IMPROVING RECOVERY FOLLOWING NEUROLOGICAL INJURY UTILIZING TARGETED PLASTICITY THERAPY

by

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I dedicate this work to my parents, Heather and Larry Darrow.

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Neurological injuries often cause permanent, significant impairments in motor and sensory function. Spinal cord injury affects 276,000 individuals in the United States and millions more worldwide, while 20 million American suffer from peripheral nerve related injuries. Both of these injuries commonly cause upper extremity motor and sensory dysfunction, which can persist for the rest of their lives. Currently, there are no consistently effective treatment options to restore sensorimotor function in patients suffering from these disabilities.

In recent years, vagus nerve stimulation (VNS) paired with rehabilitation has emerged as a possible therapeutic intervention for treating motor and sensory dysfunction following a number of neurological injuries including ischemic stroke, hemorrhagic stroke, and traumatic brain injury. This dissertation works to further these findings by investigating new injury models and new modalities for restoring further function while simultaneously optimizing aspects of the therapy for more seamless translation to the clinic. We first describe how VNS paired with motor rehabilitation can be utilized to drive significant recovery in different models of spinal cord injury at the fifth cervical level. In the same study, we go on to demonstrate the importance of

pairing VNS with neural activing driving the desired outcomes. Next, we vary the timing of VNS with the paired event in order to identify a synaptic eligibility trace of VNS which can be utilized to further optimize VNS pairings in the clinic. It was also discovered VNS paired with rehabilitation can drive plasticity in spared motor networks through the use of intracortical mapping and viral transsynaptic tract tracing. We investigated spinal cord injury at a lower level, C7, with a new bilateral injury model, and found that despite a loss in distal forelimb motor pools, VNS paired with rehabilitation was able to significantly enhance motor recovery. Generalization to similar but untrained tasks was also observed, further highlighting the potential for clinical translation. Next, we were able to demonstrate the use of VNS paired with sensory stimuli in order to restore sensory function following a model of chronic sensory loss in the forelimb, peripheral nerve injury. Not only did VNS paired with tactile rehabilitation drive significant enhancement of mechanosensory withdrawal thresholds, these VNS-mediated benefits were found to last for over two months. While generalization was not observed in a predominantly motor task, grip strength, it was observed in multiple sensorimotor functions including skilled forelimb placement, toe spread, and spontaneous forelimb use.

The findings of this dissertation clearly demonstrate that VNS therapy paired with rehabilitation can significantly improve recovery of motor and sensory function following neurological injury. We demonstrate the first use of VNS therapy to treat dysfunction after spinal cord injury. We demonstrate the first preclinical use of VNS therapy to restore somatosensory function following peripheral nerve injury. Lastly, this dissertation demonstrates the clinical utility and massive potential for translation to improve both motor and sensory function following neurological injury.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Neurological injuries often cause chronic impairment in sensorimotor function[1]. Specifically, distal motor function and sensory function are commonly lost after neurological injury or disorders which are commonly accompanied by pathological network activity. The current gold standard in the clinic for treatment in order to restore this lost function is physical rehabilitation. While rehabilitation can induce some plasticity, rehabilitation only produces modest improvements and the vast majority of patients still have chronic disability[2–5]. However, inducing targeted plasticity in specific functional networks may provide a new method to further improve recovery[6,7]. In this dissertation we provide evidence that a novel technique to drive targeted plasticity using brief bursts of vagus nerve stimulation (VNS) paired with rehabilitation can improve sensorimotor function after neurological injury[8–10]. Here we harness the benefits of plasticity-inducing VNS in order to drive recovery in sensorimotor function following multiple types of spinal cord injury (SCI) and peripheral nerve injury (PNI).

Spinal cord injury is a devastating injury to the central nervous system often leading to permanent disabilities[11]. Patients with a SCI typically suffer physically, emotionally, as well as having financial difficulties following the injury[12–14]. In the US, the incidence of SCI is among the highest in the world at approximately 40-50 cases per million[15]. SCI occurs is most common in males at 79.8% whereas females only account for 20.2%[16]. SCI patients also most commonly include patients between 15 and 29 with the second highest population being over the age of 55[17,18]. Although over the last 30 years, there has been an increase from 4.6% to 13.2% in patients over the age of 60[19,20].

The vast majority of SCI cases are due to traumatic events including traffic accidents and falls, but can also include violent crime and self-harm[21]. Specifically traffic accidents in North America accounted for 38% of SCIs between 2010 and 2014[22]. Falls accounted for around 31% of injuries as the second most common cause followed by sports-related injuries at 10-17% of traumatic SCIs[18,20]. Regardless of the cause of injury, the most common site for a SCI is in the cervical region of the spine at around 60% of the injuries with thoracic and lumbosacral at 32% and 9% respectively[19]. SCIs occurring in the cervical region of the spinal cord commonly lead to impairments in the upper extremities which tend to be highly debilitating. This demonstrates a need for effective treatments focusing on recovery of function lost due to damage in the cervical region.

Furthermore, while SCIs have clear consequences for physical and social function for patients and their families, the financial burden can be incredibly costly. The direct costs for patient care can be anywhere from $1.1 – 4.6 million US dollars per patient. These costs can be completely devastating to both the patient and their family describing a need for research focused on the prevention of SCI and recovery of function following SCI. Over the last thirty years, many neuroprotective and neuroregenerative therapies have moved from preclinical studies into clinical trials, there are still no treatments available to the patient. Subsequently, there is also no treatment available enhancing functional recovery of SCI patients[23].

Traumatic incomplete SCIs in the cervical region are the most common SCI largely debilitating a young population thus this dissertation will focus on recovering sensorimotor function following cervical SCI. In incomplete SCIs, the primary injury massively damages cells and starts secondary cascades which can lead to death of glial cells and neurons while producing

ischemia and inflammation. Following these initial insults, permanent changes occur in the organization of the spinal cord structure attempting to spare tissue surrounding the glial scar and cystic cavities produced by these secondary cascades. Over the next few weeks to months, the spinal cord has poor intrinsic recovery potential specifically with endogenous remyelination and axonal regrowth contributing to permanent neurological deficits[24]. This dissertation will investigate the recovery of function in models of incomplete SCI containing some spared tissue around the lesion site.

In Chapters 2 and 3 of this dissertation I describe a technique to improve the recovery of motor function following three different SCI injury models within the cervical region. Rat models are the most common model used in SCI research as they produce similar injury responses to that of humans and anatomically and pathophysiologically resemble the human spinal cord. The most common models of SCI used in research consist of contusion, compression, and transection models with the highest clinical relevance being the contusion model[24]. Therefore this dissertation will focus on three separate contusion models resembling SCI syndromes: Brown-Sequard syndrome (hemi-cord syndrome), posterior cord syndrome, and anterior cord syndrome. All three SCI syndromes induce damage to certain descending motor tracts while leaving some tissue spared around the injury location, a common feature of incomplete SCIs, the most common type of SCI. This opens the possibility for techniques that induce targeted plasticity to improve motor function through the reorganization of descending motor signals from the brain through the spinal cord and onto distal musculature.

Traditionally SCI research has focused on tissue regeneration, neuroprotective treatments, and cellular transplantation, but more recently there has been a large push for approaches that

induce plasticity through the use of neuromodulators. This can be described by findings over the last twenty years or so that illustrate how spinal cord injury causes permanent changes to the cortex inducing reorganization which may play a role in the loss of function following SCI[25,26]. This opens the possibility for treatments focused on inducing synaptic plasticity. Two recent neuromodulatory approaches utilizing this idea involve the stimulation of the brain or the spinal cord producing modest results[27–29]. Techniques to enhance plasticity have accrued great interest recently and hold promise for improving functional recovery[30,31].

My lab has developed a technique to induce long-lasting and robust targeted neuroplasticity. Brief bursts of electrical stimulation to the left cervical vagus nerve induce the release of neuromodulators, prominently acetylcholine and norepinephrine which are implicated in the modulation of plasticity[32–34]. Multiple recent studies have demonstrated that by temporally pairing vagus nerve stimulation (VNS) with motor or sensory activity, neurological networks specific to the paired stimuli undergo plasticity[6,7,35,36]. The underlying precise mechanism is not clearly understood, but the release of neuromodulators paired with circuit activity may enhance plasticity specific to these active circuits[37]. In the primary auditory cortex increases in area and response characteristics were observed when VNS was paired with auditory tones[36]. Pairing VNS with forelimb activity significantly increases the representation of the paired movement within the motor cortex, while animals receiving identical training without VNS did not demonstrate an expanded representation of the paired movements[35]. These studies provide evidence pairing VNS with specific stimulus has the potential to induce targeted plasticity in both the motor and systems.

VNS was approved by the US Food and Drug Administration (FDA) in 1997 for the treatment of epileptic seizures resistant to medications[38]. Although in 1952, VNS was observed to alter cortical potentials in vagotomized cats[39]. For most applications of VNS, the left vagus nerve is targeted because fibers from the right vagus nerve densely innervate the sinoatrial node of the heart[40]. In the left cervical vagus about 80% of the fibers are afferent and relay sensory information from the viscera to the brainstem[41]. In the vagus, A-fibers are the largest and fastest conducting fibers relaying visceral information; B-fibers relay efferent parasympathetic and sympathetic information; and C-fibers mainly relay afferent visceral information[42]. The vagus nerve typically carries afferent information from the viscera, receptors of the aortic arch, and reflex regulatory processes of the respiratory, digestive and cardiovascular systems[43]. These afferent projections have cell bodies that synapse at the caudal portion of the nucleus of the solitary tract (NTS)[43].

Norepinephrine (NE) could play a large role in the downstream activation of the vagus nerve as tract tracing studies have shown that anatomical connections exist from the NTS to the locus coeruleus (LC)[44,45]. VNS has been shown to provide changes to mechanistically different forms of seizures which suggests the mechanisms underlying VNS must affect different cell types and brain structures. The administrations of DSP-4, a neurotoxin specifically targeting noradrenergic cells, was shown to block the effects of VNS on suppressing seizures implicating noradrenergic signaling[46,47]. Also, the level of NE has been correlated with the effectiveness of VNS treatment for seizure suppression[48]. Therefore the release of norepinephrine from the LC due to VNS is a highly like mechanism[43,49].

Along with norepinephrine released from the LC, the nucleus basalis (NB) and subsequently acetylcholine (ACh) have been implicated in the therapeutic effects of VNS. One study demonstrated cholinergic activation of NB neurons after VNS was applied in anesthetized cats[50]. Following a lesion of the NB using a highly specific cholinergic toxin 192-IgG-Saporin, map plasticity in the motor cortex due to VNS was blocked, suggesting that Ach is necessary for VNS-mediated cortical map plasticity[51].

The above evidence provides a link to ACh and NE to regulating plasticity individually, but further evidence demonstrates that these neuromodulators work synergistically to induce facilitate plasticity when coupled with spike-timing dependent plasticity (STDP)[34,52]. STDP is derived on the Hebbian principles describing synaptic strength alterations based on the timing of pre- and post-synaptic spiking where the temporal boundaries have been clearly defined[53]. If the post-synaptic spike closely follows the pre-synaptic spike then long-term potentiation (LTP) can be induced, strengthening a synaptic connection, whereas if the pre-synaptic spike follows the post-synaptic spike then long-term depression (LTD) can be induced, decreasing the synaptic strength. Inducing LTP or LTD is dependent on the time delay between spikes, with the greatest effect seen within a time delay on the order of milliseconds depending on the cortical area[33,54,55]. More recently, studies have demonstrated that neuromodulators are regulated by STDP plasticity by changing the temporal threshold for STDP induction[56]. Another recent study have furthered the understanding of the effects on neuromodulators on STDP by applying neuromodulators after the spiking of synaptically coupled neurons to increase plasticity[57]. Although the translation of these concepts translates to systems level plasticity remain unclear[56], this evidence provides a potential underlying mechanism of pairing neuromodulator release shortly after synaptic activity.

The previous evidence demonstrates a clear link between plasticity and the release of neuromodulators, however neuromodulators alone are not enough to induce plasticity. Neural activity is combined with neuromodulator release are both required in order to produce plasticity[7]. VNS-dependent release of acetylcholine and norepinephrine may act together to alter STDP properties in order to drive plasticity in this active neural circuitry[37]. The stimulation paradigm used in this dissertation works to further understand the synaptic eligibility trace of VNS-mediated neuromodulator release. In Chapter 2 we provide evidence that the temporal pairing is essential for enhanced plasticity to provide recovery following neurological injury, corroborating previous results[58,59]. This evidence demonstrates a link between neuromodulatory regulation of plasticity and the VNS-mediated plasticity effects.

If the use of VNS paired with motor or sensory activity can be experimentally effective as a treatment for motor and/or sensory impairment in multiple models of neurological injury, VNS has a large potential for translation. VNS is currently implanted in over 75,000 patients around the world with very few suffering from side effects[60], and the current parameters of VNS used in our VNS therapy studies delivers less than 1% of the amount of stimulation currently approved by the FDA[36,61,62]. Together these findings illustrate that VNS is safe and feasible, and allowed the potential for VNS therapy to be utilized for the recovery of function after neurological injury.

In rat models of ischemic stroke, intracerebral hemorrhage, TBI, and SCI, VNS paired with rehabilitative training produced significant recovery of strength and speed[63–65]. These studies provide evidence that VNS may be a new technique for the treatment of impairments in motor function due to neurological injury. An initial pilot clinical study in ischemic stroke

survivors and a second double-blind placebo controlled trial were recently conducted to investigate if VNS paired with rehabilitation is effective in treating motor dysfunction resulting from stroke[62,66]. Both the initial safety efficacy trial and the double-blind placebo controlled trial indicated that adding VNS to rehabilitative training was successful in improving upper-limb function in stroke patients, resulting in a three-fold increase in upper extremity Fugl-Meyer scores[62,66]. Most recently a case study was conducted in a stroke patient with profound sensory loss despite improvements in motor function following the double-blind placebo controlled trial[67]. VNS paired with sensory retraining produced significant benefits in somatosensation for the patient enrolled in the case study. These results provide clear evidence that VNS enhances the beneficial effects of physical rehabilitation in both rat models and humans suffering from upper-limb dysfunction following multiple types of neurological disorders.

In Chapter 2, I describe a study designed to investigate the effects of VNS paired with the isometric pull task on improving upper limb function following two models of spinal cord injury at the cervical level 5. Clinical trials utilizing VNS to improve function after SCI are beginning shortly, but many facets of the therapy are yet to be fully understood. In this study we demonstrate that VNS therapy 1) improves function in multiple models of VNS therapy 2) improves motor recovery when reliably delivered within seconds of a successful movement, describing a synaptic eligibility trace, 3) enhances recovery only when VNS was paired with trials that approximated the desired outcome, and 4) provides the first evidence that closed-loop VNS enhances reorganization of synaptic connectivity in remaining networks in two non-overlapping models of SCI.

In Chapter 3, I describe a study designed to investigate the effects of VNS paired with the isometric pull task on improving upper limb function following a bilateral spinal cord injury at the cervical level 7. While the findings in Chapter 2 provide evidence for the utility of VNS for SCI, the injury model spares alpha motor neurons residing in C7-T1 which specifically innervate distal forelimb musculature. Since many SCI patients have damage at these levels, and damage at this level specifically causes loss of function to distal musculature, it is important to understand whether VNS can still be effective with the absence of the majority of motor neurons at this level. Substantial damage to these motor neuron pools could limit the benefits of plasticity-enhancing therapies if reorganization cannot compensate for the reduction in alpha motor neurons. Alternatively, synaptic plasticity within spared spinal networks may be sufficient to leverage remaining alpha motor neurons to support recovery. Here, we sought to model these complicating clinical features and determine whether direct damage to the distal forelimb motor pools would prevent VNS-dependent enhancement of recovery. To do so, we assessed recovery of forelimb motor function in animals that received a bilateral incomplete contusive SCI at C7/8 and underwent extensive rehabilitative training with or without paired VNS. This study demonstrates that VNS therapy paired with the isometric pull task 1) significantly improves recovery of volitional forelimb strength 2) generalizes to two similar, but untrained, forelimb tasks 3) is efficacious despite the lack of alpha motor neurons supporting the inclusion of patients with incomplete cervical SCI in future clinical trials.

In Chapters 4 and 5, I will discuss a different type of neurological injury in the periphery along with enhancing neuroplasticity to treat sensory dysfunction. PNI is one of the largest sources of lifelong disability, with over 360,000 people in the United States affected each year[68]. The upper

extremities are most commonly affected by PNIs which leads to impairments of motor function to of both the arm and hand[69]. More than half of patients with a nerve injury will recover substantial function[70]. Specifically in PNIs with full transections of nerve trunks, around 90% of adults suffer long-lasting loss of function[71]. Even with advances in surgical repair interventions, numerous patients continue to have motor and sensory disability along with secondary problems such as neuropathic pain[72].

Loss of somatosensation is a common consequence of neurological injury. After stroke, as many as 85% of patients exhibit deficits in somatosensory function[73,74]. Peripheral nerve damage also causes large deficits in somatosensation in many patients, which can often last even after surgical repair[75,76]. Currently there are no consistently effective strategies to treat sensory dysfunction, but rehabilitation that incorporates sensory retraining may provide some benefits to patients[77–81]. While most treatment methods typically focus on the recovery of motor function following damage to the nervous system, deficits in somatosensation are a large component contributing to disability[82–84]. Considering the significance of sensory loss, the development of effective treatment methods that can provide substantial recovery of somatosensory function has the potential to produce substantial benefits for patients with disability due to a wide variety of neurological damage.

In Chapter 4, I describe a study designed to investigate the beneficial effects of VNS on improving mechanosensation following peripheral nerve injury. This study was largely motivated by recent findings from a clinical case study highlighting the utility of rigorous two way translation. In the case study, VNS paired with sensory retraining therapy may improve somatosensory function in a chronic stroke patient with profound sensory loss[67]. While these data

were encouraging, the single subject and open-label design limited the applicability of these findings. Thus this study sought to further investigate these findings in an animal model of chronic sensory loss. The findings from the study demonstrate the VNS therapy paired with sensory stimuli 1) can enhance recovery of sensory function after neurological data 2) can maintain recovery of sensory function for months after the cessation of therapy 3) can generalize to other somatosensory tasks and functions.

In Chapter 5, I describe a study designed to provide further insight into the necessary components in tactile rehabilitation specifically when paired with VNS to improve sensorimotor function following peripheral nerve injury. This study is not completed, as data analysis is still ongoing.

VNS paired with rehabilitation targeted at restoring sensorimotor function provides a new promising therapy. This dissertation focuses on recovering both motor and sensory function in multiple animal models of SCI and PNI, demonstrating how VNS may be a promising tool for inducing plasticity to treat sensory and motor dysfunction due to multiple types of neurological injury in the clinic.

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CHAPTER 2

CLOSED-LOOP NEUROMODULATION RESTORES NETWORK CONNECTIVITY AND MOTOR CONTROL AFTER SPINAL CORD INJURY[1]

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Abstract

Recovery from serious neurological injury requires substantial rewiring of neural circuits. Precisely-timed electrical stimulation could be used to restore corrective feedback mechanisms and promote adaptive plasticity after neurological insult, such as spinal cord injury (SCI) or stroke. This study provides the first evidence that closed-loop vagus nerve stimulation (CLV) based on the synaptic eligibility trace leads to dramatic recovery from the most common forms of SCI. The addition of CLV to rehabilitation promoted substantially more recovery of forelimb function compared to rehabilitation alone following chronic unilateral or bilateral cervical SCI in a rat model. Triggering stimulation on the most successful movements is critical to maximize recovery. CLV enhances recovery by strengthening synaptic connectivity from remaining motor networks to the grasping muscles in the forelimb. The benefits of CLV persist long after the end of stimulation because connectivity in critical neural circuits has been restored.

Introduction

Recovery from serious neurological injury requires substantial rewiring of neural circuits. Many methods have been developed to enhance synaptic plasticity in hopes of enhancing recovery. Unfortunately, these methods have largely failed in the clinic likely due to the challenge of precisely targeting specific synapses and absence of testing in clinically-relevant models[1,2]. Real-time control of neural activity provides a new avenue to promote synaptic plasticity in specific networks and restore function after injury[3–6].

Human and animal studies demonstrate that precisely timed vagus nerve stimulation (VNS) can improve recovery of sensory and motor function. VNS engages neuromodulatory networks and triggers release of pro-plasticity factors including norepinephrine, acetylcholine, serotonin,

brain-derived neurotrophic factor, and fibroblast growth factor[7–9]. This in turn influences expression and phosphorylation of proteins associated with structural and synaptic plasticity, including *Arc*, CaMKII, TrkB, and glutamate receptors[10,11]. Engagement of neuromodulatory networks activates a transient synaptic eligibility trace to support spike-timing-dependent plasticity (STDP)[12], thus raising the prospect that closed-loop neuromodulatory strategies may provide a means to direct specific, long-lasting plasticity to enhance recovery after neurological injury. Indeed, in the absence of neurological damage, repeatedly pairing sensory or motor events with brief bursts of VNS yields robust plasticity in sensory or motor cortex that is specific to the paired experience. Moreover, the addition of VNS to rehabilitative training improves recovery in rodent models of unilateral brain injury and in chronic stroke patients, highlighting the clinical potential of closed-loop neuromodulatory strategies[7,13–16].

We tested the hypothesis that closed-loop VNS (CLV) could be harnessed to enhance recovery after spinal cord injury (SCI). To do so, we developed a real-time closed-loop neuromodulation paradigm based on the synaptic eligibility trace to deliver VNS immediately after the most successful forelimb movements during motor rehabilitation. The strategy uses a control algorithm that adaptively scales stimulation threshold to trigger a brief 0.5 s train of VNS on trials in which pull forces fall within the top quintile of previous trials (Top 20% CLV; Figure 2.1a,b, and Figure 2.1-Figure Supplement 1a). To test the hypothesis that temporal precision is required for VNS- dependent effects, we employed a similar algorithm in which stimulation was delivered on the weakest quintile of trials (Bottom 20% CLV; Figure 2.1b & and Figure 2.1- Figure Supplement 1g). Both algorithms deliver the same amount of VNS during rehabilitative training, but Bottom 20% CLV results in a significant delay between VNS and the most successful trials.

Results

To test whether CLV could improve recovery of motor function after SCI, rats were trained to perform an automated reach-and-grasp task measuring volitional forelimb strength (Figure 2.1b, Movie S1)[17]. Once proficient, rats received a right unilateral impact at spinal level C6 to impair function of the trained forelimb and underwent implantation of a bipolar cuff electrode on the left cervical vagus nerve[18]. SCI resulted in a 77% reduction in volitional forelimb strength, consistent with paresis observed in many cervical SCI patients (Figure 2.1c, PRE v. Wk 8, Paired t-test, $t(29) = 37.34$, $P = 4.4 \times 10^{-26}$, Movie S2). Top 20% CLV substantially boosted recovery of volitional forelimb strength compared to equivalent rehabilitative training without CLV (Rehab alone), demonstrating that CLV enhances recovery of motor function after SCI (Figure 2.1c; Two-way repeated measures ANOVA, Interaction; $F[6,120] = 3.88$, $P = 1.43 \times 10^{-3}$; Movies S3-4). CLV resulted in lasting recovery after the cessation of stimulation after week 11, consistent with the notion that CLV restores function in critical motor networks (Top 20% CLV; Wk 11 v. Wk 12; Paired t-test, $t(12) = -0.89$, $P = 0.38$). Despite equivalent rehabilitation and a comparable number of stimulations delivered during task performance (Figure 2.1d,e), Bottom 20% CLV resulted in substantially diminished recovery compared to Top 20% CLV (Figure 1c, Two-way repeated measures ANOVA, Interaction; $F[6,114] = 2.40$, $P = 0.03$, Movie S5) and failed to improve forelimb strength compared to Rehab alone. Together, these findings demonstrate that closed- loop neuromodulation paired with the most successful movements during rehabilitation improves recovery of motor function after cervical SCI.

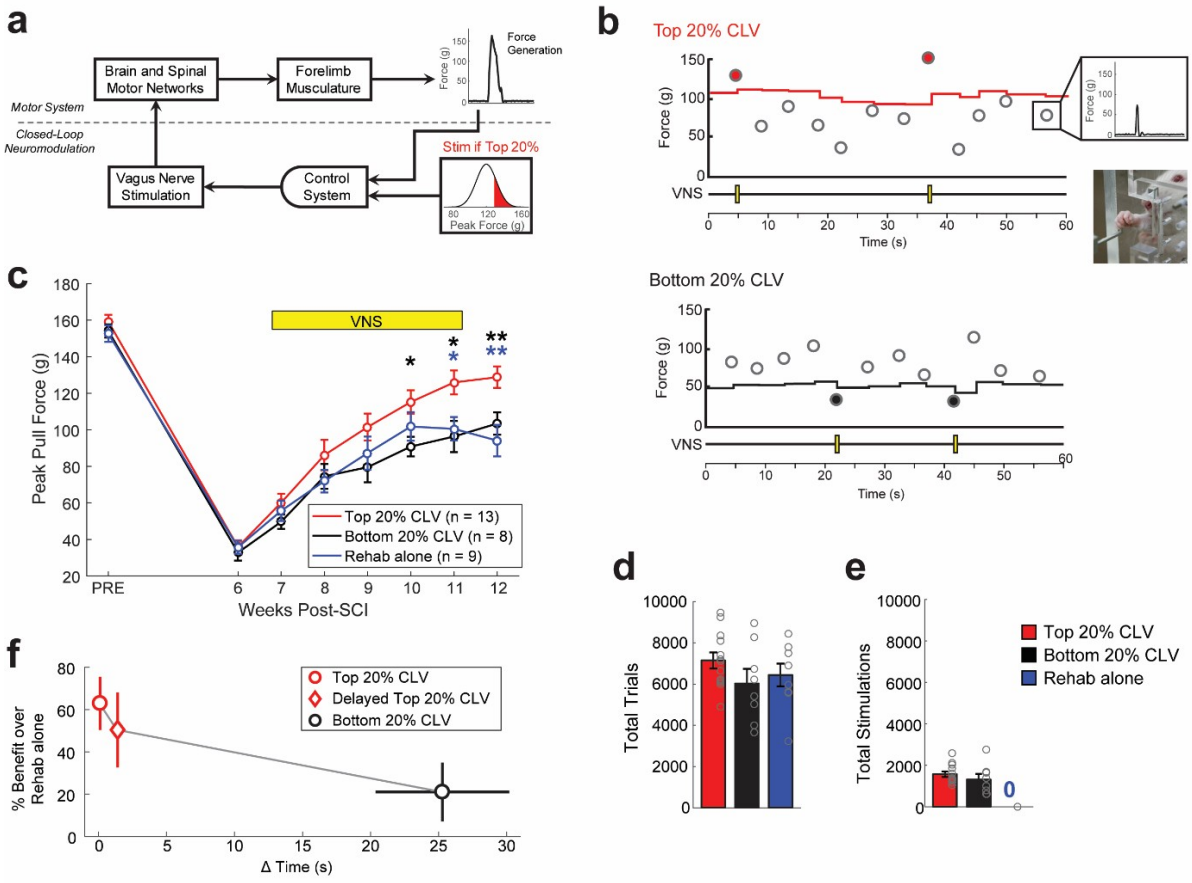

Figure 2.1. Precisely-timed closed-loop vagus nerve stimulation based on the synaptic eligibility trace enhances recovery after spinal cord injury

(a) Closed-loop neuromodulation to deliver vagus nerve stimulation to reinforce the most successful trials during rehabilitative training after SCI. (b) Top 20% CLV received a 0.5 s train of VNS on trials in which pull force falls within the highest quintile of previous pull forces. The Bottom 20% CLV group received VNS on trials in which pull force falls within the lowest quintile. Rehab alone performed equivalent rehabilitative training without VNS. Each circle represents peak pull force on an individual trial. Inset shows an animal performing the isometric pull task. See Fig. 1-Supplementary Figure 1 for more detail.
(c) Top 20% CLV significantly improves forelimb function after SCI compared to Bottom 20% CLV and Rehab alone, indicating that precisely timed VNS enhances recovery. (d,e) Differences in the intensity of rehabilitative training or the amount of stimulations cannot account for improved recovery. A significant increase in recovery is observed with Top 20% CLV after correcting for number of trials and number of stimulations (ANCOVA, effect of group; number of trials: $F[1,1] = 11.89$, $P = 0.0031$; number of stimulations: $F[1,1] = 9.57$, $P = 0.0066$). Gray circles denote individual subjects. (f) CLV delivered within 2 s of successful trials increases recovery, whereas CLV separated 25 s from successful trials fails to yield substantial benefits. This time window is consistent with the synaptic eligibility trace hypothesis. Horizontal error bars for Top 20% CLV

and Delayed Top 20% CLV are not visible because of their small size. In panel c, ** $P < 0.01$, *$P < 0.05$ for t-tests across groups at each time point. The color of the asterisk denotes the group compared to Top 20% CLV. Error bars indicate S.E.M.

The synaptic eligibility trace theory posits that neuromodulatory reinforcement must occur within seconds after neural activity to drive plasticity[12]. To clarify how temporally precise CLV must be, a subset of rats received VNS delayed approximately 1.5 seconds after the top 50% most successful trials (Delayed Top 20% CLV, Figure 2.1- Figure Supplement 1d). This short delay resulted in comparable recovery to stimulation delivered immediately after a successful trial in the Top 20% CLV group (Figure 2.1f). Stimulation in the Bottom 20% CLV group was separated by 25 ± 5 seconds from the most successful trials and failed to drive substantial benefits (Figure 2.1f, Figure 1- Figure Supplement 1g). This absence of enhanced recovery despite delivery of CLV may be attributed to either the long delay or greater variance in the timing between stimulation and the most successful trials. These findings support a temporal precision limit for CLV near 10 seconds, consistent with the synaptic eligibility trace hypothesis[12].

To determine whether more pairings of VNS with successful trials would improve recovery, we utilized an adaptive algorithm in which VNS was delivered on at least the top 50% most successful trials, resulting in 2.5 times more stimulation pairings (Top 50% CLV, Figure 2.1- Figure Supplement 1j). Top 50% CLV substantially improved recovery of forelimb function compared to Rehab alone, which provides an independent confirmation that CLV enhances recovery after SCI (Figure 2.1a, Two-way repeated measures ANOVA, Interaction; $F[6,174] = 3.56$, $P = 2.38 \times 10^{-3}$). The rate and degree of recovery were comparable in the Top 50% CLV

and the Top 20% CLV groups (Figure 2.1-Figure Supplement 1), suggesting that timing is more important than quantity of stimulation.

Plasticity in remaining networks could be harnessed to support recovery after SCI[19,20]. Unilateral SCI resulted in extensive damage to gray matter, rubrospinal pathways, and propriospinal pathways in the right hemicord while largely sparing the right dorsal corticospinal tract (CST) (Figure 2b and Figure 2-Figure Supplement 2). Thus, we used intracortical microstimulation to test the hypothesis that CLV enhances output from the corticospinal circuits to the impaired forelimb. CLV resulted in eight times more motor cortex sites that generated grasp movements in the impaired forelimb compared to Rehab alone (Figure 2c and Figure 2-Figure Supplement 4, Unpaired t-test, $t(10) = 2.28$, $P = 0.04$), providing the first evidence that CLV induces large-scale plasticity in corticospinal networks after neurological injury.

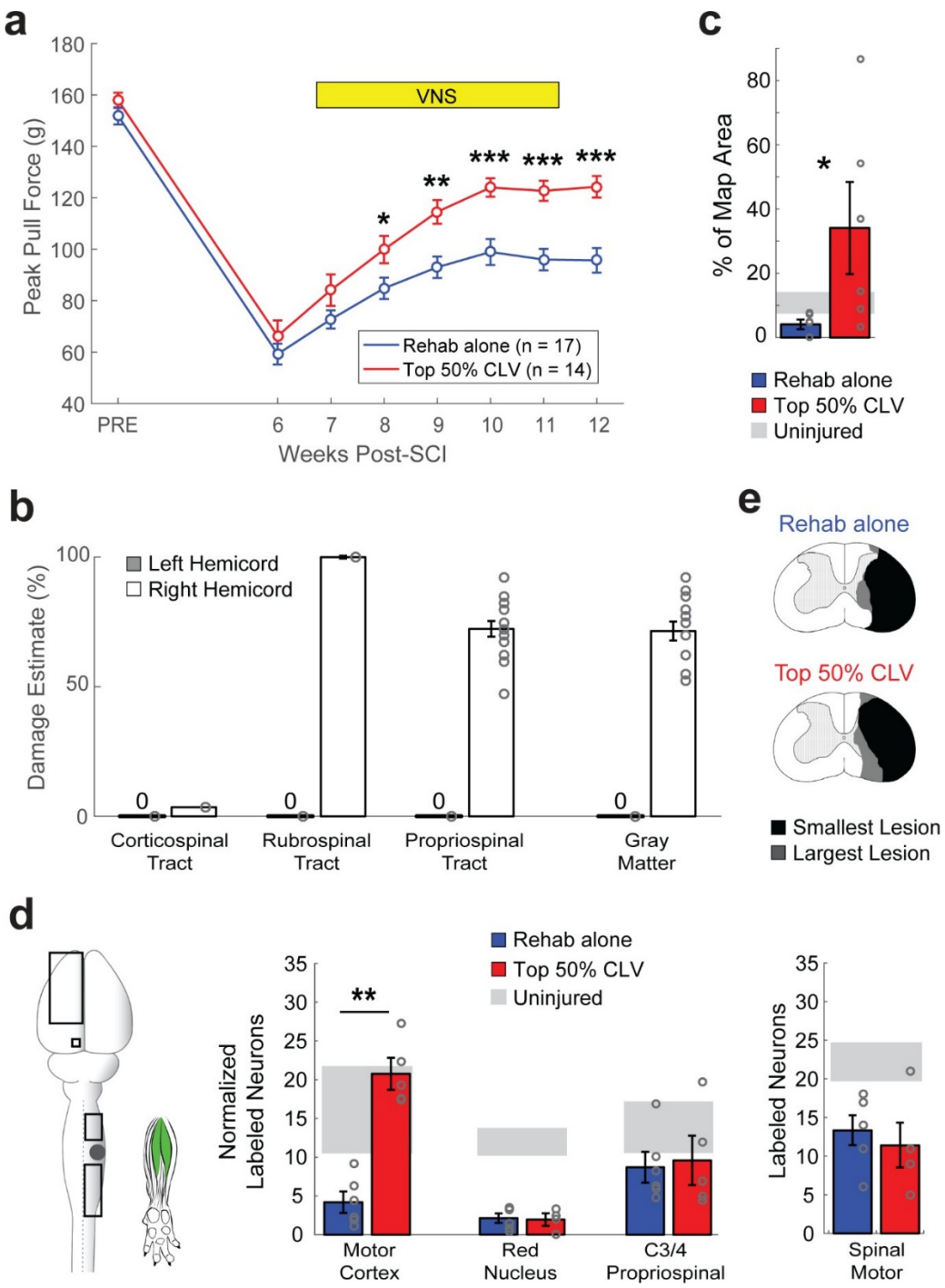

Figure 2.2. CLV enhances plasticity in spared corticospinal networks and improves functional recovery after unilateral SCI

(a) Top 50% CLV significantly improved recovery of forelimb function compared to Rehab alone. Sustained recovery was observed on week 12 after the cessation of stimulation, indicating

lasting benefits. (b) Unilateral SCI caused substantial damage to gray matter, rubrospinal, and propriospinal tracts in the right hemicord, while largely sparing the right corticospinal tract and the entirety of the left hemicord. (c) ICMS reveals that Top 50% CLV significantly increases the area of the forelimb motor cortex evoking rehabilitated grasping movements compared to Rehab alone (N = 6,6). (d) Retrograde transneuronal tracing with PRV- 152 was performed to evaluated anatomical connectivity from the left motor cortex neurons, left red nucleus neurons, and right C3/4 propriospinal neurons to grasping muscles in the trained (right) forelimb. Top 50% CLV restores connectivity and results in a significant increase in labeled neurons in the motor cortex compared to Rehab alone (N = 5,6). No changes were observed in red nucleus or C3/4 propriospinal neurons. Black boxes indicate ROIs; gray dot indicates lesion epicenter; inset shows injected muscles. (e) CLV does not affect lesion size. In all panels, gray circles denote individual subjects. In all panels, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ for t-tests across groups. Error bars indicate S.E.M.

We next tested the hypothesis that CLV improves recovery by increasing synaptic connections within the motor network controlling grasping muscles of the forelimb. We injected the retrograde transsynaptic tracer pseudorabies virus (PRV-152) into flexor digitorum profundus and palmaris longus and counted labeled neurons six days later. CLV resulted in a five-fold increase in labeled neurons in motor cortex compared to Rehab alone (Figure 2.2d and Figure 2.2-Figure Supplement 5, Unpaired t-test, $t(9) = 7.63$, $P = 3.2 \times 10^{-5}$). The magnitude of this increase in synaptic connectivity is comparable to the seven-fold increase in the number of motor cortex sites that produce grasp. CLV failed to increase neuronal labeling of spinal motor neurons, red nucleus neurons or propriospinal neurons (Figure 2.2d). Additionally, CLV did not influence lesion extent (Figure 2.2e, Figure 2.2-Figure Supplement 2). Together, these results are consistent with anatomical plasticity in the spared corticospinal network contributing to enhanced recovery when CLV is added to rehabilitative training after SCI (Figure 2.3).

The observation that CLV improves recovery and enhances functional and anatomical plasticity in corticospinal networks suggests that CLV may prove ineffective if the CST is destroyed. Given the severity and anatomical heterogeneity of damage observed in SCI patients[21], such a finding would limit the clinical utility of CLV. We therefore evaluated motor recovery in a bilateral injury model that virtually eliminates the CST on both sides of the cord (Figure 2.4b). Despite profound damage, CLV more than doubled the degree of forelimb motor recovery compared to Rehab alone (Figure 2.4a, Two-way repeated measures ANOVA, Interaction; $F[6,144] = 5.29$, $P = 7.62 \times 10^{-5}$). The observation that CLV can improve recovery following bilateral SCI suggests CLV could be clinically useful. We hypothesized that CLV enhances recovery by promoting plasticity in the rubrospinal and propriospinal pathways, which were damaged, but not eliminated, by this injury (Figure 2.4b and Figure 2.4-Figure Supplement 1). Indeed, CLV doubled the number of labeled red nucleus neurons and C3/4 propriospinal neurons compared to Rehab alone (Figure 2.3d and Figure 2.4-Figure Supplement 3, Unpaired t-test, Red Nucleus: $t(4) = 3.89$, $P = 0.018$; Propriospinal: $t(4) = 2.77$, $P = 0.05$). Consistent with the extensive damage to the corticospinal pathway, CLV had no effect on reorganization of motor cortex (Figure 2.4c and Figure 2.4-Figure Supplement 4, Unpaired t-test, $t(12) = -0.13$, $P = 0.90$) and failed to increase the number of labeled neurons in the motor cortex (Fig. 2.3d, Unpaired t-test, $t(4) = 0.83$, $P = 0.45$). These results suggest that CLV is capable of supporting recovery following SCI by strengthening anatomical connectivity within remaining pathways (Figure 2.4-Figure Supplement 5).

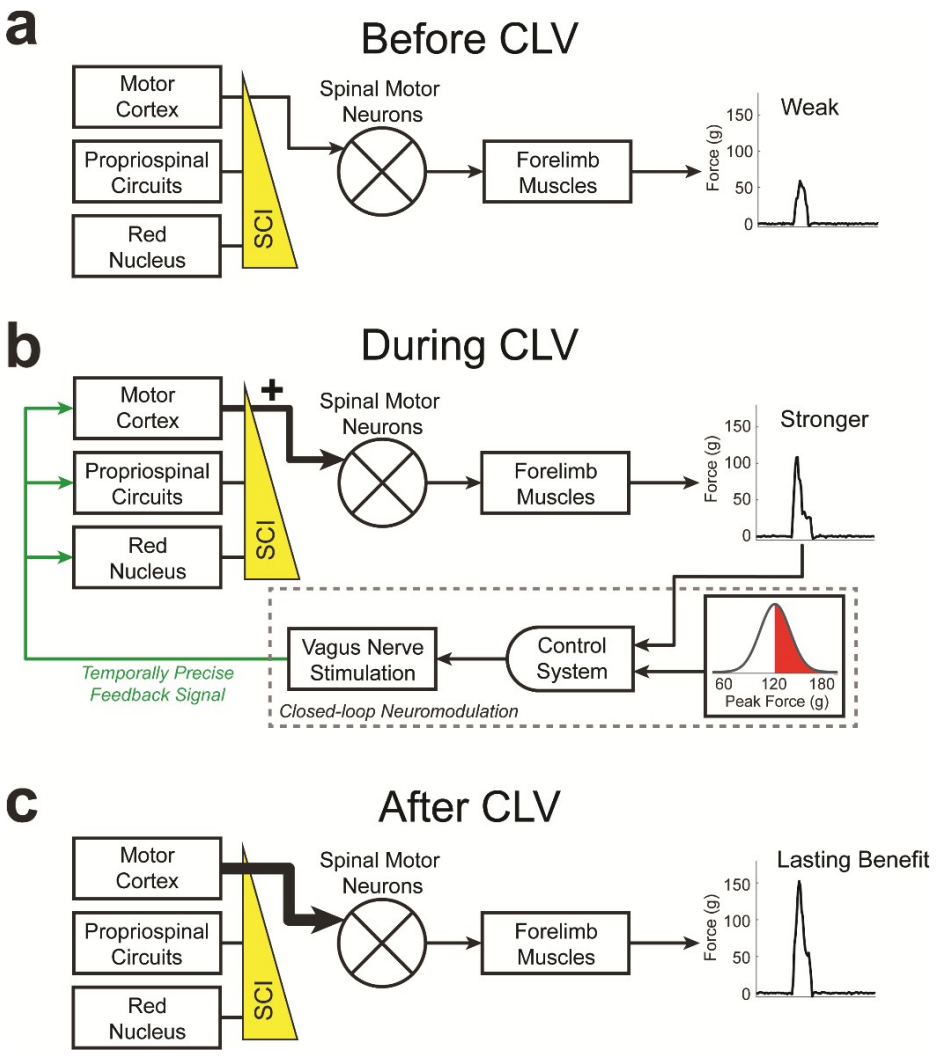

Figure 2.3. Schematic of CLV-dependent recovery after unilateral SCI

(a) After unilateral SCI, loss of motor out from rubrospinal and propriospinal networks results in forelimb paresis and impairments in motor control. (b) The addition of CLV provides temporally-precise feedback on the most successful trials to facilitate training-dependent plasticity in remaining motor networks.
(c) The benefits of CLV persist after the cessation of closed-loop stimulation because connectivity in critical neural circuits has been restored.

Discussion

In this study, we developed a novel closed-loop neuromodulation strategy to make use of the high temporal precision of the synaptic eligibility trace. We demonstrate that activation of the vagus nerve improves recovery when reliably delivered within seconds of a successful movement, and we provide the first evidence that CLV enhances reorganization of synaptic connectivity in remaining networks in two non-overlapping models of SCI. The flexibility to promote reorganization in a range of pathways is a critical benefit of CLV, given the great heterogeneity in the etiology, location, and extent of damage present in SCI patients.

Classical studies by Skinner demonstrate that adaptive reinforcement of successive approximations, or shaping, drives behavior toward a desired response[22]. This principle has been adopted for use in rehabilitation, with the intention to reinforce successively better movements[23]. We made use of this concept by applying an adaptively-scaled stimulation threshold to deliver CLV with the most successful forelimb movements during rehabilitation. Enhanced recovery was observed only when CLV was paired with trials that approximated the desired outcome, highlight the importance of timing for closed-loop stimulation to shape behavioral outcomes and maximize recovery.

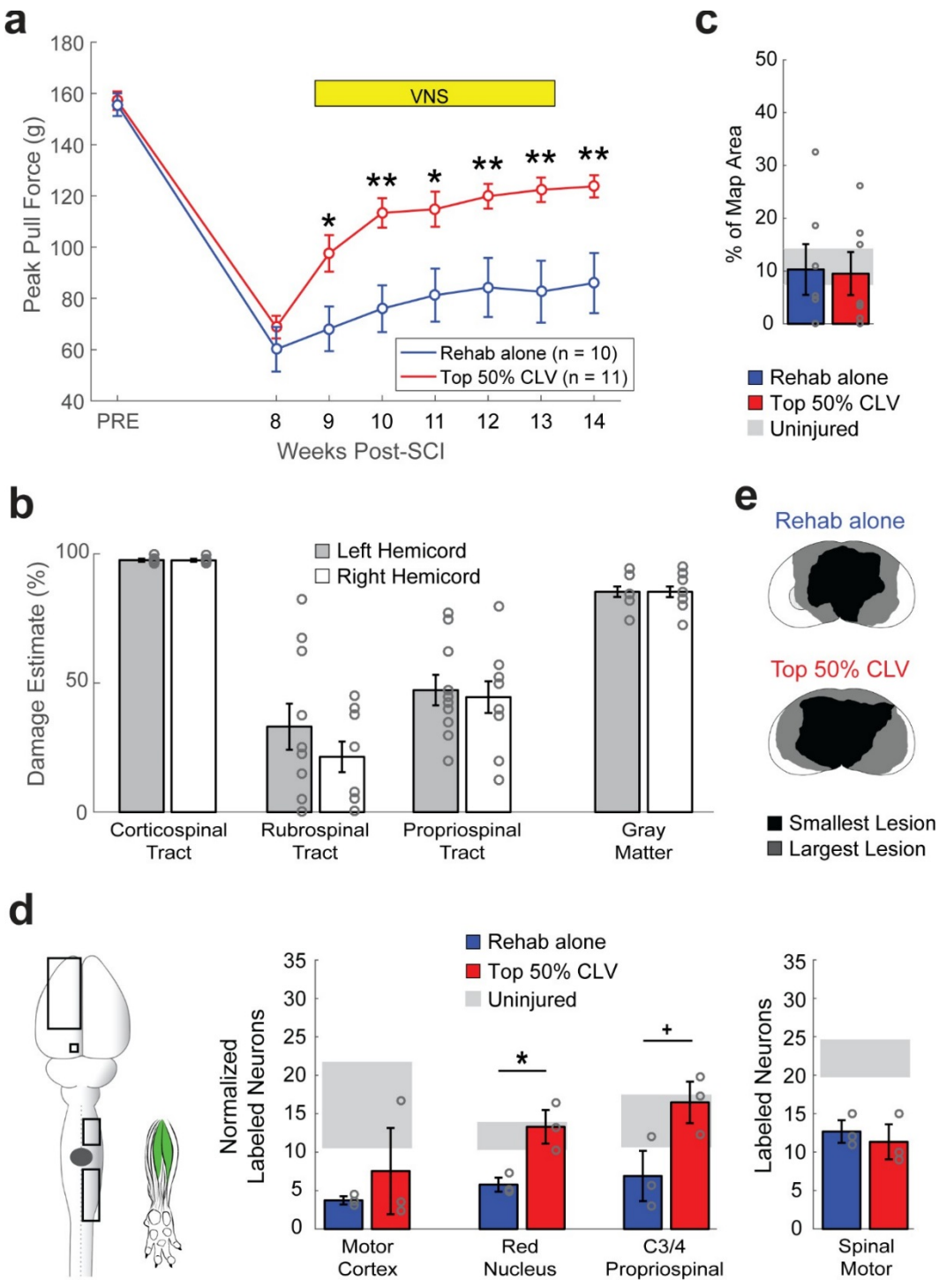

Figure 2.4. CLV enhances synaptic plasticity and recovery after bilateral SCI

(a) After bilateral SCI, Top 50% CLV substantially enhanced recovery of volitional forelimb strength compared to Rehab alone. Improved function was maintained on week 14 after the

cessation of CLV, indicative of lasting recovery. (b) Bilateral SCI resulted in virtually complete bilateral ablation of the corticospinal tract and substantial damage to gray matter. The rubrospinal and propriospinal tracts were lesioned, but partially remaining. (c) Unlike after unilateral SCI, Top 50% CLV failed to increase the area of the motor cortex evoking rehabilitated grasping movements compared to Rehab alone (N = 7,7). (d) CLV significantly increased synaptic connectivity from the left red nucleus neurons and right C3/4 propriospinal neurons to grasping muscles compared to Rehab alone (N = 3,3). Black boxes indicate ROIs; gray dot indicates lesion epicenter; inset shows injected muscles. In all panels, gray circles denote individual subjects. In all panels, ** $P < 0.01$, * $P < 0.05$, + $P = 0.05$ for t-tests across groups. Error bars indicate S.E.M.

The magnitude of neuromodulatory activation elicited by an event is directly proportional to the surprise, or unpredictability, of the event[24–26]. This phenomenon is ascribed to reward prediction error[27]. Unsurprising events fail to activate neuromodulatory systems, and even rewarding events fail to trigger neuromodulator release if they are expected. We posit the predictability and accompanying tedium of long, frustrating rehabilitation and the minimal reinforcement of practicing a previously simple motor task blunts plasticity and limits recovery after SCI. The closed-loop neuromodulation strategy developed here circumvents this by artificially engaging neuromodulatory networks and providing a repeated, non-adapting reinforcing signal typically associated with surprising consequences[7–9]. CLV drives temporally-precise neuromodulatory release to convert the synaptic eligibility trace in neuronal networks that generate optimal motor control to long-lasting plasticity[12].

CLV is a minimally-invasive, safe strategy to provide precisely-timed engagement of multiple neuromodulatory networks to boost plasticity during rehabilitation[7]. Preliminary results in chronic stroke and tinnitus patients highlight the clinical potential of CLV, while delivering less than 1% of the total FDA-approved amount of stimulation[16,28,29]. Moreover, the flexibility to deliver stimulation with a variety of rehabilitative exercises raises the possibility to design CLV-

based to target motor dysfunction of the lower limbs, somatosensory loss, and bowel and bladder issues, all of which are prevalent in SCI patients. Delineation of the timing requirements and documentation of neuronal changes driven by CLV in this study provide a framework for development of this strategy for a range of neurological conditions, including stroke, peripheral nerve injury, and post-traumatic stress disorder[7,30].

Methods

Experimental Design

All procedures performed in the study were approved by the University of Texas at Dallas Institutional Animal Care and Use Committee (Protocols: 14-10 and 99-06). Adult female Sprague Dawley rats (N = 181) used in this study were housed one per cage (12 hour light/dark cycle). Twelve experimentally naïve rats were used for control experiments. One hundred and sixty-nine rats were trained to proficiency on the isometric pull task as in our previous studies[13,14,17,31–36]. Sample sizes were based estimated effect size determined in our initial pilot studies and are consistent with comparable previous studies. Trained rats were food restricted Monday-Friday to provide task motivation (*ad libitum* access to water). Because of the cage geometry, only the right forelimb can be used to reach the pull handle to trigger a food reward. After reaching task proficiency (85% success rate on ten consecutive sessions), rats received a unilateral contusive injury (N = 128) or bilateral contusive injury (N = 41) of the cervical spinal cord. After recovery, rats received a vagus nerve cuff electrode and resumed training on the isometric pull task. In addition to the food reward, rats were dynamically allocated to balanced groups to receive a brief burst of vagus nerve stimulation (VNS) on appropriate trials. Rehabilitative training, consisting of freely performing the task, continued for six weeks. No VNS was delivered in any group on

the final week of rehabilitative training, to allow assessment of lasting effects of stimulation. Terminal motor cortex mapping or transsynaptic tracing experiments occurred the week following the end of therapy in a subset of unilateral (N = 23) and bilateral SCI rats (N = 20). Eighty-seven rats were excluded from the study due to mortality (N = 20), inability to perform the task after injury (N = 25), or VNS device failure (N = 42). Device failure included mechanical failure of the headmount or loss of stimulation efficacy, determined by a cuff impedance >25 kΩ or by the absence of a reduction in blood oxygenation in response to a train of VNS while under anesthesia (described below). This is a standard method to evaluate VNS efficacy[37,38]. Animals that failed to demonstrate a reliable drop in oxygen saturation at the end of therapy were excluded. Bilateral SCI rats were given two additional weeks of recovery time due to their larger spinal lesion and slower return to recumbency (Fig. 2.4-Figure Supplement 6). Other than therapy start time (6 vs. 8 weeks post-SCI), all training and assessment was identical for unilateral and bilateral SCI rats. All source data indexed across animals can be found in Supplementary Figures 1-4.

Volitional Forelimb Force Generation Assessment

The isometric pull task is a fully automated and quantitative assay to measure multiple parameters of forelimb force generation and was performed similar to previous descriptions[13,14,17,31–36]. Isometric pull training sessions consisted of two 30 min sessions (separated by at least 2 h) five days per week. Experimenters were blind to treatment group at all times throughout behavioral testing. Early in training, rats were encouraged to interact with the pull handle by dispensing pellets (45 mg chocolate-flavored pellets, Bio-Serv; Flemington, NJ) when they approached or touched the lever. The pull handle was initially located inside the test chamber and then slowly retracted outside of the behavioral chamber to encourage reaching with the right paw. A trial was initiated when the rats exerted at least 10 g of force on the pull handle. A trial window of 2 s started after trial initiation where the animal could receive a reward by pulling with a force exceeding a reward threshold. The reward threshold was scaled adaptively based on the median peak force of the 10 preceding trials, with a fixed bounded minimum of 10 grams and maximum of 120 g based on previous studies (Figure 1-Figure Supplement 1)[18,36]. Thus, rats received rewards on trials that exceeded either the median peak force from the previous 10 trials or 120 g. The reward threshold was set to 10 g for the first 10 trials of a training session and adaptive scaled for the remaining trials (Figure 2.1-Figure Supplement 1). This reward threshold paradigm was used for all groups at all timepoints during the study.

Rats were trained until they reached proficiency, defined as 10 consecutive sessions in which greater than 85% of trials exceeded 120 g. After reaching isometric pull task proficiency, rats were given a cervical unilateral or bilateral SCI at spinal level C6. Post-injury baseline force generation assessment occurred on week 6 for unilateral SCI and week 8 for bilateral contusion

SCI and consisted of 2 x 30 min sessions per day across 2 consecutive days (POST; Figure 2.1C, 2.2A, & 2.3A). Random group assignment was used to determine which rats received VNS for the first 75% of group assignment decisions. To ensure well-balanced treatment groups, the final 25% of rats were assigned to groups based on their post-injury performance. Rehabilitative training continued for 6 weeks with VNS delivered when appropriate. MotoTrak Software (Vulintus, Inc.) was used to record and display experimental data during the performance of the isometric pull task similar to previous studies[17,18,31,35,39]. A microcontroller board (Vulintus, Inc.) sampled the force transducer every 10 ms and relayed information to the MotoTrak software for offline analysis. For rats receiving VNS, stimulation was triggered by the behavioral software on appropriate trails during rehabilitative training. Peak pull force (maximum force generated in a trial, g) was calculated for every rat for every week of behavior. A Two-way repeated measures ANOVA was used to compared peak pull forces in each treatment condition across time, followed by *post hoc* Bonferroni-corrected unpaired t-tests where appropriate (Figure 2.1C, 2.2A, 2.4A). Percent benefit over Rehab alone was calculated as the recovery of peak pull force after therapy normalized to the average recovery observed in the Rehab alone group (Figure 2.1J). The distribution of pull forces after injury is shown in Figure 2.1-Figure Supplement 2. Behavioral data for each week for all individual subjects is available in Supplementary File 1.

Cervical Spinal Cord Injury (SCI) Surgery

Experimenters were blind to the group of the rat during surgery. All surgeries were performed using aseptic technique under general anesthesia. Rats were anesthetized with ketamine (50 mg/kg), xylazine (20 mg/kg), and acepromazine (5 mg/kg) for all procedures (i.p.). Heart rate and blood oxygenation was monitored during surgery. After achieving isometric pull task

proficiency, rats received either a right side (unilateral) or midline (bilateral) C6 spinal cord contusive impact using surgical technique from previous studies[18]. A right side or bilateral dorsal C5 laminectomy was performed for rats receiving a unilateral or bilateral SCI, respectively. The vertebral column was stabilized using spinal microforceps. For unilateral SCI, the right spinal hemicord was contused using the Infinite Horizon Impact Device with a force of 200 kilodynes and zero dwell time as previously reported (Precision Systems and Instrumentation, Lexington, KY; impactor tip diameter = 1.25 mm)[18]. For bilateral SCI rats, the midline of the spinal cord was contused with a force of 225 kilodynes and zero dwell time (impactor tip diameter = 2.5 mm). The skin overlying the exposed vertebrae was then closed in layers and the incised skin closed using surgical staples. All rats received buprenorphine (s.c., 0.03 mg/kg, 1 day post-op), enrofloxacin (s.c., 10 mg/kg, 3 days post-op) and Ringer's solution (s.c., 10 mL, 3 days post-op) immediately after surgery and continuing post-operatively. All rats were monitored daily for at least 1 week post-injury. We documented time to return to recumbency, defined as the return of the righting reflex and ability to self-feed, and plantar placement following SCI. After bilateral SCI, rats took significantly longer to return to recumbency and forepaw plantar placement compared to unilateral SCI rats (Figure 2.4-Figure Supplement 6). Therefore, bilateral SCI rats started therapy 2 weeks later. After injury, rats were hand fed twice daily and given Ringer's solution (s.c., 10 mL) for up to 1 week post-injury to maintain a healthy diet.

Vagus nerve stimulation cuff implantation surgery

A two-channel connector headmount and vagus nerve stimulating cuff were implanted on post-injury week 6 for unilateral and week 8 for bilateral SCI rats similar to previous studies[3,13–15,32–34,40,41]. Regardless of group assignment, all rats underwent implantation of the headmount and

cuff. Stimulation of the left cervical branch of the vagus nerve was performed using low current levels to avoid cardiac effects[3]. Incised skin was closed using suture. All rats received enrofloxacin (s.c., 10 mg/kg) following surgery and as needed at the sign of infection. To confirm cuff functionality and proper placement, heart rate, respiration, and blood oxygenation saturation during VNS (0.8 mA, 30 Hz, 100 μs pulse width, 1-5 s train duration) were monitored under anesthesia via pulse oximetry after cuff implant and at the end of therapy. Animals that failed to demonstrate a reliable drop in oxygen saturation at the end of therapy were excluded. Stimulation under anesthesia briefly suppressed cardiopulmonary function and was not more severe or lower threshold in SCI rats compared to intact rats.

Vagus nerve stimulation parameters

VNS was triggered by the behavioral software during rehabilitative training based on the stimulation threshold for each group, similar to previous studies[13,14,32–34]. Each stimulation train consisted of 16 x 100 μsec 0.8 mA biphasic pulses delivered at 30 Hz. An adaptive stimulation threshold specific to each CLV group was used to determine stimulation delivery during rehabilitative training (Figure 2.1-Figure Supplement 1). The stimulation threshold was adaptively scaled based on the 10 antecedent trials, with each group receiving VNS triggered on trials which fall into the appropriate range. Rats in the Top 20% CLV group (N = 13) received VNS on trials in which pull force exceeded the top quintile of the previous ten trials, with no minimum or maximum. In the majority of these subjects (N = 9), VNS was delivered immediately (~50 msec) after pull force exceeded the stimulation threshold (Figure 2.1-Figure Supplement 1A). No stimulation was delivered on the first 10 trials during a training session. In a different subset of subjects (Delayed Top 20% CLV, N = 4), VNS was delivered at the end of the

2 s trial window on trials in which exceeded the stimulation threshold, independent of the when the threshold was crossed (Figure 2.1-Figure Supplement 1D). These groups displayed comparable performance (Top 20% CLV v. Top 20% CLV Delay, Week 12, Unpaired t-test, P = 0.78) and were thus combined for analysis in Fig. 1C-E. Rats in the Bottom 20% CLV group (N = 8) received VNS on trials in which pull force failed to exceed the bottom quintile of the previous ten trials, with no minimum or maximum. VNS was delivered at the end of the 2 s trial window if pull force was below the threshold (Figure 2.1- Figure Supplement 1G). Rats in the Top 50% CLV group received VNS on trials that exceeded the median pull force of the previous 10 trials or exceeded 120 g. VNS was delivered immediately (~50 msec) after pull force exceeded the stimulation threshold Figure 2.1-Figure Supplement 1J). No groups received VNS on the final week of rehabilitative training (Week 12 for unilateral and Week 14 for bilateral SCI) to assess effects lasting after the cessation of stimulation. These parameters do not cause discomfort and do not alter reaching behavior[9,42].

Intracortical Microstimulation Mapping

Terminal intracortical microstimulation mapping (ICMS) of motor cortex was performed in a subset of unilateral SCI (Rehab alone, N = 6; Top 50% CLV, N = 6) and bilateral SCI (Rehab alone, N = 7; Top 50% CLV, N = 7) rats at the end of therapy. A group of uninjured rats were used for control (Naïve, N = 7). Rats were anesthetized with and injection (i.p.) of ketamine (50 mg/kg), xylazine (20 mg/kg), and acepromazine (5 mg/kg). A cisternal drain was performed to reduce ventricular pressure and cortical edema during mapping[9,42]. A craniotomy was then performed to expose left motor cortex. Intracortical microstimulation (ICMS) was delivered in motor cortex at a depth of 1.75 mm using a low impedance tungsten microelectrode with an interpenetration

resolution of 500 µm (100 kΩ – 1 MΩ electrode impedance; FHC Inc., Bowdin, MD; biphasic ICMS at 333 Hz, 50 ms train duration, 200 µsec pulse width, 0 – 200 µA current). Mapping experiments were performed blinded with 2 experimenters similar to previous studies[9,42]. The first experimenter positioned the electrode for ICMS and recorded movement data. The second experimenter, blind to the experimental group of the animal and electrode position, delivered ICMS and classified movements. Movement threshold was first defined. ICMS current was then increased by no more than 50% to facilitate movement classification using visual inspection. Movements were classified into the following categories similar to previous studies: vibrissae, neck, jaw, digit, wrist, elbow, shoulder, hindlimb and trunk[43,44]. The cortical area (mm^2) and movement threshold (µA) for each movement category was calculated for each group (Figure 2.2- Figure Supplement 4 and Figure 2.4-Figure Supplement 4). Based on the 500 µm inter-electrode spacing, each stimulation site eliciting a movement was counted as 0.25 mm^2. Movement area and threshold was assessed using One-way ANOVA and unpaired t-tests. Data for all movement classifications for each subject is available in Supplementary File 2.

Pseudorabies Virus Retrograde Transneuronal Tracing

Transsynaptic tracer injections using pseudorabies virus 152 (PRV-152) were performed in a subset of unilateral SCI (Rehab, n=6; VNS+Rehab, n=5) and bilateral SCI (Rehab, n=3; VNS+Rehab, n=3) rats following the respective end of therapy. A group of uninjured rats were used for control (Naïve, n=5). PRV-152 was a generous gift from the lab of Dr. Lynn Enquist and colleagues at Princeton University and was grown using standard procedures[45]. An incision was made over the medial face of the radius and ulna of the trained limb to expose the forelimb grasping muscles flexor digitorum profundus and palmaris longus. 15 µL of PRV-152 (~$8.06 \pm 0.49 \times 10^8$

plaque-forming units) was injected into the belly of each muscle across three separate sites. The incision was then closed with non-absorbable suture. We conducted detailed pilot studies to determine the optimal time of viral infection to allow for layer 5 cortical labeling. At 5 - 5.5 days post-infection we observed little to no cortical labeling for injured or uninjured animals. At 6 - 6.5 days post-infection we observed consistent layer 5 cortical labeling across injured or uninjured animals. Therefore, 6 - 6.5 days was used as our PRV-152 infection duration for our transsynaptic tracing studies. Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and transcardially perfused with 4% paraformaldehyde in 0.1 M PBS (pH 7.5) at 6 – 6.5 days after injection. The brain and spinal cord were removed. Spinal roots were kept for anatomical reference. Tissue was then post-fixed overnight and cryoprotected in 30% sucrose. Quantification was limited to the spinal motor neurons, C3/4 cervical propriospinal neurons, red nucleus neurons, and cortical layer 5 neurons because these regions exhibited consistent labeling and were specifically related to our hypotheses. The whole neuraxis from the rostral tip of the forebrain to spinal level T3 was blocked and frozen at -80 C in Shandon M1 embedding matrix (Thermo Fisher Scientific; Waltham, MA). Coronal forebrain and midbrain sections were sliced and slide-mounted at 35 μm using a cryostat (from the rostral tip of forebrain to 13 mm caudal). Coronal spinal cord sections were sliced and slide mounted at 50 μm (from C4 – T3). After coverslipping, slides were scanned and digitized using the NanoZoomer 2.0-HT Whole Slide Scanner (Hamamatsu Photonics; Japan). Tissue images were exported to a custom software program for cell counting. PRV-152 infected neurons expressed enhanced green fluorescent protein. PRV-152 neuron counts were made on every other forebrain and midbrain section (35 μm inter-slice interval) and every third spinal cord section (100 μm inter-slice interval).

Experimenters performing analysis were blind to the group of each rat. Cortical neuron counts were restricted to layer 5 of sensorimotor cortex[46]. We defined motor cortex using standard anatomical reference[47]. Our ICMS mapping studies confirm that these regions contain the cortical forelimb sensorimotor circuitry. Red nucleus neuron counts were made using standard anatomical reference[47]. Propriospinal neuron counts were made from spinal level C3 – C4 in Rexed lamina VI, VII, VIII and IX using standard anatomical reference similar to previous studies[48,49]. Back-labeled putative spinal motor neurons were located in Rexed lamina IX and counted identical to previous studies[48,49]. Sensorimotor cortex, red nucleus and cervical propriospinal neuron counts were normalized within rats to the number of putative spinal motor neurons in the lower cervical and upper thoracic spinal cord to control for any differences in injection efficacy. No differences in spinal neuron labeling were observed between CLV and Rehab alone (Figure 2.2E and 2.4E). Sensorimotor cortex, red nucleus and putative spinal motor neuron counts were analyzed separately using unpaired t-tests. Data representing raw neuron counts in each ROI is available in Figure 2.2-Figure Supplement 5, Figure 2.4-Figure Supplement 3, and Supplementary File 3.

Lesion Histology and Analysis

At the completion of experimental testing, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and transcardially perfused with 4% paraformaldehyde in 0.1 M PBS (pH 7.5). The spinal cord was removed and spinal roots were kept for anatomical reference. Spinal tissue was then post-fixed overnight, cryoprotected in 30% sucrose for 48 hours, blocked and frozen at -80 C in Shandon M1 embedding matrix (Thermo Fisher Scientific; Waltham, MA). Spinal tissue was sliced at 50 μm using a cryostat, slide mounted and stained for Nissl (gray matter) and myelin (white matter) substance similar to previous studies[18,44]. Photomicrographs were taken

at 600 μm intervals to quantify gray and white matter lesion metrics using Image J. For unilateral SCI, the rostral and caudal extent of spinal gray and white matter damage was expressed as the percentage of spared gray and white matter of the right hemicord with respect to the left hemicord (Figure 2.2-Figure Supplement 2 and Figure 2.4-Figure Supplement 1). For bilateral SCI rats, the rostral and caudal extent of spinal damage was expressed as the percentage of spared gray and white matter for each hemicord with respect to an unlesioned rostral and caudal tissue reference within animals[50]. Smallest and largest lesion areas were fitted to a schematic of spinal level C6 (Figure 2.2E and 2.3E). To calculate damage to fiber tracts, two experimenters blind to group assignment evaluated the percentage of lesioned tissue to the dorsal corticospinal tract, dorsolateral corticospinal tract, ventral corticospinal tract, rubrospinal tract, propriospinal tract, and gray matter at the lesion epicenter. The dorsal, dorsolateral, and ventral corticospinal tracts were combined to calculate total CST damage based on the proportion of fibers in each tract[51]. Data representing the damage estimates is available in Supplementary File 4.

Data Availability

All source data supporting the findings of this study are available in the online version of the paper.

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CHAPTER 3

VAGUS NERVE STIMULATION PAIRED WITH REHABILITATIVE TRAINING ENHANCES MOTOR RECOVERY AFTER BILATERAL SPINAL CORD INJURY TO CERVICAL FORELIMB MOTOR POOLS

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Abstract

Closed-loop vagus nerve stimulation (VNS) paired with rehabilitative training has emerged as a strategy to enhance recovery after neurological injury. Previous studies demonstrate that brief bursts of closed-loop VNS paired with rehabilitative training substantially improve recovery of forelimb motor function in models of unilateral and bilateral contusive spinal cord injury (SCI) at spinal level C5/6. While these findings provide initial evidence of the utility of VNS for SCI, the injury model used in these studies spares the majority of alpha motor neurons originating in C7-T1 that innervate distal forelimb muscles. Because the clinical manifestation of SCI in many patients involves damage at these levels, it is important to define whether damage to the distal forelimb motor neuron pools limits VNS-dependent recovery. In this study, we assessed recovery of forelimb function in rats that received a bilateral incomplete contusive SCI at C7/8 and underwent extensive rehabilitative training with or without paired VNS. The study design, including planned sample size, assessments, and statistical comparisons, was preregistered prior to beginning data collection (https://osf.io/ysvgf/). VNS paired with rehabilitative training significantly improved recovery of volitional forelimb strength compared to equivalent rehabilitative training without VNS. Additionally, VNS-dependent enhancement of recovery generalized to two similar, but untrained, forelimb tasks. These findings indicate that damage to alpha motor neurons does not prevent VNS-dependent enhancement of recovery and provides additional evidence to support the evaluation of closed-loop VNS paired with rehabilitation in patients with incomplete cervical SCI.

Keywords: spinal cord injury; cervical; rehabilitation; vagus nerve stimulation; vagal nerve stimulation; plasticity

Introduction

Spinal cord injury (SCI) affects 276,000 individuals in the U.S. and millions more worldwide[1,2]. The cervical spinal region is the most common site of injury, accounting for over 55% of all SCIs[3–5]. Injury to the cervical spinal cord at or above the levels containing upper limb motor neurons carries a poor prognosis, and many cervical SCI patients experience long-term loss of upper extremity function that leads to chronic disability [6–8]. The development of interventions to improve recovery of function after cervical SCI is of key importance.

Plasticity in spared networks represents a substrate for recovery after incomplete SCI[9–13]. We have developed a novel technique to boost synaptic plasticity in conjunction with rehabilitation and improve recovery after neurological injury[14,15]. This strategy involves closed-loop stimulation of the vagus nerve triggered by forelimb movement during rehabilitative training to engage neuromodulatory networks and drive specific and long-lasting synaptic plasticity in the central nervous system[16–20]. Previous studies demonstrate that VNS paired with rehabilitative training significantly improves recovery of forelimb function compared to rehabilitative training without VNS in multiple animal models of neurological injury, including incomplete unilateral and midline SCI, stroke, intracerebral hemorrhage, and traumatic brain injury[21–29]. Moreover, emerging evidence from clinical trials highlights the translational potential of VNS therapy to improve recovery of motor function in chronic stroke patients[30,31].

While initial findings support the notion that VNS paired with rehabilitative training enhances recovery after cervical SCI, the C5/6 injury model employed in these studies produced damage primarily to the white matter tracts above the distal forelimb motor pools while largely sparing the alpha motor neurons originating in C7-T1[29]. The clinical manifestation of cervical

SCI often results in damage to the spinal levels containing alpha motor neurons that control distal upper limb musculature in combination with white matter injury. Substantial damage to these motor neuron pools could limit the benefits of plasticity-enhancing therapies if reorganization cannot compensate for the reduction in alpha motor neurons. Alternatively, synaptic plasticity within spared spinal networks may be sufficient to leverage remaining alpha motor neurons to support recovery. Here, we sought to model these complicating clinical features and determine whether direct damage to the distal forelimb motor pools would prevent VNS-dependent enhancement of recovery. To do so, we assessed recovery of forelimb motor function in animals that received a bilateral incomplete contusive SCI at C7/8 and underwent extensive rehabilitative training with or without paired VNS.

Materials and Methods

Experimental Design

All experimental procedures, group sizes, outcome measures, statistical comparisons, and exclusion criteria were preregistered on Open Science Framework before beginning data collection (https://osf.io/ysvgf/). Procedures used in this study were approved by the University of Texas at Dallas Institutional Animal Care and Use Committee (Protocol 14-10). Adult female Sprague Dawley rats (n = 58) weighing approximately 300g were obtained from Charles River Laboratories. Rats were trained to proficiency on the isometric pull task, as in previous studies [21,24–26,28,32–35]. After reaching proficiency, rats underwent bilateral contusive injury at level C7/8 of the spinal cord and were implanted with a vagus nerve cuff electrode (Figure 3.1A). Eight weeks after SCI, rats underwent a post-SCI baseline assessment and were dynamically allocated to balanced groups to receive either Rehab or VNS+Rehab. Rats in the Rehab group (n = 8)

received rehabilitative training which consisted of freely performing the isometric pull task twice daily for six weeks (Weeks 9-14). Rats in the VNS+Rehab group (n = 10) received equivalent rehabilitative training, but a 0.5 s burst of VNS was paired with forelimb movement on appropriate trials during training (Figure 3.1B). No VNS was delivered during the final week of rehabilitative training (Week 14) to allow assessment of effects lasting beyond the cessation of stimulation. At the conclusion of behavioral testing, the spinal cord was removed for histological processing. Forty rats were excluded from the study based on predefined criteria listed in the study preregistration: mortality (n = 6), inability to perform the task after injury (n = 4), significant autophagia (n = 20), or VNS device failure (n = 10). All source data indexed across animals can be found in Supplementary Tables 1-5.

Isometric Pull Task

The isometric force task was used to measure volitional forelimb strength as previously described [21,24–26,28,32–35]. The behavioral training chamber consisted of an acrylic box (10 x 12 x 4.75 in) with a slot in the front right corner through which rats could access a handle manipulandum. Rats were trained to pull the handle, which was attached to a force transducer (Motor Pull Device and Motor Controller, Vulintus LLC, Sachse, TX). A trial was initiated when 10 g of force was exerted on the pull handle. If the peak pull force exceeded an adaptively scaled threshold within two seconds of trial initiation, a reward pellet (45 mg dustless precision pellet, BioServ, Frenchtown, NJ) and VNS, when appropriate, was delivered. The threshold was scaled adaptively based on the median peak force of the 10 preceding trials, with a fixed bounded minimum of 10 grams and maximum of 120 g based on previous studies[34,36].

Rats underwent training and testing according to the timeline shown in Fig. 3.1. Behavioral training sessions lasted 30 min and were conducted twice daily, five days per week, with daily sessions separated by at least 2 hr. Rats were trained until they reached proficiency, defined as 10 consecutive sessions in which greater than 75% of trials exceeded 120 g. After attaining proficiency, rats were given a bilateral contusive SCI at spinal level C7/8. Eight weeks after injury, rats returned for behavioral testing were dynamically allocated to balanced groups based on their post-injury performance. Rehabilitative training then continued for 6 weeks. For rats receiving VNS+Rehab, stimulation was triggered by the behavioral software on appropriate trials during rehabilitative training.

Bilateral Cervical Spinal Cord Injury Surgery

All surgeries were performed using aseptic technique under general anesthesia. Rats were deeply anesthetized with ketamine (50 mg/kg, i.p.), xylazine (20 mg/kg, i.p.), and acepromazine (5 mg/kg, i.p.). A bilateral dorsal C7 laminectomy was performed. The vertebral column was stabilized using spinal microforceps. The right and left lateral portions of the spinal cord (1 mm lateral from midline) were consecutively contused using an Infinite Horizons Impact Device fitted with an impactor tip diameter with a 1.25 mm at a force of 225 kilodynes and zero dwell time (Precision Systems and Instrumentation, Lexington, KY). The skin overlying the exposed vertebrae was then closed in layers and the incised skin was closed using surgical staples. All rats received buprenorphine (s.c., 0.03 mg/kg, 1-day post-op), enrofloxacin (s.c., 10 mg/kg, 5 days post-op) and Ringer's solution (s.c., 10 mL, 5 days post-op) immediately after surgery and continuing post-operatively.

Vagus Nerve Stimulation Cuff Implantation Surgery

VNS implantation procedures were performed as described in previous studies[21–29]. Seven weeks post-SCI, rats were anesthetized with ketamine hydrochloride (50 mg/kg, i.p.), xylazine (20 mg/kg, i.p.), and acepromazine (5 mg/kg, i.p.), and were placed in a stereotactic apparatus. An incision was made down the midline of the head to expose the skull. Bone screws were inserted into the skull at points surrounding the lamboid suture and over the cerebellum. A two-channel connector was mounted to the screws using acrylic. The rat was then removed from the stereotaxic apparatus and placed in a supine position. An incision was made on the left side of the neck and the overlying musculature was blunt dissected to isolate the vagus nerve. The nerve was placed into a bipolar stimulating cuff electrode, and the electrode leads were tunneled subcutaneously and connected with the two-channel skull-mounted connector. Incised skin was then sutured closed. All rats received enrofloxacin (s.c., 10 mg/kg) following surgery. Regardless of group assignment, all rats underwent implantation of the headmount and cuff. To confirm cuff functionality and proper placement, VNS-dependent activation of the Hering-Breuer reflex was assessed as in previous studies[37,38]. To do so, while anesthetized, blood oxygenation saturation during trains of VNS (0.8 mA, 30 Hz, 100 μs pulse width, up to 5 s train duration) was monitored via pulse oximetry both immediately after cuff implant and at the end of therapy. Rats that failed to demonstrate a reliable drop in oxygen saturation, indicative of a failure in stimulation efficacy, were excluded (n = 3).

Delivery of Vagus Nerve Stimulation during Rehabilitative Training

VNS parameters were equivalent to previous studies[24–26,28,35]. Each 0.5 s stimulation train consisted of 0.8 mA, 100 μsec biphasic pulses delivered at 30 Hz. Trains of stimulation were

triggered during rehabilitative training based on an adaptive threshold, as previously described[27,29]. The adaptive threshold was scaled such that stimulation was triggered when the force on a given trial exceeded the median pull force of the 10 antecedent trials or 120 g, whichever was lower. No VNS was delivered on the final week of rehabilitative training (Week 14) to assess effects lasting after the cessation of stimulation.

Cylinder Assessment

Spontaneous use of the forelimbs during exploratory activity was measured in all animals, similar to previous descriptions[39,40]. Animals were placed in a transparent cylinder and allowed to freely explore for the three minutes. Video was recorded from directly underneath the cylinder through a clear sheet of acrylic. The total number of left and right forepaw contacts with the wall of the cylinder during rearing was recorded. Assessments were performed before injury (Week -1), before therapy (Week 8), and after therapy (Week 14) by experimenters blinded to group.

Grip Strength Assessment

A custom-made grip strength meter was used to measure the grip strength of the right and left forepaws independently, similar to previous descriptions[41]. The rat was positioned over the two horizontal bars attached to separate force transducers such that each forepaw grasped a single bar. Once at least three digits on each paw had grasped the bars, the animal was pulled horizontally away from the bars in a smooth and constant motion. The peak force at which grip is released from the bar was recorded for each paw individually. Five trials were performed at each assessment, and the average of the peak grip forces were recorded. Assessments were performed before injury (Week -1), before therapy (Week 8), and after therapy (Week 14) by experimenters blinded to group.

Lesion Histology and Analysis

At the completion of experimental testing, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and transcardially perfused with 4% paraformaldehyde in 0.1 M PBS (pH 7.5). The spinal cord was removed and post-fixed overnight, cryoprotected in 30% sucrose for 48 hours, blocked, and frozen at -80°C in Shandon M1 embedding matrix (Thermo Fisher Scientific; Waltham, MA). Spinal tissue was sliced at 50 µm using a cryostat, slide mounted and stained for Nissl and myelin similar to previous studies [36,42]. Photomicrographs were taken at 150 µm intervals from approximately C5 to T2.

Statistical Analysis:

All group sizes, outcome measures, and planned statistical comparisons were included in the study pre-registration prior to beginning data collection. Performance on the isometric force task was analyzed using a two-way repeated measures ANOVA to assess effects of treatment and time, followed by *post hoc* Bonferroni-corrected unpaired t-tests where appropriate. Paired t-tests were used to compare isometric pull task measures within subjects from pre- to post-SCI and from week 13 to week 14 in the VNS+Rehab group. Unpaired t-tests at each time point were used to compare cylinder task data and grip strength across groups at each time point. Statistical tests for each comparison are noted in the text. Figures depict mean ± SEM.

Results

Rats were trained to perform the isometric pull task, an automated reach-to-grasp task that measures volitional forelimb strength [21,32,36]. Upon achieving proficiency on the task, all rats underwent bilateral contusive impact at spinal level C7/8 to impair function of the trained forelimb

(Figure 3.2). As expected, SCI at C7/8 resulted in a substantial reduction in volitional forelimb strength (Figure 3.3A; PRE v. Wk 8; Paired t-test, $t(17) = 18.79$, $p = 8.23 \times 10^{-23}$). Hit rate, defined as the percentage of trials in which pull force exceeded 120 g, was also significantly reduced by SCI (Figure 3.3B; PRE v. Wk 8; Paired t-test, $t(17) = 24.84$, $p = 8.45 \times 10^{-15}$). No differences in any measures were observed between groups prior to beginning therapy (Figure 3; Rehab v. VNS+Rehab at Wk 8; Peak force: Unpaired t-test, $t(16) = 0.99$, $p = 0.33$; Hit Rate: Unpaired t-test, $t(16) = 1.07$, $p = 0.30$).

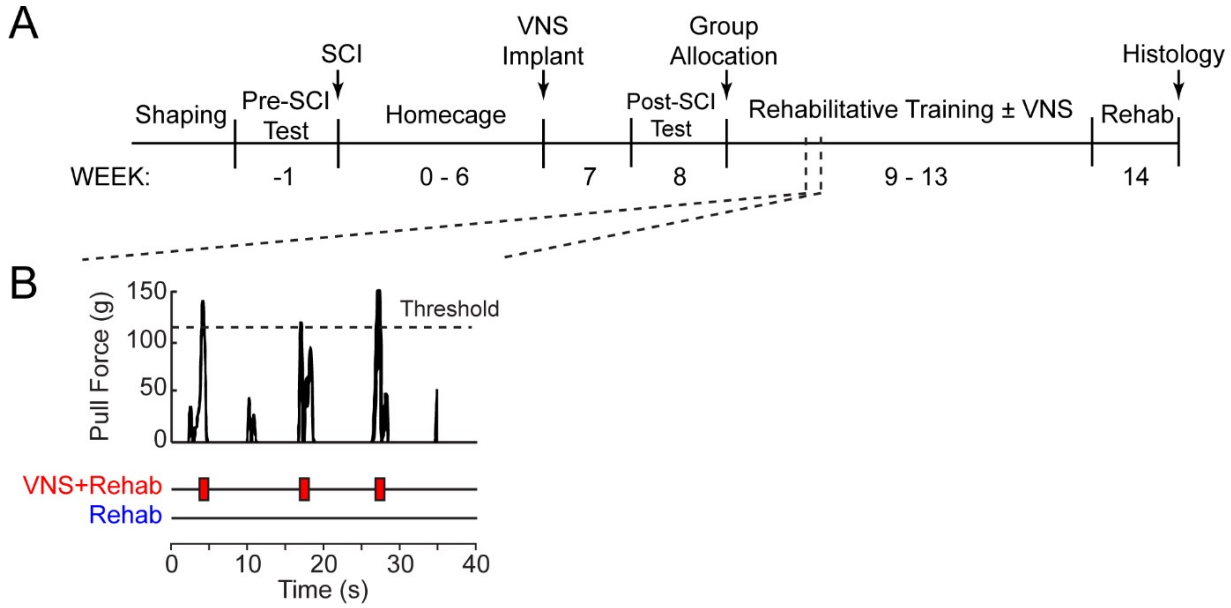

Figure 3.1. Timeline and Experimental Design

(A) Illustration of experimental timeline for each subject in the study. (B) Example of forelimb pull forces recorded during rehabilitative training. Five trials are shown. Animals in the VNS+Rehab group received a short 0.5 s burst of VNS (red blocks) paired with trials in which pull force exceeded the stimulation threshold. Animals in the Rehab group performed equivalent rehabilitative training without VNS.

We sought to determine if VNS paired with rehabilitative training would improve recovery of forelimb strength after SCI. To do so, beginning 9 weeks after injury, rats underwent 6 weeks of either rehabilitative training on the isometric pull task in which a 0.5 s burst of VNS was paired with forelimb use (VNS+Rehab; n = 10) or equivalent rehabilitative training without VNS (Rehab, n = 8). VNS paired with rehabilitative training significantly increased recovery of volitional forelimb strength compared to equivalent rehabilitative training without VNS (Figure 3.3A, Rehab v. VNS+Rehab; Two-way repeated measures ANOVA, $F[1,16] = 16.98$; $p = 8.02 \times 10^{-4}$). Post hoc tests revealed significant improvements in forelimb strength in the VNS+Rehab group compared to the Rehab group beginning on the third week of therapy (Figure 3.3A, Rehab v. VNS+Rehab at each week; Bonferroni-corrected unpaired t-tests, wks 11-14 all $p < 8.33 \times 10^{-3}$). Improved volitional forelimb strength was maintained in the VNS+Rehab group on week 14 after the cessation of stimulation, indicating lasting benefits (Figure 3.3A; VNS+Rehab, Wk 13 v. Wk 14; Paired t-test, $t(9) = 1.95$, $p = 0.82$). A similar VNS-dependent enhancement of recovery was observed for hit rate (Figure 3.3B, Rehab v. VNS+Rehab; Two-way repeated measures ANOVA, $F[1,16] = 17.67$; $p = 6.74 \times 10^{-4}$; Bonferroni-corrected unpaired t-tests at each week; wks 11-14 all $p < 8.33 \times 10^{-3}$). These findings demonstrate that VNS paired with rehabilitative training significantly enhances recovery of forelimb motor function compared to rehabilitative training without VNS after bilateral spinal cord damage at C7/8.

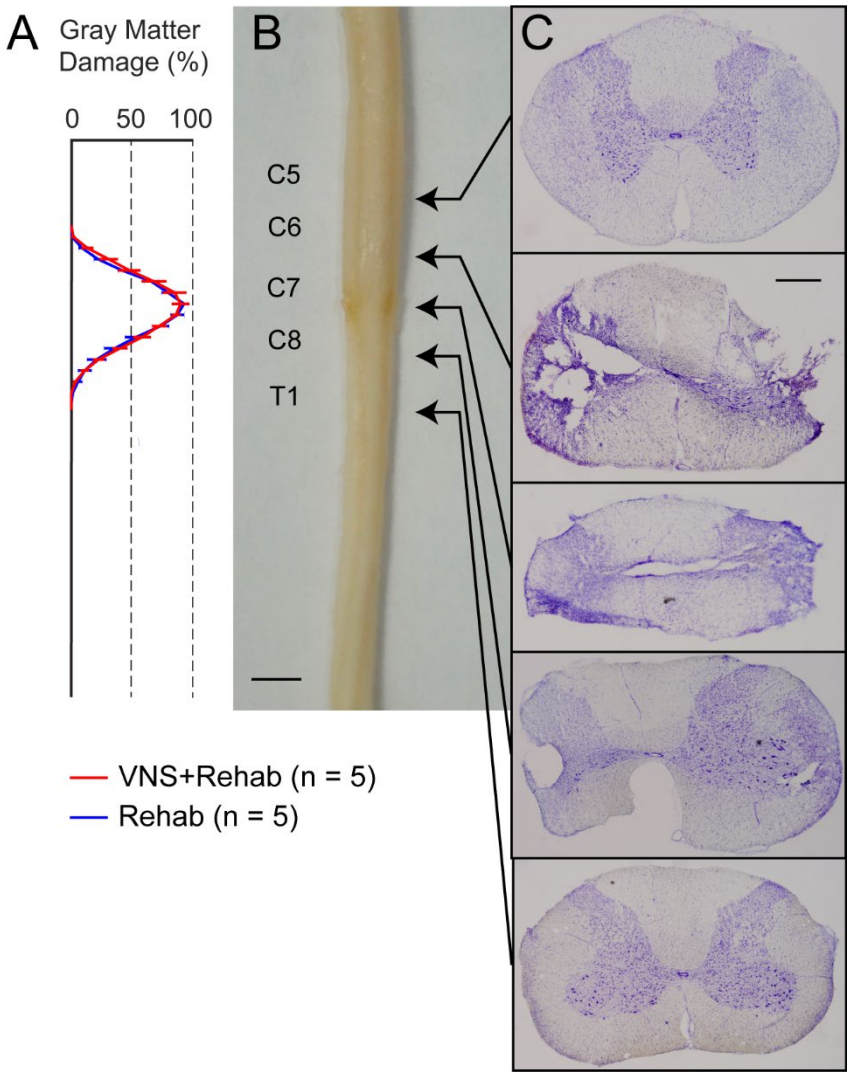

Figure 3.2. SCI causes bilateral damage between spinal levels C7 and C8.

Representative examples from a range of levels illustrating the extent of damage through the cord. Substantial damage was observed near the lesion epicenter, with minimal injury to levels above and below. Scale bars indicate 3 mm in the left gross image panel and 500 μm in the right micrograph panels.

We next assessed whether recovery was restricted to the trained task or generalized to similar, but untrained, forelimb tasks. First, we tested spontaneous forelimb use with the cylinder assessment [40]. As expected, bilateral C7/8 SCI also reduced spontaneous use of both forelimbs, as indicated by a decrease in the total number of wall touches per session (Figure 3.4A, Rehab, Pre: 95 ± 13, Post: 21 ± 5, Paired t-test, $t(7) = 5.46$, $p = 9.47 \times 10^{-4}$; VNS+Rehab, Pre: 91 ± 7, Post: 18 ± 3, $t(9) = 9.75$, $p = 4.41 \times 10^{-6}$). After the conclusion of rehabilitative therapy, the VNS+Rehab group demonstrated significantly greater restoration of spontaneous forelimb use compared to Rehab (Figure 3.4A; Rehab: 31 ± 6; VNS+Rehab: 71 ± 6; Unpaired t-test, $t(16) = 5.14$, $p = 9.87 \times 10^{-5}$). Next, we tested forepaw grip strength. Consistent with previous reports [43], bilateral C7/8 SCI results in a significant impairment in grip strength (Figure 3.4B; Rehab, Pre: 665 ± 21 g, Post: 289 ± 13 g, Paired t-test, $t(7) = 16.40$, $p = 7.64 \times 10^{-7}$; VNS+Rehab, Pre: 635 ± 24 g, Post: 285 ± 12 g, $t(9) = 16.29$, $p = 5.50 \times 10^{-8}$). VNS paired with rehabilitative training significantly improved grip strength compared to rehabilitative training alone in the trained right forelimb (Fig. 3.4B; Rehab: 369 ± 18 g; VNS+Rehab: 459 ± 24 g; Unpaired t-test, $t(16) = 3.05$, $p = 0.008$). No significant improvement in grip strength was observed in the untrained left paw (Rehab: 327 ± 22 g; VNS+Rehab: 391 ± 23 g; Unpaired t-test, $t(16) = 2.07$, $p = 0.054$). Together, these findings indicate that VNS paired with rehabilitative training yields improved recovery of motor function on similar, but untrained, forelimb tasks after bilateral C7/8 SCI.

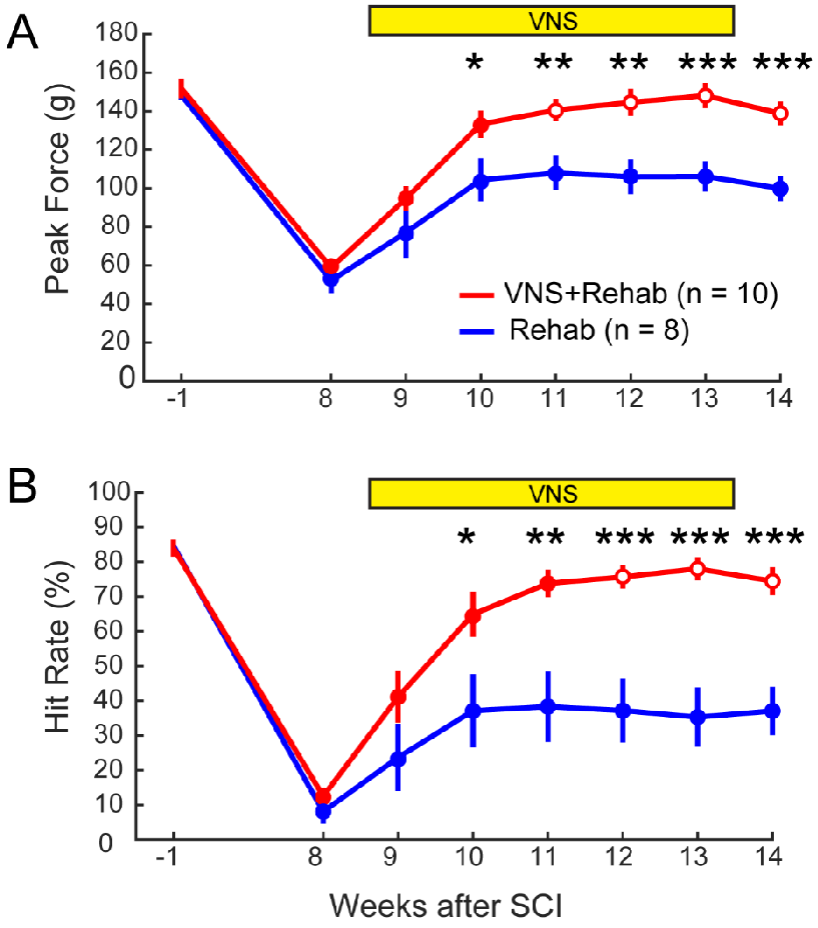

Figure 3.3. VNS paired with rehabilitative training enhances recovery after C7/8 SCI.

(A) VNS paired with rehabilitative training (VNS+Rehab) significantly improves recovery of volitional forelimb strength compared to equivalent rehabilitative training without VNS (Rehab) after bilateral SCI at C7/8. VNS-dependent benefits remain on week 14 after the cessation of stimulation, indicating lasting recovery. (B) Similarly, VNS+Rehab significantly increases hit rate on the isometric pull task compared to Rehab after SCI, measured as the percentage of trials on which peak forelimb pull force exceeds 120g. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; unpaired t-tests comparing VNS+Rehab and Rehab at each time point. Filled markers indicate a significant reduction compared to pre-SCI performance at each week (paired t-test v. Week -1, $p < 0.05$). Error bars indicate mean ± SEM.

Changes in the intensity of rehabilitative training could potentially mediate VNS-dependent recovery. No differences were observed in the total number of trials performed over the course of therapy across groups, consistent with previous reports and demonstrating that VNS does not influence the intensity of rehabilitative training (Rehab: 10,993 ± 777 trials, VNS+Rehab: 9390 ± 569; Unpaired t-test, $t(16) = 1.81$, $p = 0.09$). These findings indicate that motivation cannot account for VNS-dependent benefits on recovery.

Discussion

In this study, we demonstrate that VNS paired with rehabilitative training significantly enhances recovery of forelimb function compared to equivalent rehabilitative training without VNS after bilateral C7/8 SCI that damages distal motor neuron pools. VNS-dependent benefits last after the cessation of stimulation and extend to other similar, but untrained, forelimb measures. Improved recovery in the VNS treated group occurred without affecting intensity of rehabilitative training. The findings from this study support the potential for VNS paired with rehabilitation as a therapeutic intervention in SCI and provide and initial demonstration that damage to motor pools does not preclude VNS-dependent benefits.

Previous evidence indicates that VNS paired with rehabilitative training drives synaptic plasticity in spared networks and enhances recovery of motor function after SCI at spinal level C5/6[29]. The injury model used in earlier studies largely spared the alpha motor neurons innervating muscles in the distal forelimb, which predominantly originate in spinal levels C7 to T1[44]. Given the heterogeneity in the clinical manifestation of SCI, the present study sought to evaluate whether substantial damage to the distal forelimb motor pools would preclude VNS-

dependent enhancement of recovery. We observe that VNS paired with rehabilitative training results in significant improvements in recovery of motor function compared to equivalent rehabilitative training without VNS after damage to the distal forelimb motor pools. These findings indicate that damage to these networks is not the sole limiting factor for recovery and suggest that damage to the upper limb motor pools should not necessarily exclude patients from receiving VNS therapy. Although animal models fail to capture the variability and complexity of SCI in patients, this study extends the range of conditions over which VNS paired with rehabilitative training improves motor recovery and supports the evaluation of closed-loop VNS therapy as a post-SCI intervention.

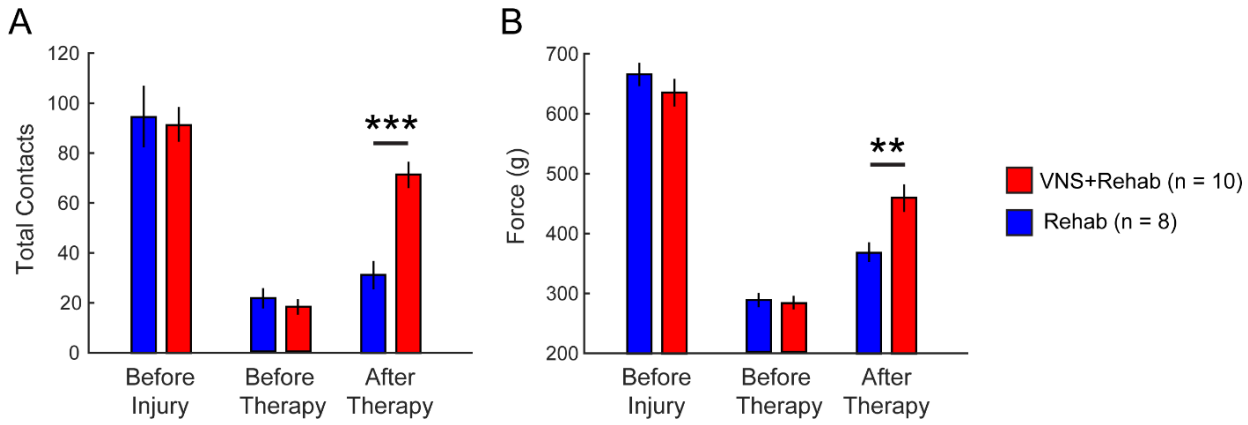

Figure 3.4. VNS-dependent recovery generalizes to similar, but untrained, forelimb tasks.

(A) SCI reduces the total number of forepaw contacts on the cylinder task. VNS paired with rehabilitative training on the isometric pull task significantly increases spontaneous forelimb use during exploration compared to rehabilitative training without VNS, indicative of a generalization of forelimb recovery to a similar task. (B) Pairing VNS with training on the isometric pull task significantly increases grip strength compared to rehabilitative training without VNS. Unpaired t-tests across groups at each time point; ** denotes $p < 0.01$, *** $p < 0.001$. Error bars indicate mean ± SEM.

Plasticity in spared brain and spinal networks is widely recognized as a substrate of recovery after SCI[9–13]. A number of studies demonstrate that VNS paired with rehabilitative training drives robust enhancement of synaptic plasticity after neurological injury, which likely underlies VNS-dependent benefits[16,27,29]. After contusive damage to the rubrospinal and propriospinal tracts, VNS paired with rehabilitative training drives substantial reorganization of synaptic connectivity in the spared corticospinal tracts to increase motor drive onto forelimb alpha motor neurons[29]. Alternatively, after damage to the dorsal corticospinal tract, VNS paired with rehabilitative training facilitates synaptic plasticity in the largely spared rubrospinal and propriospinal tracts to similarly increase motor drive[29]. The improved motor recovery observed in the present study is consistent with the notion that VNS enhances synaptic plasticity in spared motor networks to increase the drive onto the remaining alpha motor neurons controlling the distal forelimb. Considered together, these findings indicate that VNS supports synaptic plasticity to increase motor output to compensate for impairments resulting from damage to either white matter or alpha motor neurons. Incorporating regenerative strategies that restore lost connectivity with VNS to enhance reorganization in newly connected circuits may represent a novel combinatorial therapeutic regimen to intervene after complete SCI[13,45]. Future studies are needed to clarify both the nature of VNS-dependent reorganization after SCI as well as the neural mechanisms that support this plasticity.

Generalization of functional improvements to similar tasks is a key feature of effective rehabilitative therapies. In addition to task-specific enhancement of recovery observed on the isometric pull task, VNS paired with rehabilitative training yielded increased post-SCI forelimb function on two similar, but untrained, tasks. Rats that received VNS paired with task-specific

rehabilitative training on the isometric pull task demonstrated increased spontaneous forelimb use as measured by the cylinder task and improved forepaw grip strength. These findings provide an initial demonstration that VNS paired with task-specific training results in benefits that generalize to similar forelimb movements, consistent with previous studies[27]. This generalization of recovery likely arises from synaptic plasticity of inputs to spared alpha motor neurons that contribute to muscular control common across tasks. For instance, reorganization of synaptic connectivity to alpha motor neurons that exert control over digit grasp muscles would improve performance on both the isometric pull task and the grip strength task, as control of grasp musculature is a key feature in executing both tasks. In practical terms, generalization indicates that rehabilitation should include a broader range of task-specific exercises to yield the greatest benefits.

Recent clinical studies evaluating VNS to improve motor function are consistent with results from preclinical studies and highlight the translational potential of this strategy[15]. In a double-blind, placebo-controlled study in chronic stroke patients, VNS paired with rehabilitation was safe and significantly improved upper extremity function compared to the similar rehabilitation without stimulation, confirming results of a preceding open-label study[30,31]. The present study builds on the promising clinical and preclinical data and supports the potential of VNS therapy as a strategy to improve motor function after SCI.

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Competing Financial Interests: MPK has a financial interest in MicroTransponder, Inc., which is developing VNS for stroke. RLR is a co-owner of Vulintus, Inc., which makes rodent behavioral testing systems, and owner of Teliatry, which is developing a novel VNS device. All other authors declare no conflicts of interest.

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CHAPTER 4

RESTORATION OF SOMATOSENSORY FUNCTION BY PAIRING VAGUS NERVE STIMULATION WITH TACTILE REHABILITATION

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Abstract

Sensory dysfunction is a common consequence of many forms of neurological injury. Rehabilitative paradigms that incorporate sensory retraining can provide modest benefits, but the majority of patients are left with lasting sensory loss. We have developed a novel strategy that uses closed-loop vagus nerve stimulation (VNS) paired with motor rehabilitation to facilitate recovery after neurological injury. VNS drives robust, phasic activation of neuromodulatory networks concurrent with rehabilitation to enhance synaptic plasticity and support recovery. A clinical case report provides initial evidence that a similar implementation of closed-loop VNS paired with a tactile rehabilitation regimen could improve recovery of somatosensory function. Here, we sought to build on this promising initial clinical data and rigorously evaluate the ability of VNS paired with tactile rehabilitation to improve recovery in an animal model of chronic sensory loss. The study design, including planned sample size, assessments, and statistical comparisons, was preregistered prior to beginning data collection (https://osf.io/xsnj5/). VNS paired with tactile rehabilitation resulted in a significant and nearly complete recovery of mechanosensory withdrawal thresholds. Equivalent tactile rehabilitation without VNS failed to improve sensory function. This VNS-dependent restoration of sensory thresholds was maintained for several months after the cessation of stimulation, illustrating long-term benefits. Moreover, VNS paired with tactile rehabilitation resulted in significant generalized improvements in other measures of forelimb sensorimotor function, including forelimb use asymmetry and paw placement. Given the safety and tolerability of VNS therapy, these findings suggest that incorporating VNS paired with sensory retraining into rehabilitative regimens may

represent a fundamentally new method to increase recovery of sensory function after neurological injury.

Keywords: vagal nerve stimulation, peripheral nerve injury, peripheral neuropathy

Introduction

Loss of somatosensation is a common consequence of neurological injury. Damage to peripheral nerves leads to profound impairments in somatosensation in many patients, which typically persist even after surgical repair [1,2]. There are no consistently effective methods to restore sensory function, but rehabilitation paradigms that incorporate sensory retraining may provide modest benefits to some patients [3–7]. While treatment strategies tend to focus on restoration of motor function after neurological injury, deficits in somatosensation strongly contribute to disability [8–10]. Given the prevalence and significance of sensory loss, the development of effective interventions that can restore somatosensory function has the potential to yield substantial benefits for patients suffering from a wide range of neurological disorders.

We have developed a novel strategy using closed-loop vagus nerve stimulation (VNS) to enhance the benefits of rehabilitation [11]. VNS drives rapid, phasic activation of multiple neuromodulatory systems [12,13]. Engaging these neuromodulatory networks concurrent with training provides pro-plasticity feedback to support synaptic plasticity in the neural circuits activated by training [14,15]. An initial study provided a proof-of-principle demonstration that pairing VNS with tones drives robust, specific plasticity in the auditory cortex [16]. Subsequent studies have built on this premise, showing that VNS paired with motor training produces similar training-specific plasticity in motor networks [17].

After neurological injury, strategies that support plasticity in spared networks represent a potential strategy to facilitate recovery of function [18]. Based on VNS-dependent enhancement of plasticity, a number of studies have evaluated the utility of closed-loop VNS paired with rehabilitation [17]. VNS paired with motor rehabilitation improves recovery of motor function in a variety of animal models of neurological injury, as well as in patients [19–29]. Moreover, pairing VNS with various auditory sensory stimuli drives robust, stimulus-specific plasticity in auditory cortex, raising the possibility that delivery of VNS with other sensory modalities may produce similar effects [30–32]. In support of this hypothesis, a pilot study in a chronic stroke patient with substantial sensory loss reported initial evidence that pairing VNS with tactile rehabilitation improved a number of measures of somatosensory function [33].

In the present study, we sought to build on this promising initial clinical data and rigorously evaluate the ability of VNS paired with tactile rehabilitation to improve recovery in an animal model of chronic sensory loss. Additionally, we evaluated the durability of VNS-dependent effects and whether improvements in somatosensation would generalize to other measures of forelimb function. To do so, rats underwent transection and gap repair of the median and ulnar nerves in the forelimb, which produces lasting deficits in somatosensation in spite of reinnervation. Beginning 16 weeks after nerve injury, animals were randomized to receive either a tactile rehabilitation paradigm that consisted of presentation of a variety of mechanical stimuli to the ventral surface of the injured paw or equivalent tactile rehabilitation with 0.5 s bursts of VNS paired with presentation of each tactile stimulus. Mechanosensory thresholds were measured weekly throughout therapy and for 8 weeks after the cessation of therapy to identify any lasting benefits. Additionally, multiple measures of forelimb

sensorimotor function were evaluated throughout the study to examine generalization of recovery. The results from this study corroborate the findings from the pilot human study and suggest that VNS during tactile rehabilitation may improve recovery of sensory function after neurological injury.

Methods

Experimental Design

All experimental procedures, group sizes, outcome measures, statistical comparisons, and exclusion criteria were preregistered on Open Science Framework before data collection began (https://osf.io/xsnj5/). Before injury, all rats underwent baseline assessment of mechanosensory withdrawal thresholds, grip strength, and cylinder testing. All rats then underwent transection and tubular repair of the median and ulnar nerves in the right forearm and implantation of a stimulating cuff electrode on the left cervical vagus nerve. Beginning on week 16 post-injury, rats underwent baseline assessment of sensorimotor function and were dynamically allocated into two balanced groups based on mechanosensory withdrawal thresholds of the impaired forelimb. One group received tactile rehabilitation (Rehab, n = 8), comprised of 6 weeks of daily sessions in which 200 presentations of a range of tactile stimuli, including a paintbrush, a 10g filament, a copper rod, and a puff of air, were applied to the ventral surface of the injured paw. The other group received equivalent tactile rehabilitation, but a 0.5 train of VNS was paired with the delivery of each tactile stimulus (VNS+Rehab, n = 9). Mechanosensory withdrawal thresholds were measured weekly during therapy and every two weeks for two months after the cessation of therapy. Additional measures of forelimb sensorimotor function, including cylinder asymmetry, grip strength, horizontal ladder rung, and footprint analysis, were collected at

multiple time points throughout the study (Figure 4.1A). Seven rats were excluded from the study based on predefined criteria: mortality (n = 2), VNS device failure (n = 4), and autophagia (n = 1). Data from subjects excluded for VNS device failure are included as an intent to treat (ITT) analysis in the Supplemental information. All source data indexed across animals can be found in Supplementary Tables 1-7.

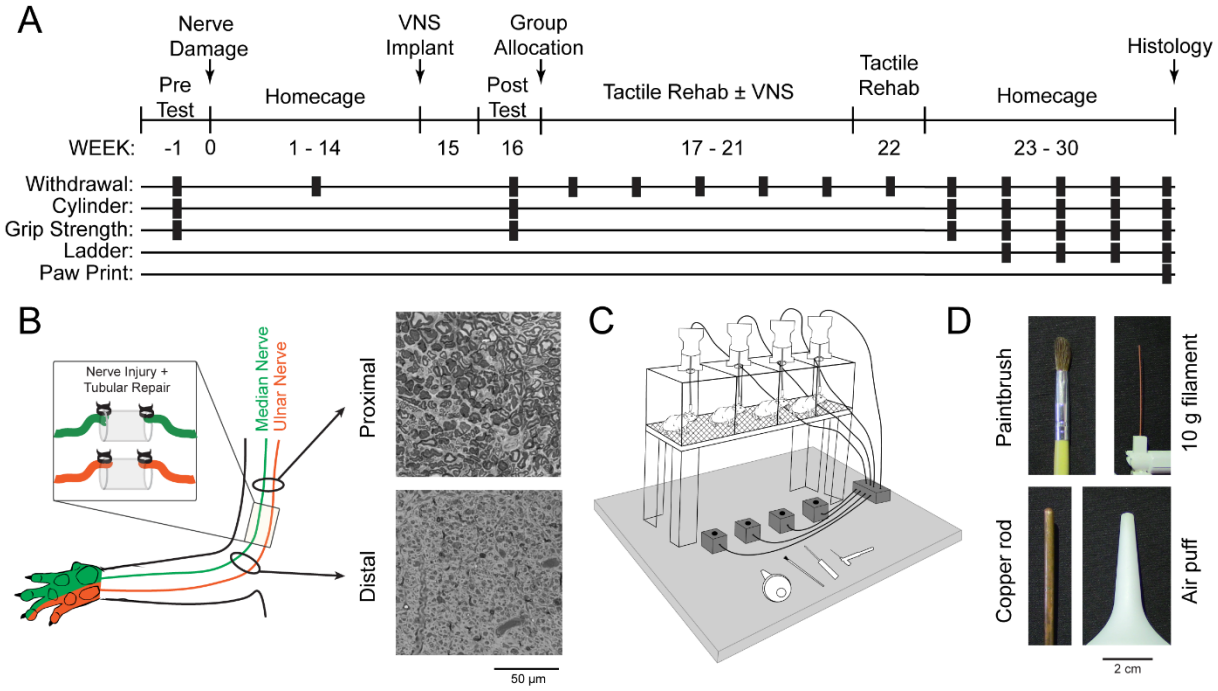

Figure 4.1. Experimental Design and Tactile Rehabilitation Paradigm

(A) Timeline of experimental design illustrating when each assessment is performed. (B) Schematic and representative images from proximal and distal cross-sections of the median nerve approximately 30 weeks after nerve transection and tubular repair. Reinnervation takes place, but the procedure results in chronic deficits in nerve architecture distal to the injury site. (C) Schematic of the tactile rehabilitation apparatus. Rats were placed in individual cages with a wire mesh floor. A variety of tactile stimuli were applied to the ventral surface of the right (injured) forepaw. A button press coincident with the delivery of the tactile stimuli initiated a 500 ms train of VNS in the appropriate group. (D) Detailed view of the devices utilized during tactile rehabilitation. The stimuli were selected to encompass a wide range of somatosensory features.

Subjects

Adult female Sprague Dawley rats (n = 24) weighing approximately 300g when they entered the study were obtained from Charles River Laboratories. The rats were housed in a 12:12 reversed light cycle environment, and behavioral training was performed during the dark cycle to increase daytime activity levels. All procedures performed in the study were approved by the University of Texas at Dallas Institutional Animal Care and Use Committee (Protocols: 14-10 and 99-06).

Forelimb Nerve Injury

Complete transection of both the median and ulnar nerves proximal to the elbow followed by tubular repair was performed as previously described [34]. Animals were deeply anesthetized with ketamine hydrochloride (50 mg/kg, i.p.), xylazine (20 mg/k, i.p.), and acepromazine (5 mg/kg, i.p.) and were given supplemental doses as needed to maintain anesthesia levels. A small incision proximal to the elbow of the right forelimb was made, and the median and ulnar nerves were carefully isolated and exposed. Both nerves were transected 1cm proximal to the elbow. Immediately following transection, the proximal and distal stumps of each nerve were sutured 1 mm from the ends of a 8 mm saline-filled polyurethane tube (Micro-Renathane 0.095" I.D 0.066" O.D., Braintree Scientific, Inc., Braintree, MA), resulting in a 6 mm gap between nerve stumps. The skin incision was sutured and treated with antibiotic ointment. All animals were given enrofloxacin (10 mg/kg) immediately following surgery and sustained release buprenorphine (1.2 mg/kg) for 6 days following injury. Animals were placed in Elizabethan collars for approximately 1 week following injury to limit autophagia.

Vagus Nerve Stimulation Implantation Surgery

VNS implantation procedures were performed as described in previous studies [20,22–28,35]. All rats underwent surgical implantation procedures to ensure blinding. Fifteen weeks after transection of the median and ulnar nerves, rats were anesthetized with ketamine hydrochloride (50 mg/kg, i.p.), xylazine (20 mg/kg, i.p.), and acepromazine (5 mg/kg, i.p.), and were placed in a stereotactic apparatus. An incision was made down the midline of the head to expose the skull. Bone screws were inserted into the skull at points surrounding the lamboid suture and over the cerebellum. A two-channel connector was mounted to the screws using acrylic. The rat was then removed from the stereotaxic apparatus and placed in a supine position. An incision was made on the left side of the neck and the overlying musculature was blunt dissected to isolate the vagus nerve. The nerve was placed into a bipolar stimulating cuff electrode, and the electrode leads were tunneled subcutaneously and connected with the two-channel skull-mounted connector. Incised skin was then sutured closed. All rats received enrofloxacin (s.c., 10 mg/kg) following surgery. Regardless of group assignment, all rats underwent implantation of the headmount and cuff electrode. To confirm cuff electrode functionality and proper placement, VNS-dependent activation of the Hering-Breuer reflex was assessed as in previous studies [36,37]. To do so, blood oxygenation saturation during trains of VNS (0.8 mA, 30 Hz, 100 μs pulse width, up to 5 s train duration) was monitored via pulse oximetry during cuff implant. The cuff electrode was replaced if rats failed to demonstrate a reliable drop in oxygen saturation during the implant surgery.

Tactile Rehabilitation and Delivery of Vagus Nerve Stimulation

Tactile rehabilitation began 17 weeks post-forelimb nerve injury and continued for 6 weeks. Sessions of tactile rehabilitation were performed once daily, four days per week, with

each session lasting approximately 1.5 hours. During each session, up to 8 animals were placed in individual acrylic chambers (14 x 15 cm) with a mesh floor (Figure 4.1C). Each session consisted of 200 touches to the ventral surface of the right (injured) forepaw with diverse mechanical stimuli (Figure 4.1D and Supplemental Video 1): a 10g von Frey filament (North Coast Medical, Gilroy, CA), a paintbrush (Kiss Products, Port Washington, NY), a 4 mm diameter copper rod (Everbilt, Atlanta, GA), and puffs of air delivered with a handheld bulb (Innovo Medical, Stafford, TX). Individual stimuli were presented in blocks of 10 with at least 10 seconds between each delivery, resulting in a total of 50 touches with each stimulus per session. Each tactile stimulation was typically 1 s in duration. The von Frey filament was applied perpendicularly to the paw and the digits. The paintbrush was applied across the paw and digits in varying directions and with an approximate upward force of 50g. The copper rod was applied to the paw with the minimal force sufficient to slightly raise the paw off the mesh floor. The handheld bulb was positioned approximately 4 cm below the paw and puffs of air were applied from multiple angles.

In the appropriate group, a train of VNS was triggered by a button press to coincide with delivery of each mechanical stimulus during tactile rehabilitation sessions. VNS parameters were equivalent to previous studies [19,22,24,25,28]. Each 0.5 s stimulation train consisted of 0.8 mA 100 μsec biphasic pulses delivered at 30 Hz. No VNS was delivered after week 21 to assess effects lasting after the cessation of stimulation. All subjects in the study, regardless of group, were implanted with the same vagus nerve stimulation (VNS) device and headmount and were connected to a stimulator cable during therapy to ensure that they were indistinguishable in

appearance. As a result, there were no visible differences between subjects to bias the experimenter administering the assessments.

Mechanosensory Withdrawal Threshold Testing

Mechanosensory detection thresholds were assessed in all animals according to standard procedures [38]. Testing was performed in an acrylic chamber (19.5 x 9.6 cm) on a wire mesh floor. For each session, animals were allowed to acclimate to the behavioral chamber for 30 min before testing commenced. Mechanical withdrawal thresholds of the left and right forelimbs were tested using a dynamic plantar aesthesiometer (Cat. No. 37450, Ugo Basile, Switzerland). The actuator filament (0.5 mm diameter) was applied to the plantar surface of the forepaw, and a linearly increasing force was applied (20 s ramp time, 50 g maximal force). The force at which paw withdrawal occurred was captured for analysis. The left and right forelimbs were alternately tested with a minimum of 1 min between consecutive tests. Trials resulting in paw withdrawal due to spontaneous exploratory activity were excluded from analysis. Assessments were performed before injury (Week -1), before therapy (Weeks 8 & 16), weekly during therapy (Weeks 17-22), and biweekly after the conclusion of therapy (Weeks 22, 24, 26, 28, and 30) by experimenters blinded to group.

Cylinder Forelimb Asymmetry Testing

Spontaneous use of the forelimbs during exploratory activity was measured in a subset of animals using the cylinder forelimb asymmetry task, similar to previous descriptions [39]. Animals were placed in a transparent cylinder (20 cm diameter) and allowed to freely explore for two minutes. Video was be recorded from directly underneath the cylinder through a clear sheet of

acrylic. The total number of both left and right forepaw contacts with the wall of the cylinder were recorded. An asymmetry index, describing the relative use of the injured forelimb, was calculated as [(right/(left + right)) x 100]. Assessments were performed before injury (Week -1), before therapy (Week 16), and biweekly after the conclusion of therapy (Weeks 22, 24, 26, 28, and 30) by experimenters blinded to group.

Grip Strength Testing

A custom-made grip strength meter was used to measure the grip strength of the right and left forepaws independently, similar to previous descriptions [40]. The rat was positioned over the two horizontal bars attached to separate force transducers such that each forepaw grasped a single bar. During testing, rats were held by the hindquarters while horizontally suspended and slowly pulled away from the module until grip broke. The peak force at which grip is released from the bar was recorded for each paw individually. Five trials were performed at each assessment, and the average of the peak grip forces were recorded. Assessments were performed before injury (Week -1), before therapy (Week 16), and biweekly after the conclusion of therapy (Weeks 22, 24, 26, 28, and 30) by experimenters blinded to group.

Horizontal Ladder Rung Testing

Horizontal ladder rung walking task was performed to assess forelimb placing, similar to previous studies [41,42]. The test apparatus consisted of Plexiglas walls that created a 1 m long alley. Metal rungs (3 mm diameter) were inserted into the base of the walls to create an irregular pattern that varied the distance of the rungs from 1 to 5 cm. The same pattern was kept consistent across all animals. The width of the alley was adjusted to approximately 1 cm wider

than an animal to prevent turning around. During testing, the apparatus was elevated 30 cm above the ground, and animals spontaneously walked the length of the alley. A video camera was positioned slightly below the horizontal plane so that paw positions could be easily visualized. Three to five trials were performed at each assessment to ensure data was collected during continuous walking. Frame-by-frame analysis of videos was performed offline and scored by a blinded experimenter, as in previous studies [42,43]. The percentage of misses or slips was calculated as the number of steps with a score of 0-2 divided by the total number of steps scored. Assessments were performed biweekly after the conclusion of therapy (Weeks 24, 26, 28, and 30) by experimenters blinded to group.

Pawprint Analysis

Pawprint analysis was performed using the stamp and paper method as previously described [41]. The forepaws of the animals were pressed into non-toxic ink, and the animals walked down a Plexiglas corridor (24 in x 4 in) with paper lining the floor. Each animal performed 1-3 trials to ensure three footprints from each paw could be analyzed. The paper was scanned and digitized, and three footprints from both the left and right paw were analyzed by a blinded experimenter using ImageJ software. Toe spread, the distance between the center of the second and fifth digits, was measured and recorded. Due to technical complications, footprint data was not collected in one rat. Assessments were performed at conclusion of therapy (Week 30) by experimenters blinded to group.

Histology

After completion of behavioral testing, segments of the median and ulnar nerves proximal and distal to the injury site were removed for histological analysis. Animals were

deeply anesthetized (ketamine hydrochloride, 80 mg/kg, i.p. and xylazine, 10 mg/kg, i.p.) and the median and ulnar nerves in the right forelimb were identified. Segments (5-10 mm) were dissected from both proximal and distal segments of each nerve and were post-fixed in 4% paraformaldehyde. After 24 hours, the nerve segments were transferred to a solution of 4% PFA and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. Four hours later, the segments were transferred to 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. Tissue segments were blocked and sliced into 4-5 um sections. Sections were mounted on slides and stained with toluidine blue before microscopic imaging at 40x magnification. The images were then analyzed for g-ratio and fiber count by blinded experimenters using ImageJ software (NIH). G-ratio was calculated as the ratio of the inner fiber diameter to the total outer diameter of the fiber.

Statistical Analysis

All group sizes, outcome measures, and planned statistical comparisons were included in the study pre-registration prior to beginning data collection. Mechanical withdrawal thresholds, cylinder task right forelimb use, and grip strength were analyzed using a two-way repeated measures ANOVA to assess effect of group, followed by *post hoc* Bonferroni-corrected unpaired t-tests where appropriate. Paired t-tests were used to compare measures within subjects from pre-injury to week 8 and week 16 pre-therapy time points, where applicable. Two-way ANOVA was used to compare footprint data, followed by unpaired t-tests. Ladder walking data was compared with an unpaired t-test. Statistical tests for each comparison are noted in the text. Figures depict mean ± standard error of the mean.

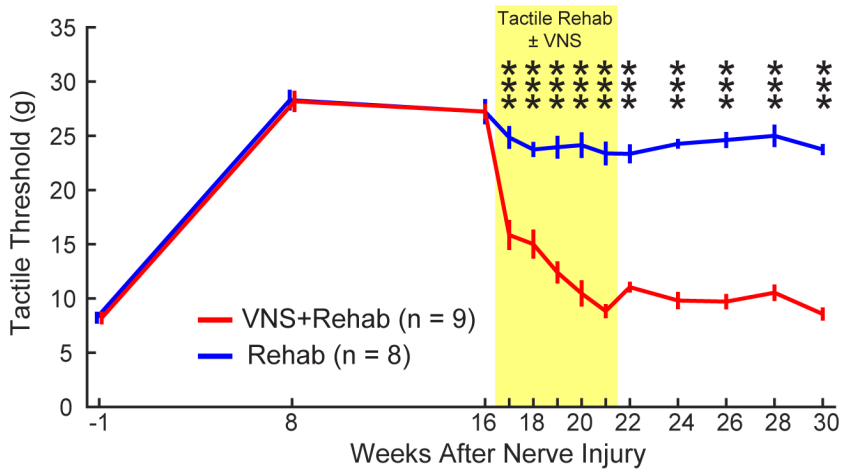

Figure 4.2. VNS paired with tactile rehabilitation restores somatosensory thresholds

Nerve damage results in chronic impairments in somatosensation in the forepaw, as indicated by a lasting increase in mechanical withdrawal thresholds. VNS paired with tactile rehabilitation (VNS+Rehab) drives robust, significant improvements in somatosensory thresholds compared to equivalent tactile rehabilitation without VNS (Rehab). The yellow shaded region denotes when tactile therapy with or without VNS was delivered. VNS-dependent restoration of somatosensory thresholds is stable, lasting many weeks after the cessation of stimulation. Unpaired t-tests across groups at each time point; *** denotes $p < 0.001$. Error bars indicate mean ± SEM.

Results

All animals underwent pre-injury baseline assessment of forelimb sensory motor function, including evaluation of mechanical withdrawal thresholds, forelimb use asymmetry, and forepaw grip strength test. Following baseline testing, all animals underwent transection and tubular repair of the median and ulnar nerves in the right forelimb. This procedure results in total denervation of the mechanoreceptors in the ventral surface of the forepaw while sparing the vast majority of innervation to the dorsal surface of the forepaw innervated by the radial nerve (Meyers 2017, Meyers 2019). Although reinnervation occurs, animals exhibit chronic disruption of nerve morphology and lasting impairments in somatosensation (Figure 4.1B).

Mechanosensory withdrawal thresholds in the right forelimb were significantly elevated 8 weeks post-injury (Figure 4.2; PRE v. Wk 8; Paired t-test, $t(16) = 29.61$, $p = 2.11 \times 10^{-15}$). Impaired somatosensation was stable when assessed at 16 weeks post-injury (PRE v. Wk 16; Paired t-test, $t(16) = 29.67$, $p = 2.04 \times 10^{-15}$). No differences in withdrawal thresholds were observed between groups prior to beginning therapy (Rehab v. VNS+Rehab at Wk 16; Unpaired t-test, $t(15) = 0.01$, $p = 0.99$).

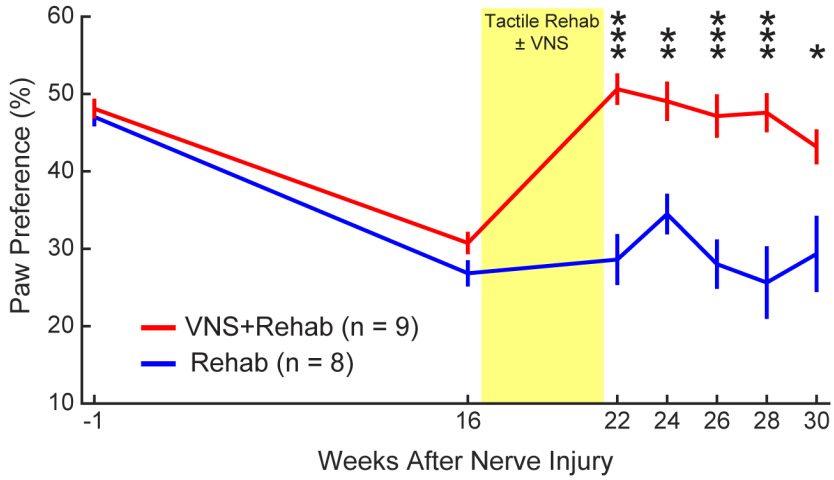

Figure 4.3. VNS-dependent recovery generalizes to an untrained sensorimotor forelimb task.

Nerve damage and resultant sensory loss produces an overreliance on the use of the uninjured forelimb during exploration, demonstrated by a reduction in preference for the injured paw. Rats that received VNS paired with tactile rehabilitation (VNS+Rehab) exhibited a restoration of paw preference compared to equivalent tactile rehabilitation without VNS (Rehab) after the completion of therapy, indicating greater volitional use of the injured forelimb. Improved forelimb use was observed for many weeks after the cessation of therapy. The yellow shaded region denotes when tactile therapy with or without VNS was delivered. Unpaired t-tests across groups at each time point; * denotes $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Error bars indicate mean ± SEM.

We sought to identify whether pairing tactile rehabilitation with VNS could improve recovery of forelimb somatosensation in animals with chronic sensory deficits. To do so, all animals underwent six weeks of tactile rehabilitation, four sessions per week, beginning on week

17 post-injury. Each session was designed based on clinical sensory retraining and consisted of delivery of 200 touches to the ventral surface of the injured forepaw with a range of mechanical stimuli (Figure 4.1.C&D). Animals received either tactile rehabilitation without VNS (Rehab, n = 8) or equivalent tactile rehabilitation with a 0.5 train of VNS delivered concurrent with the delivery of each mechanical stimulus (VNS+Rehab, n = 9). VNS paired with tactile rehabilitation resulted in significant reductions of somatosensory withdrawal thresholds compared to equivalent tactile rehabilitation without VNS, consistent with improvements in somatosensory function (Figure 4.2, Rehab v. VNS+Rehab; Two-way repeated measures ANOVA, $F[1,15] = 384.19$; $p = 4.23 \times 10^{-12}$). Post hoc tests revealed a significant improvement in somatosensory thresholds in the VNS+Rehab group on the first week of therapy that was maintained throughout the remainder (Figure 4.2, Rehab v. VNS+Rehab; Bonferroni-corrected unpaired t-tests, wks 17-21 all $p < 8.33 \times 10^{-3}$). VNS-dependent improvements in withdrawal thresholds were maintained on week 22 after the cessation of VNS (Wk. 22; Rehab v. VNS+Rehab; Unpaired t-test, $t(15) = 12.61$, $p = 2.19 \times 10^{-9}$). Moreover, improved somatosensory function was observed in the VNS+Rehab group for two months after the conclusion of tactile rehabilitation, indicative of a lasting restoration of sensation (Rehab v. VNS+Rehab; Bonferroni-corrected unpaired t-tests, wks 22-30 all $p < 8.33 \times 10^{-3}$). An intent to treat analysis including available data from the four excluded subjects reveals similar findings (Table S2). No differences in sensory thresholds were observed in the uninjured forepaw at any time point, indicating that VNS-dependent changes in withdrawal thresholds are specific to the rehabilitated paw (Two-way repeated measures ANOVA, $F[1,15] = 1.14$; $p = 0.30$). These

findings demonstrate that VNS paired with tactile rehabilitation produces substantial, stable improvements in somatosensory function in animals with chronic sensory loss.

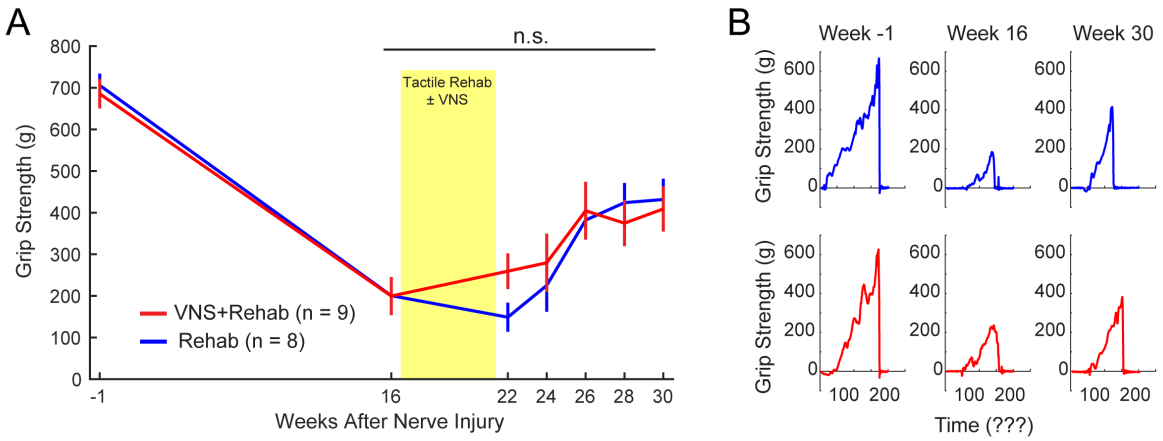

Figure 4.4. VNS paired with tactile rehabilitation does not restore motor function

(A) Grip strength is substantially reduced following nerve damage. VNS paired with tactile rehabilitation did not yield significant benefits in recovery of grip strength compared to equivalent tactile rehabilitation without VNS, suggesting that VNS therapy explicitly does not restore motor function. The yellow shaded region denotes when tactile therapy with or without VNS was delivered. (B) Representative examples illustrating grip strength at multiple time points during therapy. Unpaired t-tests across groups at each time point; n.s. denotes not significant. Error bars indicate mean ± SEM.

Sensory and motor function are highly integrated, and neurological injury often produces impairments in both. We sought to determine whether the observed VNS-dependent improvements in somatosensory function would generalize to a number of other measures of forelimb sensorimotor function. First, we assessed spontaneous volitional forelimb use during exploratory behavior with the cylinder task. As expected, nerve injury produced a dramatic asymmetry of forelimb use favoring the uninjured paw (Figure 4.3, PRE v. Wk 16; Paired t-test, $t(16) = 12.69$, $p = 9.06 \times 10^{-10}$). No differences were observed across groups before therapy (Rehab v. VNS+Rehab at Wk 16; Unpaired t-test, $t(15) = 1.77$, $p = 0.097$). At each time point

after the conclusion of therapy, animals that received VNS+Rehab demonstrated significantly greater use of the injured forelimb compared to animals that received Rehab, as demonstrated by a reduction in paw preference asymmetry (Two-way repeated measures ANOVA, $F[1,15] = 29.48$; $p = 6.95 \times 10^{-5}$; Bonferroni-corrected unpaired t-tests; wks 22-28 all $p < 0.01$).

Next, we assessed grip strength. Nerve injury produced a significant decrease in grip strength in the injured forepaw, consistent with observations from previous studies (Figure 4.4, PRE v. Wk 16; Paired t-test, $t(16) = 10.17$, $p = 2.15 \times 10^{-8}$) [41]. No differences were observed across groups before therapy (Rehab v. VNS+Rehab at Wk 16; Unpaired t-test, $t(16) = 0.02$, $p = 0.98$). No differences in recovery of grip strength in the injured forepaw were observed between the VNS+Rehab and Rehab groups, suggesting that VNS paired with tactile rehabilitation does not directly improve recovery of forelimb strength (Rehab v. VNS+Rehab; Two-way repeated measures ANOVA, $F[1,15] = 0.15$; $p = 0.69$).

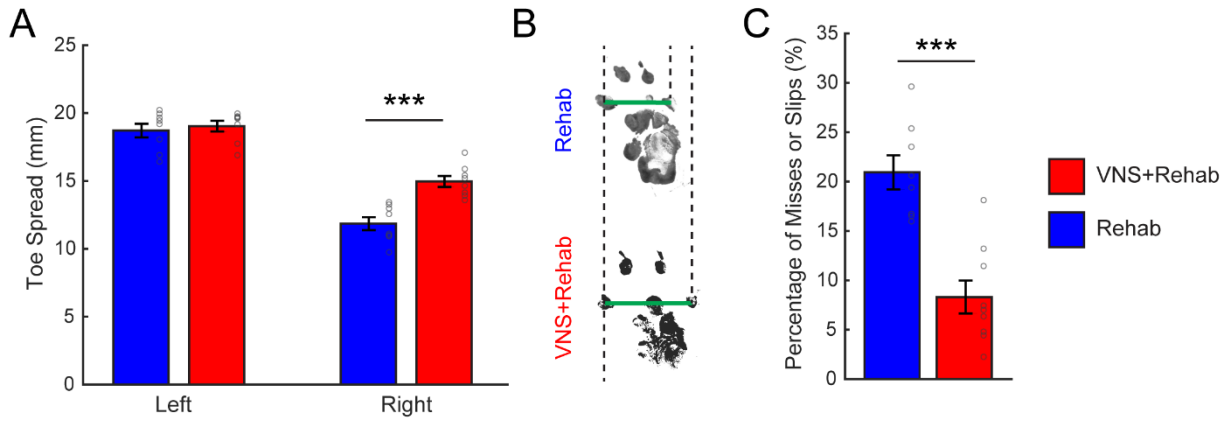

Figure 4.5. Skilled forelimb use during locomotion is improved by VNS paired with tactile rehabilitation

(A) Forelimb toe spread in the injured right forepaw was reduced compared to the intact left forepaw after nerve damage, consistent with sensorimotor dysfunction. VNS paired with tactile rehabilitation (n = 8) significantly increased toe spread compared to equivalent tactile rehabilitation without VNS (n = 8) on Week 30. (B) Representative examples of footprints

collected from the injured right forepaw after the completion of tactile rehabilitation with or without VNS. Green lines illustrate the toe spread measurement, and dotted lines are shown for alignment. (C) Additionally, rats that received VNS paired with tactile rehabilitation (n = 9) demonstrate significantly fewer misses or slips during the ladder walking assessment compared to rats that received tactile rehabilitation without VNS (n = 8). Together, these findings indicate that the benefits of VNS paired with tactile rehabilitation generalize to measures of forelimb use during locomotion. Unpaired t-tests across groups at each time point; *** denotes $p < 0.001$. Circles depict data from individual subjects. Error bars indicate mean ± SEM.

Impairments in locomotion, reflecting both sensory and motor dysfunction, arise from nerve damage [41,44]. We tested whether VNS-dependent increases in somatosensory withdrawal thresholds would improve recovery of toe spread width during walking. Nerve injury significantly decreased the length of toe spread in the impaired right forepaw compared to the uninjured left forepaw, consistent with a loss of sensorimotor function in the paw (Figure 4.5A&B, Two-way ANOVA, $F[1,31] = 147.48$, $p = 1.12 \times 10^{-12}$). VNS+Rehab resulted in significant improvements in toe spread distance in the impaired forepaw compared to Rehab alone (Figure 4.5A, Right paw, Rehab v. VNS+Rehab; Unpaired t-test, $t(15) = 4.94$, $p = 2.19 \times 10^{-4}$). Additionally, we assessed skilled forelimb placing using the horizontal ladder walking task [41,42]. Animals that received VNS+Rehab demonstrated better forepaw placement accuracy compared to Rehab, as evidenced by significantly fewer missed placements and slips (Figure 4.5C, Rehab v. VNS+Rehab; Unpaired t-test, $t(15) = 5.25$, $p = 9.62 \times 10^{-5}$). The improvements in both measures suggest generalization of the benefits of VNS paired with tactile therapy.

Changes in reinnervation could lead to improved sensory function after injury. We explored whether VNS influenced the degree of axonal regrowth in the distal nerve segment after injury. No differences in axon fiber number or g-ratio, a metric of remyelination, were observed across groups (Figure 4.6, Rehab v. VNS+Rehab; Fiber number, Unpaired t-test, $t(15) = 0.47$, $p = 0.65$; G-ratio; Unpaired t-test, $t(15) = -4.70 \times 10^{-3}$, $p = 0.99$). These findings are consistent

with previous studies that VNS does not influence peripheral nerve health or regeneration and demonstrate that peripheral changes cannot account for improved recovery [45]. Rather, this supports the notion that VNS paired with rehabilitative therapy enhances synaptic plasticity in central networks to support recovery of function [17].

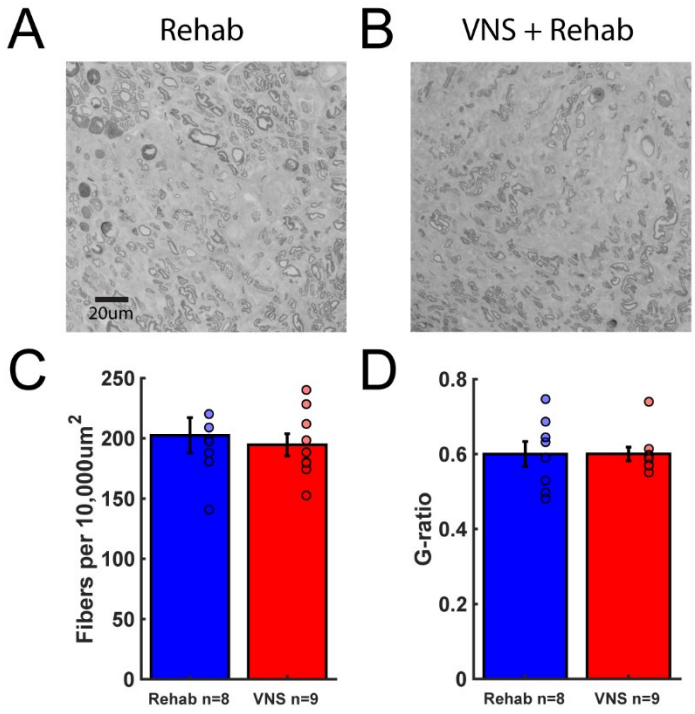

Figure 4.6. VNS does not influence peripheral nerve regeneration or health

(A, B) Example images of fibers in the median nerve distal to the site of injury in rats that received Rehab or VNS+Rehab. (C) The number of fibers in the distal segment of the median nerve is comparable between groups. (D) Additionally, g-ratio, a metric of remyelination, is not different between groups. These findings indicate that differences in peripheral nerve regeneration cannot account for VNS-dependent improvements in sensory function. Unpaired t-tests across groups at completion of study. Circles depict data from individual subjects. Error bars indicate mean ± SEM.

Discussion

Sensory loss commonly occurs following neurological injury, and there are no consistently effective methods to restore function. Here, we present findings from the first

preregistered, well-controlled study demonstrating that pairing closed-loop VNS with a sensory retraining paradigm can enhance recovery of sensory function after neurological damage. VNS paired with tactile rehabilitation resulted in robust, significant improvements in mechanosensory withdrawal thresholds compared to equivalent tactile rehabilitation without VNS in a rat model of chronic sensory loss. VNS-dependent restoration of somatosensory function was maintained for months after the cessation of therapy. Additionally, delivery of VNS paired with tactile rehabilitation significantly improved recovery on other sensorimotor measures. These findings build on the clinical success of closed-loop VNS therapy and position VNS paired with tactile rehabilitation as a novel strategy to restore somatosensory function after neurological injury. The present study was motivated by findings from a clinical case study and highlights the utility of rigorous two way translation. This case study provided initial evidence that VNS delivered during sensory retraining therapy may improve somatosensory function in a chronic stroke patient with profound sensory loss [33]. While encouraging, the inclusion of a single subject and open-label design limit the broad applicability of these findings. Thus, we sought to replicate or disprove these findings in the present well-controlled, preregistered animal study. Corroborating the initial clinical data, the current results provide confirmatory evidence that VNS paired with tactile rehabilitation can significantly improve recovery of somatosensory function. Further highlighting the clinical feasibility of this strategy, the VNS parameters employed in this study match those currently in clinical use for rehabilitation [21,29]. Considered together with the track record of safety, the findings described here support investigation of VNS paired with tactile rehabilitation to restore sensory function in a larger, controlled clinical study.

Somatosensory and motor function are fundamentally integrated, thus improvements in sensory function after neurological injury may produce concomitant benefits in motor control. Indeed, clinical evidence suggests that sensory stimulation may yield increases in motor function in stroke patients [4,46,47]. To address whether VNS-dependent enhancement of somatosensory recovery would similarly improve motor function, we evaluated a number of sensorimotor measures in animals that received VNS paired with tactile rehabilitation. Spontaneous forelimb use, as well as placement and weight bearing during locomotion, were significantly improved in animals that received VNS paired with tactile rehabilitation compared to equivalent tactile rehabilitation without VNS. This is consistent with the notion that improved somatosensory function generalizes to subsequent improvements in motor control. Alternatively, VNS paired with tactile rehabilitation did not yield benefits for recovery of grip strength. Unlike the other measures, grip strength assessment minimizes the express need for volitional motor control and coordination, instead emphasizing simple forelimb strength. The absence of a VNS-dependent improvement in grip strength may reflect the minimal contribution of sensory integration in this task, whereas measures of stepping and locomotion rely more on sensorimotor integration. Despite this lack of a somatosensory-dependent improvement in grip strength, a substantial amount of evidence in both animal models and humans demonstrates that VNS paired with motor rehabilitation can improve recovery of other aspects of motor function and control [19–29]. In response to tactile stimulation during therapy in the present study, rats would occasionally withdraw their forelimb. Although trials in which withdrawal occurred comprise a small minority of the total number of stimulations, it is plausible that VNS acts to drive plasticity in both cutaneous sensory networks and motor reflex loops engaged by tactile therapy. Thus,

recovery of withdrawal thresholds may be driven in part, by improvements in motor function. Ultimately, the optimal implementation of VNS therapy may thus involve co-delivery of both specific sensory retraining as well as motor rehabilitation. This is consistent with evidence from the initial case study, in which the subject received sequential motor rehabilitation for six weeks followed later by tactile rehabilitation for five weeks [33]. Subsequent tactile therapy provided improvements in measures of somatosensation, including stereognosis, proprioception, and detection of light touch. Thus, while a single rehabilitative regiment may provide some utility, an individualized therapy that incorporates both sensory and task-specific motor training paired with VNS is likely to provide optimal benefits for patients with both sensory and motor dysfunction.

VNS paired with rehabilitation enhances synaptic plasticity, which is believed to underlie its therapeutic benefits. VNS drives rapid engagement of the cholinergic and noradrenergic neuromodulatory networks during training to enhance training-specific plasticity [12,13]. Either depleting these neuromodulatory networks or degrading the temporal association of VNS and training prevents the effects of VNS on both plasticity and recovery, highlighting the importance of VNS-dependent plasticity in restoration of function [14–16,22,25,45]. Additionally, VNS drives other molecular changes in the central nervous system, including increased expression of brain derived-neurotrophic factor (BDNF), that may contribute to its effects [48,49]. Closed-loop VNS therapies leverage this activation of pro-plasticity neuromodulatory networks concurrent with neural activity evoked by rehabilitation to promote rehabilitation-specific changes in circuits in the central nervous system. Indeed, VNS paired with motor rehabilitation drives extensive synaptic reorganization in spared motor networks that is associated with recovery [26,27].

Moreover, VNS paired with auditory stimuli enhances stimulus-specific plasticity at multiple stations throughout the auditory network [31]. Similarly, the improved somatosensory thresholds observed in the present study are likely subserved by VNS-dependent enhancement of plasticity throughout cortical and subcortical somatosensory networks. Tactile rehabilitation produces neural activity in these somatosensory networks, and delivery of VNS drives concurrent neuromodulatory release to facilitate synaptic plasticity in neurons activated by the tactile rehabilitation. Consistent with previous findings [45], we did not observe VNS-dependent changes in median nerve health or regeneration with VNS. These findings indicate that VNS does not act through a peripheral restorative mechanism to improve sensory function. Rather, they provide further that VNS likely drives synaptic plasticity throughout the central nervous system to improve recovery [45]. Future studies directed at identifying the nature and contribution of VNS-dependent plasticity in somatosensory networks would provide a greater understanding of the underpinnings of VNS therapy and may be useful to develop individualized interventions.

Optimization of therapies is necessary for effective clinical translation. Although a robust effect of VNS paired with tactile rehabilitation was observed in this study, parametric optimization of both the electrical stimulation parameters of VNS and the mechanical stimuli utilized for tactile rehabilitation may need to be leveraged to maximize therapeutic benefits. The present study employed equivalent electrical stimulation parameters to those utilized in clinical studies of VNS paired with rehabilitation [21,29,33,50,51]. Although these parameters are effective and have been extensively optimized in other contexts [52–57], it remains possible that alternative VNS intensities or durations may yield greater benefits when paired with tactile rehabilitation. Beyond electrical stimulation parameters, previous experiments in auditory cortex demonstrate

that VNS-dependent plasticity is shaped by the features of the paired sensory stimulus [16,30,58]. Thus, the specific features of the mechanosensory stimuli presented with VNS are likely to influence the effects of the therapy. In the present study, the mechanosensory stimuli utilized in tactile therapy were selected to encompass a wide range of features and thereby activate a variety of cutaneous receptors in the paw. Although this implementation yielded a rapid, robust improvement in recovery of mechanosensory thresholds, it remains unclear whether these benefits could be replicated with a less diverse set of stimuli or could be further improved with greater stimulus diversity. Additionally, the procedural aspects of delivering tactile therapy with VNS represent an opportunity for optimization. In the initial clinical case study, the therapist used a push button to initiate VNS during manual delivery of passive tactile stimuli or during patient-initiated active object exploration [33]. The present study utilized a congruent implementation of timed VNS during manual passive tactile stimulation of the paw delivered by an experimenter. Although the current applications largely rely on manual delivery of VNS and sensory stimuli, this intervention is highly amenable to automation. Closed-loop VNS could be triggered during active object exploration either by camera tracking or acceleration sensors or during passive training by automated mechanical and vibrotactile stimuli or cutaneous electrical stimulation. An automated sensory rehabilitation paradigm, likely employed in conjunction with sensory retraining with a therapist, would provide consistency, reduced cost, and greater access to the therapy, yielding clear advantages for patients.

While the present study reveals insight into VNS-dependent restoration of somatosensory function after neurological injury, a number of limitations merit consideration. First, the primary outcome of the present study evaluated mechanosensory withdrawal thresholds, which clearly

fail to capture the full complexity of somatosensory function. Similarly, while the findings reported here indicate that VNS can improve somatosensory detection, we did not evaluate sensory discrimination, as there are few, if any, reliable assessments of forepaw tactile discrimination in rodents. However, despite the limitations of the rodent model, the restoration of tactile thresholds observed in this study corroborates the improvements in somatosensory function reported in the preceding case study, lending validity to the current results. Second, altered somatosensory perception of temperature is a frequent consequence of neurological injury [59,60]. While the present study was not designed to evaluate changes in temperature detection, VNS paired with an appropriate regimen of sensory retraining that incorporates delivery of stimuli of variable temperatures may provide a means to target restoration of temperature perception. Future studies that directly test this hypothesis, either in animal models or human subjects, should be considered. Finally, the present study does not delineate the mechanisms by which VNS therapy improves recovery of somatosensory function. A preponderance of literature suggests that VNS-dependent recovery arises from engagement of neuromodulatory circuits and subsequent enhancement of plasticity in central networks [12–15,26,27]. Future studies should prioritize identifying the nature of the synaptic changes that underlie the effects of VNS paired with tactile rehabilitation in order to optimize delivery of the therapy.

Here, we present evidence that VNS paired with tactile rehabilitation significantly improves recovery of somatosensory function after neurological injury, corroborating findings from an initial case report. VNS-dependent restoration of sensory thresholds was maintained for several months after the cessation of therapy and generalized to other measures of sensorimotor function. These findings extend previous preclinical and clinical studies showing that VNS

paired with motor rehabilitation enhances recovery of motor function and raise the prospective utility of a combinatorial approach employing both sensory and motor rehabilitation with VNS [11,21,29]. Together, these studies position VNS as a novel strategy to target sensory recovery and support the need for a well-controlled clinical study to evaluate VNS paired with tactile rehabilitation in patients with sensory dysfunction resulting from neurological injury.

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Potential Conflicts of Interest: MPK has a financial interest in MicroTransponder, Inc., which is developing VNS for stroke. RLR is an owner of Teliatry, which is developing a VNS device. All other authors declare no conflicts of interest.

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CHAPTER 5

INVESTIGATING THE COMPONENTS OF TACTILE THERAPY PAIRED WITH VAGUS NERVE STIMULATION

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Abstract

Many neurological injuries like stroke and nerve damage often cause profound impairments to sensory function. The current rehabilitation strategies that include sensory paradigms can provide some benefit, but many patients still have permanent sensory dysfunction. Previous studies have demonstrated the capability of vagus nerve stimulation (VNS) paired with tactile rehabilitation to enhance recovery of sensory function. A clinical case study describes the first evidence that VNS paired with a tactile rehabilitation paradigm could improve recovery of sensory function. More recently, a preclinical study has corroborated these findings by pairing different tactile stimuli with VNS to provide recovery of somatosensory function. This study aimed to further investigate the diversity of tactile stimuli necessary to drive significant recovery of sensory function. The study design, including planned sample size, assessments, and statistical comparisons, was preregistered prior to beginning data collection (https://osf.io/3tm8u/). VNS paired with a 10 gram von Frey filament resulted in a significant recovery of mechanosensory thresholds when compared to equivalent rehabilitation alone. Further recovery was demonstrated by VNS paired with a paint brush which resulted in a nearly complete recovery of mechanosensory thresholds and provided significantly more recovery than tactile rehabilitation alone or VNS paired with a 10 gram von Frey filament. Given the safety and feasibility of VNS therapy, these findings suggest that including a brush applied to the area with sensory dysfunction when administering VNS paired with sensory rehabilitation may provide the most benefits in sensory function following neurological injury.

Introduction

The loss of somatosensory function is common following neurological injury. As many as 85% of patients show deficits in sensory function following stroke[1,2]. Even after surgical repair, damage done to peripheral nerves can cause long-lasting deficits in somatosensation for many patients[3,4]. Currently, there are no effective treatments to recover sensory function, but rehabilitation that includes sensory components can provide some benefits to patients[5-8]. The vast majority of therapeutic interventions commonly focus on restoring of motor function following neurological injury, but impairments in sensory function play a large role in overall disability[9-11]. Due to the frequency and impact of sensory impairment, effective treatment strategies that can drive significant recovery of somatosensory function have the potential to provide substantial benefits for patients with neurological injury.

Previously, we have developed a novel strategy utilizing vagus nerve stimulation (VNS) to increase the benefits of rehabilitation[12]. VNS activates neuromodulatory circuits paired with rehabilitation to provide synaptic plasticity[13,14]. VNS paired with motor rehabilitation can drive significant improvements in motor function in multiple animal models of neurological injury and in chronic stroke patients[15-23]. In the auditory system, many studies demonstrate pairing VNS with auditory tones drives stimuli-specific plasticity, leading to the notion that VNS paired with other sensory stimuli may provide comparable effects[24,25]. Recently a case study in a chronic stroke patient with significant sensory dysfunction and a preclinical study in an animal model of chronic sensory loss, demonstrated that pairing VNS with tactile rehabilitation drove substantial benefits in multiple measures of sensory function supporting the previous hypothesis[26].

Here, we aimed to build on the recent case study and preclinical experiment to further investigate if less diversity of tactile stimuli when pairing VNS with tactile stimuli could improve recovery of somatosensory function following peripheral nerve injury (PNI). We also assessed the ability of recovery in sensory function to generalize to other measures of forelimb function. To do so, rats had transection and gap repair of the median and ulnar nerves in the forelimb, which caused chronic deficits in somatosensation despite reinnervation. Beginning 8 weeks after nerve injury, animals were randomized into three groups: 1) tactile rehabilitation paradigm with a 10g von Frey filament and paint brush, 2) tactile rehabilitation paradigm with a 10g von Frey filament paired with 0.5s bursts of VNS, 3) tactile rehabilitation paradigm with a paint brush paired with 0.5s bursts of VNS. Each group received the same amount of tactile rehabilitation applied to the ventral surface of the injured paw. The groups receiving VNS had the stimulation paired with the presentation of the tactile stimulus. Mechanosensory thresholds and spontaneous forelimb use were measured weekly throughout therapy for 6 weeks. Following the cessation of therapy, multiple measures of forelimb sensorimotor function were evaluated to examine the generalization of recovery.

Materials and Methods

Experimental Design

All experimental procedures, group sizes, outcome measures, statistical comparisons, and exclusion criteria were preregistered on Open Science Framework before data collection began (https://osf.io/3tm8u/). To begin, all rats underwent the baseline assessment of mechanosensory withdrawal thresholds, grip strength, and cylinder testing. Once baselines were established, rats had transection and tubular gap repair of both the median and ulnar nerves in the right forearm.

On week 7, animals were implanted with a stimulating cuff electrode on the left cervical vagus nerve connected to a headmount. Starting on week 8 post-PNI, rats were dynamically allocated into three groups based on mechanosensory withdrawal thresholds in the impaired limb. One group received tactile rehabilitation (Rehab, n = 8), consisting of 6 weeks of daily sessions in which 200 presentations of either paintbrush or a 10g filament were applied to the ventral surface of the injured paw. The next group received equivalent tactile rehabilitation with the 10g filament, but a 0.5s train of VNS was paired with the delivery of the 10g filament (VNS+Filament, n = 11). The last group received equivalent tactile rehabilitation, but a 0.5s train of VNS paired with the delivery of the paintbrush (VNS+Brush, n = 14). Mechanosensory withdrawal thresholds and spontaneous forelimb use on the cylinder test were measured weekly during therapy and following the last week. Additional measures of function including grip strength, horizontal ladder rung, and footprint analysis, were collected at the end of therapy (Figure 5.1A). Fourteen rats were excluded from the study based on predefined criteria: mortality (n = 5), VNS device failure (n = 7), and autophagia (n = 2). All source data indexed across animals can be found in Supplementary Tables 1-6.

Subjects

Adult female Sprague Dawley rats (n = 47) weighing 300g when they entered the study were obtained from Charles River Laboratories. Rats were housed in a 12:12 reversed light cycle environment, and behavioral training was performed during the dark cycle to increase daytime activity levels. All procedures performed in the study were approved by the University of Texas at Dallas Institutional Animal Care and Use Committee (Protocols: 14-10 and 99-06).

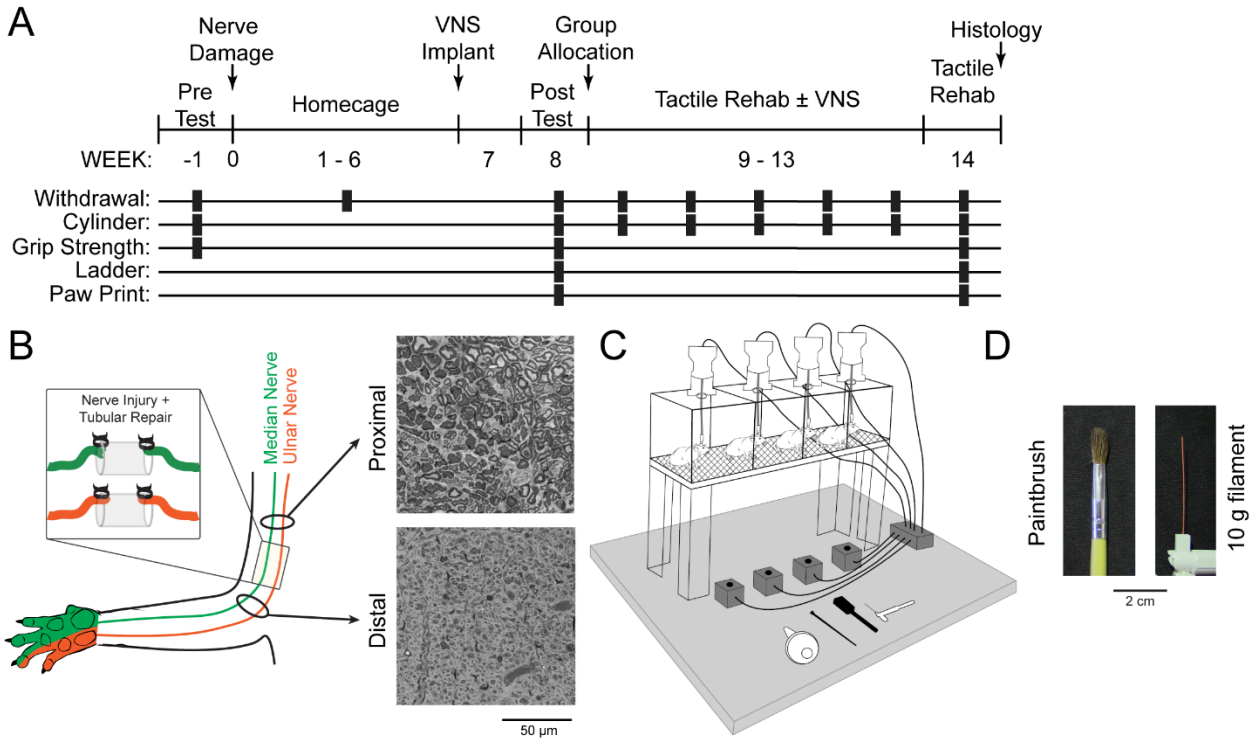

Figure 5.1. Experimental design and tactile rehabilitation paradigm.

(A) Timeline of experimental design illustrating when each assessment is performed. (B) Schematic and representative images from proximal and distal cross-sections of the median nerve approximately 30 weeks after nerve transection and tubular repair. Reinnervation takes place, but the procedure results in chronic deficits in nerve architecture distal to the injury site. (C) Schematic of the tactile rehabilitation apparatus. Rats were placed in individual cages with a wire mesh floor. A variety of tactile stimuli were applied to the ventral surface of the right (injured) forepaw. A button press coincident with the delivery of the tactile stimuli initiated a 500 ms train of VNS in the appropriate group. (D) Detailed view of the devices utilized during tactile rehabilitation. The stimuli were selected to encompass a wide range of somatosensory features.

Forelimb Nerve Injury

Complete transection of both the median and ulnar nerves proximal to the elbow followed by tubular repair was performed as previously described[27]. Animals were anesthetized with ketamine hydrochloride (50 mg/kg, i.p.), xylazine (20 mg/k, i.p.), and acepromazine (5 mg/kg, i.p.). A small incision proximal to the elbow of the right forelimb was made, and the

median and ulnar nerves were carefully isolated. Both nerves were transected 1 cm proximal to the elbow. Immediately following transection, the proximal and distal stumps of each nerve were sutured 1 mm from the ends of a 8 mm saline-filled polyurethane tube (Micro-Renathane 0.095" I.D 0.066" O.D., Braintree Scientific, Inc., Braintree, MA), resulting in a 6 mm gap between nerve stumps. The skin incision was sutured and treated with antibiotic ointment. All animals were given enrofloxacin (10 mg/kg) immediately following surgery and sustained release buprenorphine (1.2 mg/kg) for 6 days following injury. Animals were placed in Elizabethan collars following injury to limit autophagia.

Vagus Nerve Stimulation Implantation Surgery

VNS implantation procedures were performed as described in previous studies[16-21]. Fifteen weeks after transection of the median and ulnar nerves, rats were anesthetized with ketamine hydrochloride (50 mg/kg, i.p.), xylazine (20 mg/kg, i.p.), and acepromazine (5 mg/kg, i.p.). An incision was made to expose the skull. Bone screws were inserted into the skull at multiple locations. A connector was mounted to the screws using acrylic. An incision was made on the left side of the neck and the overlying musculature was blunt dissected to isolate the vagus nerve. The nerve was placed into a bipolar stimulating cuff electrode, and the electrode leads were connected with the two-channel skull-mounted connector. All rats received enrofloxacin (s.c., 10 mg/kg) following surgery. All rats underwent implantation of the headmount and cuff electrode. To confirm cuff electrode functionality, VNS-dependent activation of the Hering-Breuer reflex was assessed as in previous studies[28,29]. To do so, blood oxygenation saturation during trains of VNS (0.8 mA, 30 Hz, 100 μs pulse width, up to 5 s train duration) was

monitored via pulse oximetry. The cuff electrode was replaced if rats failed to demonstrate a reliable drop in oxygen saturation during the implant surgery.

Tactile Rehabilitation and Delivery of Vagus Nerve Stimulation

Tactile rehabilitation began 9 weeks post-forelimb nerve injury and continued for 6 weeks. Sessions of tactile rehabilitation were performed once daily, four days per week, with each session lasting approximately 1.5 hours. During each session, up to 8 animals were placed in individual acrylic chambers (14 x 15 cm) with a mesh floor (Figure 5.1C). Each session consisted of 200 touches to the ventral surface of the right (injured) forepaw with mechanical stimuli (Figure 5.1D and Supplemental Video 1): a 10g von Frey filament (North Coast Medical, Gilroy, CA) and a paintbrush (Kiss Products, Port Washington, NY). Stimuli were presented with at least 10 seconds between each delivery, resulting in a 200 touches with the stimuli per session. The von Frey filament was applied perpendicularly to the paw and the digits and only to the center of the paw. The paintbrush was applied across the paw and digits in varying directions and with an approximate upward force of 50g.

In the appropriate groups, a train of VNS was triggered by a button press to coincide with delivery of each mechanical stimulus during tactile rehabilitation sessions. VNS parameters were equivalent to previous studies[15,16,18,21]. Each 0.5 s stimulation train consisted of 0.8 mA 100 μsec biphasic pulses delivered at 30 Hz. No VNS was delivered after week 13 to assess effects lasting after the cessation of stimulation.

Mechanosensory Withdrawal Threshold Testing

Mechanosensory detection thresholds were assessed in all animals according to standard procedures[30]. Testing was performed in an acrylic chamber (19.5 x 9.6 cm) on a wire mesh floor.

For each session, animals were allowed to acclimate to the behavioral chamber for 30 min before testing commenced. Mechanical withdrawal thresholds of the left and right forelimbs were tested using a dynamic plantar aesthesiometer (Cat. No. 37450, Ugo Basile, Switzerland). The force at which paw withdrawal occurred was captured for analysis. Trials resulting in paw withdrawal due to spontaneous exploratory activity were excluded from analysis. Assessments were performed at weeks -1, 8, 9, 10, 11, 12, 13, and 14 by experimenters blinded to group.

Cylinder Forelimb Asymmetry Testing

Spontaneous use of the forelimbs during exploratory activity was measured in a subset of animals using the cylinder forelimb asymmetry task, similar to previous descriptions[31]. Animals were placed in a transparent cylinder (20 cm diameter) and allowed to freely explore for two minutes. The total number of both left and right forepaw contacts with the wall of the cylinder were recorded. An asymmetry index, describing the relative use of the injured forelimb, was calculated as [(right/(left + right)) x 100]. Assessments were performed at weeks -1, 8, 9, 10, 11, 12, 13, and 14 by experimenters blinded to group.

Grip Strength Testing

A custom-made grip strength meter was used to measure the grip strength of the right and left forepaws independently, similar to previous descriptions[32]. The rat was positioned over the two horizontal bars attached to separate force transducers such that each forepaw grasped a single bar. During testing, rats were held by the hindquarters while horizontally suspended and pulled away from the module until grip broke. Five trials were performed at each assessment. Assessments were performed at weeks -1, 8, and 14 by experimenters blinded to group.

Horizontal Ladder Rung Testing

Horizontal ladder rung walking task was performed to assess forelimb placing, similar to previous studies[33,34]. The test apparatus consisted of Plexiglas walls that created a 1 m long alley. Metal rungs (3 mm diameter) were inserted into the base of the walls to create an irregular pattern that varied the distance of the rungs from 1 to 5 cm. The same pattern was kept consistent across all animals. Three to five trials were performed at each assessment to ensure data was collected during continuous walking. Frame-by-frame analysis of videos was performed offline and scored by a blinded experimenter, as in previous studies[33,34]. The percentage of misses or slips was calculated as the number of steps with a score of 0-2 divided by the total number of steps scored. The assessment was performed at week 14 by experimenters blinded to group.

Pawprint Analysis

Pawprint analysis was performed using the stamp and paper method as previously described[33]. The forepaws of the animals were pressed into non-toxic ink, and the animals walked down a Plexiglas corridor (24 in x 4 in) with paper lining the floor. Three footprints from both the left and right paw were analyzed by a blinded experimenter using ImageJ software. Toe spread, the distance between the center of the second and fifth digits, was measured and recorded. Due to technical complications, footprint data was not collected in one rat. Assessments were performed at week 14 by experimenters blinded to group.

Histology

After completion of behavioral testing, segments of the median and ulnar nerves proximal and distal to the injury site were removed for histological analysis. Animals were

anesthetized (ketamine hydrochloride, 80 mg/kg, i.p. and xylazine, 10 mg/kg, i.p.) and segments (5-10 mm) of the median and ulnar nerves in the right forelimb were dissected and post-fixed in 4% PFA. After 24 hours, the nerve segments were transferred to a solution of 4% PFA and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. The segments were transferred to 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. Tissue segments were blocked and sliced into 4-5 um sections. Sections were mounted on slides and stained with toluidine blue before microscopic imaging at 40x magnification.

Statistical Analysis

All group sizes, outcome measures, and planned statistical comparisons were included in the study pre-registration prior to beginning data collection. Mechanical withdrawal thresholds, cylinder task right forelimb use, and grip strength were analyzed using a two-way repeated measures ANOVA to assess effect of group, followed by *post hoc* Bonferroni-corrected unpaired t-tests where appropriate. Paired t-tests were used to compare measures within subjects from pre-injury to week 8 and week 14 pre-therapy time points, where applicable. Two-way ANOVA was used to compare footprint data, followed by unpaired t-tests. Ladder walking data was compared with an unpaired t-test. Statistical tests for each comparison are noted in the text. Figures depict mean ± standard error of the mean.

Results

The results of this Chapter are currently incomplete as data is still be analyzed on multiple assessments. All animals underwent pre-PNI baseline evaluation of forelimb sensory motor function, including assessment of mechanosensory withdrawal thresholds, spontaneous

forelimb use, and forepaw grip strength. Following successful baseline assessments, all animals had PNIs in their right forelimb. This injury results in total denervation of the mechanoreceptors in the ventral surface of the forepaw while sparing the innervation to the dorsal surface of the paw innervated by the radial nerve[27]. Despite reinnervation, animals have chronic deficits in sensory function (Figure 5.1B). Mechanosensory withdrawal thresholds in the right forepaw were significantly higher 8 weeks post-injury (Figure 5.2). No significant differences in mechanosensory withdrawal thresholds were seen between groups prior to beginning therapy.

We aimed to identify if tactile rehabilitation consisting of either a 10g filament or a paintbrush paired with VNS could drive improvements in forelimb sensory function in animals with lasting sensory impairments. In order to assess this, all animals received six weeks of tactile rehabilitation, four sessions per week, beginning on week 9 post-PNI. Each session was designed based on clinical sensory rehabilitation and was comprised of the delivery of 200 touches to the ventral surface of the injured forepaw with either a 10g filament or paintbrush (Figure 5.1C&D). At week 8, animals were divided into three groups: tactile rehabilitation without VNS (Rehab, n = 8), equivalent tactile rehabilitation with the 10g filament paired with a 0.5s train of VNS (VNS+Filament, n = 11), or equivalent tactile rehabilitation with the paintbrush paired with a 0.5s train of VNS (VNS+Brush, n = 14). VNS paired with 10g filament resulted in significant reductions of somatosensory withdrawal thresholds compared to equivalent tactile rehabilitation without VNS, consistent with improvements in somatosensory function (Figure 5.2 VNS+Filament v. Rehab; $p = 3.68 \times 10^{-4}$). VNS paired with paintbrush resulted in significant reductions of somatosensory withdrawals thresholds compared to equivalent tactile rehabilitation with the 10g filament paired with VNS or equivalent tactile rehabilitation without VNS (Figure

5.2, VNS+Brush v. VNS+Filament; $p = 1.16 \times 10^{-3}$; VNS+Brush v Rehab; $p = 9.53 \times 10^{-10}$). No differences in the mechanosensory withdrawal thresholds were observed in the uninjured forepaw at any time point, indicating that VNS-dependent changes in withdrawal thresholds are specific to the rehabilitated paw (Figure S1, $p = 0.34$). These findings demonstrate that VNS paired with tactile rehabilitation including a brush produces near complete restoration of mechanosensory withdrawal thresholds in animals with chronic sensory loss.

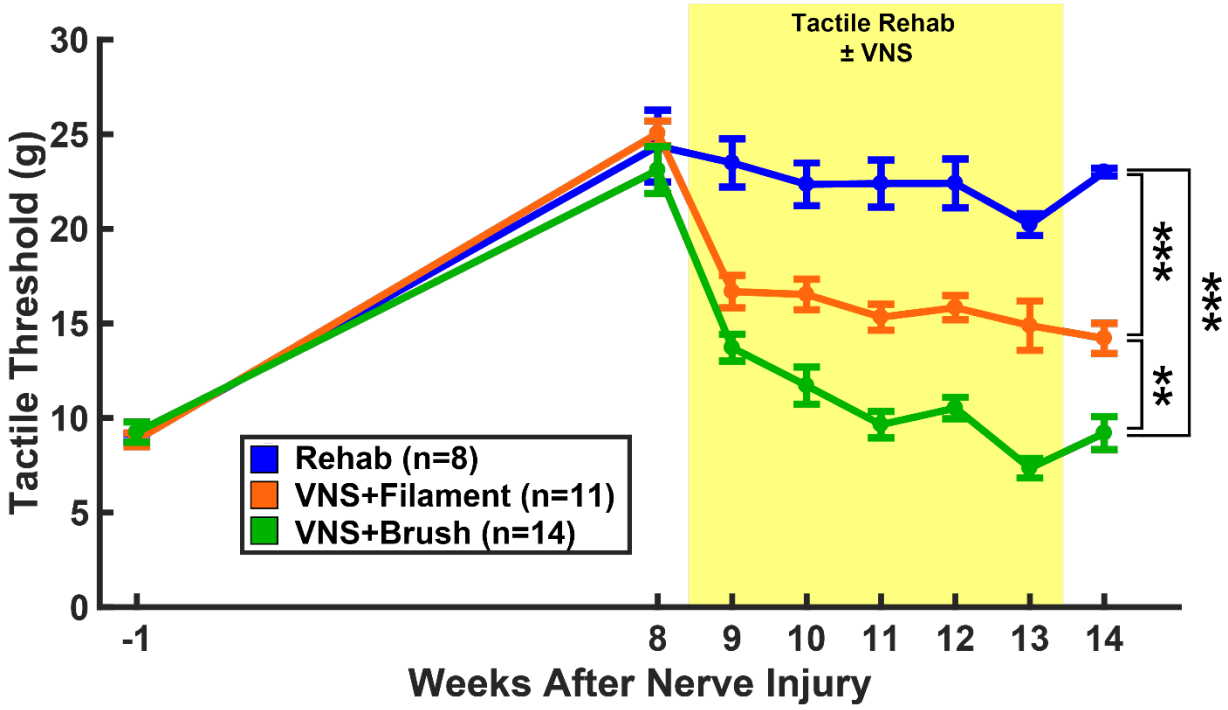

Figure 5.2. VNS paired with tactile rehabilitation significantly reduces tactile thresholds.

Nerve damage results in chronic impairments in somatosensation in the forepaw, as indicated by a lasting increase in mechanical withdrawal thresholds. VNS paired with 10g filament tactile rehabilitation (VNS+Filament) drives significant improvements in somatosensory thresholds compared to equivalent tactile rehabilitation without VNS (Rehab). VNS paired with paintbrush tactile rehabilitation (VNS+Brush) drives significant improvements in somatosensory thresholds compared to equivalent amount of 10g filament tactile rehabilitation paired with VNS (VNS+Filament) and to equivalent amount of tactile rehabilitation without VNS (Rehab). The yellow shaded region denotes when tactile therapy with or without VNS was delivered. VNS-dependent restoration of somatosensory thresholds is stable, lasting many weeks after the

cessation of stimulation. Unpaired t-tests across groups at each time point; *** denotes $p < 0.001$. Error bars indicate mean ± SEM.

Discussion

As previously stated, the results from this study are still being analyzed.

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CHAPTER 6

CONCLUSIONS

Spinal cord injury (SCI) and peripheral nerve injury (PNI) affect a large population of people every year and commonly cause permanent motor and sensory dysfunction[1–7]. Currently, there is no long-lasting and consistently effective treatment to recover sensorimotor function and subsequently improve quality of life. In this dissertation, I have illustrated that vagus nerve stimulation (VNS) paired with rehabilitation drives plasticity and improves recovery following SCI and PNI. Recently, VNS has become a powerful therapeutic tool experimentally utilized to treat a range of functional impairments due to many different forms of neurological injury[8–13]. Over the past couple of years, multiple clinical trials have demonstrated that VNS therapy is a safe, feasible, and effective for treating motor and sensory impairment following stroke[14–17]. Chapters 2 and 3 of this dissertation help describe the translational potential of VNS therapy by demonstrating a synaptic eligibility trace of VNS or the efficacy of VNS therapy when paired within seconds of targeted neural activity in motor circuitry, pairing VNS with the desired functional outcomes boosts recovery, restoring plasticity in spared motor networks in multiple models of SCI, and a generalization of VNS-dependent recovery to similar but untrained tasks. This was also the first evidence providing motor benefits following any model of SCI. In Chapters 4 and 5 of this dissertation we demonstrate the potential of VNS therapy to restore the chronic loss of somatosensation due to peripheral nerve injury. We also demonstrate further translational potential in these Chapters as VNS paired with sensory stimuli produced long-lasting VNS-mediated benefits and generalization of sensory recovery to other untrained tasks. Together, all of these studies highlight the potential for VNS therapy paired with rehabilitation

can provide substantial enhanced motor and sensory recovery after multiple models of neurological injury and thus providing the foundation for the translation of VNS therapy in the clinic.

Chapter 2: Restoring Motor Function and Network Connectivity after Spinal Cord Injury

Chapter 2 of this dissertation describes the translational potential of VNS paired with rehabilitation for enhanced recovery of motor function after SCI. Previously studies describe significant recovery of motor function following ischemic stroke, hemorrhagic stroke, and traumatic brain injury[11,18–21], but this study describes the first use of VNS to enhance motor recovery after SCI. The aforementioned studies have also not investigated the timing between VNS and the stimuli VNS is paired with. This work is incredibly important as VNS therapy alone, not paired with external stimuli or specific neural activity, does not drive plasticity in motor cortices[12]. The work described in Chapter 2 of this dissertation broadens the application of VNS therapy to treat dysfunction in more models of neurological injury by describing enhanced recovery of motor function in multiple models of SCI.

VNS Enhances motor recovery after SCI

We first demonstrate that different models of SCI, unilateral or midline contusion injuries, induce chronic motor impairments in rats on the automated isometric pull task, measuring forelimb strength[22]. Animals receiving rehabilitative training on the isometric pull task exhibited modest recovery of function over a period of 6 weeks. By pairing VNS with a matched amount of rehabilitative training on the isometric pull task, a significant enhancement of motor function was observed in both models of SCI. These results indicate, for the first time, that

VNS paired with rehabilitation can provide a significant boost of motor recovery following two separate models of SCI.

Pairing VNS with the Desired Outcome

Next we investigated whether pairing VNS with the most successful trials or pairing VNS with the least successful trials could drive significant enhancement of recovery when compared to animals receiving rehabilitation without VNS. In order to investigate this, separate algorithms were developed in order to create automated and individualized dynamic thresholds for pairing VNS, essentially developing closed-loop VNS. These control algorithms adaptively scale stimulation thresholds based on the most recently performed trials. VNS was either delivered within the top quintile of trials (top 20%) or with the bottom quintile of trials (bottom 20%). Both algorithms delivered the same amount of VNS during rehabilitative training. The results indicated that by pairing VNS with the top 20% of trials, motor recovery was significantly enhanced when compared to the group receiving rehabilitation alone, and pairing VNS with the bottom 20% of trials did not produce significant enhancement of motor function compared to rehabilitation alone. These results demonstrate that closed-loop neuromodulation paired with the most successful movements during rehabilitation improves recovery of motor function after cervical SCI. These results confirm the findings in previous classical studies performed by Skinner that demonstrate that adaptive reinforcement of successive approximations, or shaping, drives behavior toward a desired response[23]. This idea has been since adopted in rehabilitation strategies, with the intention to reinforce successively better movements[24]. This highlights the importance of the stimuli or event that VNS is paired with, and provides a new insight for further optimization of VNS therapy for use in the clinic.

The VNS Therapy Synaptic Eligibility Trace

The next finding in this study describes a way to further optimize the use of VNS therapy paired with rehabilitation by demonstrating a new possible theory of a synaptic eligibility trace of VNS. The synaptic eligibility trace theory posits that neuromodulatory reinforcement must occur within seconds after neural activity to drive plasticity[25]. To better understand how temporally precise VNS must be, a subset of rats received VNS delayed by approximately 1.5 seconds after the top 20% of the most successful trials. This short delay in VNS resulted in a comparable amount of recovery to stimulation delivered immediately after a successful trial in the top 20% group. We next furthered our understanding necessary temporal precision by analyzing the timing of stimulation in the group receiving VNS paired with the bottom 20% of trials and found that VNS was separated by 25 ± 5 seconds from the most successful trials which failed to drive substantial benefits. These findings together demonstrate a synaptic eligibility trace for VNS describing enhance recovery when paired within a few seconds of successful trials, but not driving recovery when stimulation was delayed by more than a few seconds from successful trials. More simply stated, a temporal precision limit for VNS near 10 seconds was demonstrated which is consistent with the synaptic eligibility trace hypothesis[25].

The magnitude of neuromodulatory activation elicited by an event is directly proportional to the surprise, or unpredictability, of the event[26–28]. This phenomenon is ascribed to reward prediction error[29]. Unsurprising events fail to activate neuromodulatory systems, and even rewarding events fail to trigger neuromodulator release if they are expected. We posit the predictability and accompanying tedium of long, frustrating rehabilitation and the minimal reinforcement of practicing a previously simple motor task blunts plasticity and limits recovery

after SCI. The closed-loop neuromodulation strategy developed here circumvents this by artificially engaging neuromodulatory networks and providing a repeated, non-adapting reinforcing signal typically associated with surprising consequences[30–32]. VNS drives temporally-precise neuromodulatory release to convert the synaptic eligibility trace in neuronal networks that generate optimal motor control to long-lasting plasticity[33].

Restoring Plasticity in Spared Networks

Finally, we demonstrate that VNS promotes increased plasticity in spared motor networks following two distinct models of SCI. Plasticity in remaining networks could be harnessed to support recovery after SCI[34,35]. Unilateral SCI resulted in extensive damage to gray matter, rubrospinal pathways, and propriospinal pathways in the right hemicord while largely sparing the right dorsal corticospinal tract (CST). Thus, we used intracortical microstimulation to test the hypothesis that VNS enhances output from the corticospinal circuits to the impaired forelimb. VNS resulted in eight times more motor cortex sites that generated grasp movements in the impaired forelimb compared to rehabilitation alone, providing the first evidence that VNS induces large-scale plasticity in corticospinal networks after neurological injury.

We next tested the hypothesis that VNS improves recovery by increasing synaptic connections within the motor network controlling grasping muscles of the forelimb. We injected the retrograde transsynaptic tracer pseudorabies virus (PRV-152) into flexor digitorum profundus and palmaris longus, inherently necessary for recovery on the isometric pull task, and counted labeled neurons six days later. VNS resulted in a five-fold increase in labeled neurons in motor cortex compared to rehabilitation alone. The magnitude of this increase in synaptic connectivity is comparable to the seven-fold increase in the number of motor cortex sites that produce grasp

mentioned in the paragraph above. VNS failed to increase neuronal labeling of spinal motor neurons, red nucleus neurons, or propriospinal neurons above the level of the lesion most likely due to the damage to these tracts at the site of the SCI. Additionally, VNS did not influence lesion extent, ruling out the possibility of reduced lesion size as a possible underlying mechanism of VNS. Together, these results are consistent with anatomical plasticity in the spared corticospinal network contributing to enhanced recovery when VNS is added to rehabilitative training after SCI. The observation that VNS improves recovery and enhances functional and anatomical plasticity in corticospinal networks suggests that VNS may prove to be ineffective if the CST is destroyed. Given the severity and anatomical heterogeneity of damage observed in SCI patients[36], such a finding would limit the clinical utility of VNS. We therefore evaluated motor recovery in a bilateral injury model that virtually eliminates the CST on both sides of the cord. Despite profound damage, VNS more than doubled the degree of forelimb motor recovery compared to rehabilitation alone. We hypothesized that VNS enhances recovery by promoting plasticity in the rubrospinal and propriospinal pathways, which were somewhat spared by this injury. To test this hypothesis, PRV-152 was also utilized and was injected into the same musculature as in the aforementioned hemi-contusion model of SCI. Indeed, VNS doubled the number of labeled red nucleus neurons and C3/4 propriospinal neurons compared to rehabilitation alone. ICMS mapping was also conducted in animals receiving a midline contusion SCI, and consistent with the extensive damage to the corticospinal pathway, VNS had no effect on reorganization of motor cortex. VNS also failed to increase the number of labeled neurons in the motor cortex in animals receiving PRV-152. These results suggest that VNS is capable of supporting recovery following SCI by strengthening anatomical connectivity within remaining motor pathways.

In summary, Chapter 2 of this dissertation not only describes how VNS may be a strong therapeutic tool for treating dysfunction after SCI, but also furthers our understanding of VNS therapy for better optimization to be utilized in the clinic. While this work progresses the understanding of VNS therapy, it also highlights the need for more preclinical studies investigating dose, mechanisms, and stimulation parameters of VNS for the optimization of plasticity and behavioral recovery after multiple neurological injuries or disorders.

Chapter 3: Restoring Motor Function Following Bilateral Spinal Cord Injury

Chapter 3 of this dissertation works to further the findings from Chapter 2 while also further highlighting the potential translatability of VNS therapy paired with motor rehabilitation to significantly enhance motor recovery after a different model of SCI. The previous studies above demonstrate that brief bursts of closed-loop VNS paired with rehabilitative training substantially improve recovery of forelimb motor function in models of unilateral and bilateral contusive spinal cord injury (SCI) at spinal level C5/6. While these findings provide initial evidence of the utility of VNS for SCI, the injury model used in these studies spares the majority of alpha motor neurons originating in C7-T1 that innervate distal forelimb muscles. Because the clinical manifestation of SCI in many patients involves damage at these levels, it is important to define whether damage to the distal forelimb motor neuron pools limits VNS-dependent recovery. In this study, we assessed recovery of forelimb function in rats that received a bilateral incomplete contusive SCI at C7/8 and underwent extensive rehabilitative training with or without paired VNS. This study not only provides more evidence of translation of VNS therapy for the

recovery of motor function after SCI, but also provides evidence of VNS-mediated generalization to similar but untrained tasks.

Enhanced Motor Recovery despite Distal Forelimb Motor Neuron Loss

The clinical manifestation of cervical SCI often results in damage to the spinal levels containing alpha motor neurons that control distal upper limb musculature in combination with white matter injury. Substantial damage to these motor neuron pools could limit the benefits of plasticity-enhancing therapies if reorganization cannot compensate for the reduction in alpha motor neurons. Alternatively, synaptic plasticity within spared spinal networks may be sufficient to leverage remaining alpha motor neurons to support recovery. Here, we sought to model these complicating clinical features and determine whether direct damage to the distal forelimb motor pools would prevent VNS-dependent enhancement of recovery. To do so, we assessed recovery of forelimb motor function in animals that received a bilateral incomplete contusive SCI at C7/8 and underwent extensive rehabilitative training with or without paired VNS.

VNS paired with rehabilitative training significantly increased recovery of volitional forelimb strength compared to equivalent rehabilitative training without VNS. Improved volitional forelimb strength was maintained in the group receiving VNS therapy paired with rehabilitative training for one week after the cessation of stimulation, indicating lasting benefits. These findings indicate that damage to networks surrounding motor neuron pools directly linked to distal forelimb musculature is not the sole limiting factor for recovery and suggest that damage to the upper limb motor pools should not necessarily exclude patients from receiving VNS therapy. The improved motor recovery observed in the present study is consistent with the notion that VNS enhances synaptic plasticity in spared motor networks to increase the drive onto

the remaining alpha motor neurons controlling the distal forelimb. Considered together, these findings indicate that VNS supports synaptic plasticity to increase motor output to compensate for impairments resulting from damage to either white matter or alpha motor neurons. Incorporating regenerative strategies that restore lost connectivity with VNS to enhance reorganization in newly connected circuits may represent a novel combinatorial therapeutic regimen to intervene after complete SCI[37,38]. Although animal models fail to capture the variability and complexity of SCI in patients, this study extends the range of conditions over which VNS paired with rehabilitative training improves motor recovery and supports the evaluation of closed-loop VNS therapy as a post-SCI intervention.

Generalization of VNS-Dependent Recovery to Similar but Untrained Tasks

We next assessed whether recovery was restricted to the trained task or generalized to similar, but untrained, forelimb tasks. First, we tested spontaneous forelimb use with the cylinder assessment[39]. As expected, bilateral C7/8 SCI also reduced spontaneous use of both forelimbs, as indicated by a decrease in the total number of wall touches per session. After the conclusion of rehabilitative therapy, the group receiving VNS paired with rehabilitation demonstrated significantly greater restoration of spontaneous forelimb use compared to the group receiving rehabilitation alone. Next, we tested forepaw grip strength. Consistent with previous reports[40], bilateral C7/8 SCI results in a significant impairment in grip strength. VNS paired with rehabilitative training significantly improved grip strength compared to rehabilitative training alone in the trained right forelimb. No significant improvement in grip strength was observed in the untrained left paw. Together, these findings indicate that VNS paired with rehabilitative

training yields improved recovery of motor function on similar, but untrained, forelimb tasks after bilateral C7/8 SCI.

Generalization of functional improvements to similar tasks is a key feature of effective rehabilitative therapies. In addition to task-specific enhancement of recovery observed on the isometric pull task, VNS paired with rehabilitative training yielded increased post-SCI forelimb function on two similar, but untrained, tasks. Rats that received VNS paired with task-specific rehabilitative training on the isometric pull task demonstrated increased spontaneous forelimb use as measured by the cylinder task and improved forepaw grip strength. These findings provide an initial demonstration that VNS paired with task-specific training results in benefits that generalize to similar forelimb movements, consistent with previous studies[12]. This generalization of recovery likely arises from synaptic plasticity of inputs to spared alpha motor neurons that contribute to muscular control common across tasks. For instance, reorganization of synaptic connectivity to alpha motor neurons that exert control over digit grasp muscles would improve performance on both the isometric pull task and the grip strength task, as control of grasp musculature is a key feature in executing both tasks. In practical terms, generalization indicates that rehabilitation should include a broader range of task-specific exercises to yield the greatest benefits.

Chapter 4: Restoring Somatosensory Function with VNS Therapy

Damage to peripheral nerves can lead to profound impairments in somatosensation in many patients, which typically persist even after surgical repair[41,42]. In Chapter 4 of this dissertation, I introduce a novel use of VNS to improve recovery of somatosensation following PNI causing chronic sensory loss. This study led to the discovery of multiple findings, including:

1) VNS enhances recovery of mechanosensory thresholds by pairing VNS with tactile stimuli; 2) The VNS-mediated sensory benefits are long-lasting; and 3) VNS paired with tactile stimuli can drive generalization in other sensorimotor assessments. These findings support the notion that VNS paired with sensory retraining can be significant therapeutic tool to treat sensory loss in the clinic.

Developing a Novel Therapy to Pair with VNS

The work in Chapter 4 was motivated by two significant findings from previous studies conducted within the last two years. The first was a case study conducted by Dr. Kilgard with a chronic stroke patient who had severe sensory loss[17]. The patient received VNS paired with tactile therapy in an attempt to improve his sensory function. He underwent twenty two-hour sessions of VNS paired with both passive and active tactile events. He made significant and lasting improvements in tactile threshold, proprioception, and stereognosis[17]. Specifically, the patient was able to detect tactile stimulation to his affected hand that was eight times less intense, identify the joint position of his fingers in the affected hand three times more often, and identify everyday objects using only his affected hand seven times more often, when compared to baseline[17]. The second finding came in a very recently conducted study currently under review for publication. This study found that VNS paired with motor rehabilitation drove enhancement of plasticity and motor recovery following PNI as well as a significant recovery of mechanosensory withdrawal threshold. Although the VNS-mediated effect on the mechanosensory thresholds did not recover back to baseline thresholds, the effect was significantly greater than the group of animals receiving motor rehabilitation without VNS. These findings opened the door to investigate whether pairing VNS with tactile stimuli could

further drive down mechanosensory thresholds back to normal and reciprocate the findings from Dr. Kilgard's recent case study[17].

Restoring Mechanosensory Withdrawal Thresholds

As stated previously, there are no consistently effective methods to restore sensory function, but therapy paradigms that involve sensory retraining may provide modest benefits to some patients[43–47]. In order to assess whether pairing tactile rehabilitation with VNS could improve recovery of forelimb somatosensation in animals with chronic sensory deficits, a system was developed in order to repeatedly apply tactile stimuli paired with VNS. All animals in the study underwent six weeks of tactile rehabilitation with four sessions per week. Each session was modeled after the clinical sensory retraining and consisted of 200 touches to the ventral surface of the injured forepaw with a range of mechanical stimuli.

Sensory receptors in the skin span multiple modalities including, mechanical sensation, pain, and temperature. Mechanoreceptors, which were primarily assessed in this study, involve four different categories: Merkel's disks which are slowly adapting and mediate slow pressure response, Meissner corpuscles that respond to light touch and adapt quickly to changes in textures, Ruffini endings which primarily detect deep tissue tension, and Pacinian corpuscles that detect fast vibrations. Four diverse mechanosensory stimuli were chosen for tactile therapy in order to encompass a wide range of features and thereby activate a variety of cutaneous receptors in the paw. Five weeks of pairing VNS with this diverse set of tactile stimuli resulted in significant reductions of somatosensory withdrawal thresholds compared to equivalent tactile rehabilitation without VNS. These results corroborate the initial clinical data, and provide strong

evidence that VNS paired with tactile rehabilitation can significantly improve recovery of somatosensory function.

VNS-Mediated Benefits are Long-Lasting

Next, we demonstrate that the VNS-mediated benefits of VNS on sensory function are long-lasting and maintained for 9 weeks after the cessation of VNS and 8 weeks after the cessation of tactile therapy. Previously, studies recovering motor function by pairing VNS with motor rehabilitation have only assessed if the effects of VNS on motor function have lasted for 1 week following the cessation of VNS[9,11,13,18,19]. More recently one study investigating the recovery of motor function after stroke on complex motor tasks after stroke found that the VNS-mediated benefits lasted for six weeks following the cessation of therapy[12]. The study in Chapter 4 helped to further these findings from Meyers et al by highlighting the potential of translation to the clinic, as VNS has now been shown to provide long-lasting benefits for both motor and sensory recovery following multiple neurological injuries.

Generalization of Sensory Recovery to Other Tasks

We next demonstrate that the benefits of VNS on mechanosensory withdrawal thresholds generalize to other sensory and sensorimotor tasks and function. Especially for translation to the clinic, generalization of recovery to multiple tasks and functions is very important to help reduce the amount of rehabilitation and therapy required. Generalization to similar or distant functions is typically characterized poorly, and many studies have found mixed results when investigating untrained tasks[48–54]. In Chapter 4, multiple measures of forelimb sensorimotor function were evaluated throughout to investigate generalization including cylinder forelimb asymmetry testing, grip strength testing, horizontal ladder rung testing, and pawprint analysis.

The cylinder task was utilized to assess the spontaneous volitional forelimb use during exploratory behavior. During and following therapy, animals receiving VNS paired with tactile rehabilitation demonstrated significantly greater use of the injured forelimb when compared to animals receiving rehabilitation alone. Grip strength was assessed to better understand generalization to motor circuitry, and there were no significant differences between groups when assessing the recovery of grip strength following therapy. Additionally we next tested whether sensory improvements would generalize to skilled forelimb placing using the horizontal ladder task or to toe spread during normal walking. Both forepaw placement and toe spread saw significant benefits when comparing the group receiving VNS paired with tactile rehabilitation and the group receiving rehabilitation alone.

Spontaneous forelimb use, as well as skilled forelimb placement and toe spread during locomotion, were significantly improved in animals that received VNS paired with tactile rehabilitation compared to equivalent tactile rehabilitation without VNS. These findings are consistent with the idea that improved somatosensory function generalizes to subsequent improvements in some facets of motor control. However, VNS paired with tactile rehabilitation did not have significant effects on the recovery of grip strength. This could be due to the fact that the grip strength assessment minimizes the express need for volitional motor control and coordination. The absence of a VNS-dependent improvement in grip strength may reflect the minimal contribution of sensory integration in this assessment.

These findings not only provide strong evidence for translation of VNS paired with sensory events functional recovery following neurological injury, but they also lead to the notion of combining motor rehabilitation and sensory rehabilitation into therapy paradigms for these

patients. While both VNS paired with motor rehabilitation and VNS paired with sensory retraining after yielded strong effects in recovering their respective paired functions in both preclinical and clinical studies[9,10,20,11,12,14–19], the optimal implementation of VNS therapy may involve intertwined delivery of both motor and sensory rehabilitation in order maximize the generalization to all sensorimotor function lost due to neurological injury or disorder. Besides the combination of motor and sensory rehabilitation, further investigation could assess a number topics in order to optimize the therapy including sensory discrimination, recovery of temperature sensation, VNS dosage, underlying mechanisms of sensory recovery, the automation of sensory stimuli delivery and subsequent pairing of VNS, active sensory retraining for rodents and humans, and investigation of necessary diversity of tactile stimuli.

Chapter 5: Investigating the Components of Tactile Therapy Paired with VNS

This study was primarily motivated by the results found in Chapter 4 of this dissertation, and was designed to better understand the components of tactile therapy that are necessary for the recovery of somatosensory function in a model of chronic sensory loss. Although the results of this study are still being analyzed, the completed data provides evidence that pairing either the 10g von Frey filament or the paintbrush in tactile rehabilitation with VNS drives significant recovery of mechanosensory withdrawal thresholds. Furthermore, pairing the paintbrush tactile rehabilitation with VNS provides significantly more recovery of these thresholds when compared to 10g filament tactile rehabilitation paired with VNS. These results lead to the notion that differing aspects of paintbrush vs 10g filament when mechanically applied to the forepaw are driving differing amounts of sensory recovery. The two ways in which the tactile stimuli

primarily differ are the areal amount of activation of mechanoreceptors on the forepaw and the temporal profile of activation along the forepaw. The 10g filament is applied only to the center of the forepaw while the paintbrush is applied across the forepaw in varying directions during daily tactile rehabilitation. Also, the 10g filament has a very fast temporal profile of contact with the forepaw while the paintbrush has a longer temporal profile of contact with the forepaw with varying timing of activation of mechanoreceptors as it moves across the forepaw in varying directions during tactile rehabilitation. These two differences need to be further investigated to better understand the necessary components of tactile therapy when paired with VNS in order to drive recovery of sensory function following neurological injury.

Once all data has been analyzed, generalization of recovery may help to determine the necessary components of tactile therapy in order to recovery many types of somatosensory function, not just mechanosensory withdrawal thresholds. These findings also provide evidence for the inclusion of a brush or brush-like activation of receptors in the area of impaired sensory function for patients in the clinic with neurological injury or disorder. This study, along with findings from Chapter 4, aims to provide further evidence and optimization of tactile therapy paired with VNS in order to provide the most comprehensive, efficient, and long-lasting recovery of somatosensory function following neurological recovery.

Final Conclusions

The studies in this dissertation provide strong evidence that VNS paired with rehabilitation can enhance both motor and sensory recovery following multiple models of neurological injury. We were able to extend the previous findings that VNS can enhance motor

function following models of stroke to multiple models of SCI for the first time. We were also able to demonstrate the importance of pairing VNS with the desired functional outcomes, the identification of a possible synaptic eligibility trace for the timing between VNS and the paired event, and the ability of VNS therapy to restore plasticity in different spared networks after two different models of SCI. Next we demonstrated VNS paired with motor rehabilitation could drive enhanced motor recovery in a different level of the cervical spinal cord, restore function to distal musculature despite loss of distal forelimb motorneuron pools, and can generalize to recovery in similar tasks. These results have strong implications for direct translation to future clinical trials for SCI patients.

In the second set of studies performed, we focus on recovering sensory function following a model of chronic sensory loss, PNI. We demonstrate the ability of VNS paired with a diverse set of tactile stimuli to drive significant recovery in mechanosensory withdrawal thresholds, drive long-lasting benefits for more than two months following the cessation of VNS, and generalization to other sensorimotor tasks. Taken together, these results provide strong compelling evidence for the translation of VNS paired with sensory rehabilitation to the clinic for the treatment of sensory loss following a number of neurological disorders.

To summarize, this dissertation provides significant findings in the fields of spinal cord injury, peripheral nerve injury, motor and sensory dysfunction, and plasticity research. While this work was able to answer many questions crucial for translation to the clinic, it also raises new and exciting questions and problems to investigate. In the future, experimenters could investigate recovery of complex tasks after SCI, restoration of urinary and bowel function after SCI, VNS dosage, necessary components of motor and sensory rehabilitation, the combination of motor and

sensory rehabilitation, underlying mechanisms of sensory recovery, automated sensory rehabilitation, personalized optimization of VNS delivery for individual patients, and the use of VNS to treat chronic pain.

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BIOGRAPHICAL SKETCH

Michael Darrow was born in Dayton, Ohio to Heather and Larry Darrow. He attended The University of Texas at Dallas for his undergraduate degree which he received in 2015 as a Bachelor of Science in Biomedical Engineering. His first encounter with research took place during his undergraduate career under Dr. Shalini Prasad to build bio sensors and lab-on-a-chip microfluidic devices. His curiosity and interest in neuroscience led him to start his PhD under Dr. Seth Hays in the Texas Biomedical Device Center. Currently, he is working to finish his PhD and Master's degrees in Biomedical Engineering with a focus in neuroscience. The work during his PhD is centered on improving recovery of motor and sensory function following spinal cord injury and peripheral nerve injury.

CURRICULUM VITAE

Michael Darrow, BS

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EDUCATION:

The University of Texas at Dallas — Richardson, Texas
BS in Biomedical Engineering — May 2015
Cum Laude Academic Honors

MS in Biomedical Engineering — Anticipated Graduation August 2019
Focus in Neuroscience

PhD in Biomedical Engineering — Anticipated Graduation August 2019
Enhancing Plasticity using Vagus Nerve Stimulation Improves Recovery Following Neurological Injury

RESEARCH AND ENGINEERING EXPERIENCE

2015-Present *(Dr. Seth Hays)* — PhD, Biomedical Engineering:
Conducted pre-clinical studies optimizing vagus nerve stimulation (VNS) as a treatment for dysfunction after spinal cord injury and peripheral nerve injury.
Designed custom interface hardware and software with neurophysiological data acquisition systems for rodent cortical neural recording experiments.
Developed, characterized, and implemented multiple novel, automated behavioral assessments of motor, sensory, and cognitive function in rats and mice.
Actively participated in a highly collaborative research environment that included scientists, engineers, and clinicians across the country.
Gained experience in experimental design, management, and presentation of scientific results in peer-reviewed journals and at multiple international conferences.

2012-2015 — Biomedical Microdevices and Nanotechnology Lab
Developed dynamic electrical cell substrate impedance sensing – cell sorting and trapping in microfluidic devices for cancer cell identification.
Designed custom lab-on-a-chip devices for biosensing purposes.
Skilled in mechanical CAD modeling, 3D printing, and laser cutting for rapid prototyping of custom hardware.

PROFICIENCIES AND SKILLS

Technical Skills — Solidworks; Pro-E (Creo); Microcontroller implementation: Arduino, MSP-430; MATLAB; Arduino, MSP-430; Java; Neurophysiology: OmniPlex Neural

	Data Acquisition System, RZ5 BioAmp and RPvdsEx programming; clean room trained; potientiostat for biochemical sensing;
Management	Interviewing, training, and supervising more than 75 combined laboratory technicians and students; experience organizing and managing large project teams; student mentoring (high school - graduate level); writing new Standard Operating Procedures; filing amendments to protocols; ordering lab supplies and reagents; budgeting; upholding university and laboratory safety standards and protocols
Surgical/Lab Techniques	Implantable medical devices; vagus nerve stimulating cuff electrode implants (300+); hemi-contusion cervical spinal cord injury; midline-contusion spinal cord injury; forelimb peripheral nerve injury rodent model; pseudorabies viral injections; intracortical microstimulation motor mapping; spared nerve injury forelimb model; stereotaxic surgeries; Photolithography processes; electrode fabrication; microfluidic device design and fabrication; cell impedance trapping/sensing
Imaging	Slide scanning; confocal imaging; tissue processing and embedding; immunohistochemistry; immunofluorescence; electron microscopy
Animal models	Spinal Cord Injury rat and mouse models; Peripheral Nerve Injury rat and mouse models; Stroke rat models; Behavioral Assays: Vulintus MotoTrak; Catwalk Treadscan; Hargreaves Thermal Testing; Von Frey Sensory Testing
Productivity	Adobe Illustrator, Photoshop, Premiere Pro; Microsoft Word, PowerPoint, Excel; Google Productivity Suite; Mendeley

RESEARCH INTERESTS

Implantable Medical Devices; Peripheral Nerve Injury; Spinal Cord Injury; Neuroplasticity; Sensorimotor Dysfunction; Motor Dysfunction; Rehabilitation; Neuromodulation; Vagus Nerve Stimulation

PUBLICATIONS

Ganzer, P.D., **Darrow, M.J**., Meyers, E.C**.**, Solorzano, B., Robertson, N., Adcock, K., James, J., Ruiz, A., Becker, A., Goldberg, M., Hays, S.A., Kilgard, M., Rennaker, R. (2018). Closed-loop neuromodulation restores network connectivity and motor control after spinal cord injury. *eLife.*

Hulsey DR, Mian TM, **Darrow MJ**, Hays SA. Quantitative assessment of cortical somatosensory digit representations after median and ulnar nerve injury in rats. *Exp Brain Res.* July 2019:1-8. Doi:10.1007/s00221-019-05593-0.

Darrow, M.J., Mian, T.M., Torres, M., Haider, Z., Danaphongse, T., Meyers, E., Rennaker II, R. L., Kilgard, M. P., Hays, S.A. (In Review). Improving tactile function by pairing vagus nerve stimulation with tactile stimuli following peripheral nerve injury.

Darrow, M.J., Torres, M., Sosa, Maria J., Danaphongse, T., Kilgard, M.P., Hays, S.A. (In Review). Vagus nerve stimulation paired with rehabilitation training enhances motor recovery after spinal cord injury to cervical forelimb motor pools.

RESEARCH AWARDS

Biomedical Engineering Society 90 second Oral Research Competition. First Place. 2016. ($500)
Spinal Cord Plasticity in Motor Control Symposium Poster Competition. First Place. 2017. ($200)
UT Dallas Annual Weeks of Welcome Poster Competition, Third Place. 2018. ($200)
International Spinal Cord Injury Summer School, Certificate of Completion. 2018.
UT Dallas PhD Research Small Award Program Winner. ($1000)

RESEARCH PRESENTATIONS

Darrow, M.J., Ganzer, P.D., Ruiz, A., Solorzano, B., Meyers, E.C., Kilgard, M., Rennaker, R., Hays, S.A. Optimizing Vagus Nerve Stimulation Paired with Rehabilitation to Enhance Recovery after Spinal Cord Injury. Biomedical Engineering Society. Minneapolis, MN. October 2016 (Oral Presentation)

Darrow, M.J., Ganzer, P.D., Ruiz, A., Solorzano, B., Meyers, E.C., Kilgard, M., Rennaker, R., Hays, S.A. Optimizing Vagus Nerve Stimulation Paired with Rehabilitation to Enhance Recovery after Spinal Cord Injury. Society for Neuroscience. San Diego, CA. November 2016 (Poster Presentation)

Darrow, M.J., Ganzer, P.D., Ruiz, A., Solorzano, B., Meyers, E.C., Kilgard, M., Rennaker, R., Hays, S.A. Vagus Nerve Stimulation Paired with Rehabilitation to Enhance Plasticity after Spinal Cord Injury. Spinal Plasticity Symposium. San Diego, CA. November 2016 (Poster Presentation)

Kilgard, M., **Darrow, M.J.**, Ganzer, P.D., Rennaker, R., Hays, S.A. Promoting Recovery from Cervical SCI by Pairing Rehabilitation with Vagus Nerve Stimulation. Wings for Life Conference. Austria. May 2017 (poster)

Darrow, M.J., Ganzer, P.D., Ruiz, A., Nguyen, P., Barron, L., Bilal, M., Haider, Z., Kilgard, M., Rennaker, R., Hays, S.A. Vagus Nerve S6timulation Paired with Rehabiliation Increases Plasticity and Recovery following Spinal Cord Injury. Society for Neuroscience. Washington D.C. November 2017. (Poster Presentation)

Darrow, M.J., Ganzer, P.D., Meyers, E.C., Barron, L., Bilal, M., Kilgard, M., Rennaker, R., Hays, S.A. Boosting Recovery after Neurological Injury. Ross Perot Museum Science Café. May 2018 (Oral Presenation)

Darrow, M.J., Ganzer, P.D., Ruiz, A., Nguyen, P., Barron, L., Bilal, M., Haider, Z., Kilgard, M., Rennaker, R., Hays, S.A. Promoting Recovery from Cervical SCI by Pairing Rehabilitation with Vagus Nerve Stimulation. Spinal Cord Injury and Neurotrauma Summer School by Wings for Life and International Spinal Research Trust. July 2018. Glasgow, Scotland (Poster Presentation)

PROFESSIONAL ORGANIZATIONS;

Society for Neuroscience (SfN)
Biomedical Engineering Society (BMES)