Executive Summary

Strategies to support neural plasticity in spared sensorimotor networks after spinal cord injury (SCI) have long been recognized for their potential to restore function[1]. Recently, two new neuromodulation strategies have emerged to support neural plasticity and recovery after SCI. The first new therapeutic strategy is to *direct plasticity* – these methods induce functionally beneficial plasticity in specific central nervous system (CNS) circuits underlying an impaired skill. The second new therapeutic strategy is to *increase plasticity* – these methods increase the rates and/or types of plasticity that can occur in the CNS; they thereby enhance the capacity for recovery. Both of these therapies engage plasticity mechanisms in different ways, and both have shown to be effective at restoring function after SCI.

Our collaborative team at The National Center for Adaptive Neurotechnologies (NCAN) and The University of Texas at Dallas (UT Dallas) have developed an innovative form of each type of therapy. The first therapy, H-reflex operant conditioning (HROC) developed by our NCAN team, is a *directed plasticity* neuromodulation therapy to selectively strengthen or weaken a spinal reflex[2]. The second therapy, vagus nerve stimulation (VNS) developed by our UT Dallas team, is a neuromodulation therapy to *increase plasticity* in the CNS[3]. Our teams have demonstrated that each technique alone can support recovery after SCI[4–7].

Based on these principles of *directing* and *increasing* plasticity, we propose a new therapeutic paradigm combining HROC and VNS to enhance recovery after SCI. We have called this new therapy VNS-enhanced HROC (VeHROC). In this approach, HROC drives *directed* plasticity through the modification of specific spinal circuits contributing to spasticity, and VNS *increases* the plasticity effects of HROC by synchronizing the timed release of neuromodulators. **We hypothesize that VNS can enhance the spinal conditioning effects of HROC to improve functional recovery after SCI.** As a first test of this hypothesis, we propose to develop this fundamentally novel combinatorial therapy using a clinically relevant rat model of SCI, then evaluate it in a pilot clinical trial in whom a chronic incomplete SCI has produced a spastic gait disorder.

In this project we will comprehensively characterize the effects of VeHROC in a clinically relevant rat model of SCI, investigate clinically relevant predictive biomarkers spanning multiple modalities, and leverage our unique access to a group of individuals with SCI that are already implanted with a VNS system to perform a pilot clinical trial to evaluate VehROC. This translational effort is directly focused on closing the gap to clinical translation and has the potential to yield tangible benefits for individuals living with SCI. The proposed project addresses the following FY24 SCIRP Focus Area: *Rehabilitation and regeneration—maximizing the function of the residual neural circuitry, including harnessing neuroplasticity and recovery to improve function after SCI*. Importantly, our proposed VeHROC therapy is a synergistic combination of two clinically relevant interventions. Finally, we will also integrate biomarker studies with the intervention-focused research to advance SCI treatment outcomes.

1. Background and Readiness

Spinal cord injury is prevalent and lacks effective therapeutic interventions

SCI imposes a substantial economic burden, limits operational capacity, and profoundly impacts the lives of service members and their families. The incidence of SCI is higher in service members and Veterans, particularly related to recent conflicts[8,9]. Incomplete SCI, the most common form of SCI, often results in profound neurological impairments including spasticity and weakness in the lower limbs[10–12]. Accordingly, loss of mobility is a substantial contributor to disability post-SCI, and restoration of walking is a primary rehabilitation goal for most individuals[13]. While acute trauma medical care has improved, there are no consistently effective therapies that restore function in the chronic phase of SCI.

Spasticity is a common and incredibly debilitating symptom of SCI[14]. The loss of inhibitory input from the brain to the spinal cord after SCI leads to increased excitability of stretch reflexes, or hyperreflexia[15]. The hyperactive reflex responses contribute to the abnormal muscle contractions and tightness that limits movement and characterizes spasticity[16]. Unfortunately, current spasticity treatments such as Botox and baclofen only offer temporary relief, have serious side effects, and aim to manage symptoms rather than treat the underlying cause[17]. We have chosen to focus on developing treatments for spasticity because it is debilitating and current clinical care lacks effective solutions. The development of effective spasticity treatments would yield clear benefits for individuals living with SCI.

Directed Plasticity: H-reflex operant conditioning (HROC) to restore spinal reflexes

Our NCAN team has developed an innovative *directed plasticity* therapy, H-reflex operant conditioning (HROC), to selectively strengthen or weaken a spinal reflex pathway (i.e., the knee-jerk reflex) or its electrical analog, the H-reflex (**Fig. 1**). The protocol modifies the brain's descending influence on the spinal reflex pathway so to increase (up-condition) or decrease (down-condition) the reflex. Because the conditioning changes the pathway both structurally and functionally, it affects behaviors such as locomotion that use the pathway[2,5]. In animals and people with incomplete spinal cord injury, a protocol that therapeutically modifies the H-reflex in the soleus muscle improves locomotion[4,5,18]. Specifically, in people with incomplete SCI and a spastic gait disorder, down-conditioning of the hyperactive soleus H-reflex reduces gait hyperreflexia and abnormal reflex modulation in ankle extensor muscles; this produces beneficial changes that greatly improve locomotion[4,5]. Importantly, these improvements persist after the therapy ends. Overall, HROC is a *directed plasticity* strategy to therapeutically restore spinal reflexes (i.e., reduce hyperreflexia to treat spastic gait disorder) and support recovery in individuals with spasticity after SCI.

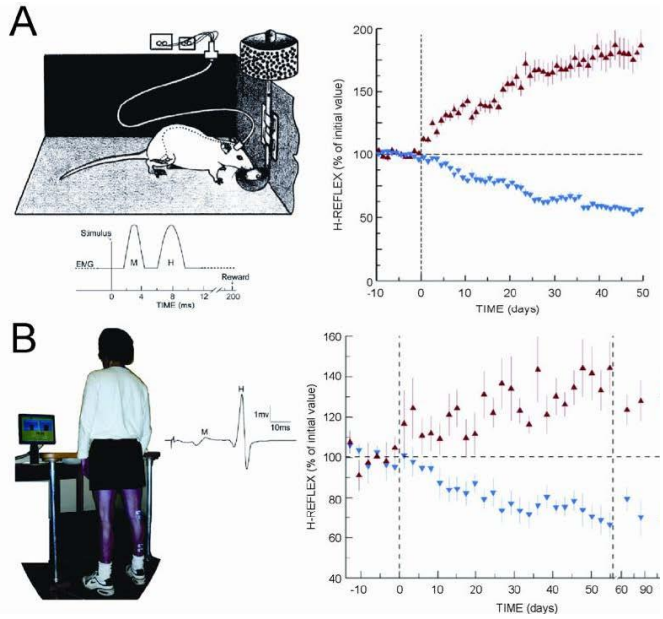

Fig. 1. H-Reflex Operant Conditioning (HROC) and its results in rats (A) and humans (B). (A; left) A rat receiving HROC. Soleus EMG is monitored 24hr/day in a rat, and a tibial nerve cuff stimulus elicits a M-response and H-reflex (bottom). The trace shows one trial. **(A; right)** The graphs show average (±SE) daily H-reflex sizes for 55 successful HRup rats (▲, red) and 72 successful HRdown rats (▼, blue). In both groups, H-reflex changed steadily over the 50 days. **(B; left)** A person receiving HROC. EMG electrodes placed on soleus, and stimulation electrodes placed over tibial nerve in popliteal fossa. The user stands facing a screen that shows the EMG level and reward cue. **(B; right)** Following the control mode (Days -10 to 0), people receive either up conditioning (red) or down conditioning (blue). H-reflex changes gradually over 56 days. Follow-up sessions highlight that the conditioning effects are long-lasting.

Increased Plasticity: Vagus nerve stimulation (VNS) to increase plasticity and recovery after SCI

Our UT Dallas team has recently developed an innovative *increased plasticity* therapy, paired vagus nerve stimulation (VNS), that greatly increases plasticity and recovery after SCI. VNS provides precisely timed engagement of neuromodulatory systems[6,19–21] that in turn drive rapid, phasic release of pro-plasticity molecules which are known to govern synaptic plasticity throughout brain and spinal networks[22–24]. Pairing the VNS-dependent release of neuromodulators with the activation of specific CNS circuits drives robust plasticity in active networks[23–26]. Importantly, the plasticity-increasing effects of paired VNS therapy must be *directed* by repeatedly pairing specific patterns of neural activity. For example, repeatedly pairing VNS with a specific movement can enhance plasticity within motor-related CNS networks.

Based on this principle of pairing VNS with neural activity, our team has pioneered the development of VNS paired with physical rehabilitation to improve motor recovery after neurological injury, including SCI, stroke, peripheral nerve injury, and traumatic brain injury (**Fig. 2A**). In this therapeutic paradigm, physical rehabilitation (i.e., therapeutic exercises) *directs* the plasticity-*increasing* effects of VNS. These improvements require engagement of neuromodulatory networks, and are subserved by widespread synaptic plasticity in cortical and corticospinal networks .

Clinical findings support the utility of this approach. Multiple clinical trials in chronic stroke patients demonstrate that VNS paired with rehabilitation significantly improves upper limb function compared to equivalent rehabilitation without VNS [7,27]. Based on a recent Phase 3 pivotal study[28], VNS therapy received FDA approval as the first neuromodulation therapy to improve arm and hand function in individuals with chronic stroke. Our team has extended these findings to SCI, recently completing a DARPA-funded early feasibility study in 19 individuals with SCI. We demonstrate that VNS is safe, feasible to deliver, and improves recovery of upper limb

function in individuals with chronic SCI (**Fig. 2b**). These efforts have culminated in our receipt of Breakthrough Device designation from the FDA for this therapy, pointing to the future clinical impact for individuals with SCI.

VNS-enhanced H-reflex operant conditioning (VeHROC) to reduce spasticity after SCI

We believe the most effective treatments for patients will be the synergistic combinations of these two classes of therapies, where the directed plasticity effects of HROC can be enhanced by the coincident release of plasticity increasing neuromodulators driven by VNS. Importantly, both HROC and VNS facilitate neuroplasticity in cortical and spinal networks to support improved recovery[20,21,29,30], highlighting that both therapies enact change in similar networks and suggests synergistic potential when combined. VeHROC can overcome multiple limitations of each component therapy, HROC and VNS, discussed below.

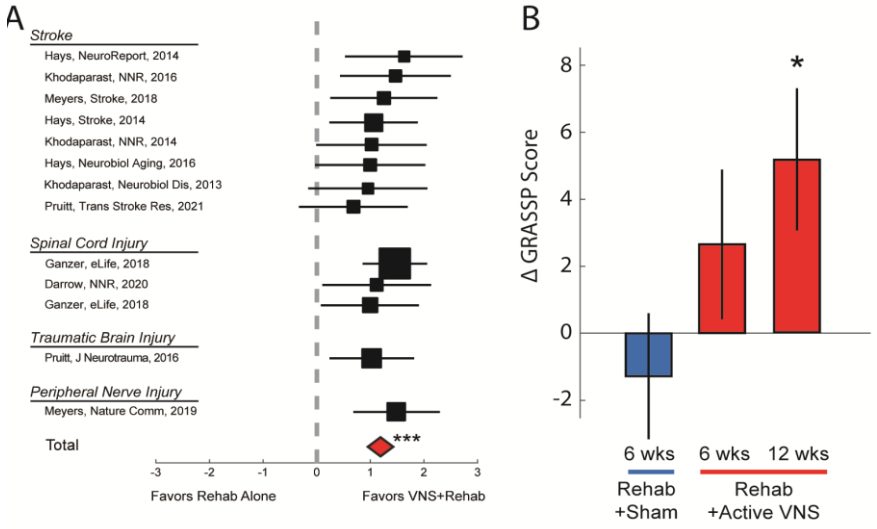

Fig. 2. VNS paired with rehabilitation enhances recovery. VNS paired with rehabilitation enhances recovery in multiple preclinical models of neurological injury **(A)**, and in humans with SCI **(B)**.

While HROC is a powerful method to direct therapeutic plasticity in spinal reflexes and improve spastic gait disorder in individuals with SCI[4,5], the technique has two key limitations that hinder future clinical utility. First, while many participants exhibit clinically meaningful recovery, residual impairments often remain, and some individuals fail to respond to therapy. Enhancing the conditioning effects of HROC with VNS may therefore improve the therapeutic effect. Second, the directed plasticity effects of HROC are slow, requiring approximately three months of therapy to produce clinically meaningful improvements. This extended timeline presents challenges for clinical translation, including limited clinician availability and hesitancy from both clinicians and patients due to the time commitment. We believe that the *plasticity-increasing* effects of VNS can overcome these limitations of HROC by both enhancing and accelerating the therapeutic effects of HROC.

Similar to HROC, paired VNS therapy has had decades of preclinical and clinical success. However, VNS has only been used in combination with physical rehabilitation to treat movement disorders (i.e., pairing VNS with upper limb therapy during massed task practice). Spasticity is a complex symptom of SCI and traditional rehabilitation protocols fail to effectively treat spasticity[31]. VeHROC extends the utility of VNS by combining it with HROC to direct the plasticity increasing effects of VNS to specific CNS circuits underlying spasticity. This project represents the first step towards combining VNS with synergistic neuromodulation therapies to more effectively direct plasticity and treat complex disorders. Finally, reducing spasticity with VeHROC therapy could lead to improvements in limb range of motion and function, thereby making subsequent VNS-enhanced rehabilitation more effective.

Our collaborative team is committed to the development and translation of innovative strategies to improve recovery after neurological injury. We have converted multiple proof-of-concept animal studies into published clinical therapies[7,27,32–34]. This project is the first step towards translating this new technology to the clinic. The proposed project incorporates rigorous, proof-of-concept preclinical studies investigating both the functional improvements of the therapy and potential predictive biomarkers to predict an individual's responsiveness to therapy. In parallel with the animal study, we will also perform a pilot clinical trial to evaluate the safety, feasibility, and early indications of efficacy in six individuals with SCI. This pilot study leverages our unique access to a group of individuals with SCI who underwent implantation of a VNS system as part of our recently completed early feasibility study (NCT04288245). The ability to enroll these individuals in a pilot study significantly reduces the cost and regulatory burden for a pilot trial evaluating VeHROC. At the conclusion of this

program, we will have thoroughly evaluated VeHROC in rats, identified predictive biomarkers to investigate in future clinical trials, and collected clinical feasibility data to pursue a larger clinical trial evaluating VeHROC.

2. Objective

The primary objective of this project is to rigorously evaluate VeHROC for improving gait in chronic SCI. VeHROC is the first in a new class of neuromodulation therapies that synergistically combine *directed plasticity* therapies with *increased plasticity* therapies to maximize recovery. The scientific premise underlying this project is that the *directed plasticity* therapy of HROC will guide the *increased plasticity* effects of VNS to enhance therapeutic plasticity and support functional recovery in brain and spinal networks.

In the preclinical study we will evaluate if VeHROC augments spinal reflex conditioning and locomotor recovery in a rat model of chronic incomplete SCI. We will perform a comprehensive, multi-modal biomarker investigation to develop predictive biomarkers to predict an individual's therapeutic recovery trajectory using measures taken at the start of VeHROC therapy. Finally, we will perform a small pilot clinical trial to evaluate whether VeHROC can reduce hyperreflexia and improve gait in individuals with a spastic gait disorder due to SCI. We hypothesize that VNS will enhance the HROC-facilitated restoration of spinal reflexes and gait function.

While our primary goal in this project is to evaluate a new neuromodulation therapy to treat spasticity by conditioning the hyperactive soleus reflex in humans with SCI, the animal study will use a conditioning protocol designed to improve gait. This approach is necessary because, unlike humans, rats do not develop a spastic gait disorder after SCI but instead experience an abnormally weakened spinal reflex. Our previous studies[18,35,36] demonstrate that conditioning this reflex in rats leads to significant improvements in gait, making it an appropriate model for testing the effects of VeHROC in the animal study. Successful completion of this study will show that VeHROC can enhance the spinal conditioning effects of HROC and drive beneficial plasticity to improve function. These positive results will provide the scientific foundation for larger clinical trials evaluating VeHROC as a therapy to condition hyperactive spinal reflexes and improve gait in individuals with spastic gait disorder after SCI.

3. Specific Aims

Aim 1 (Preclinical Study): Evaluate the efficacy of VeHROC to improve locomotion in a clinically relevant rat model of incomplete SCI.

In Aim 1, our objective is to demonstrate that VeHROC more effectively improves recovery of hindlimb function compared to either HROC or VNS alone in a clinically relevant rat model of SCI. Rats will receive a spinal contusion at T9 that produces gait deficits[35]. Rats will then be randomized into one of four treatment groups for 12 weeks: (1) HROC alone, (2) VNS alone, (3) VNS-enhanced HROC (VeHROC), or (4) no therapy (SCI only). We expect to see enhanced spinal conditioning effects in the VeHROC group, such that the rate and magnitude of change are greater compared to all other groups. Additionally, we anticipate that animals that receive VeHROC therapy will have improved locomotion, including longer steps and reduce step-cycle asymmetry (i.e., limping), and better foot-placement accuracy on a horizontal ladder test[35].

Aim 2 (Preclinical Study): Identify clinically relevant biomarkers that predict responsiveness to VeHROC.

For Aim 2, our objective is to identify collections of biomarkers that predict an individual subject's responsiveness to VeHROC. Leveraging the behavioral study in Aim 1, we will collect multiple multi-modal datasets to build a comprehensive picture of an individual's response to therapy and capacity for recovery after SCI. We will leverage recent biomarker successes in similar fields such as stroke recovery, advances in machine learning to identify complex relationships amongst multiple variables, and new dimensionality-reduction techniques that reduce the complexity of the data without loss of information. The biomarker data collected will combine electrophysiological information with next-generation miRNA sequencing. Importantly, we have limited our investigation to biomarkers that have clinical viability and can be easily measured in humans.

Aim 3 (Pilot Clinical Trial): Perform a pilot clinical trial to evaluate the safety, feasibility, and early efficacy of VeHROC in individuals with SCI and a spastic gait disorder.

In Aim 3, we will perform a single-arm pilot clinical trial to evaluate the effects of VeHROC on spinal reflexes and gait function in individuals with SCI and a spastic gait disorder (M and F, ages 18-65, >12-months after SCI, American Spinal Injury Association (ASIA) Impairment Scale (AIS) C or D). We have continuing contact with

participants with SCI from our recently completed VNS clinical trial in Dallas, and many of these participants are enrolled in ongoing follow-on studies and have expressed interest in futures studies. Leveraging this unique situation, we will recruit six participants to participate in a 12-week protocol evaluating the safety, feasibility, and early efficacy of VeHROC to reduce hyperreflexia and improve gait.

These aims build on our decades of success with each component therapy and outline an expeditious approach to accomplish the necessary steps to transition this promising therapy to larger clinical trials. Successful completion of these Aims will provide the rationale for a subsequent randomized controlled clinical trial of this potentially transformative therapy to improve the quality of life for individuals with SCI.

4. Study Design and Feasibility

4.1. Aim 1. Evaluate the efficacy of VeHROC to improve locomotion in a clinically relevant rat model of incomplete SCI.

Robust preclinical evaluation is critical to effective clinical translation. In Aim 1, our objective is to demonstrate that VeHROC, compared to HROC or VNS alone, increases the magnitude of change in spinal reflexes, and improves locomotor recovery in a clinically relevant model of incomplete SCI. Work in Aim 1 will be conducted at UT Dallas and Drs. Chen and Carp, experts in rat H-reflex conditioning, will provide their expertise and support throughout the project. *The preclinical study methods are briefly described below. Please see attachment 11: Animal Research Plan for additional details.*

4.1.A. Experimental Design.

The experimental design allows us to isolate and evaluate the effects of HROC, VNS, and their combination (VeHROC) on locomotion recovery following spinal cord injury. Adult rats (n=14 per group, balanced across males and females) will undergo baseline locomotion testing. Following this assessment, all rats receive a clinically relevant, unilateral spinal contusion injury at T9 to impair locomotion (**Fig. 3A**), as in our previous studies. After an 8-week recovery period, all animals will be implanted with a VNS cuff, soleus EMG electrodes, and a tibial nerve cuff. The rats will then be dynamically allocated to one of the following groups for 12 weeks (**Fig. 3B**):

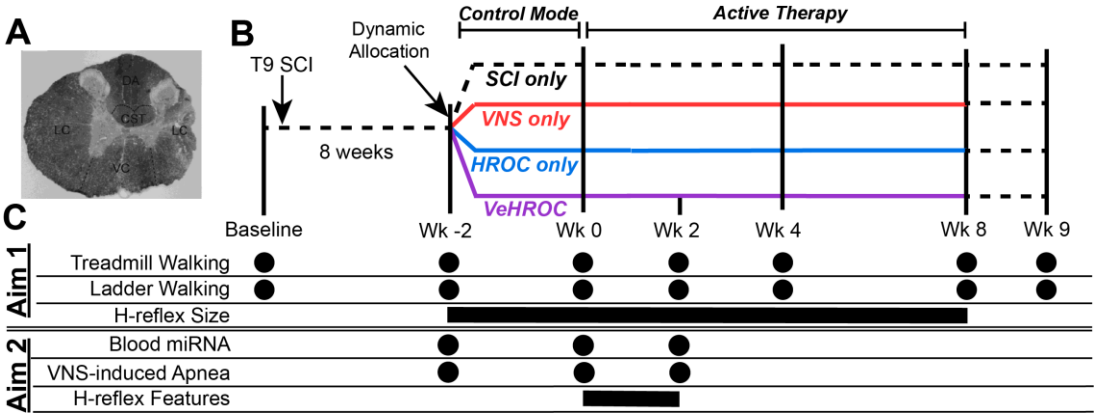

Fig. 3. Pre-clinical study overview. (A) T9 spinal section from a rat with right lateral contusion injury. Only 18% of right lateral column remains. Dorsal columns (dorsal ascending tracts (DA) & corticospinal tracts (CST)), ventral columns (VC), & left LC are intact. **(B)** Experimental timeline. Eight weeks after SCI, rats are dynamically allocated into one of 4 experimental groups. The first 2 weeks (Wk -2 to Wk 0) use the control mode of HROC to collect baseline measures. Then rats begin active therapy based on their respective group for 8 weeks. Functional assessments are performed 1 week after the end of therapy (Wk 9). **(C)** Timepoints for Aim 1 behavioral and functional assessments (*top*) and Aim 2 data for predictive biomarker discovery (*bottom*).

1. HROC only: This group receives H-reflex operant conditioning (HROC) to up-condition the weakened soleus H-reflex. We have previously shown that this conditioning protocol increases spinal reflexes and improves locomotion. The soleus EMG will be monitored 24 hours a day using the implanted electrodes while the rats move freely about their cage. When the soleus EMG activity falls within the target range, the tibial nerve cuff stimulus elicits a direct muscle response (M response) and a spinally-mediated H-reflex. If the H-reflex size is within a target range, the rat receives a food-pellet reward.

2. VNS only: Rats in this group receive the same handling and care to rats in the HROC group, with brief 0.5-second bursts of VNS delivered on a schedule that is yoked to the HROC group's successful trials. In other words, for each successful H-reflex trial in the HROC group, a corresponding VNS burst will be delivered to a rat in the VNS group. This ensures that the VNS group experiences the same timing and amount of neural stimulation as

the HROC group, but without the spinal reflex conditioning that occurs in the HROC protocol. This yoked control design allows us to isolate the effects of VNS alone, independent of the spinal conditioning provided by HROC.

3. VNS + HROC: This group receives identical procedures as the HROC only group. However, a 0.5-second burst of VNS will be administered whenever a food pellet reward is delivered during a successful HROC trial.

4. SCI only: This group does not receive any HROC or VNS during the 12-week period.

4.1.B. Expected Results. We do not expect to see modification of spinal reflexes without HROC, consistent with our previous studies[36], and thus we do not expect rats in the VNS only or SCI only groups to exhibit any spinal reflex changes. Furthermore, because VNS is not paired with rehabilitation, we do not expect to observe locomotor recovery, consistent with our previous studies demonstrating that VNS must be paired with rehabilitation to be effective[21]. We expect that the VeHROC group, compared to the HROC only group, will exhibit faster and larger changes in spinal reflexes and greater improvements in locomotion. If we observe that VeHROC provides equivalent change in spinal reflexes and equivalent recovery compared to the HROC only group, we will conclude that VNS does not enhance spinal conditioning. If we observe that VeHROC provides enhanced spinal reflex conditioning but does not yield greater locomotor improvements, we will conclude that the task is insufficient to measure the beneficial changes in spinal reflexes. This result will motivate future preclinical studies investigating VeHROC with more challenging tasks or using more sensitive outcome measures.

4.1.C. Methods

Study procedures will be guided by Drs. Yi Chen and Jonathan Carp at NCAN, experts in rodent HROC. Dr. Joseph Epperson at UT Dallas will be on site to oversee data collection and analysis for the preclinical study.

Group	Therapy	Reflex change	Locomotor Recovery	Interpretation
HROC only	HROC	+	+	HROC alone produces partial changes in spinal reflexes, and incomplete locomotor recovery.
VNS only	VNS	-	-	VNS alone does not yield reflex changes or locomotor recovery
VeHROC	HROC + VNS	+++	+++	VeHROC greatly enhances the spinal reflex conditioning and optimizes locomotor recovery
SCI only	None	-	-	No changes to spinal reflexes or recovery are expected without therapy

4.1.C.a - T9 lateral column spinal contusion. Spinal contusion injuries will be performed as in our previous studies[35]. Under general anesthesia, all animals will receive a calibrated contusion injury to the right lateral column (LC) of the spinal cord at T9 using an Infinite Horizon Spinal Cord Impactor (Precision Systems and Instrumentation). A representative example of the resulting injury can be seen in **Fig. 3A**. Additional details of the animal model protocol can be found in Attachment 11, Animal Research Plan. Before initiating treatment, we allow an 8-week recovery period post-SCI to ensure that function has stabilized[37]. This stabilization period is crucial for establishing a chronic SCI model, where the effects of spontaneous recovery have plateaued, allowing us to accurately assess the impact of subsequent therapeutic interventions.

4.1.C.b - H-reflex operant condition electrode implantation. After a 7-week post-SCI recovery period, rats will be implanted with chronic stimulating and recording electrodes to elicit and measure the H-reflex, as previously described[35,36]. To elicit the soleus H-reflex, a silicone rubber nerve cuff containing a pair of fine-wire electrodes will be placed around the posterior tibial nerve just above the triceps surae branches. To record EMG activity, a pair of fine-wire EMG electrodes with final 0.5-cm segments stripped and separated by 0.2–0.3 cm will be placed in the right soleus muscle. The wires from the nerve cuff and the muscles will be tunneled subcutaneously to a skull-mounted connector. The vagus nerve implantation surgery will then be performed (see section 4.1.C.c).

4.1.C.c - Vagus nerve implantation. VNS implantation procedures will be performed as in our previous studies[30,38–45]. This procedure is detailed in Attachment 11, Animal Research Plan. Following the implantation of the H-reflex electrodes and the VNS electrodes, incised skin will be treated with topical antibiotics, and rats will be given analgesics and antibiotics and allowed to recover for one additional week.

4.1.C.d - Soleus H-Reflex Operant Conditioning. H-reflex data collection starts one week after the implantation surgery (i.e., at least 8 weeks after SCI) and continued for 10 weeks (**Fig. 3B**). During this period, each rat is housed in a standard cage with a flexible cable attached to the skull-mounted connector. The cable connects the implanted electrodes to an EMG amplifier and stimulator. The rat has free access to water and food, except that during H-reflex conditioning the rat receives food mainly by performing the task per protocol. A custom program provides the stimulus and monitors ongoing EMG activity continuously 24 h/day, 7 days/wk, for the entire period of data collection. The stimulation paradigm and signal processing are described in Attachment 11, Animal Research Plan.

To determine the initial size of the H-reflex, data will be collected for 2 weeks under the control mode, in which the computer only records soleus EMG activity for 100 ms following the stimulus. Then, each rat will receive right soleus H-reflex up-conditioning for 8 weeks, as we have previously shown this to improve gait after this model of SCI. The software provides a 20-mg food pellet reward 200 ms after tibial nerve stimulation if the absolute value of right soleus EMG activity in the H-reflex interval is above a criterion value. The criterion value is adjusted as needed each day so that the rat receives about 700 reward pellets per day for a 300-g rat.

4.1.C.e - Delivery of Vagus Nerve Stimulation. For rats in the VeHROC group, the control software will provide a 0.5-s burst of VNS that coincides with the HROC food pellet reward described above. Rats in the VNS-only group will have a yoked control from the HROC group and receive a 0.5-s burst of VNS and food at the same time as the yoked control from the HROC group. Each 0.5-s burst of VNS burst will consist of 0.8 mA, 100 µsec biphasic pulses delivered at 30 Hz, identical to previous studies [41–44,46]. All rats in the study will be connected to the cable during therapy to make subjects indistinguishable in appearance and ensure blinding of experimenters.

4.1.C.f - Locomotor assessment. Locomotion is assessed on the treadmill and the horizontal ladder seven times in the study period, from before SCI until after treatment ends (**Fig. 3B**). Treadmill assessment includes EMG and kinematic measures and produces a detailed quantitative assessment of locomotion. We will also assess precise hindlimb control with the Foot Misplacement Apparatus System (Columbus Instruments), which quantifies the accuracy of walking across a horizontal ladder[47]. This demanding task detects persistent deficits even in rats that have regained apparently normal treadmill locomotion after SCI[35]. A full description of these assessments is available in Attachment 11, Animal Research Plan.

4.1.C.g - Data and statistical Analysis. The design has two between-subject treatment factors (HROC and VNS), each with two levels (present or absent). It includes one within-subject factor (time of assessment) and considers sex differences by including an equal number of male and female rats in each group (N=14 total rats per group). Thus, this is a 2x2 between-groups design combined with 5 times of assessment. The primary goal of this project is to test the hypothesis that HROC combined with VNS will provide better recovery after SCI than either alone. The main purpose of data analysis is to compare the impact of the different treatments on 4 measures of locomotion: step-cycle length, step-cycle symmetry, hip-height symmetry during treadmill locomotion, and accuracy of ladder walking (number of errors). Each of these dependent variables will be evaluated with mixed-effects linear models, including sex as a factor to assess sex differences in treatment effects. Significant effects will then be evaluated by post-hoc tests. Main effects of the treatments (HROC, VNS) and sex will evaluate their overall effects. The interaction between treatments and sex will test whether their effects are more or less than additive and if they differ by sex. The interaction with assessment time will test whether the effects depend on this factor (e.g., whether they persist). In regression analyses, we plan to examine the correlations of the success of conditioning (i.e., magnitude of H-reflex change) with specific locomotor functional measures (e.g., step-cycle symmetry), and we will also explore sex-specific correlations to identify any differences in recovery patterns between male and female rats. The 14-rats per group are based on power analysis of locomotor and anatomical data from rats with SCI or without SCI (e.g., [*46,47,51,52,54,55,68-71*], and preliminary studies) so that there is a 99% probability that we will detect at the $p<0.01$ level intergroup differences of the magnitudes implied by these data.

4.2. Aim 2: Identify clinically relevant biomarkers that predict responsiveness to VEHROC therapy

Predictive biomarkers hold significant clinical value by predicting an individual's responsiveness to therapy, enabling personalized treatments. In the context of neuromodulation, these biomarkers allow for tailoring stimulation parameters to patients' specific needs, improving outcomes and minimizing side effects while

enhancing clinical decision-making and cost-effectiveness. In Aim 2, our goal is to identify predictive biomarkers to determine individual responsiveness to VeHROC.

We have identified three primary biomarker targets for VeHROC, grounded in literature and clinical viability. The first target focuses on VNS-induced apnea as a marker of therapy efficacy, hypothesizing that the extent of Hering-Breuer reflex (HBR)-evoked apnea could predict VeHROC responsiveness. The second target explores neurophysiological responses during HROC, considering features like H- and M-wave amplitudes and timing as potential biomarkers. The third target involves assessing SCI and therapy related microRNAs (miRNAs) in the blood, which are involved in inflammation, neuroplasticity, and neuroprotection, and serve as minimally invasive biomarkers. Given the complexities of neural recovery after SCI, we anticipate that combining biomarkers across these modalities will yield the best predictive power, using machine learning to integrate the multi-modal data. Successful completion of Aim 2 will result in a set of biomarkers and algorithms to examine in future clinical trials of VeHROC.

4.2.A. Experimental Design. The biomarker discovery in Aim 2 will leverage the rat behavioral study from Aim 1, with data collection performed at multiple timepoints (**Fig. 3C**). VNS-induced apnea will be assessed while animals are awake and stationary in their homecage, and the HBR recruitment curve will be acquired by varying stimulation amplitude. These VNS-induced apnea measurements will allow us to monitor variability in vagus fiber recruitment over time, which we hypothesize contains information related to VNS efficacy. The EMG data recorded during HROC will be analyzed to investigate measures related to the evoked M and H-wave responses. Blood will be collected from all rats for miRNA analysis. We will perform both the blood draw for miRNA analysis and VNS-induced apnea recording during the first two weeks of therapy to investigate whether measures taken during the first 2 weeks of therapy can improve predictive power.

4.2.B. Expected Results. Our primary goal is to identify a biomarker that strongly predicts final outcomes on locomotion using the biomarker data collected at the start of therapy. Specifically, we expect that the collection of biomarkers collected within the first 2 weeks after beginning therapy (Wk 0 and Wk 2 timepoints; **Fig. 3C**) will predict with high confidence the final functional measures on locomotion at Week 11. We will investigate each biomarker in isolation, as well as the combination of all collected biomarkers. MiRNAs are small non-coding RNAs that regulate gene expression by preventing their translation into protein. MiRNA are effective biomarkers because they are tissue-specific, released into the bloodstream, remain highly stable, and reflect underlying biological processes, making them valuable indicators for monitoring disease and therapeutic effects. From the miRNA dataset, we expect to identify specific miRNAs that are associated with recovery outcomes. For the VNS-induced apnea dataset, we predict that animals in the VeHROC group will exhibit greater recovery if their minimum VNS stimulation threshold to evoke the HBR is closer to the standard VNS stimulation parameter used in this study (0.8mA). Additionally, we anticipate that apnea-related features (e.g., strength of apnea, variability of apnea response within and across sessions) and the neurophysiological features from HROC will partially predict an individual subject's responsiveness to VeHROC. Overall, we expect that the comprehensive collection of clinically-viable biomarkers that integrate miRNA expression data, VNS-induced apnea information, and HROC neurophysiological measures will best predict an individual final outcome.

4.2.C. Methods

4.2.C.a - Diaphragmatic EMG surgical implantation and recruitment of the Hering-Breuer reflex. EMG electrodes are implanted in the diaphragm in all rats from the study in Aim 1 as previously described[48,49]. This method ensures reliable and consistent dEMG recordings. Hering-Breuer reflex (HBR) thresholds using dEMG is performed as previously described[48]. The dEMG is recorded with the rat resting in its homecage. The dEMG electrodes are connected to an amplifier, filtered from 50–300 Hz, then sampled at 5 kHz using an A-D converter. The length of each dEMG burst from the peak of the inspiratory phase to its termination defines the expiratory length, and the periods between bursts define the apnea. The rate of these bursts occurring in a minute defines the respiratory rate. The vagus nerve electrodes connect to a stimulator and electrical pulses are applied (100 μs pulse duration, at 30 Hz, up to 5s). The current amplitude is increased in 0.2 mA increments, with a minimum of 20 s between each test, until either the threshold current required for recruitment of the HBR is reached or a maximum of 2 mA delivered. The average expiratory length and time between bursts is calculated over 5 s before and after the onset of VNS. These baseline measurements are used to normalize the greatest period of apnea that occurred

during the 5 s bout of VNS. The threshold for recruitment of the HBR is the minimum VNS current required to produce an apnea.

4.2.C.b - Blood collection and MicroRNA analysis. This work is led by Co-Investigator Dr. Tavares Ferreira, an expert in the proposed sequencing technologies. Blood is collected from all rats in the study described in Aim 1 at multiple time points (**Fig. 3C**) from the tail vein using standardized methods[50] and treated with an anticoagulant such as EDTA to form plasma. Next, we will extract miRNA from the plasma using Qiagen's miRNeasy Plasma Kit and then create and sequence small noncoding RNA libraries on an Illumina NextSeq2000. This step aims to identify miRNAs that are differentially expressed between groups. We will align processed reads to a miRNA database to identify known miRNAs and quantify their expression. To compare miRNA expression across treatment groups, we use generalized linear mixed models (GLMMs) accounting for fixed effects (treatment group and time points) and random effects (individual variability among rats and baseline measurements). This approach identifies miRNAs that are differentially expressed between treatment groups while controlling for intra-subject correlations and other confounding variables. We will also perform post-hoc pairwise comparisons to further explore differences between specific groups. Additionally, we will train regression models on miRNA expression data to predict outcome measures (e.g., gait asymmetry index). We aim to predict individual responsiveness to VeHROC and identify key miRNAs associated with recovery outcomes. Bioinformatics tools such as TargetScan will be used to identify potential target genes and pathways for the top expressed and top differentially expressed miRNAs. We integrate miRNA and target gene data to construct regulatory networks, using Cytoscape for network visualization and R packages like igraph for network analysis.

4.2.C.c - Biomarker-based algorithm and data analysis. This biomarker analysis will be guided by consultant Dr. Friedenberg, an expert in machine learning and statistical analyses. Inspired by recent studies that use multi-modal datasets for improved predictive modeling, we will explore statistical and machine learning approaches to combine all multi-modal features collected above to predict each subject's response to therapy. First, a dataset of input features (e.g., VNS-induced apnea, H-reflex features, and miRNA expression levels) and behavioral response variables (e.g., locomotor data) will be generated for all subjects across time. We will investigate both regression models trained to predict the outcome measure (e.g., gait asymmetry index), and classification models trained to predict categories of outcomes (e.g., responder vs. non-responder, or strong-medium-weak response). We will leverage common latent representations of biomarker features using common techniques such as canonical correlation analysis, and more advanced non-linear techniques such as CEBRA. These feature-merging methods optimize predictive power by reducing dimensionality of input data while minimizing information loss. Models will be fit with combinations of the merged latent representation of features, and the locomotion scores will be used as labels. Our primary analysis will investigate the predictive capability of biomarker data collected (Pre-Therapy to Week 2) to predict outcomes at the end of therapy using both regression and classification models. We will also evaluate sex as a biological variable by encoding sex into the models and assessing its effect alongside other predictors. Additionally, we will perform sex-specific analyses to identify any differences in recovery patterns between male and female rats. Classification models will be evaluated with cross-validation, using metrics such as accuracy, precision, recall, and AUC-ROC. Regression models will be evaluated using metrics such as mean squared error (MSE) and R-squared to assess their predictive performance. Findings will be interpreted for biological relevance, cross-referencing the biomarkers with existing literature to validate their role. This integrated analysis will help uncover complex biomarker relationships and enhance understanding of factors influencing the efficacy of VeHROC therapy.

4.3. Potential risks and mitigations for preclinical study (Aims 1&2).

The preclinical studies present several risks, each of which will be addressed with targeted strategies to ensure reliable and translatable outcomes. First, there is a risk that the therapeutic effects observed in the rat model may not fully translate to human applications due to physiological differences. To mitigate this, we will use behavioral assays closely aligned with clinical measures, apply rigorous statistical analyses, and prioritize biomarkers that are measurable in both preclinical and clinical settings. Variability in injury severity and recovery is another concern that could lead to inconsistent results. We will control for this by employing a standard spinal contusion model with precise injury parameters, randomizing animals into treatment groups, and using statistical methods to account for individual differences. The identification of clinically relevant biomarkers could be challenging due to the complexity of multimodal data. We will address this using machine learning and dimensionality-

reduction techniques, focusing on biomarkers with known clinical feasibility. Additionally, VNS-induced noise in EMG recordings is a known technical issue that could interfere with data accuracy. To counter this, we will implement filtering techniques, signal processing algorithms, and timing adjustments to isolate and remove noise from EMG signals. Pilot tests will refine these methods before full-scale experimentation begins. By applying these strategies, we aim to generate robust and translatable preclinical data that support the progression of VeHROC to human trials. Lastly, our team is comprised of experts in each of these technologies, including awake behaving electrophysiology (EMG), VNS, HROC, sequencing, and machine learning based biomarker discovery (see Letters of Collaborations for details).

5.1. Aim 3: Evaluate the safety, feasibility, and early efficacy of VeHROC in individuals with lower limb spasticity after SCI.

To evaluate the clinical utility of VeHROC, we will perform a single-arm pilot study to investigate both the spinal conditioning effects and functional improvements after VeHROC in six individuals with SCI and a spastic gait disorder. This study leverages our access to a unique population of 19 individuals who have already been implanted with a VNS system as part of our participation in our early feasibility study of VNS in SCI, and many of these participants (15/19) are enrolled in ongoing follow-on studies and have expressed interest in futures studies. We will evaluate the safety and feasibility of this approach and compare changes in spinal reflexes and improvements in locomotion from baseline to post-therapy. If VeHRC provides successful safety and feasibility outcomes, and demonstrates evidence of early potential efficacy, these findings will be used to inform a larger randomized controlled trial (RCT). *The pilot clinical trial methods are briefly described below. Please see attachment 13 Pilot Clinical Trial Plan for additional details.*

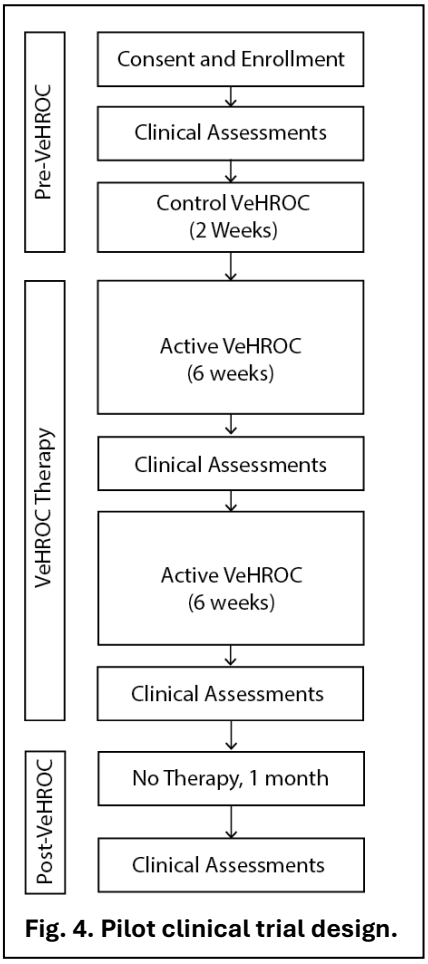

Fig. 4. Pilot clinical trial design.

5.1.A Study Initiation. Initially, we will secure regulatory approval to conduct the pilot study by amending our existing IRB and IDE protocols to incorporate the combination of HROC with VNS. Given that participants have already undergone the surgical implantation of the VNS device and the low-risk profile of HROC, we anticipate an expedited approval process. Once we have gained regulatory approvals, we will enroll six participants. These participants will be selected from those who have completed our previous study using VNS paired with upper limb rehabilitation in individuals with SCI. Of the 19 implanted participants, 8 have gait impairments, meet our eligibility requirements, and have already been involved in our other extension studies (NCT04288245). These participants have expressed interested in participating in studies for gait, thus we do not expect barriers in recruiting six of these individuals.

5.1.B Study design. After enrollment, participants will undergo baseline assessments using gold-standard clinical outcome measures including the 6-minute walk test and 10-meter walking test. All assessments will be done by a Dr. Chad Swank, DPT, who is a licensed physical therapist and was integrally involved in our previous VNS SCI clinical trial. Following baseline assessments, participants will receive 6 sessions of control VeHROC sessions across 2 weeks, followed by 36 sessions of active VeHROC therapy across 12 weeks, as previously described[51]. In the control VeHROC condition, participants do not receive visual feedback or VNS; instead, only the remaining aspects of the protocol are administered (see Attachment 13 for details). This approach eliminates both the operant conditioning and neuromodulation components, allowing for an isolated assessment of baseline effects. Functional outcomes will be assessed at baseline, at the mid-point of therapy (6 weeks), at the end of therapy (12 weeks), and at one month after the final therapy session (**Fig. 4**). The functional outcome measures for this proposed study are the 6-min walk test, gait speed measured by the 10-meter walking test[52], the Walking Index for Spinal Cord Injury (WISCI-II)[52], the Modified Ashworth Scale (MAS) to assess spasticity in the lower extremity, and spatiotemporal gait characteristics such as cadence (strides/min), stride length (cm), and step length

asymmetry[53]. The inclusion of multiple metrics is useful, as a combination of the 10MWT and the WISCI-II is recommended to provide the most valid measure of gait improvement in individuals with SCI[54].

5.1.C Delivery of VeHROC therapy. Study procedures related to clinical HROC will be guided by Drs. Jodi Brangaccio and Jonathan Carp at NCAN, experts in clinical HROC. Dr. Jane Wigginton will provide medical oversight during the trial. Dr. Joseph Epperson, an expert in delivering VNS in the clinic, will be on site to oversee devices, data collection and analysis for the VeHROC therapy. During study sessions, the HROC system is set up, including placement of the EMG electrodes on the soleus muscle and stimulation electrodes over the tibial nerve. Next the VNS power control module (PCM) is placed over the implanted device in the lateral neck, as has been used in the prior studies. The PCM powers and communicates the implantable pulse generator positioned over the vagus nerve (see Attachment 2, Regulatory Documentation; Fig. 1). At the beginning of each session, an H-reflex and M-wave recruitment curve is obtained while the person maintains a low level of soleus EMG activity. The M-wave size for H-reflex trials is chosen from the rising phase of the H-reflex recruitment curve, typically with the stimulus just above the M-wave threshold.

Participants stand in front of a screen that presents the soleus EMG activity and the H-reflex size. When the participant keeps the soleus EMG activity within a specified range for 2 s, a stimulus pulse is delivered to elicit the H-reflex. In active VeHROC sessions, the bar then changes color to either green to indicate a successful trial (i.e., when the H-reflex fell within the shaded region below the criterion value), or the bar turns red to indicate a failure (i.e., when the H-reflex fell outside of the shaded region). On successful trials, a brief, 0.5-second burst of VNS (0.8 mA, 30 Hz) is immediately delivered. The criterion value is dynamically selected so that ~50% of trials are successful. In control trials, no VNS is delivered and no visual feedback of H-reflex size is presented.

Each session contains 245 trials, split into 20 control trials, then 3 blocks of 75 active or control trials. In the control sessions, the 3 blocks of 75 trials are all control trials (no visual feedback of H-reflex size or VNS is delivered). In the active VeHROC sessions, the 3 blocks of 75 trials all provide visual feedback of H-reflex size and VNS delivery. During active VeHROC sessions, we anticipate approximately ~113 VNS deliveries per session (~50% of total trials). This is a similar dose of VNS to our previous VNS studies[28,45].

5.1.D Expected Results. The primary purpose of this study is to evaluate the safety and feasibility of VeHROC in individuals with chronic incomplete SCI. We will compare the nature and occurrence of adverse events (AEs) in the pilot clinical study with data collected during our clinical studies using VNS or HROC in individuals with SCI. Given the long records of safety and tolerability for both HROC and VNS, including in the context of SCI, we do not expect to observe an increase in the rate or change in the nature of AEs with the combined VeHROC. If we observe one or fewer serious device-related adverse events in the pilot study, we will conclude that this approach is safe. Additionally, we will evaluate feasibility based on completion of therapy sessions. If individuals complete more than 75% of the prescribed therapy sessions, we will conclude that VeHROC is feasible to deliver. These thresholds are consistent with prior FDA-regulated device studies. An observation of safety and feasibility in this study would provide a basis for evaluation in a subsequent prospectively powered, double-blinded study.

In addition to safety and feasibility outcomes, early efficacy outcomes such as reductions in H-reflex responses, walking tests, and gait kinematics will be assessed throughout the study to evaluate the magnitude and rate of improvement. We predict significant reductions in the soleus H-reflex response because participants will receive a down-conditioning protocol. Furthermore, we anticipate improvements in gait speed compared to baseline performance, as we've observed in our previous HROC study. Additionally, we will perform comparisons with subjects from our past clinical trials using HROC in individuals with SCI to compare the rate and magnitude of H-reflex change and gait improvement.

5.1.E Data and statistical analysis plan. The statistical analysis for this study is led by Dr. David Friedenberg, a statistician and data scientist at Battelle Memorial Institute. The overall purpose of this study is to evaluate the safety and feasibility of combining HROC and VNS in individuals with SCI. The primary outcome of the study is safety. Safety outcomes will be assessed in accordance with standard procedures for device-based clinical trials. Adverse events with an onset during the course of study will be recorded, tabulated by body system, first occurrence of the event, maximum severity, and strongest relationship to study treatment.

We will also collect multiple secondary measures, including changes in H-reflexes, changes in measures of spasticity on MAS, and functional outcomes through multiple gait assessments. Because this is a pilot study, it is not explicitly powered to detect significant differences in any secondary functional endpoints. However, we note that any changes observed with therapy can likely be ascribed to VeHROC, since we do not anticipate changes in H-reflexes or functional improvements in the absence of therapy. To determine for each subject whether the conditioned H-reflex size changed significantly over the 30 conditioning sessions, the average H-reflexes of the final six sessions will be compared to the average H-reflexes of the first six baseline sessions by paired *t*-test. To determine improvements for each subject on functional outcomes, including improvements in the 6-min walk test, 10-meter walking test, and WISCI-II, we will compare function before and after therapy using repeated measures ANOVAs and paired *t*-tests. For the primary safety and feasibility outcome, 6 participants yields 82% probability of observation of at least one occurrence of all events that occur in participants at a rate of 25% or greater.

5.2. Potential risks and mitigations for pilot clinical trial (Aim 3).

The clinical study involves several key risks, each of which will be mitigated through specific strategies to ensure study success and data reliability. First, recruitment and retention may be challenging due to the small, specific population required. We will leverage ongoing relationships with participants from previous VNS studies who have expressed interest in future trials, ensuring efficient recruitment. Consistent communication and support throughout the study will aid in participant retention. Given the small sample size, there is a concern that variability in individual responses may limit the study's ability to draw robust conclusions about efficacy. We will focus on collecting detailed data through within-subjects comparisons and repeated measures, which will allow us to identify trends even with the limited participant numbers. Insights from this pilot study will inform adjustments for future larger trials. Due to the open-label nature of the study, potential bias in clinical assessments could impact the validity of results. To mitigate this, we will video record all gait and spasticity assessments, and a blinded assessor will re-score the videos independently. This approach provides an additional layer of validation, reducing bias and improving data reliability. Additionally, we will use validated clinical scales (e.g., Modified Ashworth Scale) and objective gait analysis tools (e.g., timed gait assessments with fixed distances) to ensure consistent, robust measurements.

6. Study Personnel Description

This project requires expertise across pre-clinical research, clinical research, neurophysiology, spinal cord injury, devices, and regulatory affairs. The UT Dallas team includes: Eric Meyers, PhD (PI), an expert in neuromodulation therapy development and translation, who will lead project direction, regulatory communication with FDA and IRB, and fiscal management. Robert Rennaker, PhD (Co-PI), the inventor of paired VNS therapy and a recognized leader in the field, will manage regulatory approvals, VNS coordination, and therapy design. Dr. Rennaker's extensive experience includes leading successful regulatory submissions at UT Dallas for the ReStore VNS system, which is used in this project. Jane Wigginton, M.D., a highly experienced clinical trials expert and practicing physician, will oversee participant safety, enrollment, and compliance. Diana Tavares Ferreira, PhD, a neuroscientist and bioinformatician with deep expertise in RNA sequencing, will lead miRNA-sequencing experiments and bioinformatic analysis. Joseph Epperson, PhD, a skilled postdoctoral researcher who has overseen VNS administration in ongoing trials, will manage data collection and analysis. Two graduate students with hands-on experience in preclinical and clinical research will support surgeries, data collection, and analysis.

The NCAN team includes leading experts: Yi Chen, PhD, a neuroscientist and electrophysiologist with specialized knowledge in HROC, who will train staff in HROC techniques and manage the setup of data acquisition systems; Jodi Brangaccio, DPT, an expert in HROC for human studies, who will oversee human HROC procedures and monitoring; and Jonathan Carp, PhD, an experienced electrophysiologist, who will support surgical training and data acquisition.

The project is further supported by consultants who bring critical expertise to the project: David Friedenberg, PhD, a statistician and data scientist from Battelle Memorial Institute with expertise in biomarker analysis, machine learning, and statistics; Chad Swank, DPT, a highly experienced rehabilitation specialist in SCI and VNS, who will support trial design and outcome measures; and Ian Burkhart, a well-respected patient advocate and Lived Experience Consultant, whose unique insights will enhance research strategy and dissemination.