after weeks of stimulation[26,40]. The cholinergic system mediates VNS effects on the auditory cortex, but there is no direct evidence of activation of cholinergic systems. The sole phasic action of VNS relevant to TPT may be promoting noradrenergic release following activity in behaviorally relevant circuits. Activated circuitry may then be reinforced through noradrenergic action on synaptic eligibility traces of LTP[17]. Depletion of cortical norepinephrine directly affects the ability of VNS to reinforcement circuitry through pairing, thus mitigating targeted plasticity.

Despite the apparent lack of rapid serotonergic and cholinergic activation by VNS, depletion of each prevents plasticity in response to VNS pairing with motor movements in motor cortex. Considering them as permissive facilitator instead of requiring rapid activation better fits the know effects of VNS. Serotonin and acetylcholine may provide homeostatic depression of cortical circuitry through LTD[17,36]. Depletion of either may significantly slow LTD actions, resulting in an overly potentiated circuit, unable to dampen activity. Without attenuation of established circuitry, systemic shifts in representation would require orders of magnitude more potentiation to shift while maintaining functional signal to noise ratios. Cortical depletion of each drastically impacts the ability of a circuit to undergo normal palsticity[37,41–43]. Instead of promoting shifts toward specific outcomes, these systems may instead attenuate all circuitry, facilitating a generally malleable state[39]. Regardless of interpretation, the results presented in this dissertation clearly show synergy and cooperation across multiple neuromodulatory pathways is required for normative plasticity responses to VNS pairing.

Moving Forward with Mechanisms

Karl Lashley began a quest to identify specific brain regions responsible for cognitive functions in 1950[44]. His legacy persists today as groups look for engrams across brain regions[45,46]. Tacking changes to singular brain regions is often problematic, and sometimes pejoratively dubbed "new phrenology[47–49]." Motor map plasticity in primary motor cortex has been proposed to encode motor experience[50]. Motor cortex plasticity reflects recovery after stroke, and ablation of re-organized areas returns rats to pre-recovery performance[42,51,52]. Contrastingly, in other behavioral circumstances complete lesions of the motor cortex does not interfere with skilled tasks that previously required the cortex for learning[53]. The authors propose

subcortical motor circuits allow continued performance of already learned motor sequences, while the cortex "tutors" in skill learning. Recent findings using *in vivo* imaging indicate incredibly dynamic activity in motor cortex, with some populations converging and others continuing to diverge throughout learning of consistent motor behaviors[54–57]. Chasing the locus of reorganization and recovery will continue to be challenging in a potentially sparsely coded network of a sea of neurons[2].

VNS pairing drives plasticity across a broad range of brain regions. Recovery from motor injuries often involves reorganization in motor cortex[52,58,59]. Contrastingly, in a rodent model of bilateral spinal contusion, there was no significant boost in cortical reorganization observed after recovery facilitated by VNS paired with rehabilitation[11]. TPT with VNS pairing does not necessitate detailed knowledge of specific pathways or activity reinforced. When VNS is paired with fear or drug seeking extinction therapies, pathways from the prefrontal cortex to amygdala are altered in their connectivity[8,9]. Auditory stimulus pairing drives plasticity in auditory cortices[1,32,60]. VNS pairing during physical rehabilitation prompts differential reorganization based on injury type[11]. The ubiquitous central nervous system projections of the noradrenergic brainstem nuclei driven by VNS facilitate reinforcement of incredibly diverse neural pathways. The co-opting of these endogenous pathways by VNS is perhaps the greatest strength of TPT. Instead of relying on circuit-specific micro-manipulations, or receptor-specific drug application, VNS delivers endogenous neuromodulators to facilitate plasticity. Region-dependent responses could influence VNS efficacy and requirements of neuromodulatory tone. Further understanding of the response to various VNS parameters across neuromodulatory pathways can guide future thought on VNS delivery schedules and pairing paradigms.

Complete denervation of the cortex helped indicate the involvement of multiple neuromodulatory systems. The distinct role and phasic involvement of each remains unknown. Selectively silencing neuromodulatory centers utilizing optogenetics during VNS could further differentiate individual involvement[61]. Pharmacological agents targeting phasically important pathways could detrimentally impact VNS effects. If certain neuromodulators play a more permissive role in targeted plasticity utilizing VNS, pharmacological agents selectively impacting their transmission may be less detrimental to plasticity effects. Experiments determining which neuromodulatory pathways act phasically to facilitate TPT could influence clinical patient selection.

Understanding the time course of changes in response to VNS could significantly enhance treatment optimization efforts. Electrophysiological observation of cortical map changes are time consuming and often terminal. Advances in *in vivo* imaging allow sampling of large neural populations over time[54,56,62,63]. Incorporating and developing imaging techniques to monitor changes across the motor or auditory cortices throughout VNS pairing procedures would allow for analysis of the time course of changes in response to therapies. Varying pairing paradigms could influence the speed and duration of cortical reorganization, leading to important insight into optimal stimulation protocols.

Beyond cortical outcome measures, adaptation or sensitization to VNS in neuromodulatory centers could drastically impact efficacy of therapies. Further parametric characterization of responses to VNS over a long timescale could influence decisions of delivery and pairing schedules and improve understanding optimal stimulation parameters. Long term photometric recordings in multiple neuromodulatory nuclei could elucidate dynamic shifts in phasic response

patterns, and help develop further understanding of the synergistic interactions between systems[64].

Despite the dearth of knowledge regarding specific mechanisms, TPT utilizing VNS is effective across a broad range of applications. This dissertation begins to address which specific neuromodulatory pathways involved in motor cortex plasticity after VNS paired with motor movements. Continuing to elucidate the effects of VNS on neuromodulatory pathways with a focus on elucidating phasic and tonic contributions of neuromodulators, recording in neuromodulatory centers focused on characterizing responses to chronic stimulation, and the time course of plasticity, can continue to steer clinical translational efforts of TPT.

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BIOGRAPHICAL SKETCH

Daniel Hulsey grew up in Konya, Turkey, born to Keith and Nancy Hulsey. He completed his minor in neuroscience at The University of Texas at Dallas in 2012. After tasting the beauty of the brain near the end of his undergraduate career, he became involved in Michael Kilgard's research lab, where he intended to complete a Master's degree. When encouraged to pursue a PhD in cognition and neuroscience under Dr. Kilgard's mentorship, he took the opportunity to stay involved with the unique, immediately clinically relevant research. His work focused on the neuromodulatory pathways utilized by vagus nerve stimulation to direct plasticity. The depth, intricacy, and beauty of neural circuits continue to inspire his work.

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Research Interests

- Neural plasticity
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Research Experience

Graduate Research Assistant, University of Texas at Dallas, Richardson, TX
2013-Present – Dr. Michael Kilgard – mentor

- Stereotaxic injection and peripheral nerve stimulator implant surgeries
- Intracortical Microstimulation
- Immuno-toxin lesion protocol development
- Immunohistochemistry protocol development
- Extracellular recording in Locus Coeruleus
- Train and mentor undergraduate research volunteers
- MATLAB analysis of behavioral and electrophysiological data

Neural Systems and Behavior Course, Marine Biological Laboratory, Woods Hole, MA
2015 – Dr. Lidia Szczupak, Dr. John Tuthill, Dr. Randy Bruno – advisers

- Whole cell recording with sharp glass electrodes in leech ganglia
- Flight behavior and patch clamp electrophysiology in drosophila
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Masters' Research Volunteer, University of Texas at Dallas, Richardson, TX
2012-2013 – Dr. Seth Hays – adviser

- Lead histology technician
- Rodent perfusion and brain dissection
- Immunohistochemistry techniques

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- Rat motor behavioral training
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