Neuromodulation through Spinal Cord Stimulation for Functional Restoration and Rehabilitation after Cervical Spinal Cord Injury

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Abstract

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Spinal cord injury (SCI) results in permanent neurological deficits. The limited physical function impacts quality of life and socioeconomic engagement. Up to now, we have no effective interventions to restore impaired function. Activity-dependent plasticity holds great promise to promote recovery of motor and autonomic function. Neuromodulation via electrical stimulation of the spinal cord has shown growing evidence of promoting activity-dependent plasticity and functional gains following SCI. First, we review the background information about the burden and recovery process of SCI. We also summarize current advances of pharmacological, cellular, and neuromodulation approaches. Emerging evidence with stimulation technologies demonstrates

potential to facilitate neuroplasticity bridging the lesion. In the second part, we demonstrated a cost- and time-efficient experimental tool to assess forelimb function in a rodent model with severe cervical SCI. This novel strategy for the behavior task may accelerate preclinical trials. By using the behavior tasks, in the third part, we present a clinically viable brain-computer spinal interface to reanimate paralyzed forelimb function in rodents with cervical SCI. We demonstrate a stable and computationally efficient local field potential decoder enabling graded forelimb movements via epidural stimulation. Consequently, the brain-controlled epidural stimulation led to functional improvements in freely moving rats with cervical SCI. The closed-loop algorithm was implemented in an implantable size circuit capable of onboard computing, providing a clinically viable strategy to accelerate the translation of brain-computer interfaces to human use. In the fourth part, we investigate the efficacy of transcutaneous spinal stimulation paired with intensive locomotor training in two individuals with cervical SCI. We present the additive effect of transcutaneous spinal stimulation for locomotor recovery with more coordinated movements. Furthermore, we demonstrate the first evidence of transcutaneous spinal stimulation for restoring bowel function. Lastly, we discuss the potential of these neurotechnology approaches. We address the current limitations of scientific understanding and technology to guide future research to restore sensorimotor and autonomic function following cervical SCI.

This research was conducted with the approval of the Institutional Animal Care and Use Committee (IACUC) and the Human Subjects Division (HSD)

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Plain Language Summary

Spinal cord injury occurs most commonly from falls or motor vehicle accidents. The neck injury, the most common injury in spinal cord injury, results in severe disability of upper and lower limbs. It also affects many body systems including bowel and bladder, limiting quality of life and imposing financial burdens. Up to now, there is no cure for the injury. In this thesis, we describe various applications of electrical stimulation of the spinal cord after spinal cord injury to develop and test the next generation of treatments.

Testing the safety and efficacy of potential treatments for spinal cord injury in animal models is an essential step toward the use in clinics. However, research in animal models leads to significant cost and human resources. We developed a cost- and time-efficient testing task for rats with experimental spinal cord injuries to assess their upper limb movement. The concept of the behavior task may help to speed up the clinical translations of potential treatments for spinal cord injury.

Brain-computer interfaces are promising for restoring hand and arm function like typing and holding a cup in people with the neck injury. Brain-computer interfaces could enable people to control robotic arms or electrical stimulation of muscles by using their brain signals for their daily activity. In the meantime, researchers showed electrical stimulation on the injured spinal cord restored movements of paralyzed limbs in people with neck injury. Therefore, we combined these methods and created a system for brain-controlled electrical stimulation of the spinal cord to restore arm movements in rats with spinal cord injury.

We also tested non-invasive electrical stimulation of the spinal cord for walking and other body functions. We combined a conventional rehabilitation for people with spinal cord injury, walking training, with the electrical stimulation via skin-surface electrodes. We found that two people with

spinal cord injury improved their walking and bowel function. The improvement lasted at least two months after we stopped any stimulation or training. Further study is needed to understand this remarkable improvement. These positive outcomes of electrical stimulation of the spinal cord open the potential of new treatment strategies for people with spinal cord injury

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DEDICATION

I would like to dedicate my work to my great parents, Shinichi and Eiko. They have always inspired my appreciation, grit and resilience.

"I do not know what I may appear to the world; but to myself I seem to have been only like a boy playing on the sea-shore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me."

−Isaac Newton

Chapter 1. INTRODUCTION

This thesis work demonstrates the utility of electrical stimulation of the spinal cord to restore motor and autonomic function after spinal cord injury (SCI) by modulating neural activity. Neuromodulation aims to recover physical function by modifying neural connections by using electrical stimulation to enable recovery beyond what is possible with traditional physical therapy. The unifying theme of my dissertation is developing and testing novel strategies of neuromodulation for functional recovery after spinal cord injury. In this chapter, I provide an overview of traumatic cervical spinal cord injury, therapeutic interventions from neuroplasticity perspectives, and current evidence of spinal stimulation to investigate the potential development of novel rehabilitation approaches in people with cervical injury.

1.1 Traumatic Spinal Cord Injury

The spinal cord is a part of the central nervous system and plays a critical role in motor, sensory, and autonomic function. Traumatic spinal cord injury (SCI), which is the most common type of injury in the spinal cord (WHO, 2013), is widely understood to be untreatable even via standard neurosurgical techniques (Silver, 2005). Although the medical community developed surgical interventions for acute injuries, research is still ongoing to find a ‘cure’ for chronic injuries using pharmacological approaches, cell therapies, and rehabilitative interventions (Ahuja et al., 2017; Alizadeh, Dyck, & Karimi-Abdolrezaee, 2019; Schiller & Mobbs, 2012). Current treatments for chronic SCI focus on minimizing secondary complications and compensating the loss of function instead of neural recovery or restoring the impaired function (Burns et al., 2017).

The World Health Organization has estimated that 2.5 million people live with SCI worldwide (WHO, 2013). The U.S. population experiences 17,810 new SCI cases each year and nearly 40 cases per one million people, with an estimated total of 250,000 to 368,000 people with chronic SCI in America ("Global, regional, and national burden of traumatic brain injury and spinal cord injury, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016," 2019; NSCISC, 2019). Approximately 59.5% of people experiencing SCI have cervical injuries. The most frequent category, 47.2%, is incomplete tetraplegia, which results from cervical SCI. The leading causes of SCI are vehicular accidents (38.6%), falls (32.2%), violence like gunshot wounds (14.0%), sports accidents (7.8%) and medical or surgical complications (4.2%). In past decades, the mean age of patients when they got an injury significantly increased from 28.7 ± 14.1 years in 1972-1979 to 43.1 ± 18.6 years in 2015-2019 (mean ± standard deviation) (S. L. James et al., 2019; Singh, Tetreault, Kalsi-Ryan, Nouri, & Fehlings, 2014). Aging in the population with SCI has progressed as the life expectancy extends, although still significantly less than the life expectancy of people without SCI (McCaughey et al., 2016; A. K. Thompson & Wolpaw, 2015; Toda, Nakatani, Omae, Fukushima, & Chin, 2018).

The economic burden associated with SCI on the current health system is significant. The financial impact depends on the severity of the injury. In the United States, the total annual cost associated with SCI is $21.5 billion (Ma, Chan, & Carruthers, 2014). Acute care interventions, including surgical decompression and stabilization with hospitalization average 12 days, cost average $142,366, which can be covered by medical insurance (Mahabaleshwarkar & Khanna, 2014). The mean annual total medical charge per subject for all categories of SCI in the first-year was $523,089, and the charge per year after the first year was $79,759 in 2009 (DeVivo, Chen, Mennemeyer, & Deutsch, 2011). Although the medical care in the first-year post-injury shows

the highest cost, recurrent cost due to rehospitalizations and primary and secondary complications impact the injured person and their family, as well as the health care economy.

Traditionally it was believed that physical function following SCI reaches a plateau after 1.5 years (Burns & Ditunno, 2001; R. L. Waters, Adkins, Yakura, & Sie, 1998). Based on preclinical and case studies, we now understand that the injured central nervous system retains its capability for neuroplasticity in the chronic phase of injury (Brown & Martinez, 2019). However, given the current therapeutic interventions, the likelihood of further improvements after this period is limited. Research is urgently needed to develop new therapeutic approaches for facilitating physical function beyond the traditional management for SCI and promote quality of life in individuals with SCI while reducing the socioeconomic burden of this condition.

1.2 Recovery Process of Incomplete Spinal Cord Injury

Neural reorganization and modest recovery occur after SCI. The reorganization of the spinal circuits includes compensatory sprouting to denervated sites of the spinal cord and some axonal regeneration (Filli et al., 2014; Maier & Schwab, 2006; Tuszynski & Steward, 2012). The severity of SCI is heterogeneous and somewhat influenced by interventions during the acute phase. Even those individuals with complete neurological deficits are likely to have residual axonal connections below the injury (Sherwood, Dimitrijevic, & McKay, 1992). In this section, we review the recovery process after incomplete SCI.

The initial inflammatory response activates a neutrophil influx, microglia, and enzyme cascades (Fleming et al., 2006). Non-neural structures consisting of fibrotic scar tissue and pericytes are formed and inhibit regenerative activities (Göritz et al., 2011). Simultaneously, astrocytes form a border around the lesion to protect viable neural tissue. Around the lesion, viable neural tissues

can rebuild some synaptic connections (Sofroniew, 2018). Furthermore, the inflammation and secondary impacts of the injury release inhibitory molecules, such as oligodendrocyte-myelin glycoprotein, and neurite outgrowth inhibitor A, which block neural regeneration in the central nervous system (Tsintou, Dalamagkas, & Seifalian, 2015). In the chronic phase, the *RhoA/Rho kinase* pathway plays a role in negatively mediating axonal growth (X. Wu & Xu, 2016).

Spinal interneurons can bridge injured axons and connect nearby intact circuits such as propriospinal neurons by relaying supraspinal information (G. Courtine et al., 2008). The new connections potentially create compensatory routes for the transmission of neural signals. Axons of corticospinal and reticulospinal neurons can regrow short distances. They also form new synaptic connections after sprouting above the site of injury (Ballermann & Fouad, 2006; Bareyre et al., 2004). Meanwhile, the motor and sensory cortex, cerebellum, and brainstem undergo neuroplastic changes leading to some functional recovery (Lundell et al., 2011; Winchester et al., 2005). Due to the limited spontaneous recovery of the central nervous system, despite partial reorganizations in the brain and spinal cord, SCI leads to prolonged severe disabilities that include sensorimotor impairments, autonomic dysfunction, and susceptibility to secondary complications (Ahuja et al., 2017).

Conversely, spontaneous plasticity after injury can also lead to aberrant, undirected compensatory plasticity of spinal networks that may deteriorate the recovery of functional signal transmission in the nervous system (Nahar, Lett, & Schulz, 2012). Proprioceptive and other sensory information can guide the reorganization of functional circuits to bridging the lesion through spinal interneurons (Takeoka, Vollenweider, Courtine, & Arber, 2014). Proper afferent input and activity-based training with voluntary engagement may correct the undirected plasticity and result in more functional reorganization of neural networks (Beauparlant et al.,

2013). In the chronic phase, the functional recovery can be managed and compensated by rehabilitation training, but the gains are extremely limited and typically plateau after 1.5 years following injury (Fawcett et al., 2007).

Although we currently lack effective treatment that significantly improves recovery after SCI, there are many promising interventions that are being investigated in preclinical and early clinical studies. In the next section, we will review experimental treatments for SCI.

1.3 Pharmacological Approaches

Recent advances in pharmacological approaches to treat SCI reveal mechanisms that may enhance functional recovery after injury. Our emerging understanding of the recovery process after SCI suggests possible biological approaches for treatment. There are three main mechanisms that can intervene with the natural recovery process through pharmacological agents: 1) protection from the acute immune response, 2) promoting neural regeneration, and 3) activating the silent spinal networks for functional restoration.

First, **neuroprotective** pharmacological agents show the efficacy of protecting the injured spinal cord from acute immune responses and reduce subsequent cell death. For instance, Minocycline, which could reduce oligodendrocyte apoptosis and local inflammation in animal studies, is currently in the final phase of clinical trial (Casha et al., 2012; R. G. Grossman et al., 2014). Riluzole, which is expected to decrease overactivated voltage-gated sodium channels in neurons to promote cell survival, is another example of an ongoing clinical trial that may protect the injured neural structures (Wilson, Forgione, & Fehlings, 2013).

Second, some ongoing clinical trials are testing pharmacological approaches to **enhancing neural regeneration** instead of protection. Cethrin, a specific toxin inhibiting the *RhoA kinase* pathway mentioned above, could facilitate neurite and axonal growth in preclinical trials. Currently, Cethrin has been in the last phase of clinical trials (Fehlings et al., 2011). Anti-Nogo-A antibody is another neural regenerative drug that binds to and neutralizes Nogo-A, an inhibitor of axonal regrowth after SCI. This drug is in an early phase of clinical trials (Sartori, Hofer, & Schwab, 2020). Additionally, glial scar formation consisting of chondroitinase sulfate proteoglycans (CSPGs) prevents the growth of axons and neurites. An enzyme, Chondroitinase ABC (ChABC), facilitates neural regrowth by degrading the CSPGs following SCI (Bradbury et al., 2002). However, the limited half-life and stability of ChABC in *in-vivo* has slowed down the possibility of clinical translation. Researchers are currently attempting to combine gene therapy with ChABC to engineer cells to secrete the compound and enable new clinical trials (Burnside et al., 2018).

Finally, monoaminergic agents show promise for improving recovery of voluntary movements. SCI inflicts damage on descending serotonergic projections from the brainstem to the spinal cord (Ghosh & Pearse, 2014). The disrupted serotonergic system impairs walking, as well as hand and arm function. Buspirone, a 5 hydroxytryptamine (5-HT) 1A receptor agonist, is already approved by the FDA for anti-anxiety treatments and could drive global activation of 5HT1A receptors in the spinal cord. In a preliminary study, buspirone led to **enhanced volitional control** of paralyzed legs and arms in individuals with chronic SCI. Buspirone also showed efficacy in combination with transcutaneous spinal stimulation (Freyvert et al., 2018; P. Gad et al., 2017; Y. P. Gerasimenko et al., 2015). Furthermore, Spinalon, Buspirone combined with levodopa (dopamine/noradrenaline precursor), and cardidopa (decarboxylase inhibitor), has been

suggested as an activator of spinal interneurons for locomotor recovery based on outcomes in preclinical trials (Guertin, Ung, & Rouleau, 2010). The Phase II clinical trial employing Spinalon is ongoing for efficacy in individuals with motor-complete SCI since Spinalon showed safety and preliminary efficacy for people with SCI in the Phase Ia clinical trial (Radhakrishna et al., 2017)..

1.4 CELLULAR THERAPIES

In parallel with pharmacological approaches, research is focused on cell transplantation to promote neural regeneration (Gabel, Curtis, Marsala, & Ciacci, 2017). In this section, we will describe the progress of ongoing clinical trials for cellular therapeutic approaches.

Rescue mechanisms of cell transplantation fall into three categories (Yamazaki, Kawabori, Seki, & Houkin, 2020). The first mechanism is to **replace the lost cells** by cell transplantation through differentiating into a variety of cells (Gao et al., 2019). The second mechanism is to **supplement repairs and regenerations of injured neural structures through neurotrophic factors** from transplanted cells (Teng et al., 2011). The third mechanism is to **facilitate remyelination and axonal growth of host neural stem cells** in the spinal cord by cell transplantation (Katoh, Yokota, & Fehlings, 2019). All three mechanisms complement each other to drive reconstructions of neural circuits toward functional connections.

In 2020, we have 12 ongoing clinical trials of stem cell transplantation for people with SCI (Silvestro, Bramanti, Trubiani, & Mazzon, 2020). Although preclinical studies tested the efficacy of many cell types, such as olfactory ensheathing cells and Schwann cells, ongoing clinical trials mainly include four types of stem cells, umbilical cord mesenchymal stem cells, adipose tissue-derived mesenchymal stem cells, bone marrow mesenchymal stem cells, and neuronal stem cells.

Both olfactory ensheathing cells and Schwann cells could facilitate the secretion of neurotrophic factors to induce axonal growth and neural regeneration. Both cells were examined in the clinical trials. Olfactory ensheathing cells are considered as having a limited efficacy based on the completed clinical trials (L. Li et al., 2015). The clinical data of Schwann cells are currently under analysis for safety and efficacy (Anderson et al., 2017).

Mesenchymal stem cells could facilitate the cell replacement and the secretion of neurotrophic factors to reduce biological damage and impairments of functional ability in preclinical trials (Viswanathan et al., 2019). In on-going clinical trials, the mesenchymal stem cells were harvested from three sources: bone marrow, allogenic umbilical cords, and autologous fat tissues.

The bone marrow mesenchymal stem cell therapy via intrathecal injection is the most heavily studied so far. Several Phase I clinical trials demonstrated the safety and the potential of efficacy for chronic SCI (El-Kheir et al., 2014; Mendonça et al., 2014; Satti et al., 2016). Currently the three clinical trials of bone marrow mesenchymal stem cells are in Phase I/II, Phase II, and Phase II/III, respectively.

Meanwhile, three Phase II and one Phase I/IIa clinical trials have tested the efficacy and safety of allogeneic umbilical cord mesenchymal stem cells in the subacute and chronic stage of SCI. All trails used intrathecal administration of cell therapy. One Phase I clinical trial is ongoing for adipose tissue-derived mesenchymal stem cells, the CELLTOP clinical trial, using intrathecal administration. This trial recently reported an improvement in physical function in one participant (Bydon et al., 2020).

Neuronal stem cells are multipotent progenitor cells that can differentiate into various types of neuronal cells. Primarily, neuronal stem cells support the regeneration of host stem cells by becoming oligodendrocytes and remyelination of axons in the lesion (Parr et al., 2008). To date, embryonic stem cells, which may be the most promising pluripotent cells, have been tested in clinical trials (Sharp, Frame, Siegenthaler, Nistor, & Keirstead, 2010). However, significant ethical concerns have been raised, and the source of these cells is still controversial for clinical use.

Induced pluripotency stem cells may be able to overcome the immune system and foregoing ethical issues. Nonetheless, there are numerous practical barriers to induced pluripotency stem cell transplantation, such as cell survival and tumor formation. However, several preclinical studies present promising outcomes leading to ongoing clinical trials (Tsuji et al., 2019). Neuronal stem cells were studied in four clinical studies (Pereira, Marote, Salgado, & Silva, 2019). Two Phase I clinical trials and one Phase II/III clinical trial of neuronal stem cells are ongoing to test the efficacy and safety.

It is critical to address barriers of clinical trials with biological approaches in people with SCI. The heterogeneity of population with SCI is the biggest hurdle for researchers. The sample size that we can employ for clinical trials is still limited to have sufficient statistical power. The financial efficiency is extremely low as well. In the injury environment, the effect of stem cell therapy remains poorly understood, and effective cell types, dosage, administration route, and stage of SCI have yet to be established. However, data sharing and careful analysis of completed clinical trials are crucial for the future direction of the research (Silvestro et al., 2020).

In sum, stem cell therapies hold great promise of the therapeutic benefits for physical function. The clinical trials still need to overcome hurdles of ethical issues, time, and cost for running clinical trials.

1.5 NEUROMODULATION APPROACHES

Neuromodulation is an emerging approach for functional recovery following SCI. The definition of neuromodulation is a method to intervene in electrical or chemical activities in the central nervous system or peripheral nervous system to improve physical function for quality of life (Krames, Hunter Peckham, Rezai, & Aboelsaad, 2009; Sakas, Panourias, Simpson, & Krames, 2007). In this section, we present the mechanism of neuromodulation based on the basic research and clinical evidence from the perspective of activity-dependent plasticity, and current advances of neuromodulatory approaches including brain-computer interface, brain stimulation, and spinal stimulation.

1.5.1 *Activity-Dependent Plasticity*

Following spontaneous recovery after injury, the central nervous system is still capable of modulating neural networks by appropriate cues from internal and external environments for promoting functional connections. In the 1940s, Donald Hebb proposed a principle of neuroplasticity that synaptic connections can be modulated by the correlation between pre- and post-synaptic neuronal activities (Hebb, 1949). Since then, the theory was proved by showing long-term potentiation (LTP) and long-term depression (LTD) in the mature nervous systems, such as the hippocampus for memory and cerebellum for learning (Bliss & Lomo, 1973; Ito & Kano, 1982).

Subsequently, several research groups demonstrated that the timing of neural firing in pre- and post-synaptic neurons could modulate the connections of synapses resulting in structural and functional changes (Dan & Poo, 2004; Markram, Lubke, Frotscher, & Sakmann, 1997). This phenomenon is currently recognized as spike-time-dependent plasticity. The plasticity can result from the activation of the N-Methyl- d-aspartic acid (NMDA) receptors (Caroni, Donato, & Muller, 2012).

Repeating specific physical activities can also play a role in promoting neuroplasticity to improve the relevant function. Locomotor training is used as a means to induce activity-dependent plasticity following neurological accidents. Locomotor training provides effective sensory input and voluntary engagement of movement to reactivate neural networks in the injured central nervous system (V Dietz & Colombo, 1996; Susan J Harkema, Schmidt-Read, Lorenz, Edgerton, & Behrman, 2012). The active engagement for physical activity induces brain-derived neurotrophic factors (BDNFs) and tyrosine kinase B (TrkB) that can contribute to axonal growth and facilitation of neural activity through neurotransmitters (Gómez-Pinilla, Ying, Roy, Molteni, & Edgerton, 2002). This activity-dependent paradigm can promote locomotor recovery (Filipp et al., 2019).

At the cortical level, repeated skilled training can increase relevant motor outputs through the corticospinal pathways measured by motor evoked potentials via transcranial magnetic stimulation (Perez, Lungholt, Nyborg, & Nielsen, 2004). Additionally, stimulating corticospinal pathways with precise timing to induce spike-timing-dependent plasticity can improve motor function after long-term locomotor training in people with incomplete SCI (Thomas & Gorassini, 2005). The mechanisms of functional improvement also consist of reorganizations of the motor

cortex through inducing LTP in the short term, and activation of synaptogenesis in the long term (Kleim et al., 2004; Rioult-Pedotti, Friedman, Hess, & Donoghue, 1998).

At the spinal cord level, activity-dependent plasticity likely occurs via interactions of supraspinal control and propriospinal neurons (Pierrot-Deseilligny, 2002). Locomotor training may also activate central pattern generators in the spinal cord and thereby create stepping movements and modulate the impaired spinal reflexes. In people with incomplete SCI, the normalized H-reflex modulation pattern and muscle activation patterns after the locomotor training delineated the reorganization of the spinal reflexes independent of muscle properties (Ivanenko, Poppele, & Lacquaniti, 2009; Trimble, Kukulka, & Behrman, 1998).

1.5.2 *Brain Stimulation*

Brain stimulation can be used for both evaluation and intervention. There are many methods to produce stimulation of the brain, and some can enhance functional recovery. Transcranial direct current stimulation (tDCS), transcranial magnetic stimulation (TMS), and deep brain stimulation (DBS) are the major methods of brain stimulation. The stimulation induces artificial shifts of the polarization leading to activation or depression of the targeted brain regions and downstream influences on the spinal cord.

Repetitive stimulation is one method to promote neuroplasticity. First, tDCS combined with motor training of the upper extremities improves task-specific ability and unilateral hand function in people with cervical SCI (Gomes-Osman & Field-Fote, 2015a). Evidence for improving bimanual coordination, however, is still limited. Only two clinical studies investigated the effect of tDCS combined the locomotor training for locomotor recovery and did not show

any functional benefits except the lower extremity strength in people with SCI (Kumru, Murillo, Benito-Penalva, Tormos, & Vidal, 2016; Raithatha et al., 2016).

There is evidence that repetitive TMS changes the cortical excitability based on the frequency of the stimulation as well as the state of the brain (Ridding & Rothwell, 2007). The mechanism likely involves NMDA receptors and modulation of neural excitability (Huang, Chen, Rothwell, & Wen, 2007). Despite a small sample size, two clinical studies indicate a potential benefit of 10 Hz and 20 Hz repetitive TMS combined with motor training for upper limb function (Gomes-Osman & Field-Fote, 2015b). Further, a study of 20 Hz repetitive TMS combined with the robotic training reported the benefits for the locomotor recovery (Kumru, Benito-Penalva, et al., 2016).

Building on the success of TMS, researchers are combining TMS with peripheral nerve stimulation to create paired corticospinal-motor neuronal stimulation (PCMS) (Bunday & Perez, 2012). The PCMS induces presynaptic action potentials through the corticospinal tracts, and subsequently evokes activation of the postsynaptic cells immediately following arrival of the presynaptic volley. This protocol combined with motor training leads to spike-timing-dependent plasticity like LTP to facilitate function (Bunday, Urbin, & Perez, 2018). PCMS combined with activity-based training was tested in people with chronic SCI and demonstrated long-lasting functional benefits in both upper limbs and walking (Jo & Perez, 2020).

Invasive brain stimulation for SCI has also been studied in past decades. Deep brain stimulation (DBS) provides functional improvements in people with Parkinson's disease, dystonia, and essential tremor (Barbey, Bloch, & Vingerhoets, 2015). The DBS electrodes are traditionally placed in globus pallidus interna and subthalamic nucleus. For people with SCI, the importance

of the mesencephalic locomotor region (MLR) region has been suggested. The MLR relays with basal ganglia and forebrain, which project to spinal interneurons for initiating walking (Martinez-Gonzalez, Bolam, & Mena-Segovia, 2011; Shik, Severin, & Orlovsky, 1969). In rats with SCI, MLR stimulation via DBS improved walking function (Bachmann et al., 2013; Hentall & Gonzalez, 2012). Although there are potential side effects of the MLR stimulation (Hamani et al., 2016), clinical trials of the MLR stimulation for people with SCI are underway (ClinicalTrial.gov: NCT03053791). Combining the DBS with motor training or the closed-loop system is potentially a key to open new advances to promote meaningful functional recovery following SCI.

1.5.3 *Brain-Computer Interface*

Brain-Computer Interfaces (BCIs) record and decode brain signals to operate external actuators to perform the user's intended function (Donoghue, 2002). An innovative strategy using the BCI paradigm is decoding brain signals to stimulate biological targets including the brain, spinal cord, peripheral nerves, and muscles, called closed-loop BCIs (A. Jackson, Mavoori, & Fetz, 2006; Potter, El Hady, & Fetz, 2014). Such closed-loop BCIs can also strengthen synaptic connections through cortical neuron-triggered stimulation at adjacent neurons (A. Jackson et al., 2006; Rebesco, Stevenson, Körding, Solla, & Miller, 2010) via spike-timing-dependent plasticity (Caporale & Dan, 2008; Hebb, 1949).

In addition, researchers demonstrated that cortical neurons could control functional electrical stimulation of paralyzed forearm muscles to restore wrist movements in non-human primates (Moritz, Perlmutter, & Fetz, 2008) and humans with motor-complete SCI (Ajiboye et al., 2017; Bouton et al., 2016). The closed-loop BCIs with brain stimulation have been also proposed to

modulate cognition and motor control by stimulating different functional areas in the brain, such as internal capsule, brainstem, and prefrontal cortex (Little et al., 2013; Widge et al., 2019).

The concept of bridging the lesion to stimulate the spinal cord below the injury maximizes the use of the intact nervous system to restore voluntary control of paretic limbs. Researchers used brain signals to trigger spinal stimulation below the lesion, Brain-Computer Spinal Interface (BCSI), to restore impaired upper limb function (Nishimura, Perlmutter, & Fetz, 2013). In the study, the BCSI system successfully restored the paralyzed wrist movement by using the local field potentials from the motor cortex to control intraspinal stimulation below the injury in non-human primates with cervical SCI.

Furthermore, brain-triggered spinal stimulation may also facilitate the neuroplasticity after SCI. Such plasticity was demonstrated by creating artificial spike-timing-dependent plasticity in corticospinal cells using a cortical neuron to control intraspinal stimulation in a non-human primate (Nishimura, Perlmutter, Eaton, & Fetz, 2013).

Closed-loop BCI to control spinal stimulation (BCSI) can also improve walking function (M. Capogrosso et al., 2016; Cho, Squair, Bloch, & Courtine, 2019). Stereotypical movements of locomotion mainly regulated by the automaticity of the spinal cord requires less complex control compared to hand and arm movement (Wenger et al., 2014). Here researchers used epidural stimulation instead of intraspinal stimulation to reduce invasiveness. In no-human primates with thoracic SCI, spikes information from the motor cortex could reanimate the walking movement by stimulating the lumbosacral spinal cord based on the gait cycle, flexion and extension movements, through epidural stimulation on the lumbosacral spinal cord (M. Capogrosso et al.,

2016). The BCSI system was conducted on the commercially available wireless platform with the benchtop computer for processing the data.

Researchers also compared the brain-controlled stimulation and continuous stimulation via epidural electrodes for walking in rats with thoracic SCI (Bonizzato et al., 2018). The brain-controlled epidural stimulation demonstrated the superior immediate and rehabilitative effect on the locomotor recovery compared to continuous epidural stimulation, which may indicate activity-dependent plasticity induced via the BCSI paradigm.

Several scientific and technical challenges remain to advance these engineering strategies toward widespread restoration of hand and arm function after SCI. In the BSCI studies above, the decoding process used spike information which requires frequent recalibration and complex computation requiring processing in an external computer under. Our goal is to create a self-contained, implantable system with stable neural decoding and wireless power and data transfer. To accomplish this goal, we need to advance both computational and hardware efforts, but also select the appropriate spinal stimulation modality.

Intraspinal stimulation is capable of reanimating graded and synergistic functional movement in hands and arms (Moritz, Lucas, Perlmutter, & Fetz, 2007; Mushahwar & Horch, 1998; Zimmermann & Jackson, 2014). However, intraspinal stimulation has proven difficult to move through clinical translation. Epidural stimulation is a less invasive tool compared to intraspinal stimulation. However, the spatial resolution of epidural stimulation with the current implants is not sufficient for the complex movement of the hand yet (B. Barra et al., 2018; Schiavone et al., 2020).

1.6 SPINAL STIMULATION

1.6.1 *Epidural Stimulation*

Electrical stimulation of the spinal cord for physical function in neurological conditions has been used for over 50 years. In 1965, the gate control theory for pain mechanisms was proposed (Melzack & Wall, 1965), leading to the first spinal stimulation for pain management (Shealy, Mortimer, & Reswick, 1967; Wall & Sweet, 1967). A patient with a terminal stage of cancer received dorsal column stimulation at thoracic spinal segments for chronic pain management. The stimulation achieved sustained pain relief for four years. This led to a device commercialized by Medtronic in 1968. Epidural stimulation began to be used for treatment for chronic pain, with technology and stimulation parameters improving in subsequent iterations (Burton, 1977; Law, 1983). The FDA approved epidural stimulation for pain management in 1989.

Parallel efforts using epidural stimulation for physical function began in the 1970s. In more than 70 people with multiple sclerosis, implanted dorsal column stimulation for pain management surprisingly improved muscle spasticity and voluntary movements (Cook, 1976). Others found that epidural stimulation and transcutaneous spinal stimulation modulated blood flow in lower extremities (Augustinsson, Carlsson, Holm, & Jivegård, 1985; Dooley & Kasprak, 1976). Researchers found that 28% of patients with multiple sclerosis improved motor function by using epidural stimulation , while 75% showed improved bladder symptoms (Illis, Sedgwick, & Tallis, 1980).

The first epidural stimulation study for six individuals with SCI was reported in 1978. The study focused on spasticity and bowel function (R. R. Richardson, Cerullo, McLone, Gutierrez, &

Lewis, 1979; R. R. Richardson & McLone, 1978). A following study reviewed a total of 796 cases with spinal stimulation and reported 15 cases with SCI, but only three cases with chronic implantation (Siegfried, Lazorthes, & Broggi, 1981). In the report, they concluded that, due to the inconsistent results, epidural stimulation was not an effective treatment for function beyond pain control for neurological conditions. Despite the negative conclusion for epidural stimulation for physical function except for pain, several research groups continued to carefully review the findings and investigate further details of spinal stimulation. The efforts eventually led toward an innovative strategy with spinal stimulation for motor function following SCI.

Several groups demonstrated the effectiveness of epidural stimulation for spasticity and rhythmic leg movements (Barolat, Myklebust, & Wenninger, 1986; Campos, Dimitrijevic, Faganel, & Sharkey, 1981). Dimitrijevic and colleagues modulated spinal reflexes via spared connections, using the association between electrode placement and lesion for more than 50 people with SCI (M. M. Dimitrijevic et al., 1986; Dimitrijevic, Illis, Nakajima, Sharkey, & Sherwood, 1986; Dimitrijević, 1988). These scientific efforts opened a potential for spinal stimulation for motor recovery in SCI with 303 cases over the subsequent 30 years (Pinter, Gerstenbrand, & Dimitrijevic, 2000). Unfortunately, SCI demonstrated the lowest rate of positive responses compared to other neurological conditions, and further works would be needed to discover the optimal stimulation parameters and locations for therapeutic effect (Waltz, 1997).

Epidural stimulation in people with SCI could evoke gait pattern movements (Dimitrijevic, Gerasimenko, & Pinter, 1998). This suggests an existence of a central pattern generator (Grillner & Rossignol, 1978; Iwahara, Atsuta, Garcia-Rill, & Skinner, 1992). This in part fueled interest in locomotor training, which became a mainstream of the rehabilitative tool for functional recovery following SCI in the 1990s and 2000s.

In the early 2000s, the research group at the Arizona State University first demonstrated locomotor recovery using epidural stimulation with locomotor training in a case with C5-6 AIS C (Carhart, He, Herman, D'Luzansky, & Willis, 2004; Herman, He, D'Luzansky, Willis, & Dilli, 2002). Later work using showed epidural stimulation could improve motor and autonomic recovery in a person with motor-complete SCI (S. Harkema et al., 2011). Now several research groups have demonstrated that epidural stimulation with locomotor training resulted in remarkable improvements of lower extremity function for people with chronic SCI (Angeli et al., 2018; Angeli, Edgerton, Gerasimenko, & Harkema, 2014; Gill et al., 2018). These studies used tonic epidural stimulation with intensive locomotor training to modulate the excitability of the spinal cord.

In parallel, a new epidural stimulation strategy was tested using closed-loop stimulation keyed to each of three phases of the gait cycle. The goal was to activate targeted muscle groups in at an appropriate time in the gait cycle and minimize the cancellation of sensory inputs (Wagner et al., 2018). This and all prior studies used intensive rehabilitation combined with epidural stimulation.

A final group has implemented epidural stimulation at home for daily use without intensive training to test if the protocol induces recovery of motor and autonomic function (Darrow et al., 2019). They have also observed sustained restoration of voluntary movement with stimulation off, even without traditional rehabilitation (Peña Pino et al., 2020).

In addition to locomotor function, there is substantial evidence of the sustained effect of epidural stimulation on upper extremity function through cervical epidural stimulation (Lu et al., 2016; Waltz, 1997). Additional benefits appear in the autonomic system, including cardiovascular

function (Darrow et al., 2019; Susan J Harkema et al., 2018; Bonnie E. Legg Ditterline et al., 2020), bladder function (S. Harkema et al., 2011; Herrity, Williams, Angeli, Harkema, & Hubscher, 2018; Steadman & Grill, 2020), bowel function (Walter, Lee, Kavanagh, Phillips, & Krassioukov, 2018) and sexual function (Darrow et al., 2019).

1.6.2 *Mechanisms of Epidural Stimulation*

Mechanisms of neuromodulation via epidural stimulation remain enigmatic. It may act on one or more of the three primary spinal cord functions: 1) activating reflex responses to various inputs underlying functional necessities 2) learning motor control through multimodal inputs, and 3) automating the modulation of compound movements (Shik 1976, Grillner 2003, Lovely 1986, Harkema 1997). In this section, we will review the current evidence of the effect of spinal stimulation on spinal and brain networks.

Following SCI, the physiological state of the spinal cord far below the injury is impaired even though anatomically the segment is intact (Frigon & Rossignol, 2008). However, some control of spinal reflexes can persist, and the residual capability depends on the severity of the injury reducing supraspinal input and afferent connections (Courtine 2009, Edgerton 2001, Musienko 2012).

Electrophysiological studies and computational modeling are beginning to inform mechanism of epidural stimulation-induced functional recovery. A computational modelling study suggested that epidural stimulation activates large to medium diameter sensory fibers within the posterior roots (Rattay, Minassian, & Dimitrijevic, 2000). It was later verified in an animal study that the primary response was monosynaptic reflex by using paired-pulse protocol (Y. P. Gerasimenko et al., 2006).

A more sophisticated computational modelling study incorporating data from physical and pharmacological agents pointed towards mono- to polysynaptic responses via proprioceptive and tactile sensory feedback pathways without direct activation of motor neurons by epidural stimulation (Capogrosso et al., 2013). Further, epidural stimulation-evoked responses were characterized into three mechanisms based on latencies from an experimental study (Lavrov et al., 2008) and a computational simulation (Capogrosso et al., 2013): early response from direct M wave, a middle response from monosynaptic response, and late response from polysynaptic activation.

Modulation of the spinal excitability via epidural stimulation also likely combines with sensory inputs to facilitate movement following SCI (Edgerton et al., 2008). In rodents with complete transection of the spinal cord, epidural stimulation with tail pinch and loading of the hindlimbs increased cumulative muscle activity during stepping (Lavrov et al., 2015). This indicated that sensory information via intact spinal afferents could amplify the effect of epidural stimulation. In addition to reinforcing natural sensory inputs, central pattern generator also modulates the gain of muscle activities with epidural stimulation based on the phase of walking and sensory input (Lavrov 2008, Gerasimenko, Roy 2008). A simulation study also showed that sensory information from muscle spindles during stance and swinging evokes phase-specific muscle activities by controlling spinal reflex circuitry by recruiting motor neurons with reciprocal and autogenic inhibitions (Moraud et al., 2016).

Activating the spinal cord via epidural stimulation to enhance sensory inputs may explain the immediate improvement of walking with epidural stimulation in people with SCI. Longer-term improvement is also observed, and the prior modelling studies also found that phase-specific frequency of epidural stimulation modulated activities of extensor muscles and flexor muscles

with subthreshold stimulation (Capogrosso et al., 2013; Moraud et al., 2016). In a preclinical study, the recovery of late reflex responses to epidural stimulation was correlated with motor recovery following SCI (P. Gad et al., 2013; P. Gad et al., 2015). The changes in the late responses may exhibit the recovery of the spinal circuits via polysynaptic responses.

The electrical field of epidural stimulation may also modulate chemical environments, including neurotransmitters and neuromodulators, by activating neurons and glia cells of the spinal cord (Taccola, Sayenko, Gad, Gerasimenko, & Edgerton, 2018). Epidural stimulation might induce hemodynamic responses in the spinal cord (Song et al., 2019). In addition, nociceptive input induced long term potentiation and depression (LTP and LDP) in dorsal horn neurons (Garraway & Hochman, 2001). Further research is required to reveal the immediate and sustained effect of epidural stimulation.

Engaging supraspinal control is crucial to restore volitional control of movements. The descending inputs, mainly from the brainstem, include monoaminergic modulation and glutamatergic excitation for activating spinal circuits (Arber & Costa, 2018). All 12 participants with clinically motor complete cervical SCI had some volitional electromyogram (EMG) activity in leg muscles (Moss, Kilgore, & Peckham, 2011). This supports the notion of the "discomplete" spinal cord (i.e., some spared pathways), even in diagnoses of motor-complete injury (Dimitrijević, 1988). In animal studies, these spared pathways in the lesion may be sufficient to restore supraspinal control for motor outputs (G. Courtine et al., 2009). Descending tracts are undoubtedly important for voluntary control below the injury, especially when 'enabled' by epidural stimulation (Edgerton & Roy, 2012; Roy, Harkema, & Edgerton, 2012).

Even if the corticospinal tracts is completely severed, spared propriospinal and reticulospinal tracts may contribute to the restoration of volitional control in combination with epidural stimulation (Asboth et al., 2018; G. Courtine et al., 2008). These residual pathways may also promote voluntarily engagement for training tasks. The spared pathways may strengthen connections in and around the lesion as performing tasks. In the results, the activity dependent plasticity may induce recovery of leg function with stimulation off after intensive training with epidural stimulation in people with motor-complete SCI (Peña Pino et al., 2020; Rejc, Angeli, Atkinson, & Harkema, 2017; Wagner et al., 2018).

Some studies suggest the importance of preserving proprioceptive input during epidural stimulation to support the proper modulation of the spinal cord via reciprocal inhibitory circuits and voluntary control of locomotion (Formento et al., 2018). These findings led to a trial of spatiotemporal stimulation strategy to maximize the benefit from the neuromodulatory effect for functional gains (Wagner et al., 2018). Spatial selectivity and frequency-dependent recruitment of epidural stimulation have been suggested as important, although most researchers find good results with tonic stimulation (Angeli et al., 2018; Darrow et al., 2019; Gill et al., 2018). There remains much to investigate regarding the optimal methods of epidural stimulation to restore movement.

1.6.3 *High Frequency in Spinal Stimulation*

There are several remaining challenges for the use of epidural stimulation, including stimulation-induced paresthesia and aversive sensation limiting the use of certain electrode combinations (Susan Harkema et al., 2011; Reddy et al., 2016). A kilohertz (kHz) carrier frequency stimulation is a newly used method for pain management without any aversive sensation for people with SCI

(Kapural et al., 2015). The rapid depolarization and repolarization created by kHz stimulation may raise the membrane potential of larger fibers enough to evoke action potentials without depolarizing unmyelinated C-fibers (Ward & Chuen, 2009). In a preclinical study, instead of recruiting Type II afferent fiber evoking paresthesia, 10 kHz carrier frequency epidural stimulation, but not 1 kHz or 5 kHz carrier frequency, activated inhibitory neurons such as GABAergic neurons (Lee et al., 2020). The activation of inhibitory interneurons may remedy sensory processing. Nevertheless, no studies have explored epidural stimulation with 10 kHz carrier frequency for motor recovery, which could expand electrode combinations and parameter selections by avoiding aversive responses or sensations.

1.6.4 *Transcutaneous Spinal Stimulation*

Building on the success of epidural stimulation to restore movement, non-invasive transcutaneous stimulation is also being explored. Applying electrical stimulation on skin surface, transcutaneous spinal stimulation similarly activates posterior-root muscle reflexes of the spinal cord in neurologically intact subjects (Kitano & Koceja, 2009; Marsden, Merton, & Morton, 1982; Minassian et al., 2007). Several studies found that, in people with SCI, transcutaneous spinal stimulation on the lumbosacral spinal cord evoked similar activation patterns of the spinal circuits via the posterior roots as epidural stimulation (Danner, Hofstoetter, Ladenbauer, Rattay, & Minassian, 2011; Hofstoetter, Freundl, Binder, & Minassian, 2018; Ladenbauer, Minassian, Hofstoetter, Dimitrijevic, & Rattay, 2010; Sayenko et al., 2015). Recent work demonstrated transsynaptic activation of motor neurons in the cervical spinal cord via transcutaneous spinal stimulation (Milosevic, Masugi, Sasaki, Sayenko, & Nakazawa, 2019). However, one recent study raised the potential of recruiting ventral roots to activate muscles at cervical levels depending on the positions of electrodes (Y. K. Wu et al., 2020). Therefore, some

debate continues about which neural structures are best activated via transcutaneous spinal stimulation.

Several research groups have studied the effects of transcutaneous spinal stimulation on motor outputs. There is an increase of motoneuron excitability with low frequency monophasic transcutaneous spinal stimulation (Murray & Knikou, 2019). Furthermore, transcutaneous spinal stimulation induced increased subcortical motor outputs in people with chronic motor complete and incomplete cervical SCI (Benavides et al., 2020). On the contrary, stimulation enhanced the control of cortical inhibition which is associated with motor learning and cortical control of limb coordination (Perez, Lundbye-Jensen, & Nielsen, 2007).

Transcutaneous spinal stimulation for functional recovery is currently being tested, but there are three main challenges. First, the spatial selectivity of transcutaneous spinal stimulation may be less than epidural stimulation. The ability to select segments through epidural stimulation provides a large number of configurations with anode and cathode for activating different motor pools. For example, different epidural electrode locations to activate flexor and extensor motor pools separately based on the timing of gait cycles (Wagner et al., 2018). This level of selectivity may not be possible with transcutaneous spinal stimulation based on the limited spatial selectivity of transcutaneous spinal stimulation in studies in neurologically intact people (Calvert, Manson, Grahn, & Sayenko, 2019; Krenn et al., 2013; Sayenko et al., 2015). On the contrary, the less spatial selectivity of transcutaneous spinal stimulation may bring a different advantage by permitting more global activation across many spinal segments to more broadly facilitate supraspinal input (Sayenko et al., 2019).

Second, skin surface electrodes require larger current to activate distant tissues like the spinal cord. Participants may experience aversive sensation in surrounding soft tissues with the larger current. It may also evoke unnecessary muscle contractions in abdominal and back muscles that have been observed with functional electrical stimulation (Duffell & Donaldson, 2020). These effects, however, are overcome by using high carrier frequencies of stimulation.

Kilohertz carrier frequency alternating current, aka Russian current, minimizes sensory discomfort while maximally activating targeted neural structures compared to other carrier frequencies (Ward & Robertson, 1998). However, decreased muscle force was evoked with the high carrier frequency compared to low carrier frequency stimulation (Ward & Robertson, 2001). A recent study with 5 kHz carrier frequency reported no advantage of the carrier frequency with respect to the recruitment curve of the muscle activations and sensory tolerance (Manson et al., 2020). We still do not know how higher carrier frequency spinal stimulation balances activations of surrounding tissues and targeted neural structures, but the reduction in sensory side-effects appears robust (Medina & Grill, 2014)

Another potential approach is temporal interference stimulation. Temporal interference can be achieved by producing low-frequency stimulation in an area overlapping multiple electrical fields with a slightly different frequency (i.e. 5 kHz and 5.03 kHz for 30 Hz stimulation). In an animal study, Grossman et al. presented stimulating deep tissue, hippocampus, in mouse brains without activating overlying brain tissues (N. Grossman et al., 2017). It may be possible to apply this method for spinal stimulation from skin surface electrodes. Interferential stimulation is used routinely in the clinic for activation of deeper tissue for pain and postsurgical care (Fuentes, Armijo Olivo, Magee, & Gross, 2010).

Lastly, there is currently limited clinical evidence of transcutaneous spinal stimulation for physical function following SCI compared to epidural stimulation, which has been studied over 50 years. A systematic review of transcutaneous spinal stimulation for motor function (Megía García, Serrano-Muñoz, Taylor, Avendaño-Coy, & Gómez-Soriano, 2020) reported that only thirteen studies with 55 individuals with SCI were qualified for the review. Although the feasibility of transcutaneous spinal stimulation has been investigated, proper treatment protocol and efficacy remain unclear. For clinical evidence for autonomic function, only two studied examined bladder function (P. N. Gad, Kreydin, Zhong, Latack, & Edgerton, 2018; Kreydin et al., 2020) and one study cardiovascular function (Phillips et al., 2018). Therefore, transcutaneous stimulation represents a new frontier for non-invasive neuromodulation after SCI.

1.6.5 *Transcutaneous Spinal Stimulation for Sensorimotor Function*

Transcutaneous spinal stimulation for motor function in people with incomplete cervical and thoracic SCI showed substantial effect on walking function and spasticity. The stimulation induced immediate improvements of modulation of the leg EMG activity during stepping on treadmill in people with incomplete SCI (Hofstoetter et al., 2013; Hofstoetter et al., 2015). Additionally, stimulation decreased leg spasticity. In the study, one person with C7 AIS D level of injury presented a sustained improvement of spasticity at least for 17 days after transcutaneous spinal stimulation at home (Hofstoetter et al., 2020; Hofstoetter et al., 2014).

Transcutaneous spinal stimulation induces functional improvements following SCI. 10 kHz carrier frequency for 30 Hz biphasic 1 ms pulse width transcutaneous spinal stimulation as a painless method to activate the spinal cord (Y. Gerasimenko et al., 2015). Multiple segmental

spinal stimulations (i.e. T11 and L1 vertebral levels) amplified motor outputs for stepping movement in the same study.

People with motor complete SCI performed gravity-eliminated stepping training in sidelying with 10 kHz carrier frequency transcutaneous spinal stimulation and voluntary effort (Y. P. Gerasimenko et al., 2015). For the last four weeks, Buspirone, a serotonergic agonist, was administered. The participants increased the voluntary control of stepping movements. Buspirone led to further improvements of motor outputs. This study exhibited that transcutaneous spinal stimulation was capable of activating the dormant pathway of the injured spinal cord in people with AIS B and facilitated the voluntary control of paralyzed legs with several weeks of transcutaneous spinal stimulation training.

Combining an exoskeleton with transcutaneous spinal stimulation improved voluntary movements with less robotic assistance as well as cardiovascular regulation in the participant. They also showed a potential of functional gains in sitting, standing and upper extremity function via transcutaneous spinal stimulation with physical training (Freyvert et al., 2018; P. N. Gad et al., 2018; Rath et al., 2018; Sayenko et al., 2019).

Recently, a person with C8 AIS D, 21 years post-injury performed standing and treadmill walking with 10 kHz carrier frequency lumbosacral transcutaneous spinal stimulation (M. Alam et al., 2020). They presented increased volitional control of leg movements, pinprick sensory score, and improved standing ability that were sustained at least six weeks. Nonetheless, they did not show any control of the training effect.

Cervical transcutaneous spinal stimulation paired with intensive hand and arm exercise could induce immediate and sustained improvements of upper extremity function in people with

cervical SCI (Inanici, Brighton, Samejima, Hofstetter, & Moritz, 2020; F. Inanici et al., 2018). In the study, four of seven subjects showed improvements of lower extremity function in addition to their upper extremity improvements.

Thus, we postulate that cervical transcutaneous spinal stimulation may contribute to locomotor recovery in people with cervical SCI. Correspondingly, there is emerging evidence for the importance of intersegmental connections between cervical and lumbosacral spinal cord through propriospinal interneurons for locomotor function following neurological injuries (Barss, Parhizi, & Mushahwar, 2020; Islam et al., 2020; Zhou et al., 2018). Cervical transcutaneous spinal stimulation could amplify the patterned stepping movements induced by lumbosacral transcutaneous spinal stimulation when they were simultaneously delivered (Y. Gerasimenko et al., 2015).

1.6.6 *Transcutaneous Spinal Stimulation for Autonomic Function*

Restoring bowel function is one of the priorities among people with SCI (Anderson, 2004; J. S. French, Anderson-Erisman, & Sutter, 2010; van Middendorp, Allison, & Cowan, 2014). 50% of the population with tetraplegia are affected by bowel dysfunction (Liu et al., 2009). The disruption of signal transmissions in the spinal control causes bowel dysfunction leading to a severe decline in quality of life (Glickman & Kamm, 1996). The decreased bowel motility due to the impaired parasympathetic and sympathetic control can induce a loss of voluntary evacuation, constipation, and increase of the time and assistance for the bowel management. Transanal irrigation, external electrical inputs, and surgical interventions complemented by medications are common interventions (A. Krassioukov, Eng, Claxton, Sakakibara, & Shum, 2010). Intensive locomotor training decreased bowel management time (Hubscher et al., 2018). However, the

efficacy of the current approaches for bowel function is still limited (Sanders, Ijzerman, Roach, & Gustafson, 2011). There is no report on the efficacy of transcutaneous spinal stimulation for bowel function.

Neurogenic bladder dysfunction also impacts quality of life for people with SCI (Anderson, 2004; J. S. French et al., 2010; van Middendorp et al., 2014). The disruption of the centralized micturition spinal reflex causes involuntary reflex of detrusor and detrusor-sphincter dyssynergia leading to damaged urinary tracts, loss of continence, or incontinence (de Groat, Griffiths, & Yoshimura, 2015; Taweel & Seyam, 2015). The dysfunction also leads to secondary urological complications (Wyndaele, 2016). Several therapeutic approaches to date are available such as anticholinergics, botulinum toxin injection, and electrical stimulation on peripheral nerves (Janssen, Martens, de Wall, van Breda, & Heesakkers, 2017; Wyndaele, 2016).

Modulation of spinal excitability via epidural stimulation could improve the bladder management time, detrusor-sphincter synergy and voiding function (S. Harkema et al., 2011; Herrity et al., 2018; Schieferdecker, Neudorfer, El Majdoub, & Maarouf, 2019; Walter et al., 2018). Similarly, peripheral nerve stimulation via pudendal nerve could inhibit spinal reflexes modulating external urethral sphincter activity and detrusor activity in a preclinical study (Tai et al., 2008).

Furthermore, static transcutaneous spinal stimulation for lower urinary tract dysfunction has been studied in several neurological conditions, including five subjects with SCI (Kreydin et al., 2020). In the study, transcutaneous spinal stimulation improved continence by restoring detrusor function and sensation. The stimulation resulted in the improvement of bladder capacity and voiding function.

Cardiovascular dysfunction resulting from cervical SCI permeates daily activities. SCI induced cardiovascular dysfunction can lead to orthostatic hypotension, impaired cognitive function (Phillips & Krassioukov, 2015), limited activity tolerance, and increased risks of secondary cerebrovascular complications (Cragg, Noonan, Krassioukov, & Borisoff, 2013). Pharmacological approaches and rehabilitation slightly remedy limitations of physical function. However, there is no effective intervention to improve autonomic function in cervical SCI.

Transcutaneous spinal stimulation on T7-8 vertebral levels, where cardiac sympathetic pre-ganglionic neurons controlling vasoconstriction are located, to study if the stimulation could intervene blood pressure change during tilt-table tests in people with motor-complete cervical SCI (Phillips et al., 2018). They observed that stimulation during the orthostatic stress steadily maintained blood pressure with elevated heart rates, which may rule out the mechanism of autonomic dysreflexia by stimulating afferent C fibers.

With lumbosacral epidural stimulation, immediate blood pressure increase without any training was observed in people with motor-complete SCI and cardiovascular dysregulation (Aslan et al., 2018). In addition, immediate and sustained improvements of cardiovascular function in orthostatic stress trials have been demonstrated (S. J. Harkema et al., 2018; B. E. Legg Ditterline et al., 2020). Further work is needed to uncover the mechanism and efficacy of spinal stimulation for cardiovascular regulation.

Impaired thermoregulation is also predominant in people with SCI. For instance, in cold weather, people with SCI commonly present an impaired temperature tolerance (Handrakis et al., 2017). Cervical SCI deteriorates cardiovascular function, energy expenditure, and sweating (Mneimneh, Moussalem, Ghaddar, Aboughali, & Omeis, 2019; Wecht et al., 2015). However, we do not have

any effective intervention for this condition (A. V. Krassioukov et al., 2007). We anecdotally observed an improvement in thermoregulation after the transcutaneous spinal stimulation phase in a participant with cervical SCI (Inanici 2020). Transcutaneous spinal stimulation with intensive exercise may be able to intervene deteriorated function of thermoregulation.

1.6.7 *Acute Application of Spinal Stimulation*

The findings of transcutaneous spinal stimulation in people with chronic SCI raises a question of whether transcutaneous spinal stimulation is effective in the acute phase of SCI. In this section, we will review relevant evidence for the acute application of spinal stimulation after SCI.

Electrical fields on neuronal migration and outgrowth in the spinal cord have been studied in past decades (Borgens, Roederer, & Cohen, 1981). Early *in-vitro* studies found that direct current epidural stimulation promoted axonal growth toward a cathode and neurite growth toward an anode (McCaig, 1987, 1990). Based on this concept, *in-vivo* studies in a pig model with SCI presenting significant axonal growth and greater functional recovery compared to the control group but not across the lesion (Borgens, Blight, & McGinnis, 1990; Borgens, Blight, Murphy, & Stewart, 1986). A biphasic alternating current of epidural stimulation induced a different response of axonal growth from static direct current stimulation. Alternating current stimulation permitted axonal growth in the opposite direction to the electrical field and activated growth-promoting signaling pathways in neurons (Walters, 2010).

Applying alternating current for axonal growth was performed in a Phase Ia clinical trial (Shapiro et al., 2005). Within 18 days after injury, people with cervical or thoracic SCI received implantations of stimulation devices on spinous processes above and below the injury and stimulation for 15 weeks. The clinical trial concluded that all measure function improved, and

the procedure was safe. However, due to the poor experimental design and limited effect size, the trial could not proceed into the next phase of the clinical testing (Tator, 2005). Recently, a preclinical study reported the possibility of alternating electrical current for facilitating neuroplasticity in the acute phase again (J. Li, 2019). In perspectives of neural regeneration by the electrical field, transcutaneous spinal stimulation may offer a non-invasive option with similar efficacy to the stimulation on spinous processes in the previous studies.

Second, stabilizing cardiovascular regulation via transcutaneous spinal stimulation may provide a favorable sympathetic control for functional recovery during the acute stage after injury. Acute spinal cord perfusion pressure measure by mean arterial pressure (MAP) and intraspinal pressure (ISP) via intrathecal catheters (spinal cord perfusion pressure = MAP – ISP) was strongly associated with neurological recovery after SCI (Squair et al., 2017). This postulates that controlling vascular regulation and intrathecal pressure in the acute phase can facilitate neurological recovery. In people with acute SCI, spinal cord perfusion pressure above 50 mmHg improve greater in neurological function one year after injury than below 50 mmHg. As observing improvements of cardiovascular control in people with chronic SCI, transcutaneous spinal stimulation and epidural stimulation may be able to regulate acutely impaired sympathetic circuitry (S. J. Harkema et al., 2018; Phillips et al., 2018).

Lastly, acute applications of transcutaneous spinal stimulation may preserve the spinal networks by modulating the spinal networks a few hours to days after the injury. In an animal study, acute applications of epidural stimulation modulated the EMG activity in legs in the very acute stage of SCI (Taccola, Gad, et al., 2020). Spinal motor evoked potentials in tibialis anterior and soleus that disappeared in the control group could be restored with the epidural stimulation in rats with severe SCI. Despite acute inflammation, epidural stimulation imitating natural sensory inputs

potentially regulated the spinal circuits. Meanwhile, acute sacral nerve electrical stimulation at the S3 level in rats with severe SCI showed significant acceleration of colonic function and elevated level of serotonin receptors compared to the control group (Zhu et al., 2020). The findings may indicate that acute sacral nerve stimulation drives the functional recovery of the spinal cord. However, evidence for the acute application is limited. All in all, further study is needed to test the mechanisms, safety, and feasibility in clinical settings.

1.7 THESIS OUTLINE

In this introduction, we reviewed the scientific background for this thesis work. The review summarized the current evidence of mechanisms for rewiring the spinal networks through activity-dependent plasticity and efficacy of spinal stimulation for functional recovery. We highlighted the gap between fragmented understanding from animal studies and observations from clinical studies. There are two key questions: what is the clinically viable strategy to induce activity-dependent plasticity following SCI? How can we answer the question?

Thereby, **this thesis aims to develop a clinically viable approach to treating SCI by leveraging advanced neuromodulation methods and technology. As examples, I demonstrated a brain-computer spinal interface in an animal model, and transcutaneous spinal stimulation for motor and autonomic recovery in people with cervical SCI.**

In **Chapter 2**, we first establish a cost- and time- efficient task to assess forelimb function in a clinically relevant animal SCI model. We provide evidence that the novel lever task could assess impaired proximal forelimb function in rats with severely paralyzed forelimb that other traditional behavior tasks were unable to detect.

In **Chapter 3**, by using the novel lever task, we present clinically viable brain-computer spinal interface to reanimate forelimb movements in rodents with severe cervical SCI. We demonstrate stable and computationally efficient local field potential decoding of the sensorimotor cortex for forelimb movements and graded extension movement of forelimb via cervical epidural stimulation. The closed-loop system was implemented in an implantable size chip with on-board computing proposing a clinically viable wireless BCSI system.

In **Chapter 4**, we present a study investigating the efficacy of transcutaneous spinal stimulation paired with intensive locomotor training in people with chronic incomplete cervical SCI. The findings suggest the additive effect of transcutaneous spinal stimulation for locomotor recovery leading to meaningful change in activities of daily living. In addition, we present the first evidence of transcutaneous spinal stimulation for restoring bowel function.

In **Chapter 5**, we summarize the main findings in the thesis work and discussed a feasibility of the projected strategies. We also address the current limitations of scientific understanding and technology to bolster future research to restore sensorimotor and autonomic function following cervical SCI.

Chapter 2. AUTOMATED LEVER PRESS TASK ASSESSING SEVERELY IMPAIRED FORELIMB FUNCTION IN RATS WITH CERVICAL SPINAL CORD INJURY

This chapter is submitted for publication

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2.1 ABSTRACT

Although there is currently no cure for paralysis due to spinal cord injury, the highest treatment priority is restoring hand and arm function for people with cervical spinal cord injuries. Preclinical animal models provide an opportunity to test innovative treatments, but severe cervical injury models require significant time and effort to accurately assess responses to novel interventions. Also, there is no behavior task that can assess proximal forelimb functions in rats with severe C4 spinal cord injury, the most common injury level in humans. To address this gap, we developed an automated lever pressing task for rats with severe cervical spinal cord injury.

We found that the automated adaptive training required only 13.3 ± 2.5 training days to achieve behavioral proficiency. Furthermore, the task could quantify immediate and long-term improvements in severely impaired forelimb function more effectively compared to existing behavior assessments. This study demonstrates that the automated lever pressing task is capable of tracking the functional changes with various therapies in rats with severe forelimb impairments in a cost and time efficient manner.

2.2 INTRODUCTION

Spinal cord injury (SCI) results in prolonged neurological impairments that limit activities of daily living. Currently, 0.5 million people each year experience a new SCI worldwide (Bickenbach, Officer, Shakespeare, von Groote, & Organization, 2013). Almost 60% of the SCI population have cervical injuries resulting in tetraplegia and limited hand function in the U.S. (Fridén & Gohritz, 2012; NSCISC, 2019). Hand and arm function restoration is the highest priority for improving quality of life (Anderson, 2004; D. D. French et al., 2007; Snoek, IJzerman, Hermens, Maxwell, & Biering-Sorensen, 2004). However, no cure for SCI currently exists, necessitating further research to avoid lifelong care. Here we present an automated lever press task that can measure forelimb function in rats with severe cervical SCI. Our data demonstrate a wide range of forelimb function captured by the task, as well as efficient adaptive training and flexibility for testing various therapeutic interventions.

Preclinical animal models play a significant role in investigating the effectiveness and mechanism of new treatments for SCI (B. H. Dobkin & Havton, 2004; Kwon et al., 2013; Metz et al., 2000; Reier, 2004; Sharif-Alhoseini et al., 2017; A. Torres-Espín, Beaudry, Fenrich, & Fouad, 2018). Researchers have worked on the development of intervention for recovery of

upper limb function for severe cervical SCI. However, in the preclinical rodent model, we confront barriers to test new interventions in rodent SCI models.

First, there is no behavior task sensitive to changes in severely impaired forelimb function. Various behavior tasks have been developed to evaluate forelimb function in rodents such as tasks involving reaching, grasping (McKenna, Prusky, & Whishaw, 2000; McKenna & Whishaw, 1999; Montoya, Campbell-Hope, Pemberton, & Dunnett, 1991), and food manipulation (Irvine et al., 2010; Irvine et al., 2014). Additional tasks evaluate gross movement functions including weight bearing (Schallert, Fleming, Leasure, Tillerson, & Bland, 2000), grooming (Bertelli & Mira, 1993), static pull (Hays et al., 2013) and rotation(Meyers et al., 2016). Meanwhile, these measurements have limited sensitivity to functional recovery in rodents with severe cervical SCI since severe cervical injuries disabling affected forelimbs to be lifted against gravity. Limited ability to lift forelimbs leads to difficulty positioning the distal forelimb to achieve the task rewards. To address this gap, we developed a task that involves minimal antigravity movement. This novel task allows a sensitive assessment of forelimb function following severe cervical SCI.

Second, many behavior tasks are subject to experimenter bias for quantifying forelimb function. Experimenter bias must be minimized to standardize behavior assessments. This can be achieved with automated quantification of function which provides precise and immediate measurements for research studies (Sindhurakar, Butensky, & Carmel, 2019). Automated behavioral chambers are designed to control the precise timing and measurements of each element of the task under controlled conditions. Automation can reduce human monitoring bias and errors (Crabbe, Wahlsten, & Dudek, 1999; Poddar, Kawai, & Ölveczky, 2013). There are several automated variations of spinal cord injury behavior tasks including a forelimb reaching task (Nica, Deprez,

Nuttin, & Aerts, 2018), forelimb supination task (Butensky et al., 2017; Meyers et al., 2016), static pull task(Hays et al., 2013), and the CatWalk (Gensel et al., 2006). Despite their utility, these assessments are still limited in their ability to assess severe forelimb impairments. Building on these tasks, we created an automated forelimb lever press task to digitize continuous performance. The real-time feedback from this system also permits integration with novel treatments such as activity-dependent stimulation and brain-computer interfaces.

Third, training and assessing forelimb function in existing behavior tasks are costly and consume significant time. All existing tasks require extensive time to train rodents for mastering the tasks. In addition, injured animals need to have extensive care and rehabilitation to assess functional recovery in the behavior tasks. Therefore, we implemented an automated adaptive training algorithm into the lever press task to accelerate task acquisition both pre- and post-SCI. The reproducible training method and objective measurements can save significant experimental time and labor with increasing throughput (Schaefer & Claridge-Chang, 2012). Furthermore, to reduce cost for equipment, we used open-source software and commercially available parts to construct the lever press arena.

All in all, we proposed an automated lever press task to measure severe forelimb impairments and recovery following cervical SCI. By using an adaptive algorithm, we could accelerate the task learning and forelimb rehabilitation. This sensitive behavioral assessment with improved training efficiency may allow researchers to investigate novel interventions in a cost and time efficient manner. Our hope is that this novel task will accelerate the development and testing of pre-clinical interventions for severe cervical injuries and lead to improved treatments for people with spinal cord injuries.

2.3 METHOD

2.3.1 *Subjects*

Thirty-five adult female Long-Evans rats (250-360 g) participated in this study. Animals were housed with a reversed light cycle, with the room dark from 10 AM to 10 PM to encourage behavior during their active period. Animals received liquid rewards for completing each task. Access to water was otherwise restricted except for 1 hour each weekday following task training and ad libitum water access each weekend (Rowland, 2007). Bodyweight was closely monitored to prevent dehydration. All procedures were approved by the University of Washington Institutional Animal Care and Use Committee (IACUC).

2.3.2 *Behavior Arena Design*

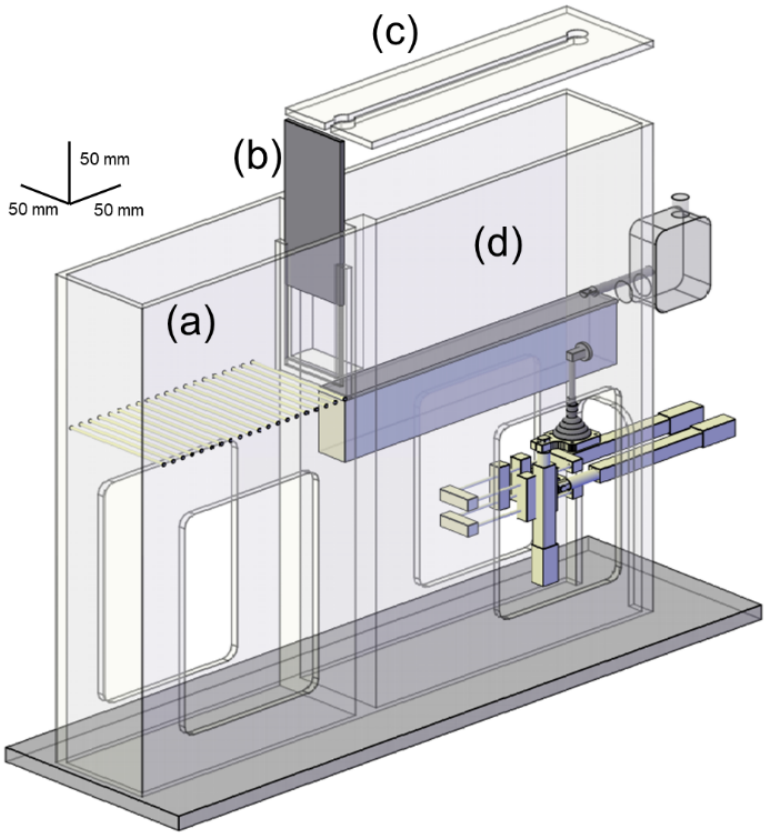
The goal of the task is to make animals produce their maximum lever press movement in order to assess forelimb impairment. The present arena was inspired by the Montoya staircase task (Montoya et al., 1991) with the aim to engage the natural foraging behavior of rodents. To further enable injured animals that cannot grasp pellets, we replaced the pellet wells of the 'staircase' with a moveable joystick lever (Figure 1). The side space allows for exploration of forelimb movements in a 2-dimensional 5 cm x 3 cm plane that extends 5 cm horizontally from the front panel of the arena and 3 cm vertically below the animal platform.

We placed an automatic joystick system consisting of a 3D-printed lever, joystick (APEM Inc., CA), and 3D-printed joystick mount attached to two linear actuators (Firgelli Technologies Inc., Canada) to position the lever at different locations beneath the platform. This arrangement allows severely injured animals to participate in the task without lifting their forelimbs against gravity

(McKenna et al., 2000; McKenna & Whishaw, 1999). Animals can instead move their forelimbs alongside their body.

We affixed a liquid reward system to the front wall of the arena which consisted of a nosepoke sensor, a 3D-printed sipper tube, and a small 12V peristaltic liquid pump (Adafruit Industries, NY). The nosepoke sensor was used to enforce body position and assure that the animals focused on the task. The rear chamber was created for animal acclimation. The track in the arena lid allowed the animals to retain free movement while being tethered with cables to head mounted connectors for related experiments.

A)



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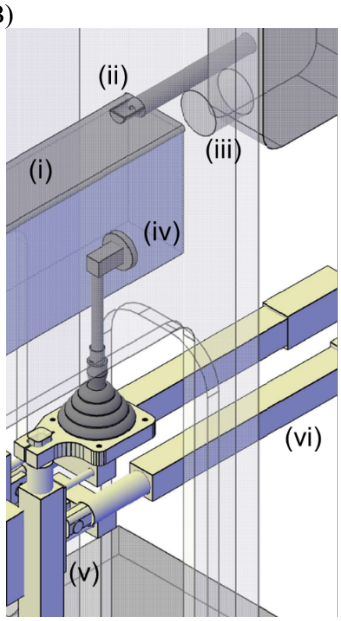

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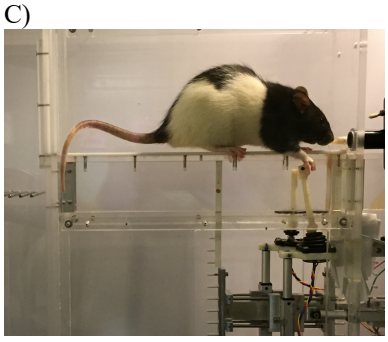


Figure 2-1. Lever arena
A) A schematic of the arena showing (a) the space for handling, (b) the wall separating the two chambers, (c) the lid with the slit for recording or stimulation cables, and (d) the training chamber during the task.
B) A close-up of the training chamber illustrating (i) the animal platform (ii) the sipper tube for liquid rewards, (iii) the nosepoke sensor, (iv) the lever mounted to the joystick, and the linear actuators for the vertical (v) and horizontal (vi) positions.
C) The lateral view of animal in the arena triggering the nosepoke sensor and pressing the lever. After the animal completes a successful press, the pump dispenses a drop of apple juice to the sipper tube. After the animal completes three successful presses, the linear actuators move the lever to the next position.

2.3.3 *Software and Data Analysis*

The lever arena was controlled by a Raspberry Pi microprocessor programmed with custom Python code. It independently performed each training step and logged behavioral data as well as training variables. Behavioral variables including nosepoke sensor output, lever displacement, lever threshold and position sampled at 60 Hz and logged in a comma separated value (CSV) file.

2.3.4 *Adaptive Thresholding*

We employed an adaptive lever press threshold to maintain difficulty and keep animals motivated. This was achieved with an algorithm which updated the lever thresholds based on the animal's maximal ability. The general concept is represented in Figure 2.

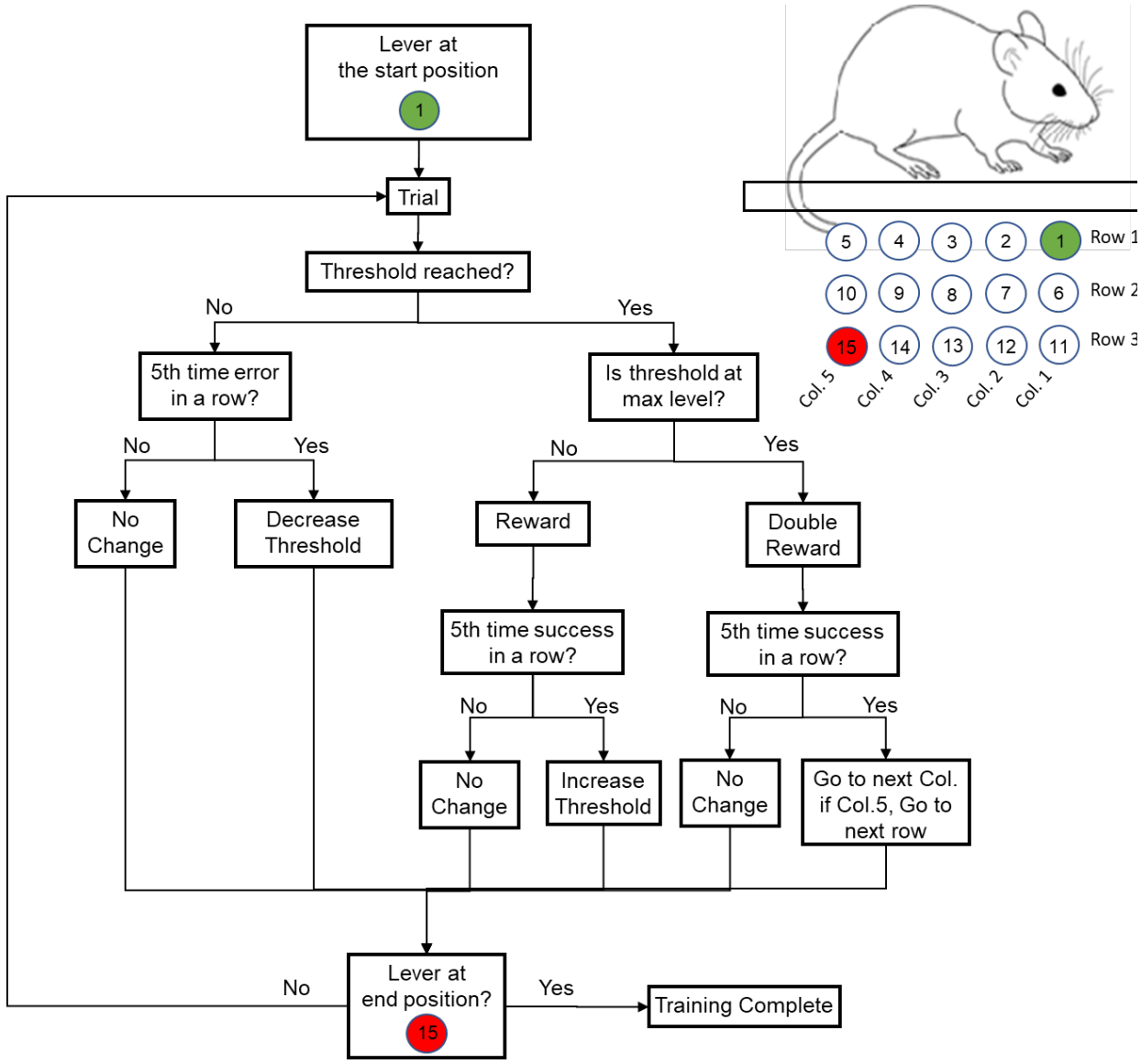


Figure 2-2. Flow chart of the finite state machine of adaptive training
Block diagram illustrates the progression of lever threshold and lever base position. The inset at top right shows the layout of the position relative to the first position.

The protocol starts with the lever base positioned close to the rat, where it is easy to reach the lever. The required lever displacement threshold is also very small, and this increases progressively as the animal gains skill. When the rat reaches the maximum threshold in the initial position, the lever base position is moved 1 cm backward to require further forelimb extension. The displacement threshold returns to its smallest value again in each new position. When the rat presses the lever 5 times beyond the threshold, the threshold increases by increments of 20% of

maximum threshold. If the rat fails to reach a certain threshold 5 times, the threshold decreases one level. When the rat presses the lever beyond the current threshold, the threshold distance is automatically incremented. When the maximum displacement threshold is reached in each position, a double reward is given to reinforce performance (Figure 2).

Once the animal completes all five horizontal positions in a row, the lever moves down vertically to the next row (1 cm lower) and returns to the forward position. Then, the same protocol will be repeated for each row. If needed, parameters of frequency of successes and failures as well as threshold increments can be optimized based on individual animal performance to achieve an adaptive, automated training sequence as detailed below.

2.3.5 *Training Protocol*

Animals are placed in the arena and the researcher selects the appropriate training step on the computer. The protocol consists of 8 different steps (Table 1). All the training steps are fully automated except for Step 2 (1-3 days) where the rat is manually assisted in learning to touch the lever. Each session lasts 5-20 minutes and are conducted under red light during the dark cycle to maximize the animal's activity level.

Pre-training Acclimatization Phase (4-5 days): When animals arrive at the facility from an outside vendor, we acclimatize them for at least 3 days. Then, we handle each animal for 5 minutes, first in their home cages and next in the arena over the course of 3 days (Pre-training Step 1). We introduce apple juice while handling animals in the arena for 2-3 days. After the handling phase, we begin water restriction by 4-hour increments per day until 24-hour restriction is achieved. Body weight is carefully monitored throughout (Pre-training Step 2).

Training Phase (10-15 days): Animals learn to associate the nosepoke with rewards by dispensing a reward when they activate the nosepoke sensor (Step 1). Next, the animals learn to touch the lever (Step 2) and press the lever backward from the starting position (Step 3). Subsequently, the animals learn to press the lever and nosepoke simultaneously (Step 4). Next, animals learn to press the lever at the maximum amplitude while doing nose spoke at all the different lever base position (Step 5 and 6). After Step 6, the animals are considered proficient.

Maintenance Phase: To maintain the lever press performance, rats perform maximum threshold presses at random starting positions. This is repeated 3 times per week for 5-10 minutes. (Step 7).

Post-injury Phase (Rehabilitative training): Post-surgery lever training starts 2-3 weeks after the implant or injury surgery. We apply one lever base position where the animals can easily place their paw in front of the lever since their lifting movement is limited. We execute the adaptive threshold algorithm with smaller increments (5-10% of maximum threshold) to maximize motivation and training effect (Step 8).

Table 2-1. Automated adaptive training protocol

Step #	Task	Criteria	Duration
Phase 1: Realization of nose spoke, lever press			
1 (fully automated)	Success for nose poke is notified with beep sound and reward.	Completing 40 trials in 10 minutes	1-2 sessions
2 (50% assist)	**Learning to touch and push the lever without nosepoke.** Each time start with 5 nosespokes to get rewards. First, the real lever is placed in low position where the animals cannot notice. Manually move a dummy lever to draw the animal's attention. Give rewards when the animal touches the dummy lever. Once rat learns to pushes the dummy lever using the ipsilateral paw, shift the animal's attention from the dummy lever to the real lever by raising the lever position to the normal position (lever threshold: 10-20% of maximum threshold).	30 successes of the real lever touch	1-3 sessions
3 (fully automated)	**Learning to push the lever on a wider range without nosepoke.** The lever is placed in the easiest position and set at the lowest threshold (lever threshold: 10-20% of maximum threshold). Lever threshold increases 10-20% of maximum threshold increment each 5 corrects push. If 5 incorrect pushes on	Reaching 80% of maximum threshold	1-3 sessions

	the lever before a successful trial, the lever threshold decrease the previous level.		
4 (fully automated)	**Learning lever push with nosepoke.** Begin with 10 nosepokes for rewards with the lever at an unreachable position. Then, the lever is moved to the same position in step 3 to start the lever push with nosepoke for rewards. Once the animal learns the association of lever push and nosepoke for rewards, the lever threshold increases 20% of maximum threshold increment each 5 correct pushes. If 5 incorrect pushes on the lever before a successful trial, the lever threshold decrease.	80% of maximum threshold	1-2 sessions
Phase 2: Obtaining the ability to push the maximum range at every lever position			
5 (fully automated)	**Learning maximum lever push with nosepoke in various positions**. In this step, the lever is set at the initial position (the most upward and front position) and will move to every position. The rat is expected to push the lever, beyond the maximum threshold, 3 times before moving to the next position. An adaptive threshold is setup to assist the rat in that task. 5 incorrect pushes lead to 20% reduced maximum threshold to maintain motivation.	Reach maximum threshold 3 times at the second last vertical position and the second row	2-4 sessions
6 (fully automated)	**Learning maximum lever push with nosepoke in all positions with minimum mistakes**. Starting from the initial position as step 5, After three maximum pushes, the lever moves to the next position.	Complete 3 maximum pushes in all 15 positions twice (maximum number of success: 90) in 15 minutes.	1-2 sessions
Phase 3: Maintenance period			
7 (fully automated)	**Maintaining the performance.** Once the animals are considered proficient, the training can be reduced to 5 min session, 2-3 times per week. At this step, the lever moves to a random position from 15 positions every 3 maximum pushes.	Maintain the training until you stop the water restriction before the surgery	until the surgery
Phase 4: Post-surgery period (1-2 weeks after the surgery)			
8 (optional: fully automated)	Retraining lever push with small threshold increment in one lever position. The lever is positioned where the injured animal can easily place in front of the lever. The lever threshold increases 5-10% of the maximum threshold (depending on the injury severity) every 2-3 pushes. If 2-5 incorrect pushes on the lever before a successful trial, the lever threshold decrease to the previous level.	until the performance reaches plateau	8-10 weeks

2.3.6 *Timeline of Experiments*

We performed three experiments. The first experiment tested whether the adaptive training protocol could accelerate the learning process. First, the protocol was tested in 24 neurologically intact animals, 9 animals with manual threshold adjustment, and 15 animals with automated and adaptive threshold adjustment. Then we applied the adaptive training algorithm into the rehabilitative training after severe SCI (Experiment 1).

The second experiment assessed the validity of the lever press task by comparing it to other established forelimb outcome measures in 6 animals (Figure 3A). This was accomplished by assessing recovery in rats with different severities of SCI who received intraspinal micro-stimulation. Experiment 2 was conducted in the early phase of this task development, so the adaptive training protocol was not applied. Instead, an experimenter set the threshold manually (Experiment 2).

As the last experiment, we tested the reanimation of impaired forelimb with brain controlled epidural stimulation in 5 animals (Experiment 3) (Figure 3B).

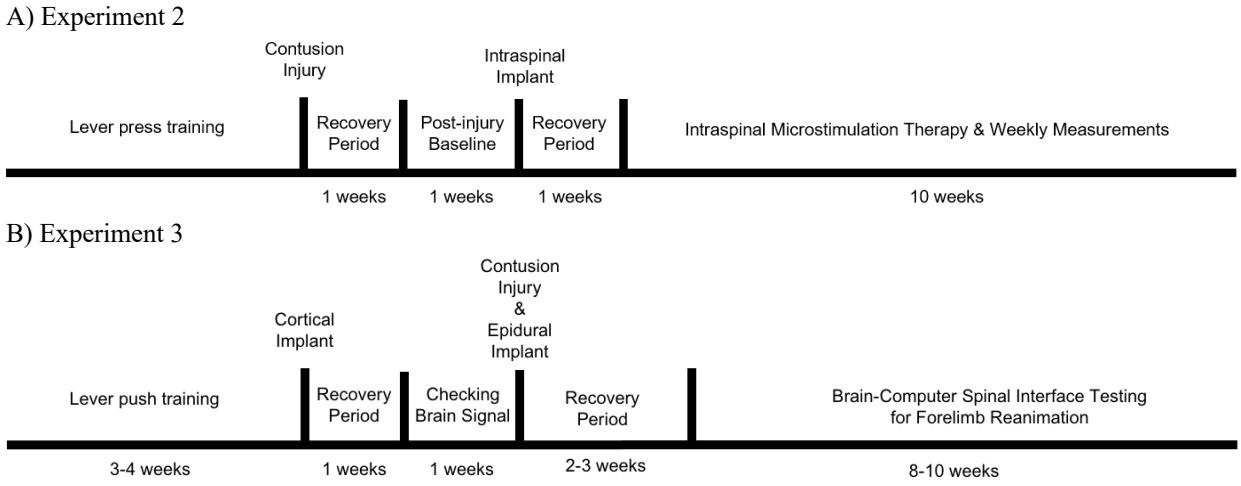


Figure 2-3. Timeline of experiment
A) Experiment 2 timeline. Training was performed 3-5 days/week throughout the experiment. After intraspinal implantation, alternate forelimb function tasks were performed once per week, specifically the Limb Use Asymmetry Task and the Irvine, Beatties, and Bresnahan test.
B) Experiment 3 timeline. The adaptive training algorithm was applied in the pre-injury period. The animals learned the lever task within 3-4 weeks. After contusion injury and epidural implants, the animals could perform brain-controlled forelimb reanimation 4-5 weeks after the recovery period.

2.3.7 *Lever Task Outcomes*

Several outcome measures were computed from the lever task, namely success rate and mean maximum range. Success rate was calculated by determining the percentage of trials that reached the predetermined maximum threshold in a session. Mean maximum range of motion was computed as the mean of the peak lever press ranges. We also recorded the continuous lever trajectory, the progression of lever thresholds, and the lever base positions.

2.3.8 *Forelimb Functional Measures*

For the Experiment 2, we used the Limb Use Asymmetry Task (LUAT) (Schallert et al., 2000) and the Irvine, Beatties, and Bresnahan (IBB) test (Irvine et al., 2010; Irvine et al., 2014). The LUAT and IBB tasks did not require training, so we assessed the animals on these tasks only once a week without any training. A LUAT score of 0.5 reflects equal use of both limbs, while scores less than 0.5 reflect decreased use of the paralyzed limb compared to the unaffected limb. Weekly scores reflect the mean of the individual scores for that week. We calculated IBB scores for two cereal types: one spherical shape and one doughnut shape. Uninjured animals generally achieved the maximum score of 9.0 on both cereal types. Weekly scores reflect the mean of the individual scores for each week, where each individual score is the mean of three trials on that cereal shape.

2.3.9 *Contusion Spinal Cord Injury*

In Experiment 2 and 3, all animals received a lateralized cervical spinal cord contusion injury. For Experiment 2, we deeply anesthetized animals with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (12 mg/kg). Each animal received a lateralized contusion injury (0.7 mm displacement, 14 ms dwell time) at spinal segments C4-C5 using a modified Ohio State

injury device (Mondello, Sunshine, Fischedick, Moritz, & Horner, 2015; Stokes & Reier, 1992). For Experiment 3, we anesthetized animals with 2-3 % isoflurane in oxygen. We conducted a lateralized C4 contusion injury using a force controlled closed-loop system with 200 kilodyne peak force (Infinity Horizon Impactor, Precision Systems and Instrumentation, LLC., Fairfax Station, VA). In both studies, animals received buprenorphine (0.05 mg/kg) for analgesia on the day of injury and twice daily for two days following spinal cord injury.

2.3.10 *Intracortical Implant*

Intracortical implantation was performed in Experiment 3. All surgeries were performed under general anesthesia using isoflurane. After a craniotomy and removal of the dura matter, a sterilized 16 electrode array of 40 μm diameter tungsten microelectrodes were inserted 1.5 mm into rostral and caudal forelimb area of sensorimotor cortex.

2.3.11 *Spinal Implant*

In Experiment 2, we implanted intraspinal 8-10 channel platinum-iridium microwire stimulating arrays as described previously with slight modifications (Kasten, Sunshine, Secrist, Horner, & Moritz, 2013). We anesthetized the animals using 2-3% isoflurane in oxygen and performed hemilaminectomies at spinal segments C6-C7. We opened the dura and inserted the wires 1.5-1.7 mm ventrally into the spinal cord. We anchored the implant to the dura with two sutures.

In Experiment 3, epidural implants were performed following the contusion injury on the same day. The sterilized epidural implant was placed on the lateralized C6 segment and secured by a dural suture. A common ground wire was inserted subcutaneously near the right shoulder. The connector was placed on the headcap shared with the cortical implant. All animals received buprenorphine for postsurgical analgesia.

2.3.12 *Intraspinal Microstimulation Protocol*

In the Experiment 2, task-dependent spinal stimulation was achieved with intraspinal microstimulation. After one week of recovery, animals performed the lever task with nosepoke-triggered intraspinal stimulation 3-4 days per week. Nosepoke triggered a biphasic 300µs 10-pulse train of 50 Hz stimulation at the pre-determined amplitude producing approximately 1 cm elbow extension movement. We confirmed stimulation settings prior to each session. The animal's unstimulated lever press ability was assessed on the remaining 1-2 sessions each week.

2.3.13 *Brain Controlled Epidural Stimulation Protocol*

We delivered amplitude modulated spinal stimulation train based on local field potentials of the forelimb sensorimotor cortex to restore forelimb movement after severe SCI in experiment 3. A Tucker Davis Technology (TDT) system (Alachua, FL) was used to synchronize the lever arena with brain data and stimulation signals. The forelimb movement intention was detected from multichannel-local field potential (200-400 Hz single band) power of forelimb sensorimotor cortex via a canonical correlation analysis decoder as previously described (Samejima 2020). The decoded signals controlled epidural stimulation to evoke the forelimb extension movement required to press the lever with the paralyzed forelimb.

2.3.14 *Statistics*

Statistics were computed using custom R scripts (R version 3.3.1). For Experiment 1, training days were compared between the control group and the adaptive training group using the non-parametric Wilcoxon rank sum test. To evaluate the influence of lever position, LUAT, and IBB scores on lever press performance, we defined the performance of each animal at each individual

lever position as the response variable. Spearman's rank-order correlation analysis was used to assess the relationship between the lever success rate, IBB and post-injury date.

2.4 RESULTS

2.4.1 *Adaptive Training*

The adaptive threshold algorithm facilitated quick and sustained learning in rats in Experiment 1. Figures 4A & B illustrate examples of adaptive threshold training for two animals. Figure 4A depicts the performance of a rat during a training session in Step 3 of the training phase. The session started at the second row and first horizontal position. The rat needed to reach the maximum lever threshold three times to move to the next step. If the rat failed to reach the threshold five times, the threshold decreased. The rat quickly learned the maximum threshold lever press in one session.

Figure 4B represents an example of how the adaptive threshold strategy could maximize the lever press performance in the later training phases. The training session started at the second vertical position in Step 5. The first three vertical position were easy for the rat as the animal could reach maximum threshold quickly. Some errors were observed in the fourth horizontal position. Eventually, the animal spent the longest time in the fifth position with several failed attempts. The animal was motivated but was unable to reach the maximum threshold in the last position.

To assess the benefits of the adaptive threshold algorithm, nine uninjured animals were trained using a manual threshold (control group), and 15 animals were trained using an Adaptive Threshold (AT group). Figure 4C compares the number of days required to reach proficiency for the two groups. Training was considered complete when the animal was able to press three times

beyond the maximum threshold at each position with two rounds of all positions. The adaptive threshold protocol required 13.3 ± 2.5 days to reach proficiency, significantly fewer than 19.8 ± 1.6 days for the control group ($p < 0.001$).

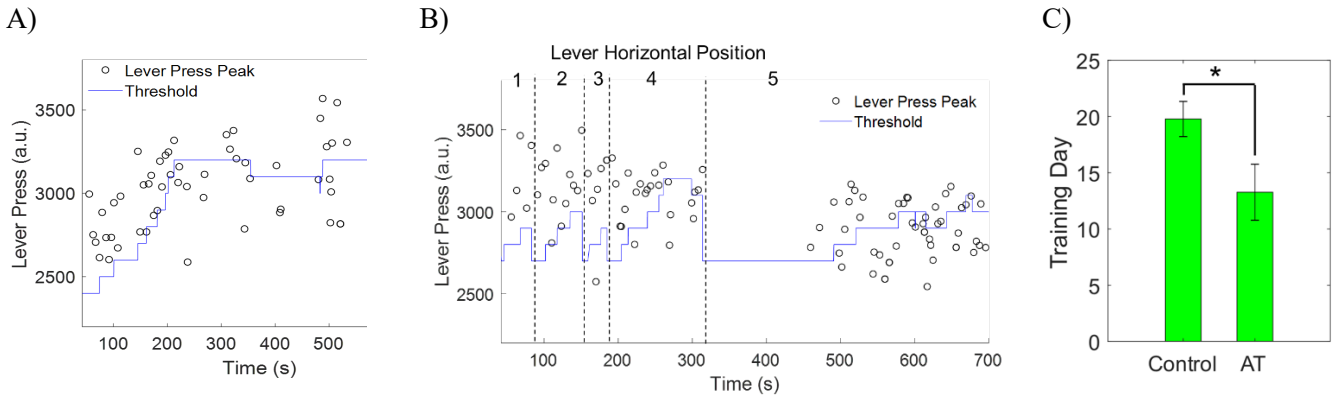


Figure 2-4. Adaptive training
A) An example session during training step 3. The blue line shows the threshold value and the empty circle shows peak lever press range for each trail. During step 3 the goal is to teach the rat to press the lever to the maximum distance. The lever position is stationary, and the nosepoke sensor is off. The animal increased the lever press range as the threshold progressed and then performed consistently above the maximum threshold of 3200 arbitrary units (a.u.). **B)** An example session in Step 5. The goal of step 5 is to train the rat to press the lever beyond the maximum threshold with nosepoke at all five lever horizontal positions. Position 1 is the most rostral, and position 5 the most caudal. The animal easily produced maximum displacement at horizontal positions 1-3 which allowed the animal to skip some threshold levels. The animal progressed through all levels in position 4. The animal struggled but gradually improved pressing the lever beyond the threshold at horizontal position 5. **C)** Training days. The adaptive threshold group (AT; N = 15) required significantly fewer days to task proficiency compared to the manual threshold training (Control; N = 9), mean ± SD, * $p < 0.001$.

We employed adaptive threshold training with smaller increment to rehabilitate forelimb functions in a different cohort of rats after severe SCI (Figure 5). The strategy created an environment that engaged rats with severe forelimb impairments to perform the task with prolonged motivation(Miltenberger, 2011). Figure 5 demonstrates the effectiveness of the adaptive threshold paradigm to train the animal's maximum peak lever press range in one session even after injury.

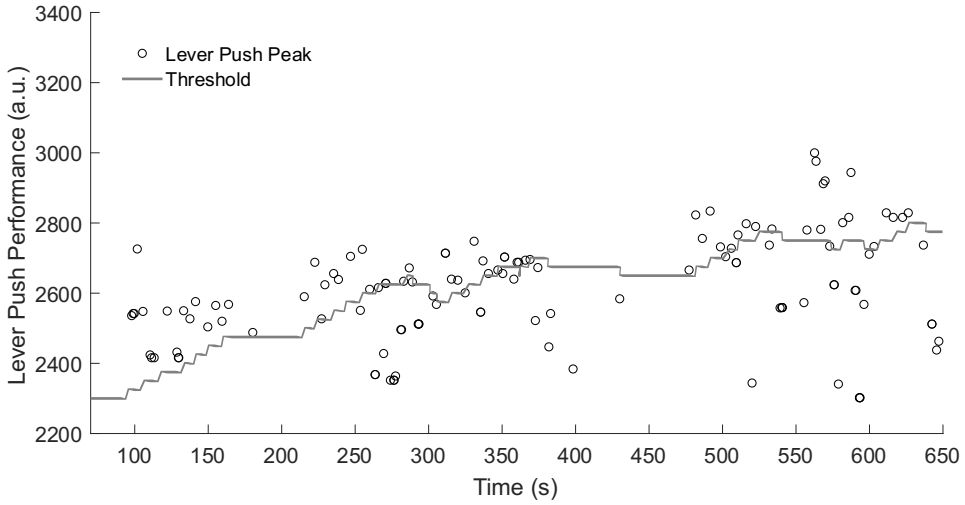


Figure 2-5. Rehabilitative training using adaptive thresholding after injury
Example session of the rehabilitative training after the contusion injury. The training was performed without any stimulation. The lever base was placed in position 3 where the animal could reach. The threshold increments were set much smaller compared to the pre-injury training. This enabled the animal to gradually progress during each session (one session shown).

2.4.2 *Comparing to Standard Outcomes*

We assessed the lever press success rates immediately after the contusion injury and up to 11 weeks post-injury. Figure 6A illustrates the possible lever positions. Figure 6B presents the lever success ratios before injury at each of the 15 positions. Figures 6C and 6D show the animals' mean lever press performance at each lever position at the second week after the injury and at the conclusion of the study (Week 11). We calculated an individual animals' weekly success ratios by using the mean of the animals' weekly success ratios as the group mean without any stimulation at the time. Darker colors indicate greater success ratios at a given position. The animals showed greater lever press success ratios at proximal lever positions compared to distal lever positions. The animals demonstrated good recovery in the first and second rows but still limited performance in the third row (Figure 6D).

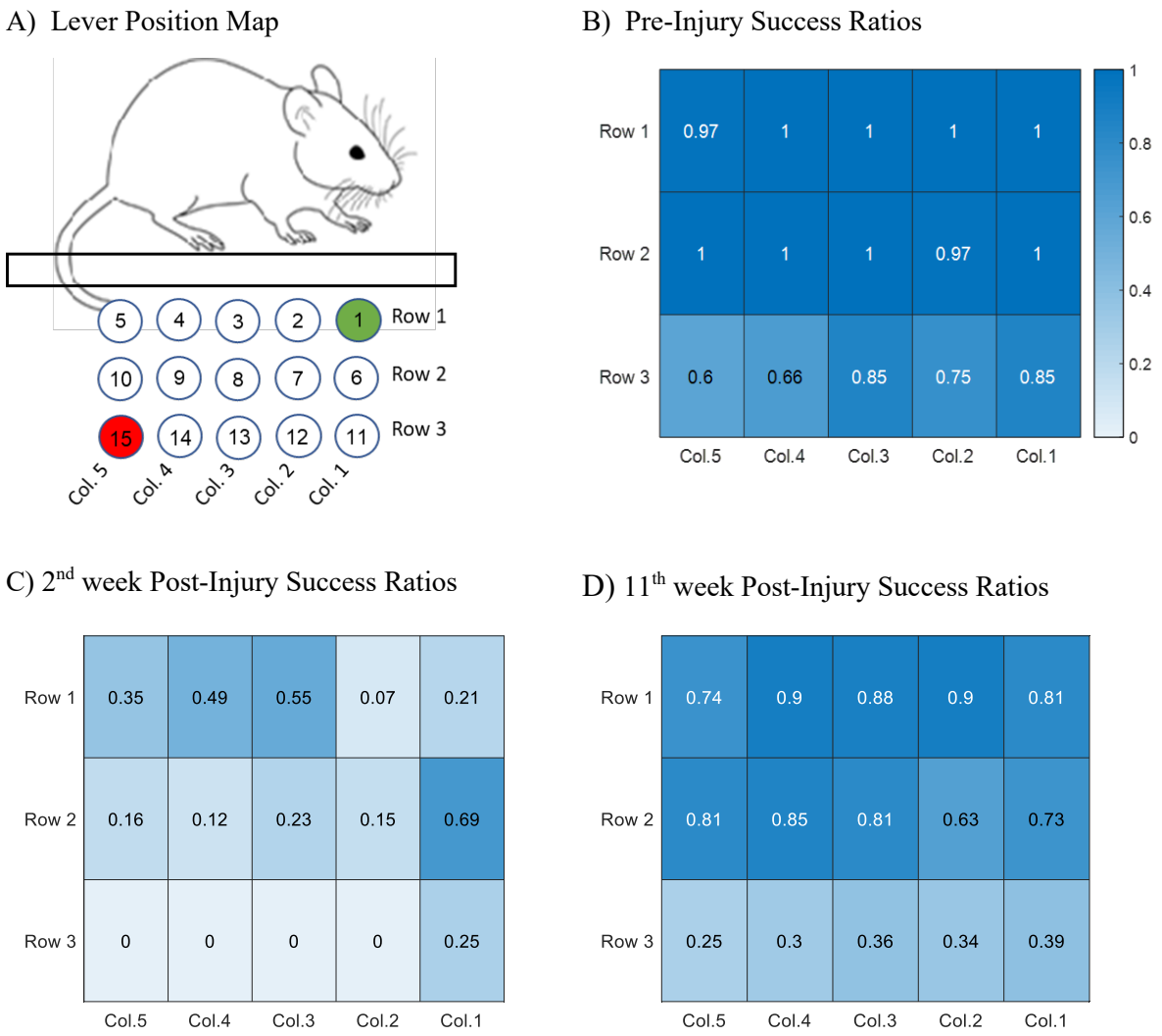


Figure 2-6. Lever performance variability
A) 15 lever positions are shown. The proximal and distal positions were determined relative to the platform. The rostral and caudal positions were determined relative to the front wall. **B)** Lever press success ratio patterns in the 15 lever positions (N = 6 animals). The heat maps show mean scores at each lever position before injury. The 15 individual squares correspond to the 15 lever positions, shown in their approximate location relative to the rat. Darker shades indicate greater lever success ratios, while lighter shades indicate fewer lever successes. **C)** Lever performance pattern at the second week after the contusion injury. The performance at all positions was declined compared to the pre-injury performance. **D)** Lever performance pattern at the 11th week post injury. The performance showed the improvement at the Row 1 and 2 positions but limited at the Row 3 positions.

Animals exhibited very similar trend of the performance on the lever task at the distal lever position to the LUAT and IBB scores (Figure 7A-C). Nevertheless, the lever press performance at the proximal lever positions showed the improvement over time (Figure 7D). This

demonstrates the ability of the lever task to detect forelimb functional changes in animals that were not detected by other functional assessments.

We further quantified the relationship between the measures using a correlation analysis which revealed a significant linear relationship between lever success performance at the proximal positions and training dates in the animals (Figure 7D). The correlation analysis between the lever press performance at the proximal lever positions over time demonstrated a moderate association, R value 0.45. On the other hand, the lever press performance at the proximal lever positions and IBB had a weak association, R value 0.1. Therefore, the lever success ratios could reflect the proximal forelimb function that traditional measurements could not detect.

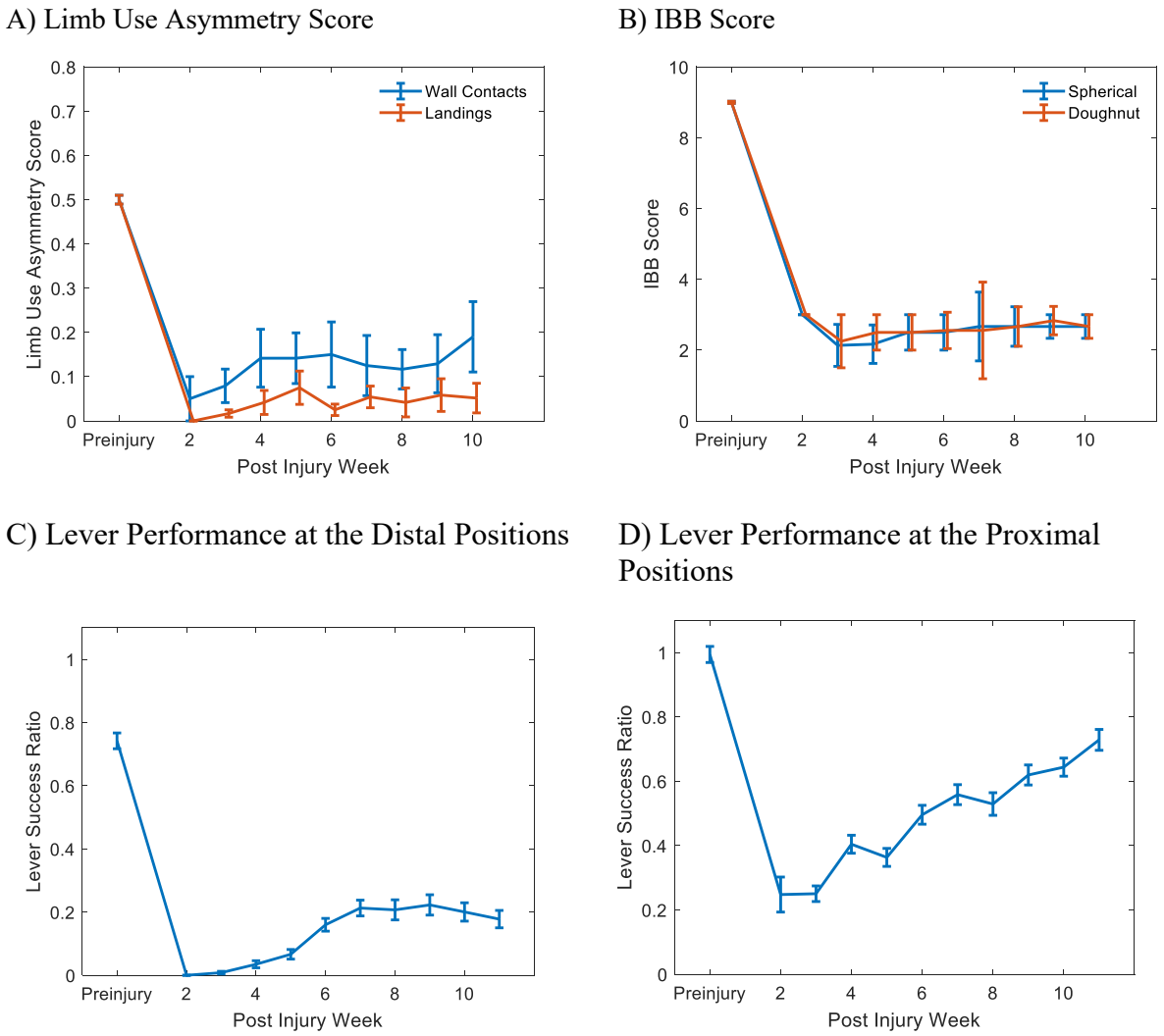


Figure 2-7. Weekly traditional functional scores and lever press success ratios
A) A) Scores on the Limb Use Asymmetry Task (LUAT). for the group each week. A score of 0.5 indicates equal limb use. Blue lines indicate wall contact scores; green lines indicate landing scores. **B)** Mean IBB scores for the group each week for the spherical and doughnut shaped cereal. 9.0 is the maximum possible score. **C)** Weekly performance on the lever task at the distal positions (Row 3: Position 11-15), and **D)** performance on the lever task at the proximal positions (Row 1 & 2: Position 1-10) illustrates recovery not captured by the LUAT and IBB measures.

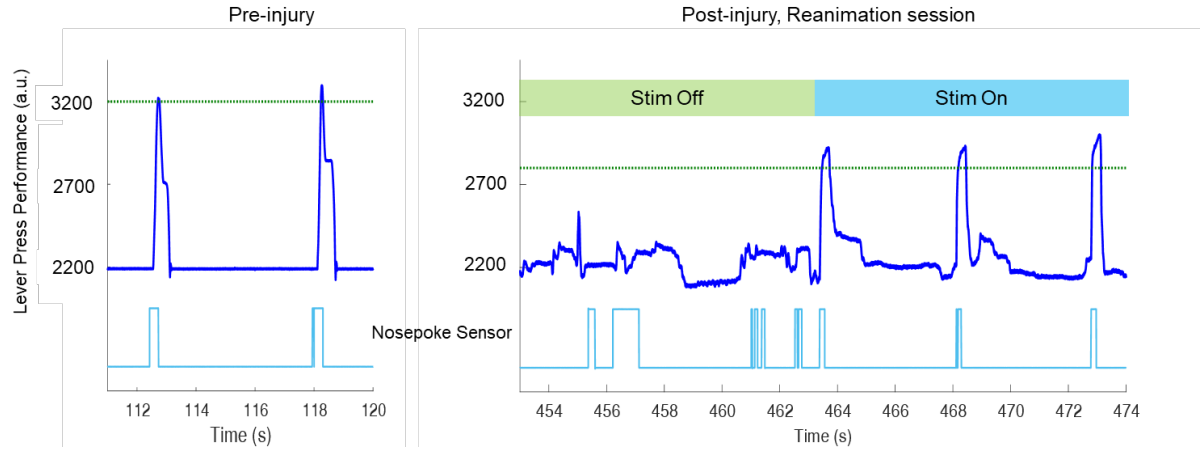
2.4.3 *Detecting the Immediate Effect of Reanimation*

Finally, we tested the immediate reanimation performance on the lever press task using rats with severe SCI via a brain-computer spinal interface (BCSI), as well as any long-term therapeutic

effect of using the BCSI system. Brain-controlled epidural stimulation evoked significant forelimb extension movements. The peak lever press range in the stimulation-on trials was significantly higher than the stimulation-off trials. Moreover, in this example, the animal at 62 days post-injury demonstrated that the reanimated lever trajectory was comparable to the pre-injury presses (Figure 9A).

Five animals participated in 16.6 ± 2.7 sessions of the epidural stimulation via the BCSI over 89.4 ± 11.9 days after injury. Following all sessions, the rehabilitative effect on the forelimb function was measured as the peak lever displacement without stimulation active. We determined the pre-injury lever peak range as 100% forelimb function. Compared to the function right after the contusion injury, the lever task detected significant long-term functional improvement ($p = 0.03$).

A)



B)

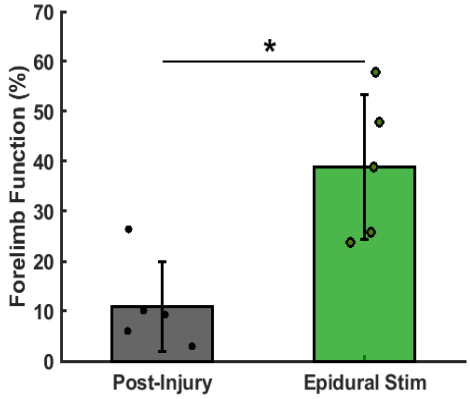


Figure 2-8. Reanimation performance and rehabilitative effect of brain controlled epidural stimulation
A) The effect of reanimation by the brain computer spinal interface. The left figure shows examples of the lever trajectory pre-injury. The green line represents the reward threshold. The right figure demonstrates one example of a reanimation session in an injured animal at 62 days post-injury condition. Without stimulation, the animal was unable to reach the reward threshold. Using brain controlled epidural stimulation ('Stim On') the animal could reach the threshold. **B)** Long-term functional improvement for animals involved in the Experiment 3 (N = 5). Maximum lever displacements without stimulation were compared immediately after the contusion injury and after received 16.6 ± 2.7 sessions of epidural stimulation via the BSCI system. The lever task could detect the therapeutic benefit of using the BSCI, even after stimulation was turned off. Mean ± SD, *p = 0.03.

2.5 DISCUSSION

We describe an adaptive lever press task enabling quantification of proximal forelimb movement in rats with severe cervical spinal cord injury. We implemented an automated training algorithm which accelerated training on this new task. For preclinical tests, this method may offer the ability to evaluate immediate and long-term effects of various interventions for forelimb function following severe cervical SCI.

The lever task enables us to measure functional improvement even in rats with severely impaired forelimb function. Given the high prevalence of severe tetraplegia (McKinley, Santos, Meade, & Brooke, 2007), the development of therapies and measures for severe upper limb impairments is essential (Cenci, Whishaw, & Schallert, 2002). To our knowledge, no assessment aims to provide a specific quantified measure of proximal forelimb function in rodents (Fouad, Ng, &

Basso, 2020). One method of validating a novel task is through comparison with established behavior tasks. The comparison allows for an assessment of efficiency and sensitivity (Sloan et al., 2015).

The lever press success ratios at the proximal lever positions had moderate associate with time since injury but not IBB. Considering almost all animals had limited dexterity but the lever task could capture the changes over time, the task may be more sensitive to the proximal forelimb function than the traditional tasks. Furthermore, according to the Experiment 3, a different property of the lever task, the peak range of lever press, could reflect the immediate functional gains and long-term therapeutic effect of the brain controlled epidural stimulation in the animals with severe cervical SCI.

Automation of behavioral training and performance evaluation can accelerate preclinical research (Poddar et al., 2013). Furthermore, a previous study revealed the efficacy of adaptive threshold training to accelerate task acquisitions in pre- and post-injury (Meyers et al., 2016). Our algorithm combined automation with an adaptive threshold algorithm using open source software. The automated training significantly shortened the training duration compared to the experimenter-controlled threshold, comparable to other recent forelimb automated tasks (Bjånes & Moritz, 2018; Meyers et al., 2016).

Furthermore, the adaptive training demonstrated its effectiveness with rehabilitative training in animals with severe cervical SCI. The adaptive threshold was successful in motivating and encouraging improved performance for animals after injury. The automation strategy of the ‘just right’ challenge follows clinical practices by rehabilitation therapists (Woodbury et al., 2016)

and may provide a good model for preclinical testing (Abel Torres-Espín, Forero, Schmidt, Fouad, & Fenrich, 2018).

The automated algorithm using the open source software can provide flexibility of the behavior task. The software and structure of this lever arena offers the ability to design optimal training protocols for each animal. For example, the software provides flexible adjustment of lever threshold, lever base position, amount and timing of rewards. These programable parameters can be utilized to maximize performance for animals with different injury levels and abilities. The real-time recording of performance can additionally be synchronized with brain signals to decode behavior (Aoki, Tsubota, Goya, & Benucci, 2017) and deliver stimulation.

It is important to recognize several limitations of this lever arena. First, although the starting lever position explores a 2D workspace, the lever movement at each position is one degree-of-freedom. Future iterations may modify the lever sensor to capture force or out-of-plane movements. Second, the lever task is conducted in an isolated arena. Home cage training is preferred to assess natural behaviors(Fenrich et al., 2016; Poddar et al., 2013), however this comes at the cost of decreased flexibility. This is the first generation of the lever arena, and future iterations may provide options to assess forelimb functions in rodents in their home cages and with a range of neurological conditions and deficits.

2.6 CONCLUSION

We demonstrated an automated task to train and quantify proximal forelimb function. This novel lever press task detected impairments and recovery in rats with severe of spinal cord injuries. The task could additionally track immediate and long-term recovery with spinal stimulation

therapies. The flexible design allows for new therapies to be tested in a time and cost-effective manner.

2.7 Conflicts of interest

The authors have no conflicts of interest.

2.8 Funding

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2.9 Acknowledgments

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Chapter 3. CLOSED-LOOP BRAIN-COMPUTER SPINAL INTERFACE RESTORES UPPER LIMB FUNCTION AFTER SPINAL CORD INJURY

This chapter is submitted for publication.

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3.1 ABSTRACT

Brain-computer interfaces (BCIs) for treatment of spinal cord injury (SCI) are being used to reanimate paralyzed limbs. Most approaches stimulate the peripheral nerves or muscles which has limitations. To overcome these challenges, here we show that a BCI can control spinal stimulation and improve forelimb function in rats with cervical SCI. We decoded forelimb movement intention via multichannel local field potentials in sensorimotor cortex using a canonical correlation analysis, which is both less computationally complex and more stable over time than spike-based algorithms. We then used this decoded signal to modulate epidural stimulation and restore volitional forelimb movement. Finally, we implemented the efficient closed-loop algorithm in a miniaturized on-board computing platform. This Brain-Computer Spinal Interface (BCSI) approach utilized recording and stimulation hardware that is already being used in separate clinical trials, so it may readily translate to human subjects as a potential solution to upper extremity paralysis.

3.2 INTRODUCTION

Spinal cord injury (SCI) results in lifelong functional impairments. The majority of people with SCI have tetraplegia affecting upper extremity (Dewan et al., 2018; NSCISC, 2019). The highest priority for improving the personal quality of life in people with tetraplegia is restoring hand and arm function (Anderson, 2004; J. S. French et al., 2010). Although there are several on-going clinical intervention studies for SCI, there is currently no effective treatment for paralyzed hands and arms following SCI.

To restore function to paralyzed upper limbs, researchers have begun creating artificial connections between the brain and paralyzed muscles or the spinal cord below the injury (Ajiboye et al., 2017; Bouton et al., 2016; C. Ethier, E. R. Oby, M. J. Bauman, & L. E. Miller, 2012; Moritz et al., 2008; Nishimura, Perlmutter, & Fetz, 2013; Zimmermann & Jackson, 2014). Stimulation of peripheral nerves and muscles can produce muscle contractions, but often generates non-physiological recruitment order of muscles causing fatigue and aberrant movements (Enoka & Duchateau, 2008; Kern et al., 2002). Alternatively, intraspinal micro-stimulation (ISMS) provides naturalistic recruitment of muscle fibers with functional synergistic movement (Bamford, Putman, & Mushahwar, 2005; Moritz et al., 2007; Mushahwar & Horch, 1998; Zimmermann, Seki, & Jackson, 2011). However, the clinical use of ISMS has proceeded slowly due to its invasiveness.

Epidural stimulation, while sacrificing spatial precision compared to ISMS (B. Barra et al., 2018), demonstrate similar evoked movement to ISMS (Tao et al., 2019) and is a clinically approved hardware for pain control. Neuromodulation via epidural stimulation show growing evidence of its efficacy for immediate and long-term gains in paralyzed upper limbs in humans (P. Gad et al., 2018; F. Inanici et al., 2018; Lu et al., 2016). Brain controled-epidural stimulation in animal models has also resulted in successful reanimation of leg movements and potential therapeutic effects for locomotion (Bonizzato et al., 2018; M. Capogrosso et al., 2016; N. Wenger et al., 2016).

Here we demonstrate a brain-computer spinal interface (BCSI) that decodes movement intention from the motor-cortex and subsequently stimulates the cervical spinal cord via epidural stimulation below the injury.

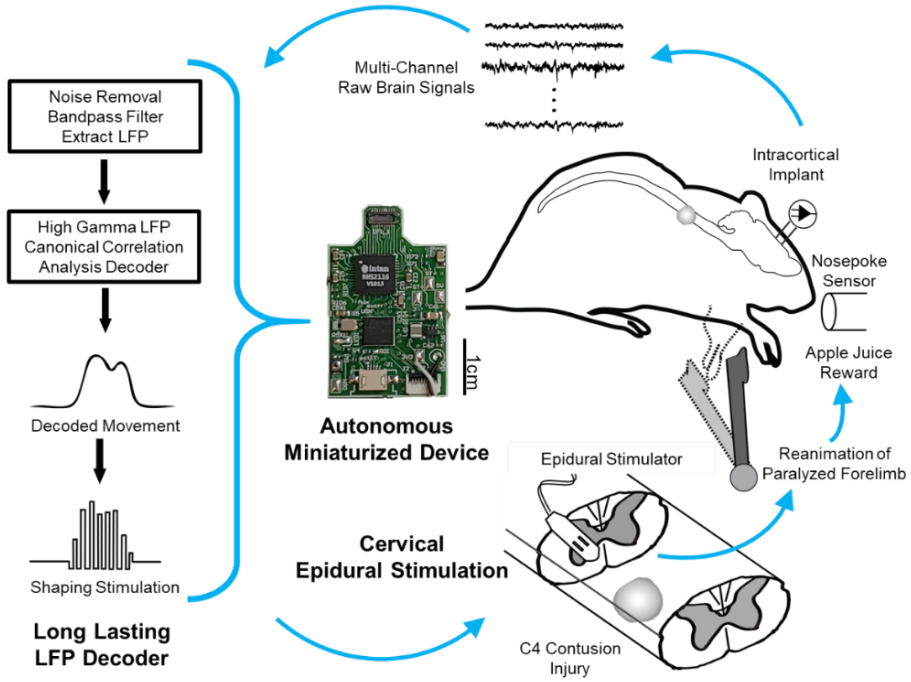
While several studies have demonstrated artificial connections between the brain and spinal cord (Nishimura, Perlmutter, & Fetz, 2013; Zimmermann & Jackson, 2014), the brain decoding performance is often not stable over time. Several competing methods of brain decoding balance electrode invasiveness, limited hardware longevity, signal non-stationarities, and spatial resolution (Barrese et al., 2013; Campbell & Wu, 2018; Simeral, Kim, Black, Donoghue, & Hochberg, 2011). Spikes, the electrical signature of action potentials from single neuron, have historically been used for neural decoding as they demonstrate a high accuracy for predicting intended movement (Brandman, Cash, & Hochberg, 2017). Spike decoding additionally presents high spatiotemporal resolution in humans (Ajiboye et al., 2017; Bansal, Truccolo, Vargas-Irwin, & Donoghue, 2012; Bouton et al., 2016). Nevertheless, spike decoding has limited signal stability requiring frequent recalibration by researchers (Perge et al., 2014).

To overcome the limitations associated with spike-based decoding, we used intracortical local field potentials (LFPs) which have multiple potential advantages. LFP activity correlates with movement-related spike activity in the high gamma band with stable decoding performance over months (Flint, Scheid, Wright, Solla, & Slutzky, 2016). Furthermore, LFP decoders also have lower bandwidth requirements than spikes, which translates to a lower computational complexity (Slutzky, 2018). Real-time control of epidural stimulation based on LFP decoding is a new approach to restore functional movements after cervical SCI. Finally, we realized the minimum required processing pipeline based on the LFP decoder in a miniaturized device using a field-

programmable gate array (FPGA) (Ranganathan et al., 2019). The circuit performed on-board computing with low power consumption at an implantable scale for rodents.

In summary, we developed a BCSI capable of robust decoding performance with low-computational complexity using LFP decoding to control epidural stimulation (Figure 1a). Animals learned to use the BSCI to reanimate forelimb movement after severe SCI (Figure 1b). Our results suggest a feasible approach to accelerate clinical translation of autonomous BSCI for people with cervical SCI.

a



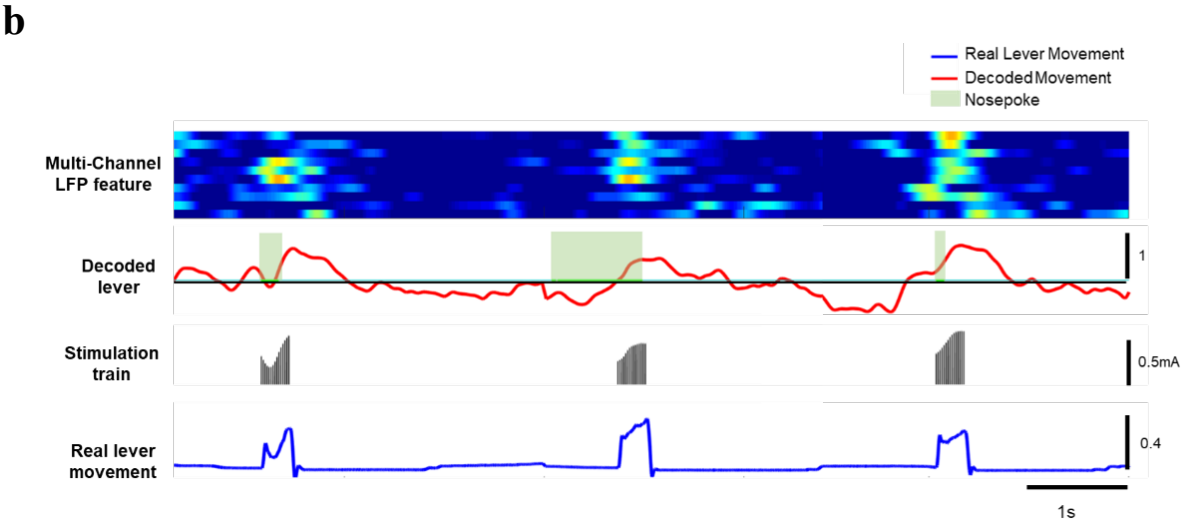


Figure 3-1. Brain-Computer Spinal Interface System for rodents with cervical spinal cord injury
a) Design of BCSI system. Multi-channel brain signals record neuronal population activity and are separated into high gamma band local field potentials (LFPs). The LFPs predict decoded movement via a canonical correlation analysis. The decoded movement controls the epidural stimulation amplitude in real time. All signal processing and stimulation can be implemented in the miniaturized, modular Field-Programmable Gate Array-based interface with embedded computing. Stimulation is delivered via cervical epidural stimulation implanted below the injury to reanimate forelimb movement after spinal cord injury. This permits successful forelimb and lever movement, triggering delivery of reward. **b) Performance of LFP controlled spinal stimulation.** Multi-channel high gamma (200-400 Hz) LFP power over time (top row). Based on the LFPs, movement intention is continuously decoded (2nd row). LFP power increases are visible immediately before and during the lever presses. When the decoded lever movement rises above a predefined threshold (green line), proportional stimulation is delivered to the spinal cord (3rd row). The stimulation amplitude is proportional to the decoded movement when above threshold. Stimulation via epidural electrode at the C6 spinal segment restore forelimb movements capable of moving a lever (bottom row). The continuous trajectories of the lever reflect the real-time modulation of the stimulation (bottom two rows).

3.3 METHOD

3.3.1 *Animals*

Eleven female Long Evans rats (250-360g) participated in this study. All animal procedures were conducted in accordance with the National Institutes of Health guidelines for the care and use of experimental animals and were approved by the University of Washington Institutional Animal Care and Use Committee (IACUC).

3.3.2 *Experimental Overview*

Animals were trained on a novel lever pressing task described below. After three to four weeks animals could complete 60 lever presses to maximum displacement within ten minutes. Animals were then implanted with intracortical electrodes followed by a one-week recovery period. We performed brain recording and offline decoding of the lever movements based on multi-channel LFPs. Subsequently, the animals received a right lateralized C4 contusion injury and implantation of epidural electrodes over the right C6 spinal segment. After a two- to three-week recovery period, we conducted retraining for the lever task in the injured condition and refitting of the LFP decoder. Lastly, we tested the efficacy of LFP-controlled epidural stimulation in animals with severe SCI. LFP decoding stability across conditions was analyzed in seven of eleven animals. Brain-controlled epidural stimulation system was tested in five of eleven animals following SCI. One animal was involved in the both decoding stability and BCSI system tests.

3.3.3 *Lever task training*

A novel lever task was created to allow animals with a severely impaired forelimb to engage in the task while using a head-mounted external recording and stimulation system. The behavior arena consisted of a translucent acrylic box with a gap in both sides of the central platform similar to the Montoya staircase (Montoya et al., 1991). A movable joystick in the right gap and provided a two-dimensional workspace for the starting position of a lever that was pressed backward by extending the elbow. A nosepoke sensor and a fluid tube for reward were placed on the front wall (Figure 1a).

The task involved placing the nose in front of the nosepoke sensor to put the animals in a consistent position relative to the lever. The voluntary nosepoke additionally served to signal engagement

with the task. To receive a reward, the rat had to reach and press the lever backward to a predetermined threshold while simultaneously aligned with the nosepoke sensor. The lever press required elbow extension to complete. Animals were given an apple juice reward for successfully pressing the lever. Task training was performed five to twenty minutes each day, five days per week for three to four weeks to achieve proficiency before surgery. The rats were water restricted between sessions. After each session, they were given one-hour unrestricted water access per day and free water over weekends.

3.3.4 *Cortical surgery*

All surgeries were performed using sterile technique and 2-3% isoflurane anesthesia in oxygen. Body temperature was maintained at 37 °C using a heating pad during surgeries and until fully recovered. Baytril (0.05mg/kg) was administered preoperatively. After a craniotomy and removal of the dura mater, sterilized 16-wire tungsten microelectrode arrays (40 μm diameter, 200 μm space between adjacent wires) were inserted into rostral and caudal forelimb area of sensorimotor cortex (the middle of the implant was placed 1.5 mm rostral and 2.5 mm lateral to bregma) and advanced to 1.5 mm below the brain surface to record pyramidal neuron activity in layer V (Supplementary figure 1b). Buprenorphine (0.5 mg/kg) was administered twice per day for three days postoperatively.

3.3.5 *Spinal surgery*

After the C4 unilateral laminectomy, the right lateralized C4 contusion injury was performed using a force control closed-loop system, the Infinite Horizon Impactor (Precision Systems and Instrumentation, LLC., Fairfax Station, VA). The impact force was set at 200 kdyn, and the severity of the injury was consistent across animals.

Spinal epidural stimulating electrodes were constructed as follows (Monzurul Alam et al., 2017). Two bundled electrode wires (11 μm diameter, AS631, Cooner wire) were stabilized on a polyimide sheet (5mm x 1.5mm x 46 μm) by epoxy. One mm of Teflon insulation was removed from each of the two wires. After the C7 unilateral laminectomy, the sterilized epidural implant was placed between the C5-C6 lamina and the dura mater from the caudal side of C6. Subsequently, the caudal side of the epidural implant was sutured to the dura over the dorsal aspect of the right C6 (Figure 1a). A loop of wires covered by a catheter was formed near their site of insertion to provide stress relief. A common ground wire was inserted subcutaneously near the shoulder on the right forelimb. The connector was placed on the headcap shared with the cortical implant.

3.3.6 *Motor evoked potential recording with nerve cuff and epidural stimulation*

A separate group of five animals was used to test recruitment curves of triceps activation by radial nerve stimulation and epidural stimulation(Monzurul Alam et al., 2017; Nikolaus Wenger et al., 2016). These spinal motor evoked potential (MEP) tests were performed during a terminal surgery using urethane anesthesia. Urethane anesthesia was chosen because of its ability to maintain spinal reflexes(Hara & Harris, 2002). The epidural stimulator was implanted on the dura at the right C6 spinal segment and the ground wire implanted in the shoulder muscle as described above. Bipolar electrodes were then inserted in the right triceps for electromyographic (EMG) recording (AS631, Cooner wire). After the right radial nerve was exposed around the outlet from the brachial plexus, a nerve cuff (Micro Cuff Sling, CorTec, Germany) was placed around the nerve for bipolar stimulation. Single 500μs stimulation pulses of monophasic (cathodic) and biphasic (cathodic-first, charge balanced) were delivered at 2 Hz for a total of 30 pulses per parameter tested. We performed the experiments with three stimulation conditions including regular epidural

stimulation, epidural stimulation with a 10k Hz carrier frequency, and nerve cuff stimulation (NCS) with various current intensities using an analog stimulus isolator (Model 2100 Isolated Pulse Stimulator, AM System, Sequim, WA).

The signals were amplified (1000 x) using the Tucker Davis Technologies (TDT) RZ5 system (Alachua, FL). MEPs were analyzed offline using custom MATLAB scripts. Triceps EMG signals were filtered (4th order Butterworth band-pass; 30-1000 Hz). A single trial of MEP was defined as evoked responses during the 30 ms following each stimulation event. MEPs were subsequently processed to compute maximum peak to peak amplitude and calculate recruitment curves in triceps muscles.

The slope of recruitment curves was computed as follows. The raw value of each peak to peak amplitude was normalized by the maximum mean amplitude (from a set of 30 pulses) observed during the entire recording from each animal. A Boltzmann sigmoid function was fit to the normalized recruitment curve to compute the slope for NCS, as well as epidural stimulation both with and without the 10 kHz carrier frequency (Marc Klimstra & E Paul Zehr, 2008).

3.3.7 *Data acquisition and signal processing*

Amplified neural data were recorded using the TDT multichannel data acquisition system at 24.4 kHz. Continuous lever and nosepoke signals were recorded in to the same system. We collected at least 50 lever presses each recording session. For both offline analyses (MATLAB) and online analysis (TDT system), we used the following decoding procedures (Figure 4).

First, a common average reference (CAR) filter was used to increase the signal-to-noise ratio (SNR) of the recorded signal (Ludwig et al., 2009). The CAR filter removed common voltage

components mainly produced by the noise content across all channels. Then, we filtered the 16-channels of brain data (4th order Butterworth, band-pass filter, forward) to the frequency band of interest. The 200-400 Hz band and a 0-100 ms lag time were selected since the parameters demonstrated the highest correlation with the lever movement in offline analyses (Supplementary Figure 2).

An outlier removal algorithm was applied to cap particularly high voltages greater than 3 times the standard deviation of each LFP signal. The filtered signals were rectified, and low pass filtered (4th order Butterworth, 2.5 Hz) to obtain multi-channel envelopes. The envelope of each channel was a continuous signal that represented the changes in spectral power of LFPs in response to output movement. To obtain the highest movement related spectral components, a canonical correlation coefficient (CCA) filter was applied on the multi-channel envelopes during the lever task (Khorasani, Foodeh, Shalchyan, & Daliri, 2018). Offline analysis was performed using the signal processing method demonstrated in the previous study (Khorasani et al., 2018).

When using the decoded signal to trigger epidural stimulation, we used a sample-and-hold method for 2 ms after the stimulation event to remove the stimulation-induced artifact before applying the previous signal processing. We ensured that using the 2 ms sample holding method did not produce negative effects on the decoded signals. Additionally, the intracortical signals may contain significant noise due to unusually high impedance or failing connections on the cortical electrodes. Thereby, we removed channels containing irregularly high amplitude signals, power spectrums not obeying 1/f frequency filtering, and movement-related artifacts visible in spectrograms as high amplitude transient broadband events.

3.3.8 *Post-injury decoding*

Following the contusion injury, the animals presented severe right forelimb paresis (weakness) showing minimum lever press movement (IBB scale: 1.6 ± 0.93 (mean ± SEM), N = 5, 40-60 days post-injury)(Irvine et al., 2014). To obtain CCA weights after injury, we recreated the decoded movement based on the residual ability of extension movement in the paretic forelimb. The residual lever press movement combined with nosepoke sensor activation was used to identify movement intention. An artificial bell-shaped lever signal was inserted at time points where the nosepoke sensor was activated, immediately followed by a low amplitude lever push. This strategy for severe upper limb paresis served as a proxy for imagined movements used successfully in human subjects with severe SCI (Ajiboye et al., 2017; Bouton et al., 2016; Collinger et al., 2013; Hochberg et al., 2012).

3.3.9 *Online stimulation protocol*

The obtained CCA weights for each channel were applied in the online, closed-loop decoding algorithm. The decoded movement signal was z-score normalized to have zero mean and unit standard deviation. This normalized signal was mapped between the motor threshold and maximum stimulation amplitude to control epidural stimulation. Whenever the decoded signal crossed a predetermined threshold epidural stimulation was delivered, provided the animal was also activating the nosepoke sensor. This assured both a consistent body position, and engagement with the task. The epidural stimulation was either monopolar biphasic square-wave pulses with 400μs cathodic-first current or charge balanced asymmetric square-wave pulses with 400μs cathodic-first and 4ms anodic at 1/10th current amplitude (Koivuniemi & Otto, 2011). Pulses were delivered in 15-40 pulse trains at 50-100 Hz. The specific parameters were determined based on

the movement response in each animal. Specifically, we scaled the stimulation current amplitude to evoke the movement in each experiment (300μA-1mA).

Two of five animals demonstrated aversive responses including freezing and/or withdrawing behaviors to epidural stimulation. For these animals, we applied a 10 kHz carrier frequency (Y. P. Gerasimenko et al., 2015; Fatma Inanici et al., 2018) with the monophasic stimulation consisting of 50 μs cathodic pulses with 50 μs intervals in a 400 μs train. Similar to the aforementioned method, 15-40 pulses were delivered at 50-100 Hz with an overlaid 10kHz frequency within each pulse to control the stimulation-induced sensation. Due to the technical limitation of the pulse generator, we could not insert prolonged anodic pulses right after the cathodic pulses to make it charge-balanced stimulation.

We also sought to exploit the modulation of the current amplitude with the change of the decoded movement in three animals that did not require high carrier frequency. We scaled the decoded signal from a predetermined threshold to the maximum decoded movement amplitude into the range 0 to 1. We multiplied the current difference by the mapped decoded signal to define continuous stimulation current patterns.

3.3.10 *Functional Assessment*

During the functional assessment trials with BCSI, catch trials with stimulation off were randomly interleaved at 20-30% probability. To quantify functional changes, peak lever movements of each intended lever press were compared between the stimulation on condition and the catch trials.

3.3.11 *Simulation Study on the Autonomous Closed-Loop System*

We restructured the modular FPGA-based device, the Neural Closed-Loop Implantable Platform (NeuralCLIP) (Ranganathan et al., 2019) for this study. Our goal was to test if this device could locally record and process brain signals to detect an event, as well as trigger stimulation

The structure of the NeuralCLIP consisted of the Intan Technologies RHS2116 microchip (Los Angeles, CA) for recording and stimulation. It contains 16 channels and a 16-bit ADC that is controlled via a serial peripheral interface (SPI), and a low power FPGA (AGLN250, Microsemi, Aliso Viejo, CA). The FPGA processed data through a synchronous and modular data pipeline consisting of the decoding operations. In this study, we added rectifier, downsampler and smoothing filters to the previous work to make the process more efficient and promote low power consumption.

To test the NeuralCLIP's utility, pre-recorded brain signals from both pre- and post SCI animals were provided as inputs to the NeuralCLIP. The CCA weights were determined on the TDT computer and then implemented on the FPGA prior to signal processing. The signals were processed through the data pipeline (Figure 8a) located on the FPGA. The decoded movement produced by the NeuralCLIP were compared to the pre-set threshold, and if greater than the threshold used to trigger stimulation pulses.

3.3.12 *Statistical procedures*

All data are reported as mean ± SEM. Statistical evaluations were performed by two-way ANOVA with Bonferroni correction for parametric data. For all other data, significance was assessed using

the non-parametric Wilcoxon signed-rank test. All analyses were performed with SPSS software (Chicago, IL). Differences were considered significant at p-value < 0.05.

3.3.13 *Data availability*

The data that presents the findings of this study will be made available by the authors on reasonable request.

3.4 RESULTS

3.4.1 *Decoding performance and signal stability*

To develop a robust brain-computer spinal interface, we first identified the association between the intra-cortical LFP features and forelimb-driven lever movement. Brain activity signals were acquired via 16 channel intracortical recording electrode arrays (Supplementary Figure 1). After filtering for a range of frequencies, we observed that signal power in frequencies above 200 Hz was most associated with forelimb movement, and was greater immediately before and during the lever press (Figure 2a).

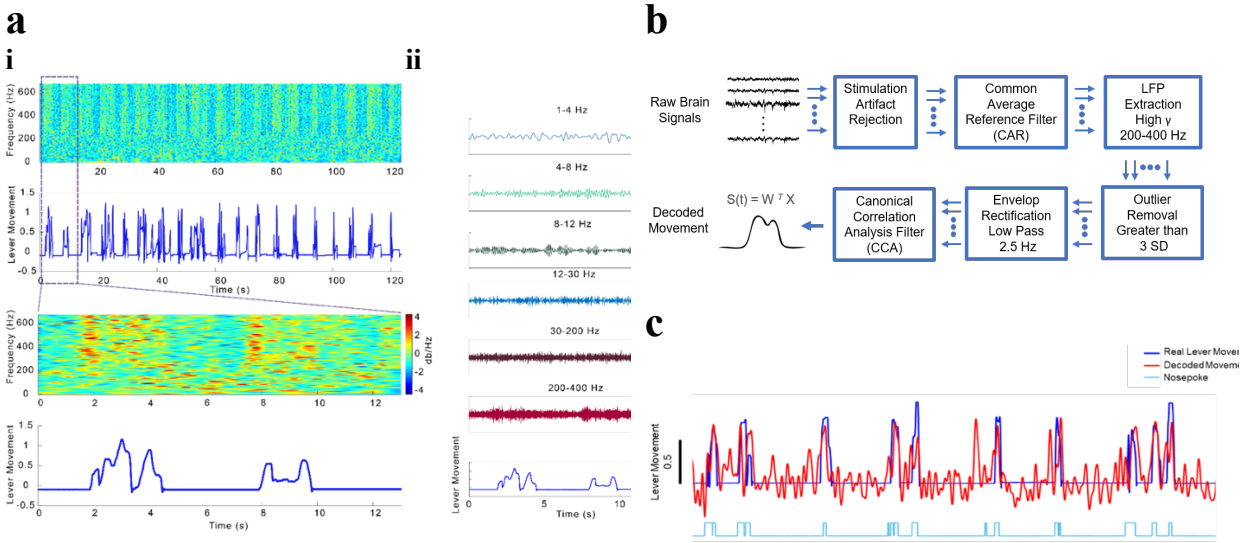


Figure 3-2. Development of LFP decoder for lever movement
a) Time-frequency association of LFP signal and lever movement. i: A power Spectrogram of one LFP channel was plotted with lever movements during the behavioral task. High power in 200-400 Hz sub-band was observed during lever presses. **ii:** The sub-bands of the LFP signal were plotted. **b) Structure of movement intention decoder.** Based on the results of the offline analysis we selected 200-400 Hz envelopes of multi-channel LFPs as features with a 0-100 ms lag time to refine the decoding performance. Signals were then processed online as follows. A common average reference filter was applied to increase the signal to noise ratio of the recorded brain signals. Local field potential (LFP) features were extracted by applying a band-pass filter. Artifacts with significant voltage amplitude were rejected to increase decoding performance. After removing outliers greater than 3 standard deviations (SD), the obtained envelopes were multiplied by the derived canonical correlation analysis (CCA) weights to decode animal intention for forelimb movement in real-time. Finally, when epidural stimulation was triggered by the decoded movement, stimulation artifacts were removed using the sample-and-hold method.
c) Example of pre-injury decoding performance. The decoded movement (red line) based on multi-channel high gamma LFPs produced high correlation coefficients, R-value = 0.59, with the lever trajectory (blue line). This was observed across all animals.

We then used a canonical correlation analysis (CCA) filter with 200-400 Hz frequency (high gamma) band to maximize information available from all cortical recording channels (Figure 2b). The decoder performed well when trained and tested on the same day in an uninjured animal (Figure 2c). The LFP decoder consistently produced predictions that were highly correlated with the real lever trajectory and comparable to the previous studies (Perge et al., 2014; Slutzky, Jordan, Lindberg, Lindsay, & Miller, 2011). In addition, the high gamma band decoder performed comparably to a decoder utilizing multiple frequency bands (Supplementary Figure 2).

To evaluate the stability of the decoding model, we assessed the decoder performance across the entire study. Models trained on the first recording day (the cross-day model) were compared against models trained at the beginning of every session (the same day model). The cross-day model performance was comparable to the same day model over 30 days based on a 5-fold cross-validation (Figure 3).

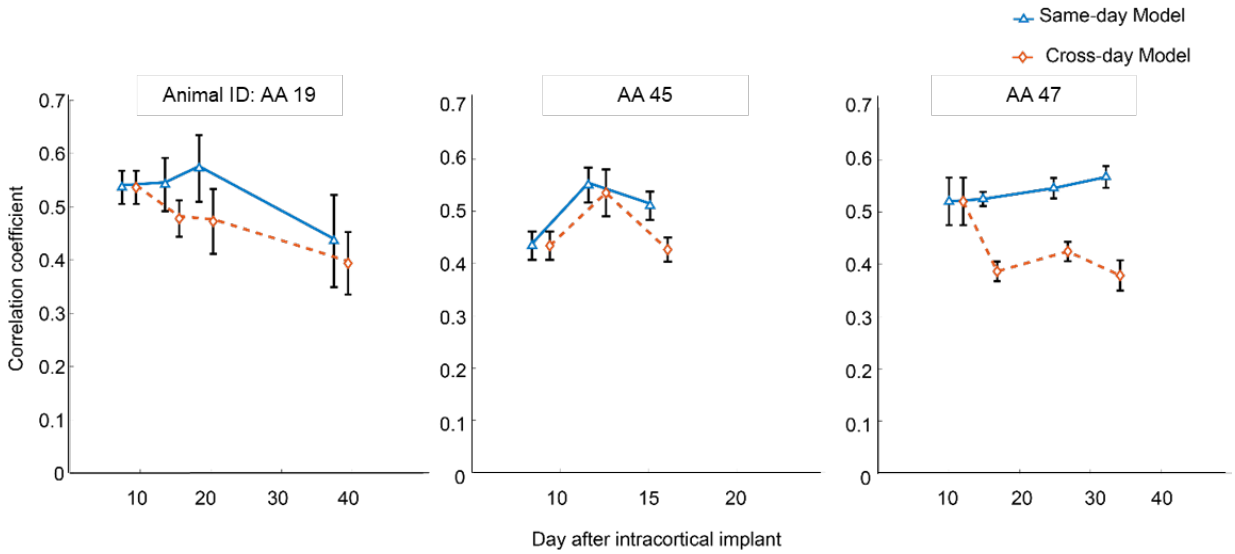


Figure 3-3. Long term decoding stability without calibration
Stability of LFP information for decoding forelimb movement was compared between models trained on the same day and models trained only on the first experimental session. Each data point is mean ± SEM of the correlation coefficient (R-value) of the test set obtained from 5-fold cross-validation analysis. Same-day calibration of the decoder produced marginally better decoding accuracy compared to the cross-day model. Even without same-day re-calibration, however, the LFP-based decoder could still produce sufficient decoding performance for the task even after 30 days.

Next, we compared the average power increase of high gamma LFPs during forelimb movement to generate lever presses in the pre- and post-injury conditions. Figure 4a illustrates an example of LFP power in the multi-channel high gamma band and lever displacement across time both before and after spinal cord injury. In Figure 4b, the positive values indicate higher power in LFPs right before lever press peaks compared to after the peaks. We were able to detect a power difference in the high frequency LFPs between the before and after the lever press in both pre- and post-injury conditions across all animals (Figure 4b). This result demonstrates the robustness of the decoder using high-gamma LFP bands.

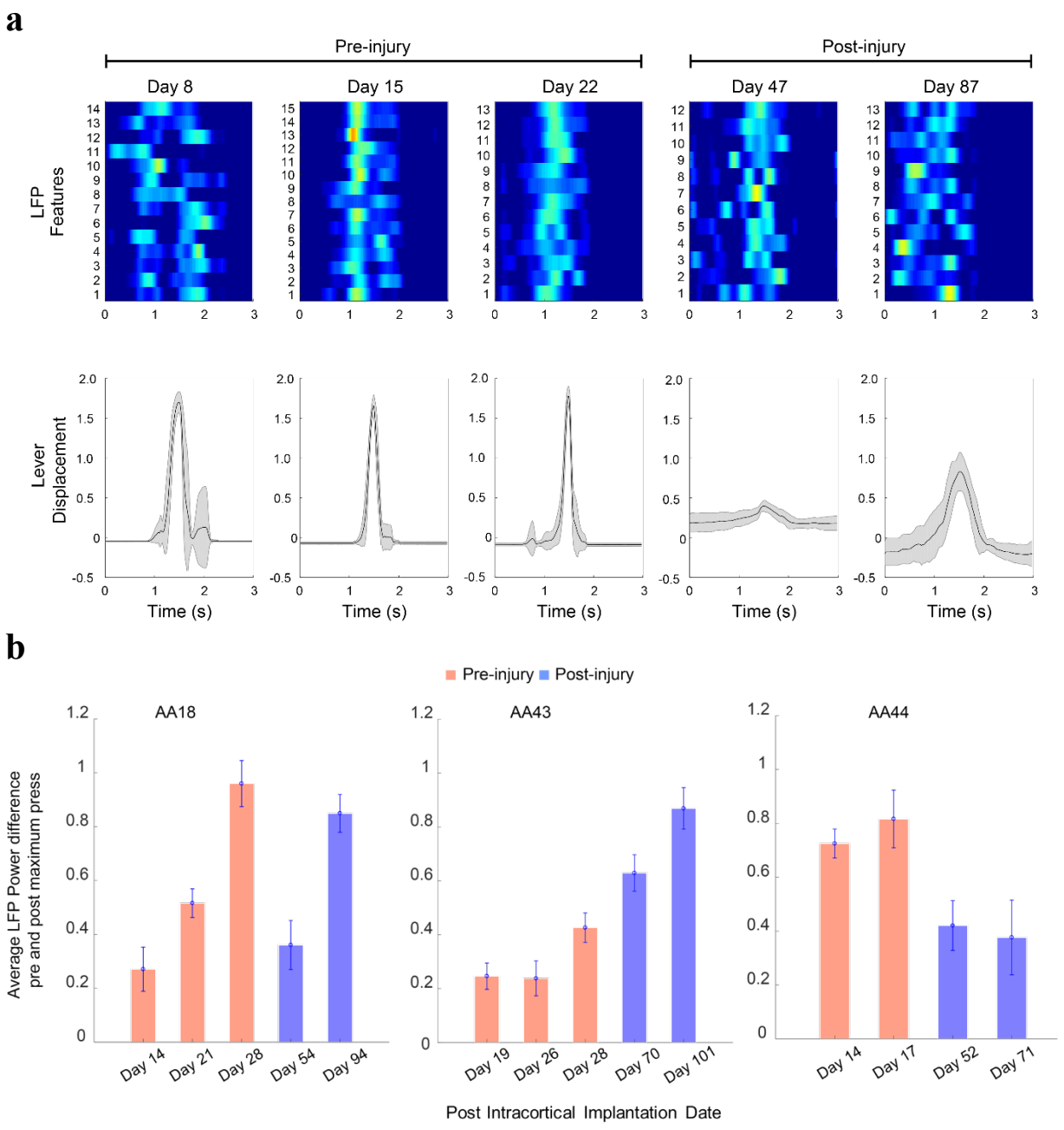


Figure 3-4. Sustained LFP Power in pre- and post-injury conditions

a) Increased high gamma LFP power during lever task in pre- and post-injury conditions. Average multi-channel 200-400 Hz LFP features (top) are synchronized with lever presses (bottom) across many days after intracortical array implantation. In both pre- and post-injury conditions the high gamma LFP power in multiple channels increased before lever press and during holding of lever, followed by returning to the baseline when the animal released the lever. **b) High gamma LFP power difference before and during lever presses across animals.** Differences between power in the 200-400 Hz band before and after maximum lever presses in pre- and post-injury conditions are shown. Each bar shows the difference of LFP power before the peak of the press and after the peak of press averaged over all trials in each session (mean ± SEM).

3.4.2 *Epidural stimulation recruitment curve*

We next compared the ability of epidural stimulation to recruit muscles in a graded manner. We compared muscle recruitment between epidural and nerve cuff stimulation using spinal motor evoked potentials (MEPs) by acute surgery in five animals without any injury. Single pulse nerve cuff stimulation produced triceps activity with less than 500 μA current and rapidly reached maximal muscle activity with only small increases in stimulus amplitude. Conversely, epidural stimulation, both with and without a 10 kHz carrier frequency, demonstrated more graded recruitment of the muscle activity (Figure 5).

The mean slope of the recruitment curve for nerve cuff stimulation was 20-fold steeper than the mean slope for epidural stimulation in the triceps (0.21 ± 0.097 vs 0.009 ± 0.004, p-value = 0.004). This demonstrates that epidural stimulation produces more graded recruitment than nerve cuff stimulation. Epidural stimulation using a 10 kHz carrier frequency required more current to achieve the same activation as epidural stimulation without a carrier frequency. Although the average recruitment curve tended to be less steep when using 10 kHz waveforms compared to traditional epidural stimulation waveforms, the difference was not significant (0.004 ± 0.001 vs 0.009 ± 0.004, p-value = 0.132).

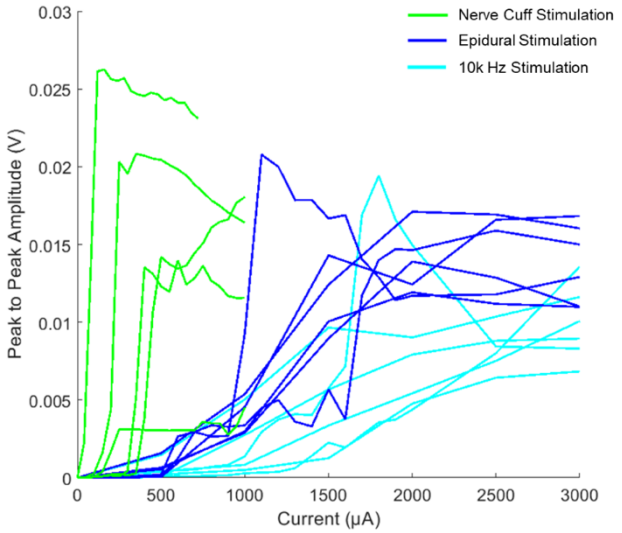


Figure 3-5. Spinal motor evoked potential recruitment curve
Triceps motor evoked potential recruitment curves were produced by either radial nerve cuff stimulation (green lines), C6 epidural stimulation (blue lines) or C6 epidural stimulation with a 10 kHz carrier frequency (cyan lines). The response is plotted as the average of the peak to peak amplitude evoked by different stimulus current amplitudes. Nerve cuff stimulation has very steep recruitment and thus poor grading of force compared to both methods of epidural stimulation.

3.4.3 *Epidural stimulation: Sensory benefits of high carrier frequency*

To deliver brain-controlled epidural stimulation to restore forelimb movements, we explored a range of stimulation parameters to produce both effective and comfortable stimulation. These were tested in five animals who received a cervical spinal contusion injury that caused sustained and severe forelimb impairments. All animals showed synergistic forelimb extension movements in the paralyzed limb by stimulation from one epidural electrode. Two of the five animals, however, demonstrated aversive behaviors when using 50-100 Hz biphasic or monophasic epidural stimulation. We discovered that adding a 10 kHz carrier frequency superimposed on each 400 μs pulse reduced the adverse effect while still successfully reanimating lever press

movements. We summarized the successful stimulation parameters for each animal in Supplementary Table 1.

3.4.4 *Brain controlled epidural stimulation reanimation*

We then tested whether the closed-loop BCSI could improve the paralyzed forelimb function following severe cervical SCI. Intracortical LFPs were used to decode the animals' intention to move. The decoder in turn modulated the epidural stimulation delivered to the lateralized C6 vertebral spinal cord to produce forelimb extension movement. In catch trials, the stimulation was briefly turned off to control for lever press performance in the absence of stimulation. When the stimulator was off, the animals struggled to press the lever and failed to reach the reward threshold. Despite failing to perform the task, the movement intention was still visible as the increased activity in the decoded movement aligned with a trained nosepoke behavioral response that was required during performance of the lever press (Figure 6).

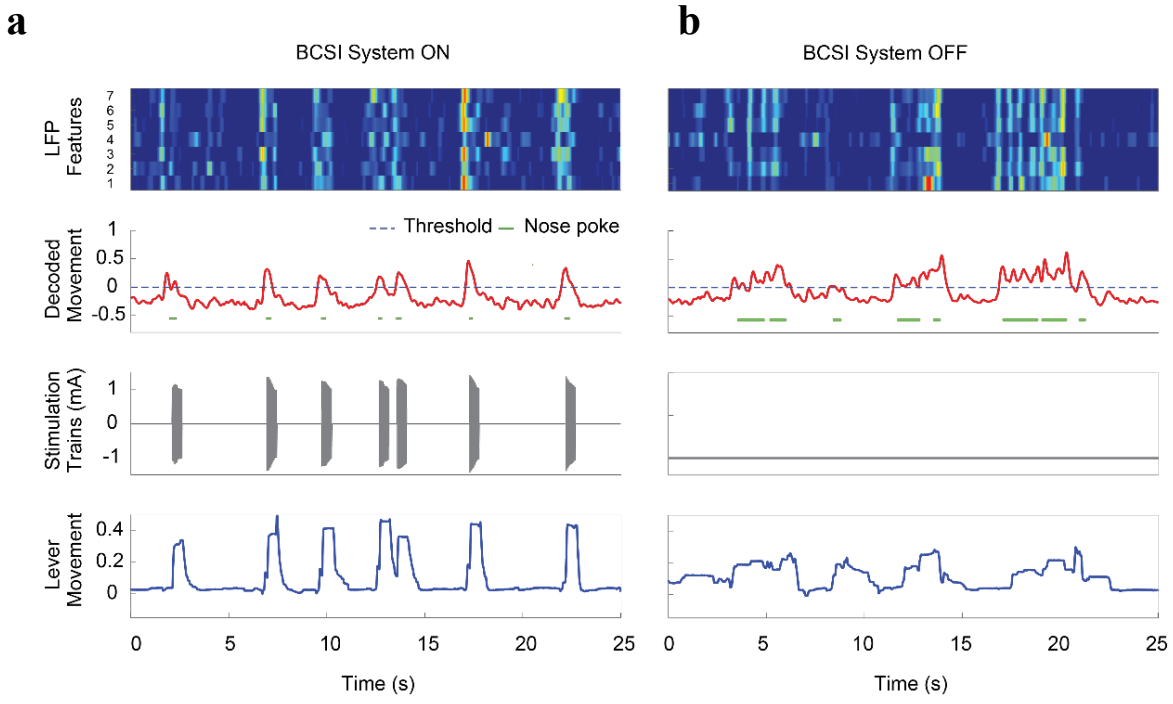


Figure 3-6. Multi-channel high gamma LFP power during the testing of the BCSI system
a) Stimulation on trials. An animal performed the lever task with LFP controlled epidural stimulation. Each band on the top row represents the LFP power of each channel. During the intended movement, the power of the high gamma band increased in the majority of channels. **b) Catch trials**. During the stimulation off period, the animal still demonstrated periodic high gamma power increases correlated with nose-poke behavior indicating intention to press the lever presses.

3.4.5 *Proportional LFP modulation of epidural stimulation*

We further tested the ability of three animals to modulate the stimulation current amplitude based on the decoded movement. In all these animals the reanimated lever movement reflected the proportionally modulated stimulation. Our goal was to demonstrate graded muscle contraction with a modulated current intensity of epidural stimulation (Figure 7a). The remaining two animals required the 10 kHz carrier frequency which was not proportionally modulated due to technical limitations of our pulse generator.

3.4.6 *Functional improvement*

Lever presses were successfully improved in all five animals tested post-injury using the BCSI system. To quantify the functional improvement, we compared the average peak lever range under three conditions: pre-injury, post-injury with the BCSI system (stimulation on trials), and post-injury with stimulation off (catch trials). Peak lever presses were found to be significantly different in each condition ($F(2, 645) = 275.4$, $p < 0.001$). The lever press performance after the injury with no stimulation (0.21 ± 0.01) was significantly lower than the pre-injury performance (0.81 ± 0.02). Average reanimated forelimb movement by the BCSI was 38% greater (0.29 ± 0.01) greater than the stimulation off post-injury trials ($p < 0.01$; Figure 7b).

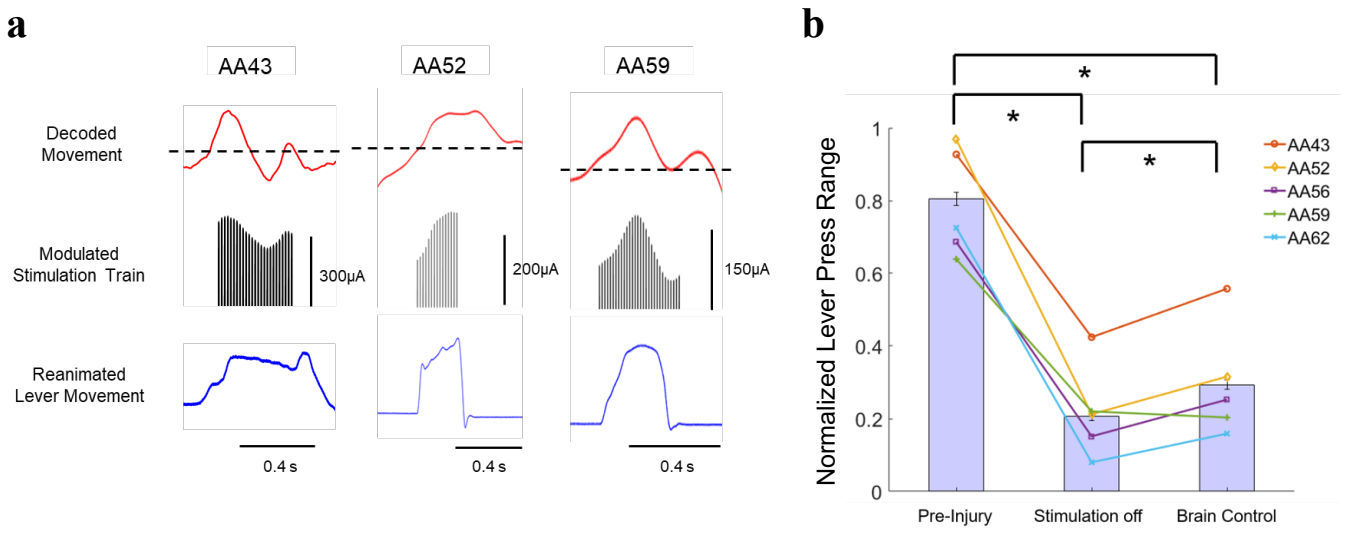


Figure 3-7. BCSI Reanimation and Functional Improvement
a) Reanimated lever movement reflecting proportional modulation of stimulation. The stimulation current amplitude was continuously scaled based on the decoded movement amplitude. The proportional control of epidural stimulation produced graded forelimb movement. The lever trajectory reflected the graded stimulation effect in all three animals tested. The dashed lines are stimulation thresholds. **b) BCSI system improved forelimb function.** The mean peak lever press range significantly improved with brain-computer spinal interface system. Average reanimated forelimb movement by the brain-computer spinal interface was 38% greater compared to the stimulation off post-injury trials (N = 5; * p-value < 0.01).

3.4.7 *Closed-loop system simulation implemented in an autonomous miniaturized device*

Finally, we evaluated the performance of an autonomous decoding system on the Neural Closed-Loop Implantable Platform (NeuralCLIP). The NeuralCLIP was configured with signal processing blocks to implement our CCA decoder on an FPGA architecture(Ranganathan et al., 2019) and system-on-chip which is small enough to be worn by a rodent or implanted under the skin of human subjects. To test the NeuralCLIP's ability to perform the necessary signal processing blocks, prerecorded data of brain signals in animals, before and after injury, were used as inputs to the onboard FPGA. To improve detection of movement intention from the previous work (Ranganathan et al., 2019), a rectifying block was placed between the bandpass filter and CCA. Also, a downsampler followed by a smoothing filter was placed after the CCA output to improve

threshold detection for decoded movement. (Figure 8a). The refined NeuralCLIP could detect the lever press movement intention from the LFPs and matched the real lever movement in the uninjured condition (Figure 8b) and the nosepoke signals with the minimum lever movement in the injured condition (Figure 8c). These results suggest that an efficient decoder can run on an implantable device and provide the necessary processing to control spinal stimulation and promote functional restoration after spinal cord injury.

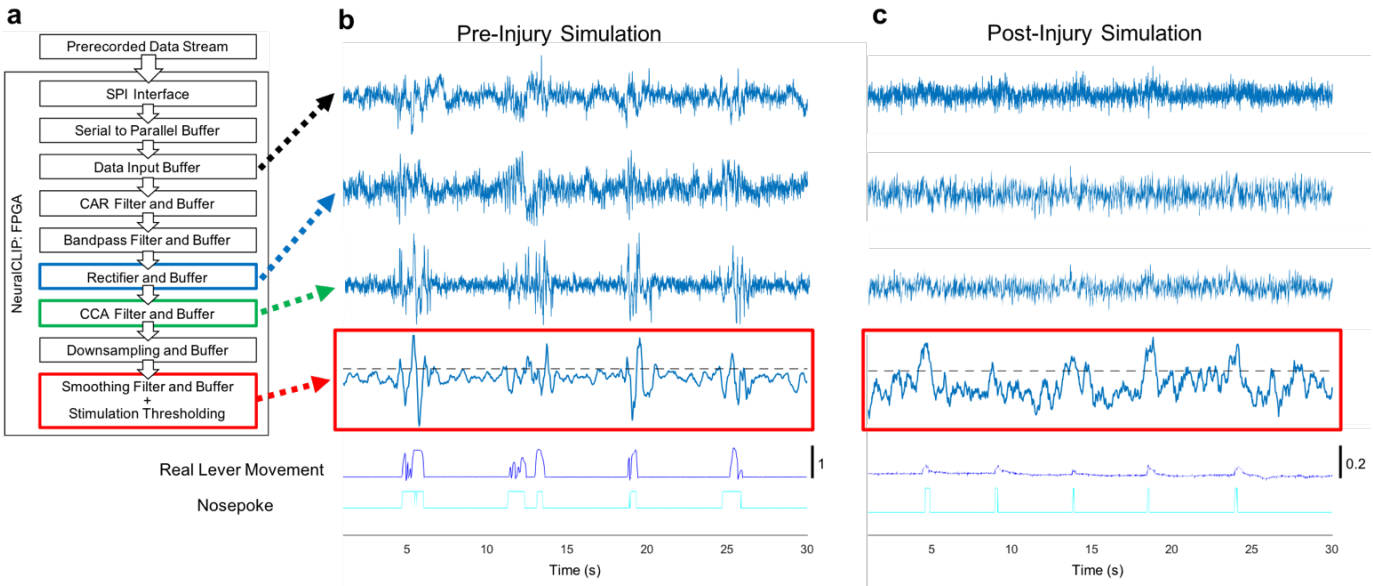


Figure 3-8. Autonomous miniaturized platform decoded movement intention to trigger stimulation pre- and post-injury a) FPGA Pipeline Blocks of NeuralCLIP. The Neural Closed-Loop Implantable Platform (NeuralCLIP) processed the pre-recording LFP signals from the motor cortex and identified intention and trigger stimulation. The functional block diagram presents the pipeline processing. Modifications from the original NeuralCLIP pipeline include a rectifier, downsampler and smoothing filter to improve the decoding performance while maintaining the low power consumption. **b) & c) Stimulation of Decoding the Lever Movement in Pre- and Post-injury Condition.** The CCA-decoded lever movement was created by 4 cortical channels. The first row shows one channel of raw brain signal input. The second row shows the rectified single channel signal after common average reference (CAR) filter and 200-400Hz band-pass filter. The third row illustrates the multichannel CCA filtered signal. The decoded lever movement followed by down sampling and smoothing filter (red box) could trigger the stimulation when the animal intended to move by crossing the pre-determined threshold. The decoded movement was aligned with the ground truth, the real lever movement and nosepoke signals (bottom). The NeuralCLIP could decode the forelimb movement intention with the pre-recorded brain signals recorded in the pre- and post-injury (Day 35) condition in an example animal.

3.5 DISCUSSION

Here we demonstrate a brain-computer spinal interface (BSCI) for restoring functional upper limb movement following cervical spinal cord injury. Our main findings are 1) intracortical high gamma local field potentials provided a stable prediction of forelimb movement over many days before and after spinal cord injury, 2) brain-controlled epidural stimulation evoked graded synergistic forelimb movement proportional to the stimulation current, and 3) the efficient closed-loop algorithm could be implemented on a miniature device with onboard computing. Our results inform a pathway to clinical translation of the BCSI application to human trials as recording and stimulation techniques used here have already been applied to humans in separate clinical trials.

3.5.1 *Local field potential decoding*

We found that the high gamma LFP decoder provided accurate and stable predictions of forelimb movement trajectory over time. LFPs reflect the summation of multiple neural sources near the recording area (Buzsaki, Anastassiou, & Koch, 2012). Previous studies have shown a high correlation between multiunit spike activity and LFPs allowing accurate prediction of movement-related information (Flint, Wright, Scheid, & Slutzky, 2013). One study reported similar decoding performance using LFP activity compared to multiunit spikes due to greater signal stability of LFPs in non-human primates. LFP decoders have also shown sufficient stability for motor intention decoding without recalibration over several months in human (Milekovic et al., 2018). In a previous study, the CCA decoder used here maintained high performance with a lower computational complexity compared to principal component analysis or correlation coefficient-based methods (Khorasani et al., 2018).

In the present study, we demonstrated that the frequency band between 200-400 Hz provided the highest accuracy for decoding forelimb lever press movement compared to other frequency bands (Supplementary Figure 2). Using the CCA decoder on multichannel LFPs in the 200-400 Hz band, we demonstrated accurate and stable decoding over many days. This even included before and after a contusion injury, following which the decoder could be used to modulate epidural stimulation and restore functional movements (Figure 4). This presents a substantial advantage over using spike information for control of external devices, as spikes-based decoders often require daily spike sorting procedures and frequent validations (Ajiboye et al., 2017; Bockbrader, 2019; Bouton et al., 2016).

An additional benefit of using a CCA with a single frequency band of LFPs is reduced computational complexity. Despite the limited spatial accuracy of LFPs, the advantages of the signal stability and computational efficiency in the proposed decoding strategy allowed us to implement the complete closed-loop system on a miniature device small enough to be implanted in human subjects. This may accelerate clinical translation of closed-loop BCI systems by eliminating the needs for cabling or high bandwidth wireless data transfer (Arjona, Rosenthal, Smith, & Moritz, 2019). Next, we selected a clinically viable stimulation strategy to be controlled by the output of the CCA decoder for upper limb reanimation.

3.5.2 *Epidural stimulation for upper limb reanimation*

Upper limb movements are less rhythmic with more supraspinal control compared to the lower extremities (Volker Dietz & Fouad, 2013). Many current BCI applications implemented robotic arms and functional muscle stimulation via a large number of electrdes in people with tetraplegia (Wodlinger et al., 2014). Direct muscle stimulation has shown credibility for functional gains

(Ajiboye et al., 2017; Bouton et al., 2016). However, direct muscle stimulation evokes a non-physiological recruitment of muscle fibers leading to rapid muscle fatigue (Merletti, Knaflitz, & De Luca, 1990). Intraspinal microstimulation (ISMS) recruits muscle in a more natural order (Bamford et al., 2005; Holinski et al., 2016; Mushahwar & Horch, 1998) . Here we extend these findings to epidural stimulation and demonstrate graded recruitment of muscle activity for forelimb extension that is superior to radial nerve stimulation (Figure 5). Our results suggest that epidural stimulation may achieve fatigue resistant and naturalistic contractions and be a viable strategy to reanimate functional movements of the upper limb.

Reanimating naturistic upper limb movement is a primary goal of functional restoration. We created a protocol which linearly modulated stimulation current amplitude based on the decoded movement signal (Figure 7a). We have observed that spinal stimulation combined with proportional modulation can mediate synergistic forelimb movements from single electrodes (Moritz et al., 2007; Sunshine et al., 2013). This is a marked improvement over direct muscle stimulation which requires many stimulating electrodes and computationally complex control schemes for restoring paralyzed arm and hand function (Ajiboye et al., 2017; Bouton et al., 2016; Christian Ethier, Emily R Oby, Matthew J Bauman, & Lee E Miller, 2012; Moritz et al., 2008). Even our simple LFP decoder-controlled epidural stimulation system could modulate forelimb movements with their intention and improved the outcomes of the lever press task (Figure 6, Figure 7b).

3.5.3 *Noxious sensations of epidural stimulation were controlled using a high carrier frequency*

During the epidural stimulation for reanimating forelimb movements, we observed that two of five animals exhibited aversive behaviors during epidural stimulation during standard frequency stimulation (50-100 Hz). This limited their participation in the behavioral task and made the BCSI initially unusable in these cases. This mirrors a clinical challenge of lumbosacral epidural stimulation inducing paresthesia and aversive sensation for rehabilitation training and pain management in people with SCI (Susan Harkema et al., 2011; Reddy et al., 2016). This aversive sensation limits the use of certain electrode combinations and parameters during epidural stimulation in humans.

For pain management, epidural stimulation with a 10 kHz has shown more effective pain reduction without paresthesia in clinical trials (Kapural et al., 2015; Reddy et al., 2016). In addition, rehabilitation for motor function has been performed with 10 kHz carrier frequency transcutaneous spinal stimulation in human with SCI (P. Gad et al., 2018; Y. P. Gerasimenko et al., 2015; Fatma Inanici et al., 2018). Prior to our study here, however, there is no report of directly evoking limb movements through 10 kHz carrier frequency epidural stimulation. Our data on spinal motor evoked potentials revealed that the recruitment patterns of epidural stimulation with a 10 kHz carrier frequency for triceps was similar to epidural stimulation without the carrier frequency despite requiring more current to achieve the same level of activation (Figure 5). Thus, we applied the high carrier frequency to epidural stimulation for the two animal that experienced the aversive sensation. By incorporating this 10 kHz carrier frequency, we successfully controlled the aversive

behaviors and implemented the BCSI to improve functional movements in all animals tested (Figure 7b).

High carrier frequency epidural stimulation may modulate recruitment patters of afferent fibers and activation of interspinal neurons (Al-Kaisy et al., 2014; Arle, Mei, Carlson, & Shils, 2016; Lee et al., 2020), but the mechanism of sensory control remains unclear. Use of kilohertz carrier frequency is a promising method to control sensation with epidural stimulation for motor function and overcome a significant challenge in human trials of epidural stimulation for movement.

3.5.4 *Hardware application*

Current BCIs requires external desktop computers to perform computation for decoding and control of stimulation or actuators even though some BCI systems are now wireless (Marco Capogrosso et al., 2016). This limits the use of the BCI system outside research laboratories and presents challenges to translation to home use or community applications (Zickler, Halder, Kleih, Herbert, & Kübler, 2013). Onboard computing on implantable systems is one solution. However, the computational power on the miniaturized chip is limited due to the current technological capability. Thus, implantable onboard computing systems combined with low computational complexity algorithms are an ideal approach to eliminate the need of external desktop computers with the current technology.

We previously presented the NeuralCLIP, a modular FPGA-based device, that can provide scalable autonomous processing and stimulation in a size appropriate for untethered animal use or implantation (Ranganathan et al., 2019). In the present study, we adjusted the pipeline processing on the FPGA for our CCA algorithm and stimulation strategy. The CCA-based algorithm for LFP

decoding was successfully implemented on the autonomous FPGA and triggered stimulation based on neural data recorded both before and after the injury (Figure 8). Further, the measured power consumption (Ranganathan et al., 2019) can be provided by wireless power transmission (B. H. Waters et al., 2018). However, we have not yet performed an in-vivo study with this implantable system. Further study is needed to test the implementation of wireless power sources on the miniaturized board and confirm that heat dissipation is safe for surrounding tissue. Nonetheless, this may be the first demonstration of a complex decoder operating on a device with the potential for complete implantation within the body

3.5.5 *Limitations*

Restoration of daily activity using the paralyzed arms and hands requires multiple degree of freedom (DOF) movement. This study required animals to perform only one DOF movement. Using epidural stimulation to increase the variety of forelimb movements may require a wider implant with a larger number of channels to cover multiple spinal segments and dorsal root entry zones (Beatrice Barra et al., 2018). This requires substantial advancements in technology that are already underway (Minev et al., 2015), and also an increased physiological understanding of spinal stimulation.

In this study, the LFP decoder created a single DOF movement prediction for reanimation. It is likely that LFPs also contain the information for at least three-dimensional movement of the arm and hand (Andrew Jackson & Hall, 2017). Future work is needed to adapt decoding strategies that can extract three- or more dimensional multi DOF movement with low power consumption.

3.6 CONCLUSION

We found that brain-controlled epidural stimulation below the injury restored volitional control of a paretic forelimb in rats with severe cervical SCI. The low computational complexity of the algorithm combined with a clinically applicable and efficient stimulation could be implemented in a miniaturized autonomous closed-loop system. The BSCI strategy demonstrated in the present study may overcome many of the current barriers to translating BCIs into clinical approaches for upper limb restoration following SCI.

3.7 ACKNOWLEDGEMENTS

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3.8 AUTHOR CONTRIBUTIONS

S.S., A.K., V.S., M.R.D., and C.T.M. conceived and designed the experiments, S.S., N.M.T, and A.K. performed the experiments, and S.S., A.K., and C.T.M. wrote the paper. All authors contributed to its editing.

3.9 COMPETING INTERESTS

The authors declare no competing interests.

3.10 SUPPLEMENTARY INFORMATION

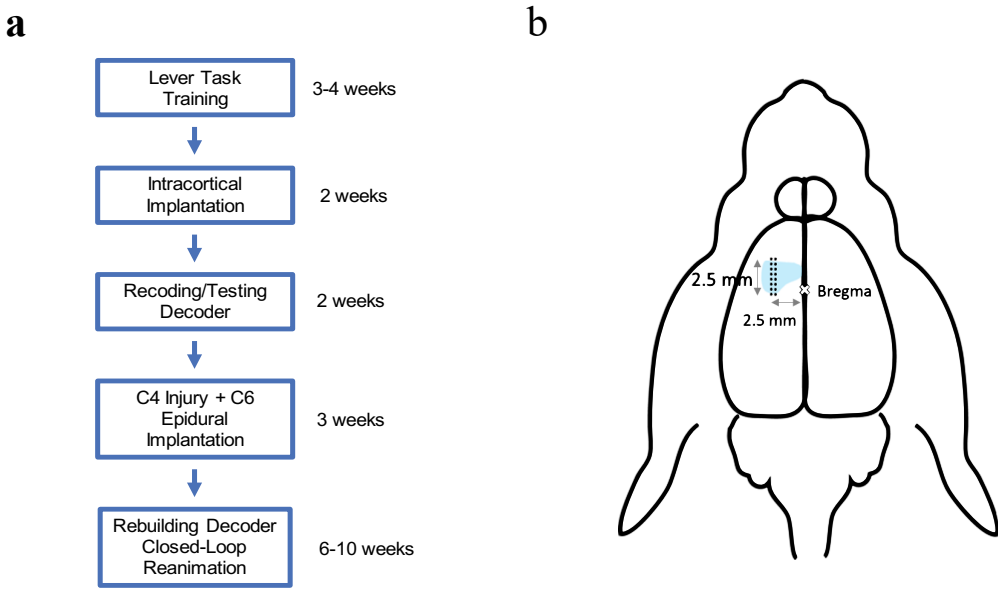


Figure 3-9. Supplementary Figure 1
a) Experimental design. Animals were trained to proficiency on the lever task prior to intracortical implantation. After one-week of recovery, local field potentials (LFPs) were recorded from the brain and decoded offline. Subsequently, animals received a C4 contusion injury and C6 epidural implantation on the right side of the spinal cord. Animals were then retrained on the lever task and the CCA decoders were retrained if needed. Finally, we evaluated the closed-loop reanimation performance of the brain-controlled spinal stimulation interface. **b) Cortical implant in sensorimotor cortex.** Animals received custom intracortical 8 x 2 microarrays, 2.5 mm length, in the left forelimb sensorimotor cortex (blue area). The center of the forelimb area is located 1.5 mm rostral and 2.5 mm lateral to bregma. The arrays were inserted 1.5 mm below the brain surface to record the activity of pyramidal neurons in layer V.

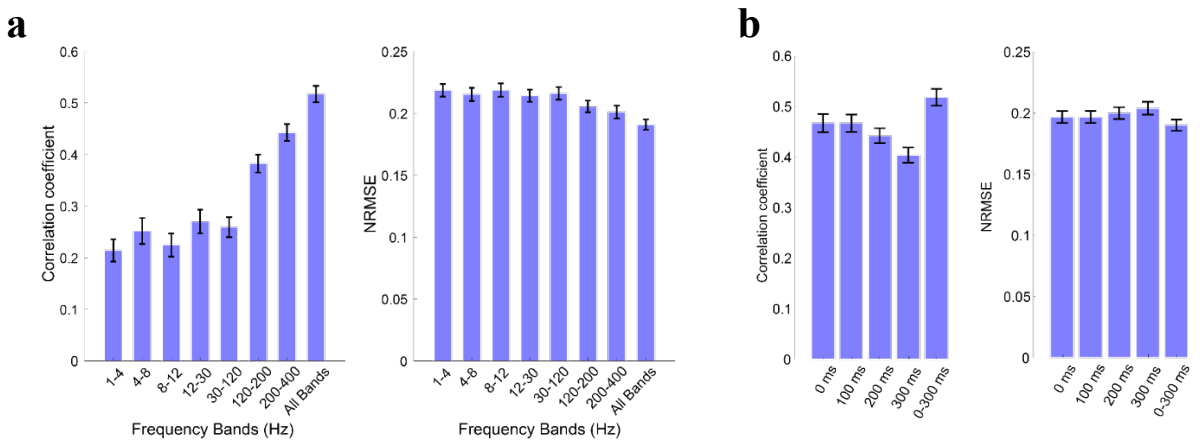
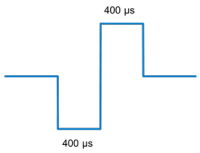
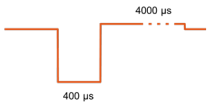
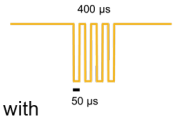
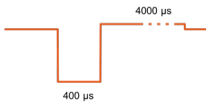
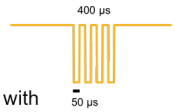


Figure 3-10. Supplementary Figure 2. Comparison of decoding performance of separate sub-band and temporal features

Offline analyses of correlation coefficient and normalized root mean squared error (NRMSE) was conducted to evaluate the accuracy of the decoder. 5-fold cross validation was used in these analyses. All data was obtained in pre-injury conditions.

a) Decoding performance with different frequency bands. 200-400 Hz envelopes of multi-channel LFPs showed the highest correlation coefficients and the lowest NRMSE compared to other features ($P<0.001$; R-value: 0.45 ± 0.016) and were also comparable to decoder performance with all sub-bands combined (R-value: 0.51 ± 0.016). **b) Decoding performance with different lag time.** High gamma LFP envelopes of multi-channel LFPs with a 0-100 ms lag time showed the highest correlation coefficients and the lowest NRMSE compared to other features ($P<0.001$). The outcomes were also comparable to decoder performance with all lag-time LFP information combined. Each bar shows mean ± SEM of correlation coefficients and NRMSE for seven animals.

Table 3-1. Supplementary Table 1. Stimulation Parameters of Epidural Stimulation

Animal ID	Stimulation Parameters (train, frequency, train/pulse width, current, additional control)	Waveform	Perception of regular frequency stimulation	Closed loop system trial date
AA43	30 pulse train, 50 Hz, 400 µs, 600-1.1mA, Proportional modulation	Biphasic 	Tolerated well	47 days post-injury
AA52	30 pulse train, 50 Hz, 400 µs, 600-800 µA, Proportional modulation	Monophasic 	Tolerated well	42 days post-injury
AA56	20 pulse train, 50 Hz, 400 µs, 800 µA, 10 kHz carrier frequency	Monophasic with 10 kHz carrier frequency 	Requiring 10k Hz carrier frequency to manage sensation	62 days post-injury
AA59	40 pulse train, 100 Hz, 400 µs, 450-750 µA, Proportional modulation	Monophasic 	Tolerated well	63 days post-injury
AA62	30 pulse train, 100 Hz, 400 µs, 1.1 mA, 10 kHz carrier frequency	Monophasic with 10 kHz carrier frequency 	Requiring 10k Hz carrier frequency to manage sensation	24 days post-injury

The stimulation parameters were optimized depending on the behavioral responses. AA43, AA52, and AA59 performed proportional modulation of charge balanced symmetric or asymmetric pulses based on the decoded signals. AA56 and AA62 required a 10 kHz carrier frequency during monophasic pulses to evoke sufficient forelimb extension movements and control freezing or withdrawing behaviors indicating aversive sensation from epidural stimulation. Due to the technical limitations of the pulse generator, we could not produce a balanced change or proportional modulation of the stimulation current for the stimulation with 10 kHz carrier frequency, but stimulation trains were brief and not repeated over many days.

Chapter 4. RECOVERY OF WALKING AND BOWEL FUNCTION BY TRANSCUTANEOUS SPINAL STIMULATION WITH INTENSIVE TRAINING IN TETRAPLEGIA

This chapter is submitted for publication.

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4.1 ABSTRACT

Epidural stimulation paired with intensive locomotor training has led recovery of motor function and autonomic control following spinal cord injury (SCI), with impressive impacts on quality of life and participation for people with SCI. Due to invasiveness and cost of epidural stimulation, however, it is not always the best approach for the diverse population with SCI. Transcutaneous spinal stimulation is a non-invasive alternative to epidural stimulation, but the clinical evidence

for the efficacy is currently limited. To investigate the effect of cervical and lumbar transcutaneous spinal stimulation on walking and autonomic function, two 64 years old male participants with chronic incomplete cervical SCI participated in the study. They underwent two months of intensive locomotor training and two months of transcutaneous spinal stimulation paired with intensive locomotor training. The improvement in six minutes walking distance during the transcutaneous spinal stimulation phase was three-fold greater than the locomotor training alone in both participants. The improvements reduced their risk of falls and increased their ability to engage in daily home exercises. Gait analysis revealed distinct but different responses to transcutaneous spinal stimulation among individuals. Both participants experienced improved sensation and normalized the ability of bowel management. One participant, furthermore, eliminated the need of intermittent catheterization after the transcutaneous spinal stimulation phase. Our results suggest that non-invasive spinal stimulation strategy may promote neural recovery of locomotor and autonomic function beyond the traditional rehabilitation program for people with chronic cervical SCI.

4.2 INTRODUCTION

Spinal cord injury (SCI) results in prolonged neurological impairments limiting participation in essential activities of daily living. Currently, more than 0.6 million new people each year experience SCI worldwide (Dewan et al., 2018). People with cervical SCI prioritize the ability of arm/hand use, walking, pain relief, bowel/bladder control, and sexual function to improve quality of life (Anderson, 2004; Ditunno, Patrick, Stineman, & Ditunno, 2008; J. S. French et al., 2010; Lo, Tran, Anderson, Craig, & Middleton, 2016). Nearly half (47.2%) of people with SCI have incomplete cervical injury in the United States (NSCISC, 2019). Cervical SCI commonly presents mobility impairments, neurogenic bowel and bladder function, autonomic dysreflexia, and secondary complications such as constipation, urinary tract infection, and sleep disturbance (Charlifue, Weitzenkamp, & Whiteneck, 1999; A. Krassioukov, 2009; A. Richardson, Samaranayaka, Sullivan, & Derrett, 2019). The secondary complications can be partly explained by a lack of standing and walking activities, leading to deteriorating function in other body systems (V. Dietz, 2010; Dunn et al., 1998; Hicks, 2020).

Activity-based training, including overground and treadmill training, is the most common clinical practice for locomotor recovery following SCI (Alexeeva et al., 2011; Behrman, Ardolino, & Harkema, 2017; Behrman & Harkema, 2000; B. Dobkin et al., 2006; Edelle C Field-Fote & Roach, 2011; Filipp et al., 2019; Susan J Harkema et al., 2012; Hornby et al., 2020). A recent study also demonstrated the efficacy of locomotor training on autonomic function following SCI (Hubscher et al., 2018). Nevertheless, the level of recovery has yet to approach the functional goals in the population with SCI. There is a potential for promoting functional recovery using neurotechnology to assist in sending neural signals across the lesion in the spinal

cord (Grégoire Courtine & Sofroniew, 2019; Ereifej et al., 2019; N. D. James, McMahon, Field-Fote, & Bradbury, 2018).

Over the last four decades, research has demonstrated that there is dormant connectivity in the damaged spinal cord that can be activated using an extrinsic stimulus, recovering some physical function (M. M. Dimitrijevic et al., 1986; S. J. Harkema, 2008; Minassian et al., 2004; R. R. Richardson, Siqueira, & Cerullo, 1979; Waltz, 1997). It is hypothesized that electrical stimulation of the spinal cord via implanted electrodes, termed epidural stimulation, recruits large-medium diameter afferent fibers. This, in turn, is able to activate other neural structures, including spinal interneurons, to modulate the physiological state of the spinal cord below the injury (Capogrosso et al., 2013; Formento et al., 2018; Rattay et al., 2000; N. Wenger et al., 2016). Following the serendipitous discovery of the effects of epidural stimulation on motor and autonomic recovery after motor-complete SCI (S. Harkema et al., 2011), several independent research groups have shown that epidural stimulation paired with locomotor training resulted in remarkable improvements of lower extremity function in people with chronic SCI (Angeli et al., 2018; Gill et al., 2018; Wagner et al., 2018). Furthermore, there is substantial evidence of the efficacy of epidural stimulation on the autonomic system, including cardiovascular (Darrow et al., 2019; Susan J Harkema et al., 2018; Bonnie E. Legg Ditterline et al., 2020), bladder (S. Harkema et al., 2011; Herrity et al., 2018), bowel (Walter et al., 2018) and sexual function (Darrow et al., 2019).

Epidural stimulation, however, is invasive with the associated risks of surgical and implanted device complications (Taccola, Barber, Horner, Bazo, & Sayenko, 2020; Walsh, Machado, & Krishnaney, 2015). The intervention can also be costly due to the prolonged training period and surgical implants (Shipley & North, 2018). Thus, people with SCI, stake holders, and researchers

are considering alternative methods of spinal stimulation that lead to the restoration of physical function for the heterogeneous population of people with SCI.

Electrical stimulation of the spinal cord applied at the skin surface, transcutaneous spinal stimulation, is a non-invasive method for promoting functional recovery in individuals with SCI. Transcutaneous spinal stimulation on the lumbosacral spinal cord has facilitated lower extremity function in people with chronic complete and incomplete SCI (M. Alam et al., 2020; P. Gad et al., 2017; Y. P. Gerasimenko et al., 2015; Hofstoetter et al., 2015; Minassian et al., 2016). Transcutaneous spinal stimulation on the lumbosacral spinal cord activates similar spinal circuits as epidural stimulation (Hofstoetter et al., 2018; Sayenko et al., 2015). Additionally, recent studies revealed immediate improvements of blood pressure control and bladder function with transcutaneous spinal stimulation (P. N. Gad et al., 2018; Kreydin et al., 2020; Phillips et al., 2018).

Activation of afferent inputs via epidural or transcutaneous spinal stimulation paired with intensive training appears to promote neuroplasticity beyond conventional therapies, thus qualifying as a method of neuromodulation (Krames et al., 2009). The mechanism of electrical neuromodulation of the spinal cord remain unknown (Edgerton & Roy, 2012; Taccola et al., 2018). To date, we have very limited evidence of the immediate and long-term effects of transcutaneous spinal stimulation paired with locomotor training on locomotor and autonomic function compared to the effect of locomotor training only.

Previously, we provided evidence that cervical transcutaneous spinal stimulation paired with intensive training of the hand and arm promotes immediate and sustained improvements of upper extremity function in people with cervical SCI (Inanici et al., 2020; F. Inanici et al., 2018). Besides

the beneficial effects on upper extremity function, cervical transcutaneous spinal stimulation induced improvements in lower extremity function in four out of seven participants. Thus, we postulate that cervical transcutaneous spinal stimulation may contribute to locomotor recovery in people with incomplete cervical SCI. Correspondingly, there is emerging evidence for the importance of connections between cervical and lumbosacral spinal cord through propriospinal interneurons for locomotor function following neurological injury (Barss et al., 2020; Islam et al., 2020; Zhou et al., 2018). Cervical transcutaneous spinal stimulation could amplify the patterned stepping movements induced by lumbosacral transcutaneous spinal stimulation when they are delivered as the same time (Y. Gerasimenko et al., 2016).

In the present study, we evaluated the feasibility and efficacy of stimulating the cervical and lumbosacral spinal cord simultaneously via transcutaneous spinal stimulation during intensive locomotor training on improving physical function. We hypothesized that transcutaneous spinal stimulation paired with intensive locomotor training would provide greater functional gains in locomotor and autonomic function compared to intensive locomotor training only for people with chronic, incomplete cervical SCI.

4.3 METHODS

All study procedures were approved by the University of Washington Institutional Review Board approval (IRB). The study protocol was registered with ClinicalTrials.gov (NCT03026816). Patients with chronic SCI were recruited if they met all inclusion and exclusion criteria (Supplementary Table 1) and provided informed consent prior to enrolling in the study.

4.3.1 *Participants*

We enrolled two participants with incomplete cervical SCI with neurological and mobility challenges (Supplementary Table 2).

Participant 1 (P1) was 64 years of age, had a C4 American Spinal Injury Association Impairment Scale (AIS) D injury after a bodysurfing accident, and was 3.5 years post-injury. At the baseline, P1 was a wheelchair user and required maximum physical assist for daily activities. P1 joined the current study more than one year after completing a transcutaneous spinal stimulation study for upper extremity study (F. Inanici et al., 2018).

Participant 2 (P2) was 64 years of age and experienced a C6 AIS D injury after a mountain bike accident 4.5 years prior. He had not participated in any previous spinal stimulation study. P2 was ambulatory with one forearm crutch and contact guard assist in the community but with a history of frequent falls. During this study, there were no side effects related to transcutaneous spinal stimulation or the rehabilitation protocol. However, P2 had a fall during the second week of the locomotor training only. The incident occurred before any application of the stimulation. The fall caused a repeat nasal bone fracture. P2 rested for 1.5 months, and the study was resumed after receiving clearance from his primary physician and the IRB. There were no further complications. Medications for each participant remained constant throughout the study (P1: 40 mg of Baclofen daily, P2: no medication).

4.3.2 *Experimental Design*

We assigned the two participants in a prospective, two-arm cross-over study to evaluate the effect of transcutaneous spinal stimulation with intensive locomotor training (Figure 1). This study design was conducted to control for and assess the difference between the accumulative effect of locomotor training and the additive effect of transcutaneous spinal stimulation. We began with repeated baseline measurements once per week for four weeks to evaluate each participant's functional variability over time and control for learning effects of the assessments. After this was completed, both participants began the intervention period with four weeks of intensive locomotor training.

For P1, we alternated four-week transcutaneous spinal stimulation with locomotor training followed by four weeks of locomotor training only. For P2, we continued the locomotor training for an additional four weeks, followed by four weeks of transcutaneous spinal stimulation combined with locomotor training. Both participants completed the intervention with four weeks of transcutaneous spinal stimulation with locomotor training (P1: A-B-A-B design, P2: A-A-B-B Design; Figure 1). During the entire intervention phase, we measured walking function via standardized assessments every week. We also conducted gait analysis using 3D motion capture and electrophysiological recordings every month. After completing four months of interventions, both participants returned monthly for repeat functional measurements and gait assessment to evaluate sustained changes of motor and autonomic function for at least two months after the completion of the intervention phase. During the follow-up period, both participants performed instructed home exercise programs. The home exercise programs consisted of 30-minute walking exercise at least three times per week. Physical assistance from caregivers and assistive devices was provided as needed.

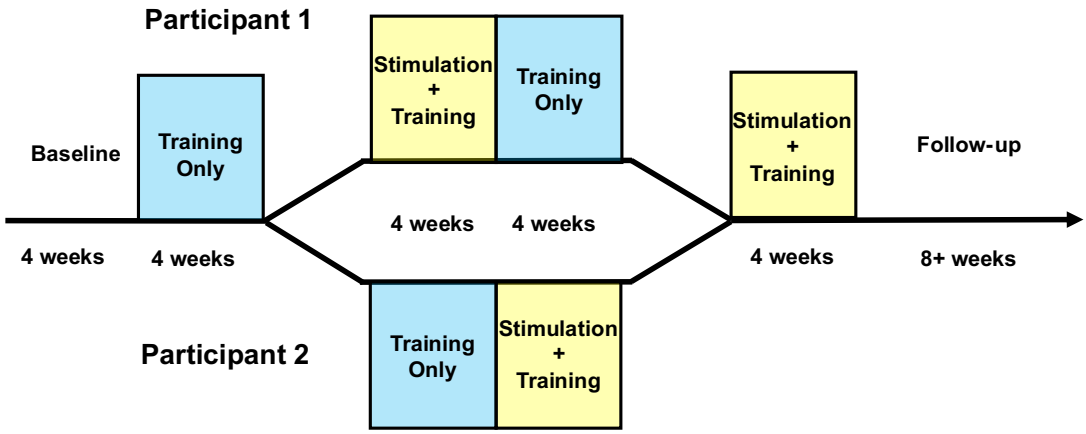

Figure 4-1. Experimental Design and Timeline
Study timeline illustrating the one-month baseline period (left) followed by the four week-intensive locomotor training in both participants. After four weeks of training, Participant 1 (P1) received transcutaneous spinal stimulation with intensive locomotor training for four weeks. Subsequently, P1 repeated the same order of the interventions, with four weeks of training and four weeks of transcutaneous spinal stimulation with training (A-B-A-B design). Participant 2 (P2) continued the intensive locomotor training only for four more weeks. Then, we added the transcutaneous spinal stimulation to the locomotor training for eight weeks (A-A-B-B design). Participants were followed at least two months without any stimulation or supervised exercise to examine sustained benefits from the intervention. Stimulation: Transcutaneous Spinal Stimulation; Training: Locomotor Training;

4.3.3 *Locomotor Training*

Participants received intensive locomotor training, including overground walking and treadmill training with a safety harness (Figure 2A and B). The training focused on high intensity, fast speed, long-distance, and less weight bearing on arms and trunk (Behrman & Harkema, 2000; Hornby et al., 2020). We provided sensorimotor feedback with manual assistance during treadmill training using a robotic body weight supported harness system (KineAssist, Woodway Inc; Figure 2B) (Behrman et al., 2005; S. Harkema et al., 2011; Patton et al., 2008). The training focused on increasing speed and requiring less body-weight support (from 10-26% of body weight) with and without manual assistance.

All training sessions were conducted by experienced physical therapists for 1.5 to 2 hours per day, three to four times per week, throughout the protocol. During the stimulation phase,

transcutaneous spinal stimulation was applied during intensive locomotor training. The hours in each intervention protocol were 60 hours in P1 and 62 hours in P2 in the locomotor training phase, and 62 hours in P1 and 64 hours in P2 in the transcutaneous spinal stimulation with locomotor training.

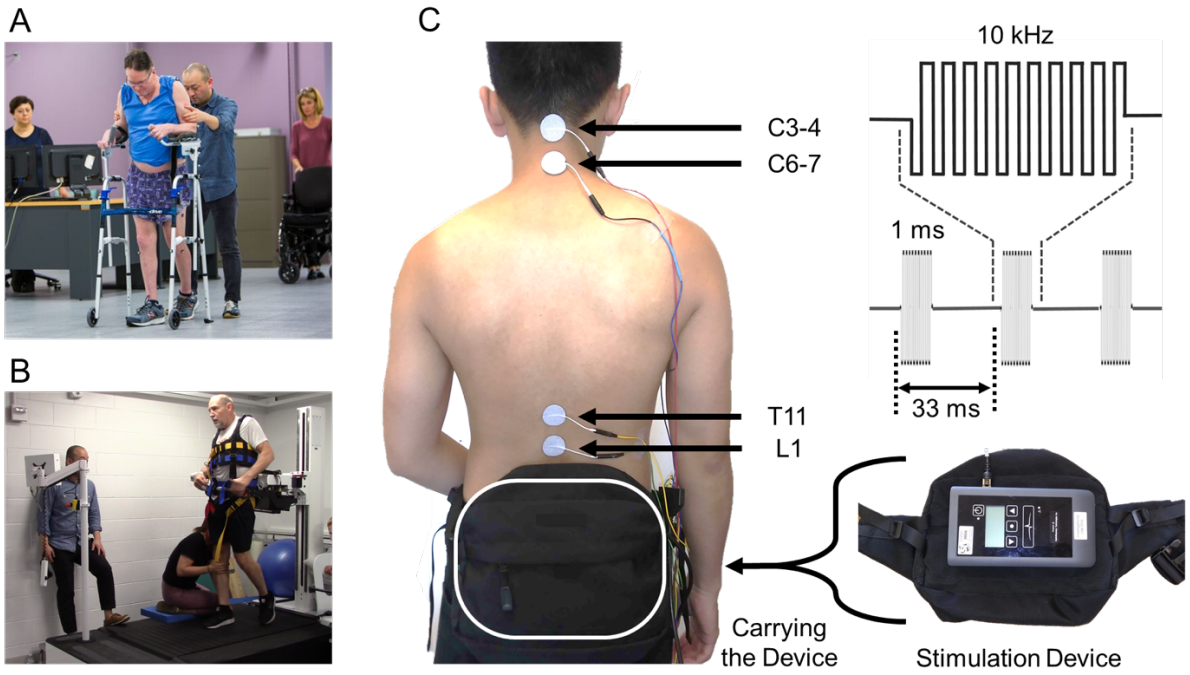

Figure 4-2. Combined Cervical and Lumbosacral Transcutaneous Spinal Stimulation with Intensive Locomotor Training
A: Intensive overground locomotion training with platform walker. **B:** Intensive treadmill training with bodyweight support and manual assistance of leg swing at maximum treadmill speed. **C:** Electrode locations: four cathode electrodes were placed above and below the cervical spinal cord injury (C3-4, C6-7) and at the T11 and L1 vertebral levels **(Left).** Anodes were placed at bilateral iliac crests. Participants wore a fanny pack to carry the stimulation device during locomotor training. Biphasic, rectangular, 1 ms pulses at a frequency of 30 Hz, filled with a carrier frequency of 10 kHz **(Right Top)**.

4.3.4 *Transcutaneous Spinal Stimulation*

The stimulation waveform used biphasic, rectangular, 1 ms pulses at a frequency of 30 Hz, filled with a carrier frequency of 10 kilohertz (kHz) (P. Gad et al., 2017; Y. P. Gerasimenko et al., 2015) (Figure 2C). The 10 kHz carrier frequency permitted currents over 100 mA without discomfort. Stimulation was delivered via four 2.5 cm round electrodes (Axelgaard, ValuTrode

Cloth) placed midline at C3-4, C6-7, T11 and L1 spinous processes as cathodes. Two 5 × 10 cm rectangular electrodes (Axelgaard, ValuTrode Cloth) were placed symmetrically over the iliac crests as anodes. The transcutaneous spinal stimulator (GTX Medical BV NeuroRecovery Technologies, Inc.) was portable and housed in a small fanny pack to allow intensive locomotor training. Participants received the stimulation for 1.5 to 2 hours per session during the treadmill training and overground training. The current amplitude was set to subthreshold intensity that does not cause visible muscle activation and also determined based on the parameters enabling maximum voluntary movement and maximum treadmill walking speed. The stimulation produced a tingling sensation around the stimulation electrodes. Neither participant, however, reported any paresthesia in the lower limbs, only an occasional warm sensation of the limbs.

4.3.5 *Functional Measures*

We collected the Six-Minute Walk Test (6MWT) for walking endurance as the primary outcome measure every week (van Hedel, Wirz, & Dietz, 2005). As secondary measures, we used the International Standards for Neurological Classification of Spinal Cord Injury (ISNCSCI) to assess neurological motor and sensory function (Kirshblum & Waring, 2014), and Ten-Meter Walk Test (10MWT) to assess walking speed (Rossier & Wade, 2001). Muscle spasticity was assessed for the bilateral lower extremities using the Modified Ashworth Scale (MAS) in a supine position (Bohannon & Smith, 1987; Sangari, Lundell, Kirshblum, & Perez, 2019). The MAS was performed in knee flexion, knee extension, and ankle plantar dorsiflexion with the knee in an extended position. We used a scale of 0-4 for each movement with a 1+ converted into a score of 1.5. Then the MAS scores were summed across the three movements per leg, with a maximum score of 24 per subject. Walking Index for SCI II (WISCI) (Burns, Delparte, Patrick, Marino, & Ditunno, 2011; Dittuno & Ditunno, 2001) and Berg Balance Scale (BBS) (Wirz,

Müller, & Bastiaenen, 2010) complemented the assessment of mobility level at baseline and post-intervention. All functional assessments were conducted by experienced physical therapists, and ISNCSCI examination was performed by a neurosurgeon. All functional measures were recorded without any stimulation to eliminate the effects of ongoing stimulation.

4.3.6 *Motion Capture*

Three-dimensional motion capture data were captured at 120 Hz using a 10-camera Qualysis system over an 8-meter walkway (Qualysis AB, Gothenberg, Sweden). Subjects performed more than five gait trials at a self-selected speed in each session with rest breaks provided as needed. We employed a lower extremity modified conventional gait model (CGM) to determine marker placement (Kadaba, Ramakrishnan, & Wootten, 1990). The kinematic and spatiotemporal data were analyzed using custom MATLAB scripts (MathWorks, Inc., Natick, MA). The kinematic data were processed in OpenSim, an open-source musculoskeletal modeling and dynamic simulation program (Delp et al., 2007). A gait cycle was defined as the period from heel-strike (HS) to subsequent ground contact in the same limb. Toe-off (TO) events were used to define the transition between the swing phase and the stance phase. These events were extracted based on the velocity of the toe marker.

Based on the extracted phase timestamps of HS and TO, the data were normalized to percent gait cycle, HS to next HS by linear interpolation with at least five gait cycles per leg. Selected spatiotemporal parameters included stride length, step length, cadence, and step time. Interlimb coordination was assessed using a published method (Thibaudier, Tan, Peters, & Trumbower, 2020). Spatial interlimb coordination was quantified as spatial asymmetry index (spatial AI): the mean difference between left and right step length divided by the mean sum of left and right step

length. Temporal interlimb coordination was quantified as temporal asymmetry index (temporal AI): the mean difference between the left and right step time divided by the mean sum of left and right step time. Spatial and temporal AIs were computed in all steps in each session. The range of these index values is between 0 and 1, where lower scores reflect more symmetric gait.

From the video kinematics, we computed the excursion of the hip, knee and ankle joints in the sagittal plane during the entire gait cycle. Angular component of coefficient of correspondence (ACC) was computed for the hip-knee cyclogram consistency using circular statistics (Tepavac & Field-Fote, 2001). The ACC is a vector coding method computing the average coefficient of correspondence of the hip-knee cyclogram in all steps in each session with a range of 0-1: 0 means no correspondence across cycles, and 1 means all cycles are identical (Awai, Bolliger, Ferguson, Courtine, & Curt, 2016; E. C. Field-Fote & Tepavac, 2002). The toe markers were used to quantify the variability of toe trajectory with the normalized toe tolerance area. The toe trajectory in each swing phase was divided into 10% increments of the horizontal excursion, normalized to the overall toe length of the swing trajectory. We then computed the two-dimensional 95% tolerance ellipses of the data points in each 10% increment of the swing trajectory (Grasso et al., 2004; Ivanenko, Grasso, Macellari, & Lacquaniti, 2002). The normalized toe tolerance area was computed as the mean area of all ellipses.

4.3.7 *Electrophysiological Assessments*

To assess the state of sensory pathways, we measured the somatosensory evoked potentials (SSEP) using a Cadwell Sierra Summit system (Cadwell Industries, Inc., Kennewick, WA). We stimulated the tibial nerve at the posterior side of medial malleolus and recorded cortical potentials between Cz' (2 cm posterior to Cz) and Fz (Curt, Van Hedel, Klaus, & Dietz, 2008).

We began stimulation using an intensity just below the sensory threshold and increased the amplitude by 5 mA steps to 40 mA and repeated the recordings to recruit all sensory nerves. P40 latencies from an average of 300 responses were extracted for each stimulation intensity.

To assess the difference between the transcutaneous spinal stimulation with and without 10 kHz carrier frequency, we measured the spinal motor evoked potentials (sMEPs) evoked by the following waveforms. The first waveform was a charge-balanced monophasic 1 ms pulse width waveform without carrier frequency (Supplementary Figure 5A Top). The second waveform was identical to the first except for the addition of the10 kHz carrier frequency (Supplementary Figure 5A bottom). Here we used the monophasic waveforms due to a technical limitation of the stimulator used for the training that could not produce an equal rectangular biphasic waveform with and without the 10 kHz carrier frequency.

We delivered each of the two waveforms at 2 Hz and increased current in 10 mA step up to the tolerable level. We collected at least twenty responses for each current amplitude and waveform pair. This assessment was performed in four participants with cervical SCI, including P1 and P2. We had two additional participants for the sMEP cross-sectional study only. This included a 44 years old male with C5 AIS D (LEMS: Right 19 and Left 40), 13 years since injury and a 42 years old male with C7 AIS D (LEMS: Right 34 and Left 43), 26 years since injury. We used the surface electromyogram (EMG) signals from soleus muscles on each side through the Delsys Trigno EMG wireless system (10-1000Hz; Delsys, Boston, MA). The EMG data were recorded at 1.1-1.9 kHz and pre-amplified with a gain of 1000x.

The sMEP data were analyzed offline through custom MATLAB scripts. The soleus EMG signals were filtered using a 4th order Butterworth filter with a band-pass of 30-500 Hz. A single

trial of the response was defined as an evoked response during the 5-30 ms time window after each stimulation event. Subsequently, we processed the EMG signals to compute the maximum peak to peak amplitude. The raw value of each peak to peak amplitude was normalized using the maximum mean amplitude. A Boltzmann sigmoid function was fit to the normalized recruitment curve to compute the maximum activation amplitude for the monophasic pulses with and without the 10 kHz carrier frequency (Supplementary Figure 5B) (M. Klimstra & E. P. Zehr, 2008). We then determined the activation threshold current as 10% of the maximum activation (Capogrosso et al., 2018).

4.3.8 *Bowel and Bladder Assessments*

We quantified changes in bladder and bowel function using validated patient-reported outcome measures; the Neurogenic Bowel Dysfunction Score (NBDS) for bowel function (Krogh, Christensen, Sabroe, & Laurberg, 2006), and the Neurogenic Bladder Symptom Score (NBSS) for bladder function following SCI (Welk et al., 2018; Welk et al., 2014).

4.3.9 *Statistical Methods*

The current study was a preliminary case series. The outcome measures are presented as means ± standard error of mean (SEM) unless otherwise noted. When possible, we provide comparisons with available data such as minimally detectable change (MDC). For the sMEP data with all four paricipants, significance was assessed using the non-parametric Wilcoxon signed-rank test. Differences were considered significant at a p-value < 0.05.

4.4 RESULTS

4.4.1 *Locomotor Recovery and Rehabilitative Effect of Stimulation*

Both participants presented substantial improvement of walking ability during the transcutaneous spinal stimulation phase compared to the locomotor training phase. The improved walking function was maintained at least two months after completing the intervention.

The distance walked for 6 minutes (6MWT) increased more during transcutaneous spinal stimulation than during locomotor training alone in both participants (Figure 3A and B). The average change of the 6MWT distance relative to the baseline in both subjects was approximately three-fold greater after transcutaneous spinal stimulation compared to locomotor training alone (changes in each phase:12.5 m vs. 42.1 m in P1, 48.5 m vs. 129.5 m in P2; Figure 3C). The LEMS paralleled the substantial improvement during the transcutaneous spinal stimulation phase (Supplementary Figure 1C). We also observed a continuous improvement in walking speed measured by the 10MWT throughout the study (changes in each phase: 0.19 m/s vs. 0.14 m/s in P1, 0.52 m/s vs. 0.47 m/s). (Supplementary Figure 1A&B).

Both participants required less assistance during walking and balance measured by WISCI and BBS after the training protocol (Supplementary Table2). P1, notably, improved WISCI from 8 to 14 after all the intervention. Eventually, P1 could walk with a quad-cane and contact guard assist at the completion of the rehabilitation program compared to only able to walk with a platform walker and maximum physical assist at the baseline (Supplementary Figure 2, Supplementary Video 1). Furthermore, P2 improved BBS from 37 to 52, which indicated a transition from high to low fall risk (Wirz et al., 2010). These results indicate substantial benefits from the entire

rehabilitation program since the improvements were largely beyond the MDCs (Burns et al., 2011; Lam, Noonan, & Eng, 2008).

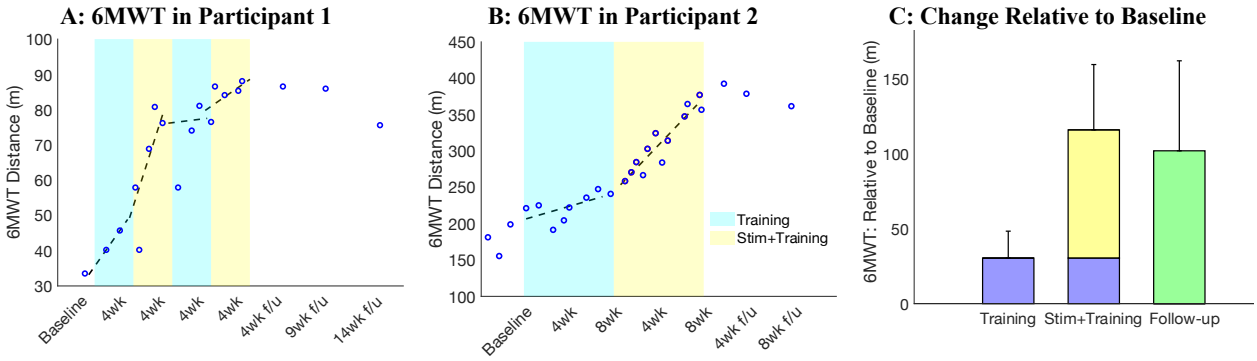

Figure 4-3. Six-Minute Walk Distance

Transcutaneous spinal cord stimulation combined with intensive locomotor training enabled two participants to improve their Six-Minute Walk Test (6MWT) distance 3-fold greater compared to training alone. The improved function was maintained for at least two months after the intervention phases. **A:** Participant 1 (P1) improved waking distance during the first four-week transcutaneous spinal stimulation and maintained this improved function for at least nine weeks without any further skilled intervention. **B:** Participant 2 (P2) demonstrated improvement of the 6MWT distance at a much faster rate during transcutaneous spinal stimulation than locomotor training alone. **C:** The average change of the 6MWT distance relative to the baseline in each intervention phase for P1 and P2 combined. The transcutaneous spinal stimulation with intensive locomotor training increased walking distance approximately three-fold compared to locomotor training alone. Stim: Transcutaneous Spinal Stimulation; Training: Locomotor Training. Empty circles present the individual data point. Dashed lines represent the linear regression during each intervention. Error bars indicate standard error of the mean.

4.4.2 *Spatiotemporal and Kinematic Outcomes*

At baseline, both participants presented with impaired gait patterns in step length, stride length, step time, cadence, and joint excursion. After completing the rehabilitation program, P1 improved all gait parameters except for bilateral step time and cadence. P2 improved all gait parameters except for the right hip joint excursion (Figure 4 and Supplementary Table 3). Almost all of these improvements in gait parameters were maintained throughout the follow-up phase (Figure 4).

A: Participant 1		
Baseline	**Post Stim + LT**	**Follow-up**

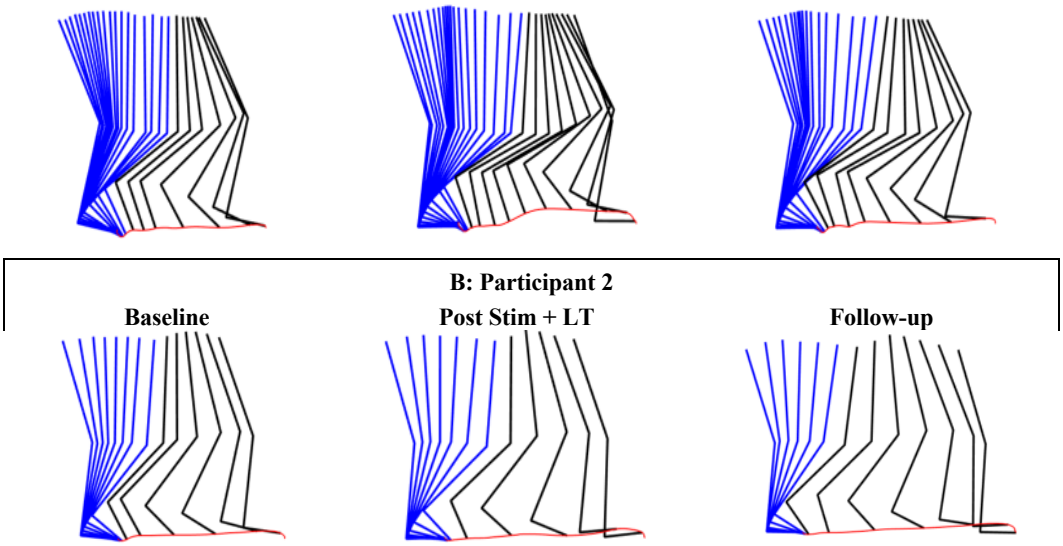

Figure 4-4. Comparisons of Right Leg Stepping Patterns at Baseline, after the Transcutaneous Spinal Stimulation Phase, and in Follow-up
Representative, color-coded stick diagram of average right leg movements during stance (blue), and swing (black) together with successive trajectories per 0.05 sec of the joint endpoint. The red line indicates toe trajectory. **A:** Participant 1 demonstrated improved step length after the transcutaneous spinal stimulation and maintained the improvement throughout follow-up. **B:** Participant 2 improved step length during stimulation, and further improvement during follow-up. Stim: Transcutaneous Spinal Stimulation; LT: Locomotor Training;

We compared kinematic changes resulting from two months of locomotor training only to two months of transcutaneous spinal stimulation with locomotor training. For P1, the locomotor training alone did not change the average stride length in either leg but did improved the spatial AI (Figure 5A blue bars). Conversely, stride length improved during the transcutaneous spinal stimulation phase (Figure 5A yellow bars) likely driven by the observed increase in bilateral hip joint excursion (Supplementary Figure 3). However, the improved hip joint excursion and stride length were not associated with a further improvement of spatial AI (Figure 5A). Locomotor training alone increased step cadence. Conversely, the transcutaneous spinal stimulation phase slowed the cadence; however, P1 improved the temporal AI during the transcutaneous spinal stimulation phase (Figure 5A yellow bars). Adding transcutaneous spinal stimulation to the locomotor training maintained the improved spatial interlimb coordination and contributed to the improvement of the temporal interlimb coordination with longer stride length and slower swing.

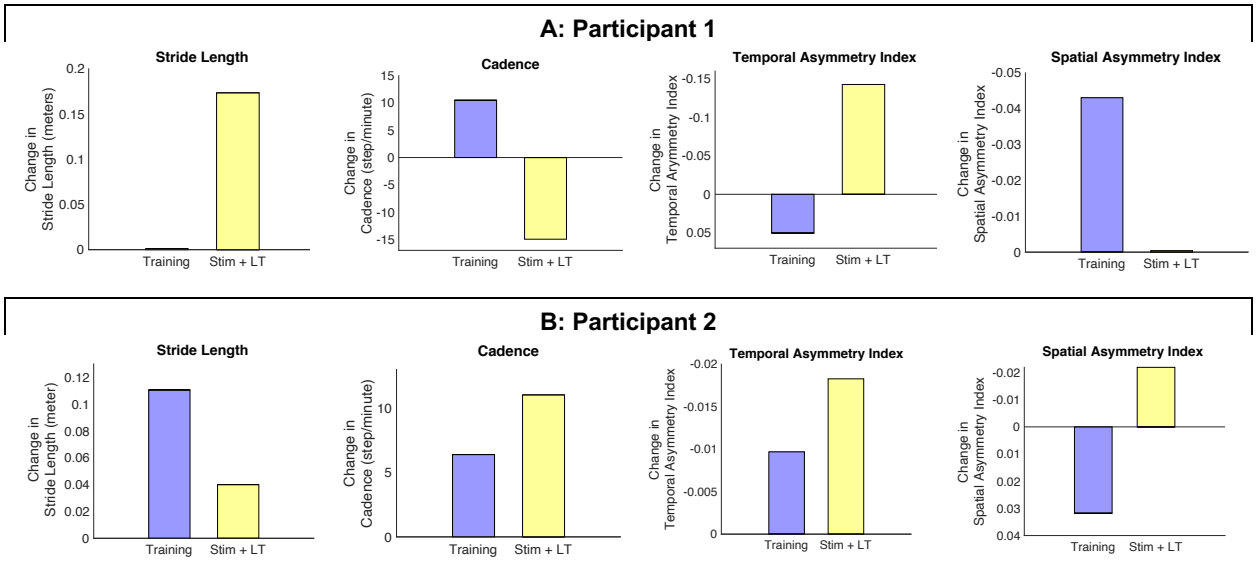

Figure 5. Comparisons of Spatiotemporal Changes between during the Locomotor Training Only Phase and the Transcutaneous Spinal Stimulation Phase.
Changes in gait kinematics during each phase of the intervention. Values of the y-axis for temporal and spatial asymmetry indices are reversed because a negative value indicates improved interlimb coordination. Participant 1 improved stride length temporal interlimb coordination during the transcutaneous spinal stimulation phase **(Top)**. Participant 2 improved cadence and interlimb coordination, especially temporal asymmetry index during the transcutaneous spinal stimulation phase **(Bottom)**. Blue bar: change during the locomotor training only phase. Yellow bar: change during the transcutaneous spinal stimulation phase; Stim: Transcutaneous Spinal Stimulation; LT: Locomotor Training;

Participant 2 responded differently in their spatiotemporal outcomes compared to P1. There was only an increase in the right hip excursion in the locomotor training only phase (Supplementary Figure 3). The changes led to improved temporal AI but declined spatial AI (Figure 5B purple bars). Subsequently, the transcutaneous spinal stimulation phase improved left hip excursion but step length less so (Figure 5B yellow bar). P2 also increased the cadence when comparing the transcutaneous spinal stimulation phase to the locomotor training only phase (Figure 5B). Consequently, the transcutaneous spinal stimulation phase improved both temporal and spatial

AI greater than the locomotor training only phase (Figure 5B yellow bar). During follow-up, P2 maintained his improved stride length and cadence (Supplementary Table 3).

The changes in step length and step time with stimulation turned on and off during the same gait analysis session were inconsistent in both P1 and P2 (Supplementary Figure 4). Lastly, to study intralimb coordination, we used the hip and knee ACC and normalized toe tolerance area in each lower extremity. We found slight improvements in the endpoint variability in P2. However, the rest of the outcomes showed inconsistent responses to the rehabilitation program in both participants (Supplementary Table 4).

4.4.3 *Temporary Improvement of Spasticity with Transcutaneous Spinal Stimulation*

Leg muscle spasticity improved only after transcutaneous spinal stimulation with locomotor training and not after the locomotor training alone. The Modified Ashworth Scale (MAS) sum score was similar or slightly worse with the locomotor training only relative to the baseline (+ 2 in P1 and + 3 in P2; Figure 6A). Less spasticity was observed after transcutaneous spinal stimulation relative to the baseline (-5 in P1 and -2 in P2). The improved muscle tone, however, returned close to the baseline level two months after the intervention. The observation indicated that transcutaneous spinal stimulation could induce a meaningful but temporary improvement of spasticity in the lower extremities.

4.4.4 *Sensory Recovery*

Both participants demonstrated recovery in the sensory system. In P1, we could not differentiate the effect of transcutaneous spinal stimulation and locomotor training since we measured the ISNCSCI after the first transcutaneous spinal stimulation phase and the second stimulation phase. However, P2 demonstrated much greater improvement in the pinprick sensation during

the transcutaneous spinal stimulation phase (+ 13 points) than during the locomotor training only phase (+ 4 points). In both participants, the pinprick sensation showed improvements after the intervention (+ 20 points in P1 and + 17 points in P2; Supplementary Table 2). The light touch sensation showed improvements only in P1.

The P40 latencies of the tibial SSEPs showed a delayed response at baseline in both participants. After the intervention, both participants had shortened latencies closer to normal sensory nerve conduction (2.87 ± 1.25 ms; Figure 6B).

4.4.5 *Recovery of Autonomic Function with Transcutaneous Spinal Stimulation*

Both participants presented clinically meaningful improvements in autonomic function after the transcutaneous spinal stimulation with locomotor training. First, we investigated the effect of the transcutaneous spinal stimulation on bowel function. The changes of the NBDS indicate notable improvement, including the category of the impairment severity from severe to moderate (-4 points) and severe to very minor (-9 points) in P1 and P2, respectively (Figure 6C). Indeed, both participants decreased the bowel management time from 31-60 minutes to less than 30 minutes after completing all intervention. P2 also reported a normalized frequency of bowel movement from 2 to 3 times per week to the daily management that P2 had never achieved since his injury.

At baseline, both participants used intermittent catheterization. The NBSS did not show any apparent change in either participant (Supplementary Table 3). Nevertheless, throughout the protocol, P1 experienced a decrease of the residual urine volume (estimated 125ml to 30ml). Eventually, two months after completing all interventions, P1 could eliminate the use of intermittent catheterization. He continued to manage bladder voiding without intermittent catheterization the next six weeks until the endpoint (Supplementary Table 2). Recently, we

found that P1 maintained his improved bladder management for 1.8 years since he stopped using the intermittent catheterization in the study. He did not suffer from any urinary tract infections since then either. Whereas, P2 experienced no improvement or deteriorative effect on bladder function throughout the intervention and follow-up phases. Lastly, P2 reported that he could find improved tolerance against the cold weather. Anecdotally, he could stay with more comfort and longer time in low-temperature environments during the transcutaneous spinal stimulation phase than before.

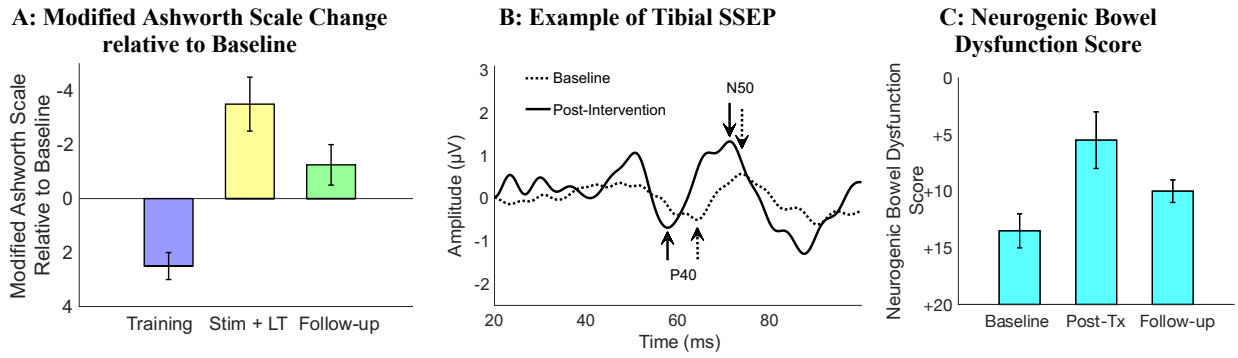

Figure 4-5. Improved Muscle Spasticity, tibial SSEP P40 latency, and Bowel Function after Transcutaneous Spinal Stimulation with Locomotor Training
A: The Modified Ashworth Scale (MAS) sum scores in bilateral knee flexion, knee extension and ankle dorsiflexion improved after the two months of the transcutaneous spinal stimulation with locomotor training. The y-axis is reversed, and upward shows negative values, which indicate decreased spasticity, relative to the baseline value. Stim: Transcutaneous Spinal Stimulation; LT: Locomotor Training. **B:** Example of somatosensory evoked potential (SSEP) with right tibial nerve stimulation at 28 mA during the baseline (dotted line) and at 30mA after all interventions (black line; P1). Arrows present P40 and N50 of tibial SSEP during the corresponding study phases. The tibial SSEPs occur earlier and with larger magnitude after the intervention compared to baseline. **C:** The Neurogenic Bowel Dysfunction Score improved after the two-month locomotor training and the two-month transcutaneous spinal stimulation with locomotor training both in Participant 1 and Participant 2. Higher bars illustrate the lower score which indicate better bowel function. Post-Tx: Post-treatment. c Error bars indicate standard error of the mean.

4.4.6 *Stimulation Parameters*

We quantified how stimulation current changed over the course of the intervention. The required stimulation intensity over lumbosacral electrodes decreased over time to less than a half of

current to induce the maximum walking ability after two months of transcutaneous spinal stimulation (Figure 7 Black empty circles) in both P1 and P2. The stimulation intensity delivered to the cervical electrodes was lower than the lumbosacral stimulation throughout study since the participants reported some disturbance of lower extremity function with a higher current at the cervical level. The cervical level stimulation current also reduced over time in both participants for optimal outcomes (Figure 7 Blue crosses).

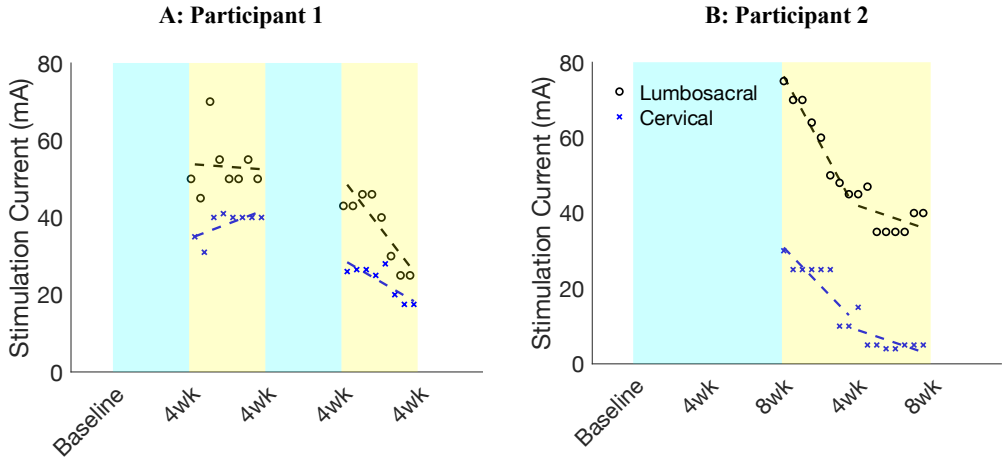

Figure 4-6. Stimulation Current at Spinal Cord
A: Stimulation current change in Participant 1. The y-axis shows the stimulation current of transcutaneous spinal stimulation. Black empty circles and blue crosses show average simulation current intensity of two electrodes at lumbosacral (T11 and L1) and cervical levels (C3-4 and C6-7), respectively. The current was required to achieve the maximum function in training decreased notably during the second month of the transcutaneous spinal stimulation with training. **B:** Participant 2 showed the rapid decrease of the required current intensity achieving the maximum function in the first four weeks. Stim: Transcutaneous Spinal Stimulation; Training: Locomotor Training; Circles and crosses present individual data points. Dashed lines present the linear regression models every month.

Finally, we studied the effect of single pulse transcutaneous spinal stimulation with 10 kHz carrier frequency on leg muscles. First, we tested whether the transcutaneous spinal stimulation with 10 kHz carrier frequency at L1 vertebral level could activate soleus muscle in people with incomplete cervical SCI. The carrier frequency increased the activation threshold relative to the stimulation without carrier frequency (44.3 ± 12.2 mA vs 132.5 ± 16.3 mA, $p = 0.0062$; Supplementary Figure 5D). The induced reflex responses in EMG activity, however, were very

similar between both parameters. This demonstrated that the stimulation with 10 kHz carrier frequency could evoke responses in four subjects with various levels of severity of SCI even though the required current was larger.

4.5 DISCUSSION

In the current study, our preliminary findings suggest that transcutaneous spinal stimulation with intensive locomotor training could lead to greater recovery of walking and autonomic function than performing intensive locomotor training only in people with incomplete cervical SCI. The improved walking distance of the Six-Minute Walk Test (6MWT) during the transcutaneous spinal stimulation phase was three-fold greater compared to locomotor training alone. The locomotor recovery was sustained for at least two months after the intervention and permitted enhanced walking activity in the community. Additionally, both participants demonstrated notable recovery of bowel dysfunction, including normalized bowel management time and frequency. Lastly, Participant 1 (PA) no longer required intermittent catheterization for at least one year after completing the study.

4.5.1 *Walking function*

Prior to this study, P1 presented with severely impaired walking ability, unable to safely walk at home or in the community. Participant 2 (P2) was classified as high fall risk during walking with a forearm crutch, his primary form of mobility. Both participants improved the distance walking in 6 minutes much more during the transcutaneous spinal stimulation than during the locomotor training alone (Figure 3A and B).

After completing the spinal stimulation program, P1 was able to complete walking exercises at home with a caregiver, and P2 had a much lower fall risk. These changes in community walking

were complemented by improved scores in the Walking Index for SCI (WISCI) and Berg Balance Scale (BBS) (Supplementary Table 2). Importantly, both participants maintained the regained function in the follow-up phase. In the results, P1 could participate in daily home walking exercise programs with caregivers after the study to maintain the function. Excitingly, after the intervention, P2 was able to manage the stairs and participate in more challenging activities such as vertical jumping for the first time since injury (Supplementary Video 2).

4.5.2 *Gait Analysis*

The gait parameters suggest that transcutaneous spinal stimulation with locomotor training could improve impaired gait patterns via different mechanisms than simple intensive locomotor training. Gait patterns in people with SCI are impaired often because of spasticity, abnormal synergistic leg movements, and sensory deficits (Scivoletto et al., 2008). Currently, reinforcing compensatory gait strategies through conventional training could promote walking function, but the strategies reach functional plateau about 1.5 years after the onset of the injury (Fawcett et al., 2007). Although high-intensity locomotor training shows some potential for improving leg coordination instead of amplifying the aberrant compensatory strategies (Ardestani et al., 2018; Holleran et al., 2018; Leech, Kinnaird, Holleran, Kahn, & Hornby, 2016), the ability to normalize the impaired gait patterns is still limited with traditional care (Awai & Curt, 2014; Krawetz & Nance, 1996). We characterized leg coordination and kinematic parameters during both transcutaneous spinal stimulation and the locomotor training in both participants.

During locomotor training alone, the spatial interlimb coordination improved but not temporal interlimb coordination in P1. During the transcutaneous spinal stimulation phase, P1 also had a faster improvement of hip joint excursion and stride length, causing an increase in step time.

Regardless of the slower step time, P1 improved the temporal interlimb coordination with transcutaneous spinal stimulation. Similarly, P2 demonstrated substantial improvement of the temporal interlimb coordination with transcutaneous spinal stimulation compared to intensive locomotor training only. Previous studies reported that temporal interlimb coordination may be primarily regulated through the spinal cord, brainstem and subcortical areas (Malone, Bastian, & Torres-Oviedo, 2012). This regulation mechanism may allow the automaticity of the rhythmic gait pattern to be directly affect by transcutaneous spinal stimulation (Y. Gerasimenko et al., 2015). Thus, transcutaneous spinal stimulation paired with intensive activity-based training may enhance control of reciprocal and autogenic inhibitory circuitry in the spinal cord and modulation of subcortical inputs for locomotor recovery (Aiko K. Thompson & Wolpaw, 2019). In stroke, people with better interlimb coordination demonstrated less energy cost for walking (Awad, Palmer, Pohlig, Binder-Macleod, & Reisman, 2015). Thus, the improved interlimb coordination and walking distance in six minutes in people with chronic SCI suggest that one benefit of transcutaneous stimulation may be an improvement of energy-efficiency in walking.

Meanwhile, the changes of intralimb coordination measured by the angular component of the coefficient of correspondence (ACC) and end-point variability (Sohn 2018, Holleran 2018) were inconsistent among participants (Supplementary Table 4). The improved *interl*imb coordination but not *intra*limb coordination in people with chronic injury in this study does not follow the previous report that the neural recovery of SCI was attributed by the intralimb coordination when the locomotor recovery in the acute to subacute stage (Awai et al., 2016). Both intralimb and interlimb coordination, meanwhile, are likely influenced by the presence or absence of an assistive device while walking (Thibaudier et al., 2020). P1 used a platform walker during motion capture sessions, which may influence the regression in spatial component of walking

patterns. Lastly, although we investigated the movement in the sagittal plane in this study, prior studies have presented the effect of lateral stabilization on the metabolic cost during walking as well (Matsubara, Wu, & Gordon, 2015). Further research is needed to investigate an association of neural recovery with limb coordination through biomechanical analyses.

4.5.3 *Spasticity*

The results of the MAS sum scores revealed a potential anti-spastic effect of the transcutaneous spinal stimulation with locomotor training (Figure 5A). Spasticity is commonly understood as increased reflex excitability from Ia afferent input with presynaptic and postsynaptic regulatory mechanisms following a disrupted communication between the brain and spinal cord (Adams & Hicks, 2005). The standard care for spasticity includes exercise, antispasmodic medications, and surgical interventions such as rhizotomy and tendonectomy (Elbasiouny, Moroz, Bakr, & Mushahwar, 2010). However, there is no agreed upon clinical intervention. In the early phase of the epidural stimulation research in the 1980s, studies reported a reduction of spasticity via epidural stimulation following SCI (R. R. Richardson, Siqueira, et al., 1979; Siegfried et al., 1981). Although the mechanisms of spinal circuitry have been studied in animal models (Bennett et al., 1999; Simpson, Robertson, & Goodman, 1993), the clinical efficacy of epidural stimulation on spasticity is not well understood. Conversely, transcutaneous spinal stimulation demonstrated immediate but temporary reduction of spasticity (Estes, Iddings, & Field-Fote, 2017; Hofstoetter et al., 2020; Hofstoetter et al., 2014). A recent study (Hofstoetter et al., 2020) illustrated the effect of repetitive transcutaneous spinal stimulation on leg spasticity. Our stimulation intervention led to a similar temporary improvement in spasticity as observed previously, despite several differences between the current study and the previous studies in stimulation parameters and the use of activity-based therapy (Barolat et al., 1986; Hofstoetter et

al., 2020; Pinter et al., 2000). With consistent outcomes now observed across several different research groups, further may lead to an effective program using transcutaneous spinal stimulation to durably improve spasticity.

4.5.4 *Sensory improvement*

Our findings demonstrate modest sensory recovery due to stimulation treatment. The tibial SSEPs to assess dorsal column-medial lemniscus pathways revealed shorter P40 latencies after completing the stimulation program compared to the baseline in both participants. The parameters of the tibial SSEPs may reflect the functional status in SCI (Spiess, Schubert, Kliesch, & Halder, 2008), and shortened latencies suggest an improved somatosensory transmission function. Similar latency changes were reported in a previous study using epidural stimulation and intensive training (Angeli et al., 2014). The potential improvement of the proprioception pathways could contribute to the faster rate of locomotor recovery reflected in the walking distance here and in previous work (Chisholm, Qaiser, Williams, Eginyan, & Lam, 2019). Notably, the ISNCSCI revealed consistent improvements of pinprick sensation in both participants. We found that P2 showed a much greater improvement of pinprick sensation during transcutaneous spinal stimulation than during locomotor training alone. Another case report shared a similar change of pinprick sensation with the transcutaneous spinal stimulation with locomotor training in a person with cervical SCI (M. Alam et al., 2020). Further investigation is required to uncover the promising effect of transcutaneous spinal stimulation on sensory function.

4.5.5 *Autonomic function*

In the current study, we found that transcutaneous spinal stimulation combined with locomotor training improved bowel and bladder function. The disruption of the signal transmissions in the spinal control in someone with SCI causes bowel dysfunction leading to a severe decline in quality of life (Glickman & Kamm, 1996). Decreased bowel motility is due to the impaired parasympathetic and sympathetic control that can induce a loss of voluntary evacuation, constipation, as well as increased time and assistance needed for the bowel management. The disruption of the centralized micturition spinal reflex causes involuntary reflex of detrusor and detrusor-sphincter dyssynergia leading to damaging urinary tracts, loss of continence, or incontinence (de Groat et al., 2015; Taweel & Seyam, 2015). Most importantly, this dysfunction is a significant cause of urological complications in someone with a spinal cord injury (Wyndaele, 2016). Efficacy of current available conservative and invasive approaches for both bowel and bladder function do no provide satisfying outcomes for people with SCI (Sanders et al., 2011).

In the present study, both participants had improved bowel function after the stimulation (Figure 5C). In addition, this normalized time and frequency of bowel management were sustained at least two months without any intervention in P2. Previous research has shown that 80 intensive locomotor training sessions could decrease the bowel management time (Hubscher et al., 2018); however, there is no report on the efficacy of transcutaneous spinal stimulation for bowel function. Future studies will benefit from a larger sample size a more comprehensive questionnaire (Krogh, Perkash, Stiens, & Biering-Sørensen, 2009), as well as an assessment of the abdominal muscle function to better understand the mechanisms of bowel function that changed (Korsten et al., 2004).

With respect to the bladder function, we observed inconsistent changes of the NBSS in both participants. Nevertheless, P1 was able to eliminate the use of intermittent catheterization after the transcutaneous spinal stimulation phase and continued to manage his bladder without intermittent catheterization for 1.8 years after the completion of the intervention. Since then, he also had no urinary tract infection, which significantly improved his quality of life (Theisen et al., 2020). Similarly, a recent study tested multiple sessions of transcutaneous spinal stimulation for lower urinary tract dysfunction in several neurological conditions including five subjects with SCI (Kreydin et al., 2020). They observed improved continence by restoring detrusor function and sensation. Several reports demonstrate that the modulation of the spinal excitability through epidural stimulation could improve the bladder management time, detrusor-sphincter synergy and voiding function (S. Harkema et al., 2011; Herrity et al., 2018; Schieferdecker et al., 2019; Walter et al., 2018). Overall, our findings provide additional evidence of the possible efficacy of transcutaneous spinal stimulation with locomotor training to durably improve bowel and bladder function.

Thermoregulation dysfunction is also predominant in people with SCI. In cold weather, people with SCI commonly have impaired temperature tolerance (Handrakis et al., 2017). Cervical SCI alters cardiovascular function, energy expenditure, and sweating that affects temperature regulation (Mneimneh et al., 2019; Wecht et al., 2015). However, the mechanisms and effective interventions for treating thermoregulation dysfunction remain unclear (A. V. Krassioukov et al., 2007). In the current study, P2 reported impaired tolerance for low temperatures since his injury. After stimulation, he reported that he could tolerate much longer time in low temperature environment throughout the transcutaneous spinal stimulation intervention compared to pre-intervention period. In the previous study, our group also observed a similar improvement in

thermoregulation in another subject with cervical SCI (Inanici et al., 2020). Based on these observations, transcutaneous spinal stimulation with intensive exercise may be beneficial to restore thermoregulation. Further studies are needed to explore this area and also include additional objective measures.

4.5.6 *Neurological Mechanisms of Spinal Stimulation*

The precise mechanisms of transcutaneous spinal stimulation that lead to functional recovery are unknown. Related epidural stimulation recruits myelinated afferent axons, activating spinal interneurons and efferent pathways via monosynaptic and polysynaptic circuits (Capogrosso et al., 2013; Moraud et al., 2016). Transcutaneous spinal stimulation likely activates similar pathways, including posterior roots (Hofstoetter et al., 2018; Ladenbauer et al., 2010). To induce an immediate response, the afferent pathways activated with subthreshold spinal stimulation may modulate the physiological state of the spinal circuitry, allowing residual descending inputs from the brain and proprioceptive information to bypass the lesion (Chakraborty, Truong, Bikson, & Kaphzan, 2018; Jankowska, 2017; Taccola et al., 2018). Repetitive spinal stimulation and activity-based training may reactivate spared connections in the lesion to allow for the reorganization of neural circuits (G. Courtine et al., 2008; Flynn, Graham, Galea, & Callister, 2011) and facilitate neuroplasticity through spike-timing dependent plasticity (Jo & Perez, 2020; Urbin, Ozdemir, Tazoe, & Perez, 2017). Consequently, the long-term applications of spinal stimulation with activity-based training can strengthen the functional connections between the brain and spinal cord, as shown in animal models (Asboth et al., 2018; van den Brand et al., 2012).

In addition to benefits when the stimulation was active, we also observed carry-over effects on walking and autonomic function that persisted long after the stimulation and rehabilitation protocol. As performance improved with the transcutaneous spinal stimulation and locomotor training, the intensity of the stimulation current required to activate the spinal networks was reduced in both participants (Figure 7). This observation parallels the decreased level of stimulation for subsequent standing training sessions in a prior study (Sayenko et al., 2019). Moreover, we observed improved walking function several months after the rehabilitation program ended in both subjects. These findings open a possibility that the known mechanisms of motor and autonomic recovery seen in epidural stimulation may be similar to the mechanisms of in non-invasive stimulation.

The single pulse charge-balanced monophasic stimulation with 10 kHz carrier frequency increased activation threshold (Supplementary Figure 5) but led to very similar responses compared to stimulation without the carrier frequency in people with SCI. Both participants noted no discomfort over the course of the training with the transcutaneous spinal stimulation. Our use of a 10 kHz carrier frequency minimizes sensory discomfort and maximally activates the targeted nervous system compared to higher or lower carrier frequencies or without a carrier frequency (Ward & Robertson, 1998). Together, there is a potential that we can amplify the effect of transcutaneous spinal stimulation by designing stimulation protocols for targeted activation (Wagner et al., 2018).

4.5.7 *Limitations*

Here we show a safe application of simultaneous cervical and lumbosacral spinal stimulation paired with locomotor training in two participants without any adverse effects. The optimal

parameters of transcutaneous spinal stimulation for restoring physical function remain undetermined. We delivered a continuous 30 Hz biphasic 1 ms pulse width with 10 kHz carrier frequency with the motor subthreshold current on C3-4, C6-7, T11 and L1 spinous processes in our stimulation protocol. The parameters were determined based on the previous studies (Y. P. Gerasimenko et al., 2015; Hofstoetter et al., 2015; Minassian et al., 2016). However, optimizations of parameters and electrode locations have not been rigorously investigated.

We did not perform a direct comparison of the efficacy of lumbosacral spinal stimulation (M. Alam et al., 2020; P. Gad et al., 2017; Y. P. Gerasimenko et al., 2015; Hofstoetter et al., 2015; Minassian et al., 2016) versus combined cervical and lumbosacral spinal stimulation in the present study. Several studies highlight the importance of cervicolumbar pathways for lower extremity function via arm exercise and the cervical spinal stimulation in SCI and stroke (Barss et al., 2020; Islam et al., 2020; Klarner et al., 2016; Zehr et al., 2016). We need further studies to test whether the cervical transcutaneous spinal stimulation can amplify the effect of the lumbosacral transcutaneous spinal stimulation on lower extremity function.

The population with SCI is heterogeneous. We have not employed this rehabilitation paradigm in people with motor-complete SCI. Our previous study of the cervical transcutaneous spinal stimulation demonstrated its efficacy for upper limb motor function such as pinch force in people with motor-complete cervical SCI (Inanici et al., 2020; F. Inanici et al., 2018). The majority of subjects who show clinically motor complete SCI are considered to have some descending input below the injury (Kakulas & Kaelan, 2015; Sherwood, Dimitrijevic, & McKay, 1992), so they may be able to leverage those residual pathways to restore function through this non-invasive strategy. In future research, it is important to investigate responders and non-responders to this intervention to understand its potential impact.

There are a limited number of spinal stimulation studies for the recovery of physical function in people with cervical SCI (M. Alam et al., 2020; Angeli et al., 2018; Hofstoetter et al., 2015; Wagner et al., 2018). Furthermore, the age range in these studies is often limited, for example from 23 to 48 years old. The current study tested the efficacy of non-invasive spinal stimulation in two 64 years old males with incomplete cervical SCI. Although this initial report contains a limited sample size and injury severity range, the findings in older people with SCI expands previous studies by providing evidence of transcutaneous spinal stimulation for the aging population.

4.6 CONCLUSION

Our findings suggest a potential of a novel rehabilitation strategy using transcutaneous spinal stimulation for walking function after cervical SCI. Two participants with incomplete tetraplegia experienced a sustained increase in walking ability in daily living after the rehabilitation program. Furthermore, the transcutaneous spinal stimulation with intensive exercise induced substantial improvement of autonomic function, including bowel and bladder function.

4.7 SUPPLEMENTARY INFORMATION

Table 4-1. Supplementary Table 1. Inclusion and Exclusion Criteria

Inclusion Criteria • spinal cord injury (T12 or higher) at least 1-year duration • 21 and 70 years of age • Having difficulty with leg functions and mobility in activities of daily living • stable medical condition without cardiopulmonary disease or frequent autonomic dysreflexia • capable of performing simple cued motor tasks • cleared for gait training by primary physician of the subject
Exclusion Criteria • autoimmune etiology of spinal cord dysfunction/injury • history of additional neurologic disease, such as stroke, MS, traumatic brain injury • peripheral neuropathy • rheumatic diseases • significant medical disease; including uncontrolled systemic hypertension with values above 170/100 mmHg; cardiac or pulmonary disease; uncorrected coagulation abnormalities or need for therapeutic anticoagulation. • active cancer • cardiovascular or musculoskeletal disease or injury that would prevent full participation in physical therapy intervention • unhealed fracture, contracture, pressure sore, or urinary tract infection or other illnesses • pregnant • dependent on ventilation support • implanted stimulator • depression or anxiety based on the Center for Epidemiologic Studies Depression Scale (CESD) (score >16/60) and General Anxiety Disorder-7 item Questionnaire (score >9/21), respectively. • alcohol and/or drug abuse. • cognitive impairment based on Short Portable Mental Status Questionnaire (SPMSQ) (score >2/10). • unable to read and/or comprehend the consent form. • unable to understand the instructions given as part of the study • established osteoporosis and taking medication for osteoporosis treatment. • has bone mineral density T scores ≤ -3.5 in anteroposterior lumbar spine, proximal femur, distal femur and/or proximal tibia measured by DEXA • low-energy fracture history before or after spinal cord injury • History of orthopedic surgery in lower extremities that may be a confounding factor for interpretation of the results (such as tendon transfer, tendon or muscle lengthening for spasticity management, arthrodesis, etc.) • fixed lower extremity joint contractures • is taller than 80 inches and/or more than 350 pounds body weights, due to the limitation of the experimental equipment such as the body weight support system • history of severe allergy (i.e. allergic reaction that could not be treated with antihistaminic medication) • malabsorption syndrome, primary hyperthyroidism, and/or hypogonadism

Table 4-2. Supplementary Table 2. Neurological and Functional Characteristics

	Participant 1 64 years old Male 3.5 years since Injury		**Participant 2** 64 years old Male 4 years since Injury	
	Baseline	Post-Intervention	Baseline	Post-Intervention
NLI & AIS	C4 AIS D	C4 AIS D	C6 AIS D	C6 AIS D
UEMS (R | L)	13 | 21	**16 | 22**	22 | 25	22 | 25
LEMS (R | L)	19 | 19	**21** | **25**	21 | 25	**23** | 25
Light Touch (R | L)	33 | 32	31 | **34**	48 | 47	**52** | **52**
Pinprick (R | L)	18 | 23	**25** | **36**	40 | 41	**49** | **49**
Tibial SSEP P40 Latency (R Stim| L Stim) (ms)	64.06 | 60.47	**57.50** | **58.28**	55.63 | 54.53	**54.22** | **53.21**
WISCI II	8	**14**	17	**19**
Berg Balance Scale	2	**9**	37	**52**

Both participants improved lower extremity muscle strength and sensation, tibial SSEP conduction velocity, and walking and balance ability. Bold values mark improvements; NLI: Neurologic Level of Injury; AIS: American Spinal Cord Injury Impairment Scale; UEMS: Upper Extremity Motor Score; LEMS: Lower Extremity Motor Score; R | L: Right | Left; Tibial SSEP: Tibial nerve stimulation induced Somatosensory Evoked Potential recorded at Cz'-Fz; ms: milliseconds; WISCI II: Walking Index for Spinal Cord Injury II;

Table 4-3. Supplementary Table 3. Spatiotemporal and Kinematic Outcomes at Baseline and after All Intervention

		Participant 1			Participant 2		
	Unit	Baseline	Post-Intervention	Week 14 Follow-Up	Baseline	Post-Intervention	Week 8 Follow-Up
Step Length (R)	m	0.49 ± 0.01	**0.57 ± 0.01**	**0.53± 0.01**	0.55 ± 0.01	**0.62 ± 0.01**	**0.71 ± 0.01**
Step Length (L)	m	0.37 ± 0.04	**0.46 ± 0.02**	**0.42 ± 0.01**	0.56 ± 0.01	**0.63 ± 0.01**	**0.72 ± 0.01**
Stride Length	m	0.86 ± 0.03	**1.03 ± 0.02**	**0.94 ± 0.02**	1.10 ± 0.01	**1.25 ± 0.01**	**1.43 ± 0.01**
Step Time (R)	sec	2.15 ± 0.23	**2.06 ± 0.07**	**2.06 ± 0.12**	0.69 ± 0.01	**0.59 ± 0.01**	**0.61 ± 0.01**
Step Time (L)	sec	1.27 ± 0.07	1.48 ± 0.04	1.42 ± 0.05	0.73 ± 0.01	**0.59 ± 0.01**	**0.65 ± 0.01**
Cadence	step/min	39.0 ± 3.2	34.5 ± 1.4	36.5 ± 1.9	84.9 ± 0.9	**102.3 ± 0.7**	**96.5 ± 1.0**
Hip Joint Excursion (R)	deg	44.4 ± 1.7	**45.9 ± 1.2**	40.9 ± 1.2	27.5 ± 0.5	**29.5 ± 0.2**	**34.9 ± 0.5**
Hip Joint Excursion (L)	deg	47.3 ± 0.9	**54.6 ± 1.8**	**50.1 ± 0.8**	44.1 ± 0.4	**47.4 ± 0.5**	**50.6 ± 0.5**
Knee Joint Excursion (R)	deg	59.4 ± 1.8	**65.9 ± 1.1**	56.3 ± 1.3	33.1 ± 0.8	**35.2 ± 0.7**	**36.5 ± 0.6**
Knee Joint Excursion (L)	deg	57.6 ± 2.4	**71.3 ± 1.6**	**62.0 ± 1.5**	43.4 ± 1.0	42.4 ± 1.1	43.3 ± 0.9
Ankle Joint Excursion (R)	deg	16.2 ± 1.7	**21.1 ± 1.1**	15.4 ± 1.0	29.7 ± 0.6	**38.5 ± 0.9**	**44.9 ± 0.4**
Ankle Joint Excursion (L)	deg	30.2 ± 3.4	**37.1 ± 1.9**	**34.8 ± 1.5**	23.5 ± 1.0	**30.7 ± 0.5**	**40.0 ± 0.8**
Spatial AI	a.u.	0.143	**0.099**	**0.109**	0.005	0.015	0.007
Temporal AI	a.u.	0.255	**0.163**	**0.182**	0.030	**0.002**	0.035

Means ± Standard Error of Mean. Bold values mark improvements after completing the rehabilitation program and improved or sustained parameter during the follow-up compared to the baseline measures. R: Right; L: Left; AI: Asymmetry Index; ACC: Angular component of coefficient of correspondence, Toe Tolerance Area: Normalized Toe Tolerance Area; a.u.: artificial unit;

Table 4-4. Supplementary Table 4. Intralimb Coordination and Endpoint Variability at Baseline and after All Intervention

		Participant 1			Participant 2		
	Unit	Baseline	Post-Intervention	Week 14 Follow-Up	Baseline	Post-Intervention	Week 8 Follow-Up
Hip-Knee ACC (R)	a.u.	0.84 ± 0.01	0.81 ± 0.01	0.80 ± 0.01	0.90 ± 0.01	**0.93 ± 0.01**	0.90 ± 0.01
Hip-Knee ACC (L)	a.u.	0.83 ± 0.01	**0.88 ± 0.01**	0.82 ± 0.01	0.95 ± 0.00	0.95 ± 0.00	0.93 ± 0.01
Toe Tolerance Area (R)	cm^2/cm	0.06 ± 0.00	**0.05 ± 0.00**	**0.02 ± 0.00**	0.04 ± 0.00	**0.03 ± 0.00**	**0.03 ± 0.00**
Toe Tolerance Area (L)	cm^2/cm	0.08 ± 0.00	0.10 ± 0.00	**0.05 ± 0.00**	0.04 ± 0.00	**0.03 ± 0.00**	0.04 ± 0.00

Means ± Standard Error of Mean. Bold values show improved parameters after all the intervention and improved or sustained parameters during the follow-up compared to the baseline measures. R: Right; L: Left; AI: Asymmetry Index; ACC: Angular component of coefficient of correspondence, Toe Tolerance Area: Normalized Toe Tolerance Area;

Table 4-5. Supplementary Table 5. Bladder and Bowel Patient-Reported Outcome Measure

	Participant 1				Participant 2			
	Baseline	Post-1st Stim+LT	Post-2nd Stim+LT	Week 14 Follow-up	Baseline	Post-2-month LT only	Post-2-month Stim+LT	Week 16 Follow-up
NBSS								
Incontinence	8	0	7	0	0	0	0	0
Storage & Voiding	7	6	11	7	3	5	5	3
Consequences	9	4	5	4	4	1	0	0
QOL	3	2	3	2	1	1	2	1
Bladder Management Method	Intermittent catheterization by a caregiver during daytime & condom catheter at night			**Condom catheter at night only**	Intermittent self-catheterization			
NBDS	15	13	**8**	**11**	12	14	**3**	**9**
Reported Defecation Frequency and Time	Self-manage 31-60 min x 7 per week		**Self-manage less than 30 min x 7 per week**	Self-manage 31-60 min x 7 per week	Self-manage 31-60 min x 2-3 per week		**Self-manage less than 30 min x 6-7 per week**	

Bold values show improved parameters after all the intervention and improved or sustained parameters during the follow-up compared to the baseline measures. NBSS: Neurogenic Bladder Symptom Score; NBDS: Neurogenic Bowel Dysfunction Score; QOL: Quality of Life; Stim: Transcutaneous Spinal Stimulation; LT: Locomotor Training

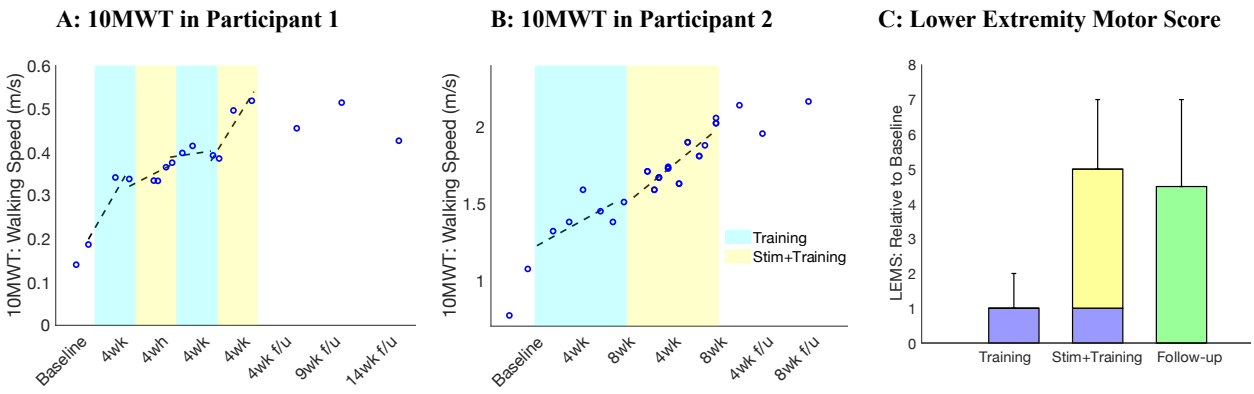

Figure 4-7. Supplementary Figure 1. Increased Walking Speed Progress and lower limb strength
A: Walking speed measured by Ten Meter Walk Test (10MWT) progressed during both the intensive LT and the transcutaneous spinal stimulation with LT. The improved function was maintained at least three months after the intervention phases. The progress of 10MWT walking speed in P1. **B:** The progress of the walking speed in P2. Stim: Transcutaneous Spinal Stimulation; LT: Locomotor Training. Circles present individual data points. Dashed lines present the fitted linear regression models in each phase. Error bars indicate standard error of the mean. **C:** International Standards for Classification of Spinal Cord Injury Lower Extremity Motor Score (LEMS) was recorded at the end of each phase. The LEMS increased during the Stim + Training phase compared to the Training phase and was sustained without further intervention in Participant 1 and 2 (P1 and P2).

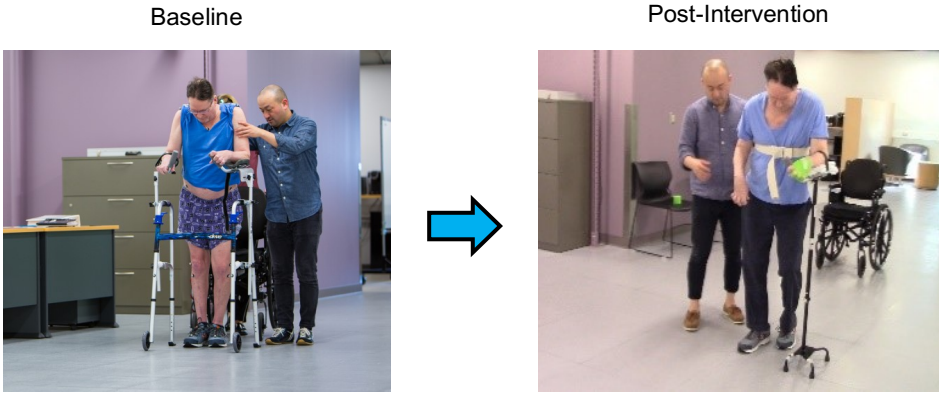

Figure 4-8. Supplementary Figure 2. Progress of Walking Function in Participant 1
Intensive overground locomotor training in Participant 1 (P1). P1 could progress from walking with the platform walker (Left) and maximum physical assist to walking with the quad-cane and contact guard assist level after completing all intervention (Right).

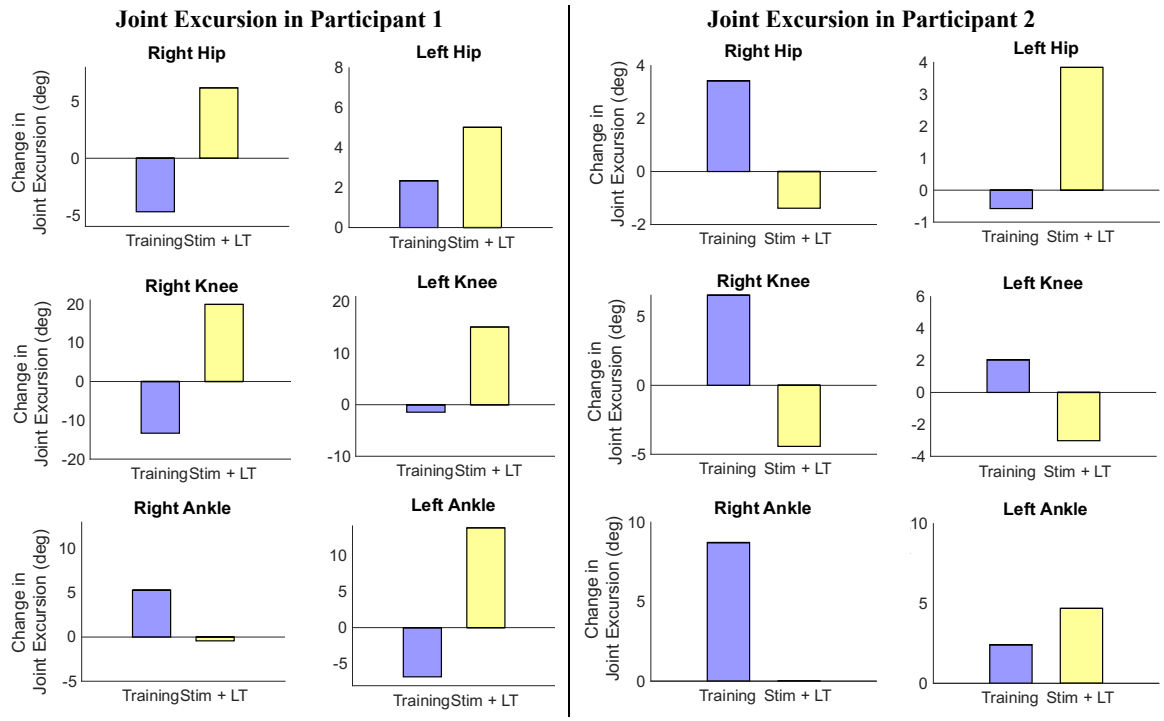

Figure 4-9. Supplementary Figure 3. Comparison of Joint Excursion between The Locomotor Training Phase and The Stimulation Phase

Change of joint excursions in each phase in Participant 1 **(Left)**. Change of joint excursions in each phase in Participant 2 **(Right).** Stim: Transcutaneous Spinal Stimulation; LT: Locomotor Training.

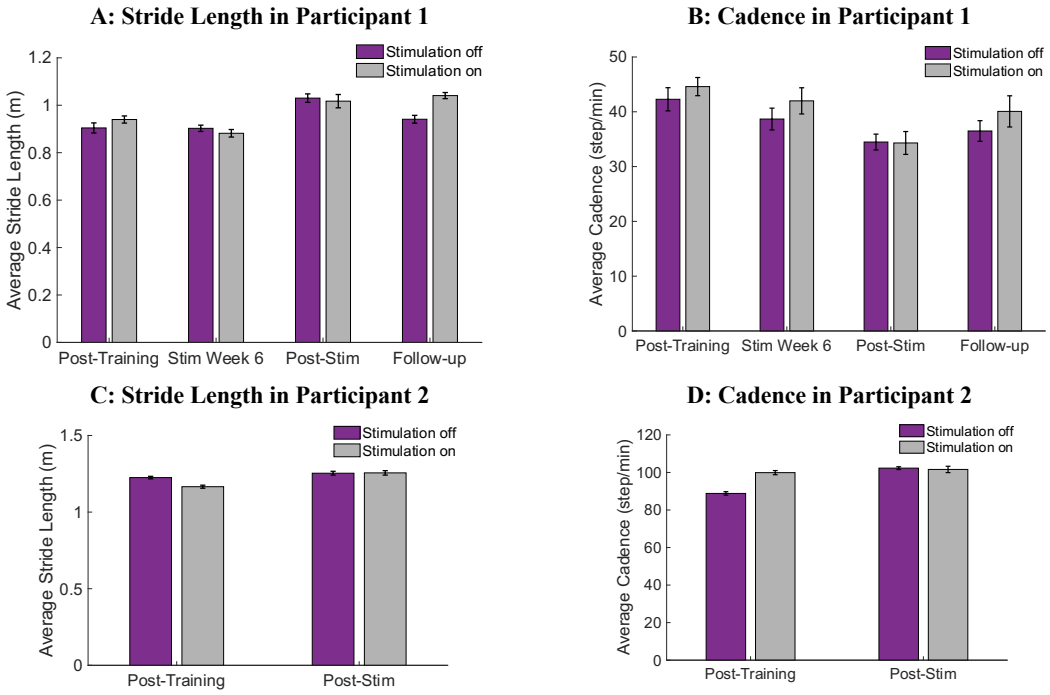

Figure 4-10. Supplementary Figure 4. Immediate Effect of Transcutaneous Spinal Stimulation on Stride Length and Cadence

The comparisons of step length and cadence between self-selected walking with transcutaneous spinal stimulation on and off on the same day. **A:** Stride length with and without transcutaneous spinal stimulation (Stimulation on: Left bar and Stimulation off: Right bar) after locomotor training only phase (Post-Training), in the 6th week of transcutaneous spinal stimulation with locomotor training (Stim Week 6), after transcutaneous spinal stimulation with locomotor training (Post-Stim) and in the last follow-up visit (Follow-Up) in Participant 1. **B:** Cadence in Post-Training, Stim Week 6, Post-Stim and Follow-up in Participant 1. **C**: Stride length in Post-Training, and Post-Stim in Participant 2. **D**: Cadence in Post-Training, and Post-Stim in Participant 2. Error bars indicate standard error of the mean.

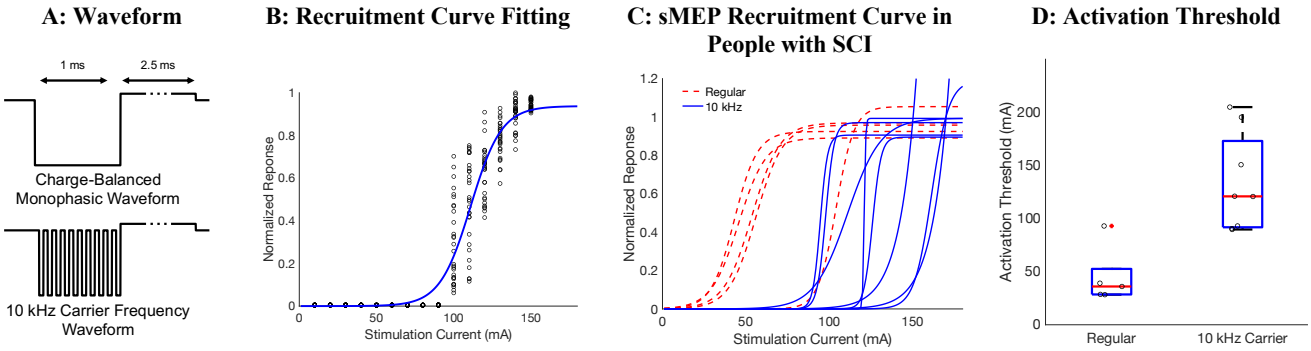

Figure 4-11. Supplementary Figure 5. Comparison of Spinal Motor Evoked Potentials between Charge-Balanced Monophasic Pulse with and without 10 Kilohertz Carrier Frequency

A: To test the effect of 10 kilohertz (kHz) carrier frequency, we used the charge-balanced monophasic waveform without carrier frequency (Top) and the charge-balanced monophasic waveform with 10 kHz carrier frequency (Bottom). We employed these waveforms since we could not produce a biphasic waveform without carrier frequency in the device due to the technical limitation. **B**: An example of fitting a sigmoid curve on the spinal motor evoked potential (sMEP) data of the soleus muscle. The empty circles show individual peak to peak amplitude data points, and the blue line shows a sigmoid curve fit in the data. Based on the sigmoid curve, we determined the activation threshold at the 10% of maximum activation. **C**: All sigmoid curves of the sMEPs in both waveforms of the stimulation. The charge-balanced monophasic waveform with 10 kHz carrier frequency (10 kHz carrier, blue line) (n = 5 in 3 subjects) required higher current to activate the soleus muscles compared to the charge-balanced monophasic waveform without carrier frequency (Regular, red dash line) (n = 8 in 4 subjects). **D**: The activation threshold was significantly higher with the 10 kHz carrier (132.5 ± 16.3 mA) than with the Regular waveform (44.3 ± 12.2 mA, p = 0.0062). Error bars indicate standard error of the mean.

Chapter 5. DISCUSSION AND CONCLUSION

This thesis investigated new approaches using spinal stimulation for activity-dependent plasticity of the spinal networks in both an animal model and people with cervical SCI. We realized a brain-computer spinal interface to reanimate upper limb movements in rats with SCI. In addition, we demonstrated a novel therapeutic strategy using transcutaneous spinal stimulation to improve walking and autonomic function in people with chronic incomplete SCI. Our research addressed critical scientific questions to develop further effective approaches for functional recovery. In this chapter, we briefly summarize the main findings and contributions of this thesis work to the scientific community. Subsequently, we discuss emerging questions and future work based on these findings.

5.1 SUMMARY OF MAIN FINDINGS

For impaired upper limbs, brain-controlled intraspinal stimulation could reanimate wrist movements in animal models (Nishimura, Perlmutter, & Fetz, 2013; Zimmermann & Jackson, 2014). Furthermore, brain-controlled epidural stimulation could promote spatiotemporal control of paralyzed limbs via the wireless system for locomotor recovery (M. Capogrosso et al., 2016) and potential of durable activity-dependent plasticity (Bonizzato et al., 2018). However, it was unknown whether brain-controlled epidural stimulation can reanimate upper limb movements following cervical SCI. In order to seek a clinically viable application of reanimating upper limb movements through brain signal decoding and epidural stimulation, we tested a decoding and spinal stimulation method in animal models (Chapter 3). We also simulated the closed-loop algorithm on the current miniature computer platform to propose an autonomous closed-loop

system for independence from the laboratory settings. This required a new behavioral paradigm described in Chapter 2.

In Chapter 2, we report on an adaptive lever pressing task enabling quantification of functional forelimb movement in rats with severe forelimb impairment. We implemented automated adaptive thresholding controlled by open-source software. The adaptive algorithm significantly shortened the training duration compared to the experimenter-controlled protocol. The adaptive thresholding was successful in motivating and encouraging animals. The adaptive thresholding strategy accelerated the rehabilitative process after the injury that mirrors rehabilitation in clinical settings. Furthermore, we found that automation leads to less labor, time, and experimenter bias. This concept of the behavior task may improve preclinical studies for the next generation of interventions after SCI.

In Chapter 3, we explored a clinical viable BCI system to reanimate impaired forelimb function following severe cervical SCI by using the behavior task introduced in Chapter 2. Our main findings are: 1) intracortical high gamma local field potentials provided a stable prediction of forelimb movement over many days before and after spinal cord injury, 2) brain-controlled epidural stimulation evoked graded synergistic forelimb movement proportional to the stimulation current, and 3) the efficient closed-loop algorithm could be implemented on an implantable commercially available device with onboard computing. Based on the findings, we are one step closer to feasible translation of the BCI application to human trials as recording and stimulation techniques used here have already been employed in separate clinical trials.

We next turn to testing interventions in human subjects in Chapter 4. Previously we demonstrated that a currently available non-invasive spinal stimulation method, high carrier frequency

transcutaneous spinal stimulation, with intensive training for arm and hand function, led to significant benefits for daily activities compared to the same amount of intensive training in people with cervical SCI (Inanici et al., 2020; F. Inanici et al., 2018). Other research groups presented transcutaneous spinal stimulation could induce more volitional leg movement and improved bladder function (Y. P. Gerasimenko et al., 2015; Hofstoetter et al., 2015; Kreydin et al., 2020). Therefore, we tested whether transcutaneous spinal stimulation with intensive locomotor training could bring greater improvement of walking ability and autonomic function compared to performing only locomotor training in incomplete cervical SCI.

We investigated the effect of transcutaneous spinal stimulation with intensive locomotor training for motor and autonomic function. Our findings suggest that transcutaneous spinal stimulation with intensive locomotor training leads to greater recovery of walking and autonomic function than performing the same amount of intensive locomotor training alone for people with incomplete cervical SCI. Specifically, improved walking ability during the transcutaneous spinal stimulation phase was three-fold greater compared to the locomotor training only phase. Importantly, locomotor recovery was sustained at least two months after the intervention and enhanced engagement in community walking with fewer falls. Notably, both participants demonstrated the meaningful recovery of bowel function, including normalized bowel management time and frequency. Lastly, one of the participants reported improved bladder function, in that he no longer required intermittent catheterization more than one year after completing the study.

5.2 CONTRIBUTIONS

The novel lever press task introduced in Chapter 2 is the first behavior task to measure proximal forelimb function in rats with and without severe cervical SCI. We also implemented adaptive

threshold routine controlled by the open-source software to accelerate learning and rehabilitation. The scripts and materials of the behavior training strategy will be available online. These can be adapted in the other behavior tasks for time- and cost-efficiency.

The brain-computer spinal interface presented in Chapter 3 is the first closed-loop system to reanimate upper limb movements by using LFP-controlled epidural stimulation following severe cervical SCI. The multichannel LFP decoder was stable over weeks, even after the injury. Epidural stimulation at C6-C7 recruited triceps activity in a more graded manner compared to the radial nerve stimulation. Furthermore, we found that applying 10 kHz carrier frequency to epidural stimulation alleviated aversive behavior due to high current epidural stimulation for reanimating forelimb movements. The combination of onboard computing with a computationally efficient decoder and efficient stimulation method will both help to accelerate clinical translation of BCIs into daily life outside the laboratory environments.

The study in Chapter 4 is the first study designed to investigate the efficacy of combining cervical and lumbar transcutaneous spinal stimulation with intensive locomotor training for people with cervical SCI. The outcomes highlight the potential of combined therapy compared to the locomotor training only on functional recovery. This study also demonstrated for the first time improved bowel function with transcutaneous spinal stimulation in people with SCI, which is one of the highest priorities in the population (Anderson, 2004; J. S. French et al., 2010). No adverse events were observed with simultaneous cervical and lumbosacral transcutaneous spinal stimulation during locomotor training.

Lastly, the participants were 64 years-old males. In the previous spinal stimulation studies for locomotor recovery, the age range was limited, from 23 to 48 years old. The results may expand

evidence of transcutaneous spinal stimulation for the aging population with SCI, who may have a poorer prognosis than the younger population with SCI (Engel-Haber, Zeilig, Haber, Worobey, & Kirshblum, 2020). Overall, this thesis work provides additional evidence of the new neuromodulatory strategies with spinal stimulation for rehabilitation and functional recovery after cervical SCI.

5.3 PERSPECTIVES

The medical community has advanced interventions for functional recovery from the consequence after SCI. However, the efficacy of these interventions for SCI is still far from what people with SCI seek. Neuromodulation via epidural stimulation opened a door for a breakthrough for functional recovery in chronic SCI. In this section, we will discuss future directions to promote clinical translations.

5.3.1 *Suggestion for a 'Closed-Loop' between Basic Experiments, Computational Models, and Clinical Research*

In this thesis work, we created a new closed-loop platform for neuromodulation in an animal model. We also learned the effect of transcutaneous spinal stimulation on functional recovery in clinical trials. Investigating biological mechanisms in preclinical models is essential to develop new interventions. Meanwhile, learning important questions from clinical trials drives the next step of meaningful experiments in preclinical experiments. Therefore, we suggest iterating the loops of basic science and clinical research with careful analysis of the outcomes. This process, however, is costly and requires long time commitment.

Computational simulation may mitigate the cost and time by catalyzing the barriers of clinical translation for new interventions. Simulation using computational models can provide more

insights into how extrinsic inputs interact with intrinsic variables to predict clinically relevant outcomes. The simulation may highlight our lack of understanding of essential elements in the neural circuits as well (Capogrosso & Lempka, 2020; Khadka et al., 2020). As integrating computational simulations, feedback between basic experiments and clinical trials would advance our knowledge and technology faster and more efficiently toward clinical applications.

5.3.2 *Clinical Translations of Brain-Computer Spinal Interface*

Spatiotemporal modulation of epidural stimulation for walking based on external sensors was feasible in people with cervical SCI (Wagner et al., 2018). Preclinical studies reinforced that spatiotemporal modulation induced more locomotor recovery compared to continuous epidural stimulation (N. Wenger et al., 2016). Brain-controlled epidural stimulation could foster sustained locomotor recovery (Bonizzato et al., 2018; M. Capogrosso et al., 2016). In addition, previous review reported that the association of supraspinal control with responsiveness to locomotor recovery by gait training in people with SCI (Edelle C Field-Fote, Yang, Basso, & Gorassini, 2017).

These studies suggest the potential of compensating the significant loss of supraspinal control by the closed-loop BCI system. Consistent brain-controlled spinal stimulation may facilitate more spike-time-dependent plasticity than continuous spinal stimulation with intensive training. As we presented the brain-computer spinal interface for upper limb function, we may be able to promote neuroplasticity for upper extremity function by using brain-controlled epidural stimulation in people with cervical SCI.

To accelerate clinical translations, we can use less invasive methods to extract brain signals such as electrocorticogram. For the less invasive methods, the field potentials from the surface of the

brain, which is similar to our decoder in the study, can be great sources of movement intention. Along these lines, it is also important to study the effect of brain-controlled transcutaneous spinal stimulation on functional recovery compared to continuous transcutaneous spinal stimulation.

5.3.3 *Development of New Strategies with Transcutaneous Spinal Stimulation*

We presented the efficacy of transcutaneous spinal stimulation with activity-based therapy in this thesis and our previous studies (Inanici et al., 2020; F. Inanici et al., 2018). However, to validate the findings, we need to have a double-blinded randomized control study with a larger sample size. Sham stimulation can also be used to control the placebo effect.

Furthermore, our participants anecdotally reported the recovery of autonomic function not limited to bowel, bladder, and cardiovascular function. Further research with empirical measures, such as urodynamics and ultrasound, is needed to validate the autonomic effect and underlying mechanisms. The non-invasive, and cost-efficient advantages of transcutaneous spinal stimulation can lead to a rapid clinical translation. Thus, it is worth conducing large-scale clinical trials involving the direct comparison between transcutaneous spinal stimulation and epidural stimulation for functional recovery.

The preclinical findings provide us a hint of how we can tackle the physiological mechanisms to develop effective protocols. The increased activity of the late responses of the EMG reflects polysynaptic reflexes during walking (P. Gad et al., 2013). In addition, 300 - 500 ms long-lasting response increases after the cutaneous stimulation when spasticity increased in the muscle in a preclinical study (Bennett, Sanelli, Cooke, Harvey, & Gorassini, 2004). Neuroplasticity with the peripheral nerve stimulation by using the H-reflex analysis in people with chronic SCI (A. K. Thompson, Chen, & Wolpaw, 2013; Aiko K. Thompson & Wolpaw, 2019). Therefore, clinical

electrophysiological tests such as the walking EMG, H-reflex, and spinal motor evoked potential tests with functional outcomes may be useful to study insights into the changes in the spinal networks with transcutaneous spinal stimulation.

Although we used 30 Hz continuous stimulation with 10 kHz carrier frequency, parameters of transcutaneous spinal stimulation have not been thoroughly investigated. We have space to optimize the parameters to improve functional outcomes. Furthermore, the safe dosage of the stimulation has to be investigated when we bring the therapy to clinics and home settings. For electrode locations, cervico-thoracic transcutaneous spinal stimulation could modulate spinal inhibitory interneurons at the lumbosacral spinal cord via heteronymous pathways (Barss et al., 2020; Zewdie, Roy, Okuma, Yang, & Gorassini, 2014). There is a potential of cervical transcutaneous spinal stimulation amplifying the effect of lumbosacral transcutaneous spinal stimulation for locomotor and autonomic recovery. Further research is required to reveal the mechanisms for the combined stimulation.

5.3.4 *Time Window of Recovery and Combining with Other Therapies*

Designing the protocol of neuromodulation with optimal timing is also critical for enhancing functional recovery following spinal cord injury. Most clinical studies using spinal stimulation were conducted in the chronic phase of injury to separate spontaneous recovery from the therapeutic effects of interventions. The immediate applications of spinal stimulation after the onset of SCI may lead to greater functional recovery by normalizing the cardiovascular system (Phillips et al., 2018; Squair et al., 2017) and activating the spinal networks (Taccola, Gad, et al., 2020). Further research in preclinical models is needed to investigate the potential of acute application of transcutaneous spinal stimulation.

Combining spinal stimulation with other modalities is another promising approach. Combining a monoaminergic agent with transcutaneous spinal stimulation augmented the effects on upper and lower limb function in the chronic phase (Freyvert et al., 2018; P. Gad et al., 2017; Y. P. Gerasimenko et al., 2015). Pharmacological approaches, such as anti-Nogo antibody, and stem cell transplantations may be able to augment the efficacy of spinal stimulation. However, combining effective interventions may have unforeseen effects on each therapy. When researchers simultaneously applied anti-Nogo antibody therapy and intensive locomotor training in rats with SCI, the benefits were even less than the untreated group. However, when staggering two therapies in the acute phase, the functional recovery was much greater than the control group (Chen et al., 2017; Maier et al., 2009). Future work must thoroughly test the combined effect of different therapies in the acute, subacute, and chronic recovery phases in animal models.

5.4 General Conclusions

In this thesis, we demonstrated the clinically viable strategy of brain-computer spinal interfaces for upper limb function following severe cervical SCI. We also uncovered the potential of transcutaneous spinal stimulation with intensive training for the recovery of walking and autonomic function in people with cervical SCI. From the currently available evidence, we discussed the potential mechanisms and strategies of neuromodulation via spinal stimulation to promote functional recovery for clinical translations.

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