

RESEARCH STRATEGY

Background and Motivation: Spinal cord injury (SCI) causes life-long neurological impairment, with loss of sensory and motor function distal to the point of injury. There are approximately 300,000 patients living with SCI in the United States, and currently no effective treatment. This patient population is reaching normal life expectancy, and it has become an urgent priority to devote considerable research effort to SCI, especially for chronic cases. Repairing SCI after trauma is challenging because of the poor ability of supraspinal axons to regenerate and reconnect to severed descending pathways below the level of injury. The loss of these vital inputs reduces the generation and regulation of motor output. However, approximately 10% of Asia A (i.e. the most severe) patients show trace motor unit activity, though not strong enough to drive visible contraction [2, 3].

Humans and animal models with less complete or partial injury show spontaneous recovery, most likely due the sprouting of endogenous brainstem or propriospinal circuits and synaptic reorganization at multiple locations [4-6]. This plasticity provides supplementary drive to activate motor units no longer receiving sufficient descending input. Although the extent of recovery after SCI is often correlated to the amount of spared descending tracts, training-dependent plasticity and sprouting can reorganize indirect descending pathways and propriospinal connections. The plastic potential of propriospinal interneurons can be utilized to enhance functional recovery with activity-based therapies [7]. An important contributor to recovery after SCI is sensory activity entering through dorsal roots, as verified in experiments where dorsal root lesion greatly reduced the overall recovery [8]. A recent study using a mouse mutant with specific genetic ablation of muscle spindles demonstrated that proprioceptive information is vital to recovery after hemisection of the spinal cord [9]. Other studies have demonstrated importance of tactile and Ib fibers in recovery as well [3]. Collectively, these works demonstrate that sensory input is vital for locomotion recovery after SCI [10, 11].

The improvements in function seen in humans drive the critical need to understand the mechanisms underlying recovery. Epidural stimulation was first used and approved by the FDA for suppression of intractable pain via stimulation of large diameter axons in the dorsal horn [12]. It was subsequently identified to reduce spasticity [13] and allowed voluntary mobility across the knee or ankle in several SCI patients, indicating its utility in supplementing motor activation [14]. In a seminal paper by Harkema et al. [15], it was demonstrated in humans that treadmill training in conjunction with electrical epidural stimulation (EES) enhanced standing, stepping and volitional movement of leg when in a supine position, which was followed up with similar demonstrations in 3 additional complete injured individuals [16]. The locomotor training was shown to bolster use-dependent plastic changes in sensorimotor circuits caudal to the injury site [17].

These studies hypothesize that afferent stimulation most likely acts to increase the excitability of large diameter sensory afferents (LDSA), ultimately driving locomotor responses and plasticity [18] (**Figure 1**). Unfortunately, electrical stimulation is not specific to neuronal phenotypes and the precise populations involved are unknown. Genetic ablation models suggest 1a proprioceptive axons are necessary for spontaneous recovery, but issues with transgenic lines used for this study include “gait ataxia, increased frequency of perinatal mortality, scoliosis,

Premise: Current understanding of EES and open questions

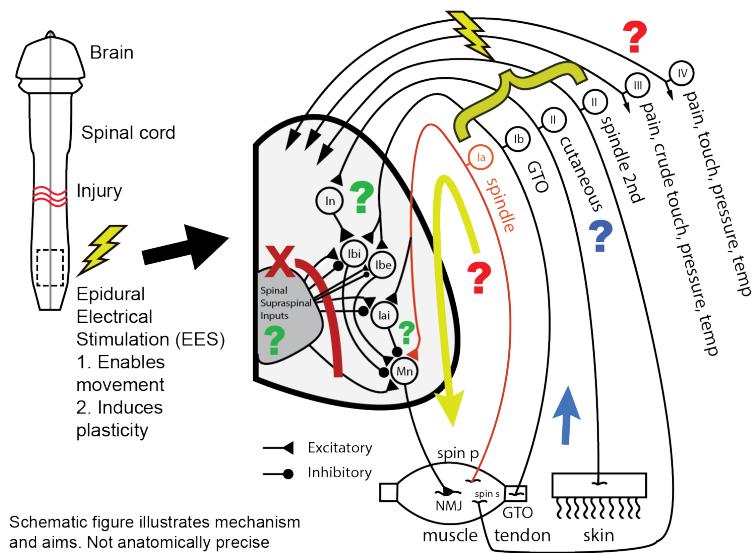


Figure 1. Scientific Premise. Stimulation of the spinal cord caudal to an injury can improve functional outcomes after SCI (left; stylized rat spinal cord). Reduced descending inputs (red X and line) are thought to be supplanted by stimulation of afferent pathways (yellow lightning bolt) that combine with task relevant afferent signals (blue arrow) to provide inputs to the remaining local spinal circuitry that are 1) large enough and 2) patterned well enough to induce the local spinal circuits to drive motor neurons with strong and task relevant commands. Simulations and experimental studies suggest that stimulating large diameter afferents (yellow overbrace) and perhaps spindle feedback only (orange pathway) is sufficient. However, it has not been possible to experimentally validate which neurons are excited (red and blue question marks; SA1). The sufficiency or necessity of different types of afferent is also unknown, because those of similar diameter (e.g. Ia's vs Ib's; II-cutaneous vs II-proprioceptive) cannot be selectively stimulated electrically (blue question mark; SA2). Finally, the remodeling of spinal circuitry that underlies enhanced recovery with afferent stimulation is only just being understood; genetic dissection of these circuits is crucial to understanding these mechanisms of plasticity (green question marks; SA1 and 2; Hypotheses 2-5).

resting tremors and ptosis” [19], make them non-ideal for studies of locomotor function and recovery [9]. Computational modeling studies corroborated with electrophysiological and pharmacological data of afferent populations suggest that type Ia/Ib/II proprioceptive and type II cutaneous afferents are all affected and may work in concert to enhance recovery [10, 11, 20].

With recent developments in synthetic neurobiology, there are new approaches to neuromodulation on timescales ranging from milliseconds (e.g. optogenetics) to hours (e.g. chemogenetics), as well as viral and transgenic methods to restrict their expression to defined neural groups or phenotypes (e.g. motor, proprioceptive, or nociceptive) [21, 22]. To modulate LDSA in general, as well as proprioceptors specifically, we will target them with a combined surgical and viral approach to express chemogenetic designer receptors exclusively activated by designer drugs (DREADDs) within only LDSA or proprioceptive neurons of the dorsal root ganglia [23]. In addition, co-expression of a genetic tracer (mCherry) will support axonal tracing of the afferents within the spinal cord [24]. The goal in this work is to use the power of these genetic tools to accelerate the dissection of the mechanisms underlying enhanced recovery, not to replace or supersede EES as a method of rehabilitation. In the future, it is possible that these techniques would become safe, FDA approved, and have advantages for rehabilitation, but the focus here is on basic, mechanistic understanding.

DREADDs are M3-muscarinic receptors mutated for selective activation by clozapine N-oxide (CNO), an inert molecule devoid of constitutive activity that readily passes through the blood brain barrier. Although the mechanism of action of DREADDs has been questioned [25], careful controls for potential side effects of CNO, such as those we include in this proposal, mitigate this problem. These G-protein coupled receptors are further mutated to interact with selective downstream signaling pathways to either depolarize (hM3Dq) or silence

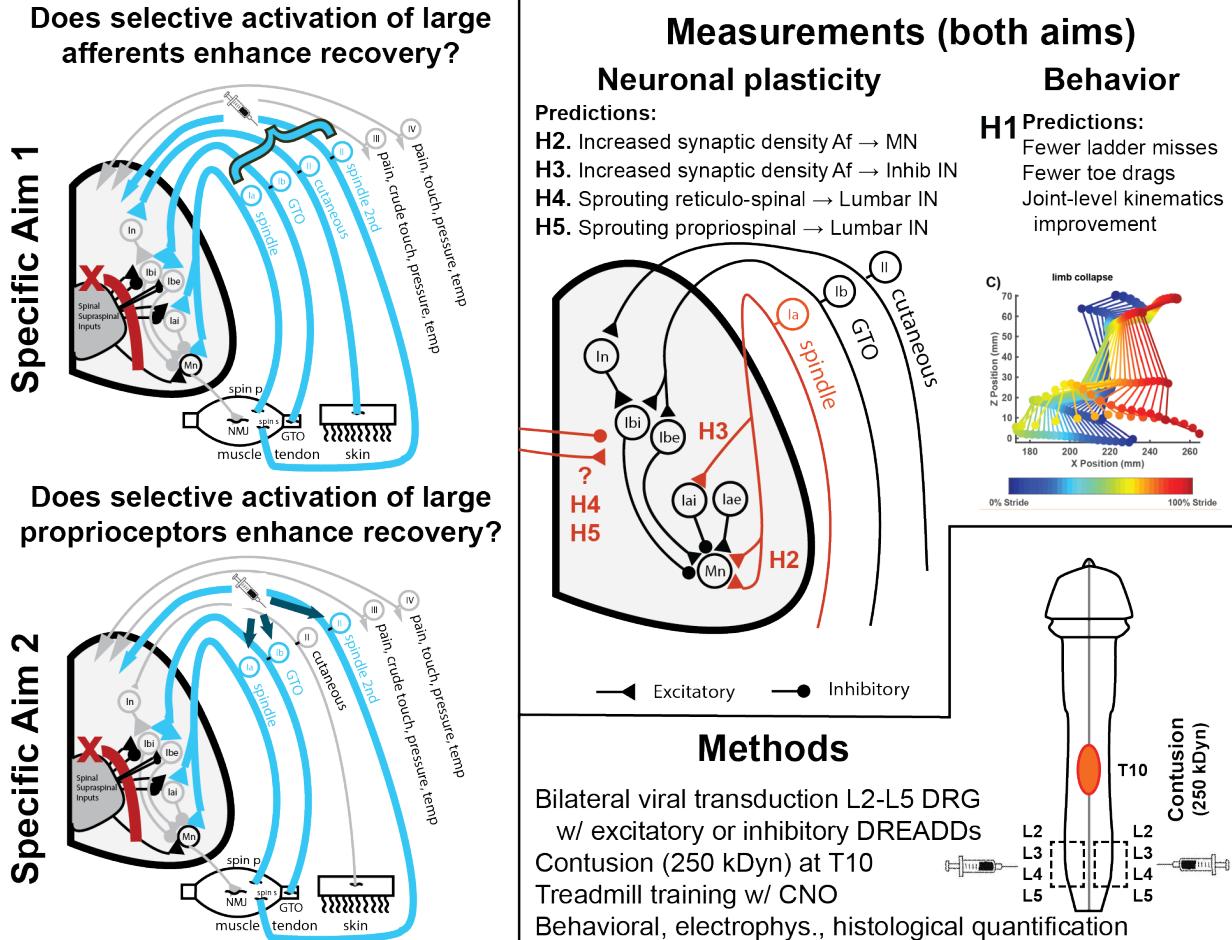


Figure 2. Aims and Hypotheses. Our aims (left) are to determine whether 1) all LDSA, and 2) large proprioceptive afferents only, are sufficient to enhance recovery from SCI. This is critical information to understand the necessity and sufficiency of different afferent classes in driving functional enhancement and plasticity. We hypothesize that excitation of both sets of afferents will improve locomotion (H1; right), and that genetic tracing will reveal mechanisms of plasticity to be increased synaptic density between (H2) afferents and MN, and (H3) between afferents and inhibitory IN, alongside sprouting of (H4) descending reticulo-spinal projections, and (H5) propriospinal projections. Af = Afferent, IN = Interneuron, MN = Motoneuron

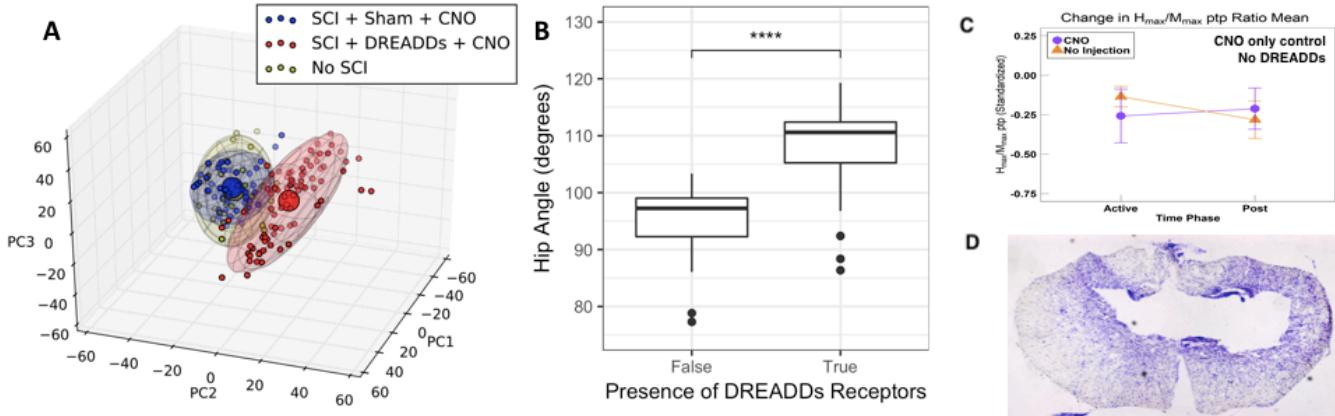


Figure 3: DREADDs activation of afferents causes significant changes in kinematics in a contusion injury model. **A)** PCA analysis of 28 kinematic variables from no-injury (yellow), contused with CNO administration and sham DRG surgery (blue), and contused with DREADDs afferent activation by CNO (red). Small points = strides; large points = means; first 3 PCs explain 77% of the variance; ellipsoids denote $\pm 2\text{SD}$ along principal axes of subgroup; n=11 rats, data pooled from 3, 4, and 6 weeks after moderate contusion (200 kDyn). DREADDs activation (4 mg/kg) caused an increase in PCA2, which depends most strongly on a more extended hip. These data are consistent with our injection into L1 L2 and L3 that supply muscles primarily about the hip, and simulation results suggesting afferent stimulation extends joints (Danner and Rybak, pers. comm.). The recovery of sham treated to no-injury is likely due to relatively mild injury and data collection starting at 3 weeks post-injury, and is consistent with the literature [1]. Furthermore, CNO injections in absence of DREADDs in naïve animals had no effect on H-reflex (**C**), suggesting **A** is not the result of CNO normalizing SCI. The proposed work will use more severe injury (250 kDyn) and gather data from 2 weeks. **B)** Mean hip angle for SCI+DREADDs+CNO versus SCI+Sham+CNO animals. **C)** PTP H_{max}/M_{max} ratio mean is not significantly different across phases within the same group (2-way ANOVA on data baselined to the pre-condition, Mean \pm SD, p=0.83 and p=0.31 for No Injection control (n=7) and CNO (n=6) groups, respectively). **D)** Nissl staining of contused lumbar spinal cord from a DREADDs transduced animal, used to quantify the extent of the contusion in subsequent analyses.

(hM4Di) neurons. They have been selectively expressed in a number of animal models to induce depolarization or hyperpolarization of neurons, affecting behavioral outcomes [23]. Unlike optogenetics which modulate neuronal activity on short time intervals and usually require a tether [26], if properly expressed and activated, DREADDs can provide sustained (~1.5 hours) but reversible neural excitation or inhibition of defined neural populations in freely moving animals during treadmill training. Using this approach, we can also label the affected neural populations and their axonal projections in post-mortem histological analyses, and characterize the higher order neurons they influence.

SIGNIFICANCE:

The significance of this grant is derived from three key aspects (**Figures 1 and 2**):

1) To determine whether activation state of LDSA neurons during treadmill training influences recovery of locomotion after T10 spinal cord contusion injury [9, 27]. This is the working hypothesis behind enhanced recovery with epidural stimulation, and while there is evidence to support this claim, it is ultimately not possible to know precisely which neurons were stimulated, and to what degree. Using our previously developed approach to selectively express DREADDs in LDSA, we can confirm or refute their role in enhanced recovery, because our combined viral and surgical technique targets the LDSA only, and the DREADDs receptors that modulate neuronal firing co-express a fluorescent marker to definitively identify which neurons were activated after the experiment.

2) To determine the role of proprioceptors *in isolation* in driving functional recovery post-SCI. It has been hypothesized that proprioceptive neurons are the primary driver of enhanced locomotor function [28]. We will use previously developed viral tools, in conjunction with a transgenic animal line, to selectively express DREADDs in *only* proprioceptive DRG neurons. We can compare locomotor outcomes to experimental controls and animals where all LDSA were excited via DREADDs to decouple the respective roles of proprioceptors and cutaneous afferents in enhancing recovery from SCI. Previous studies have demonstrated that minimal cutaneous afferent input is necessary for precision foot placement and weight bearing, but it is unknown whether increasing their excitability has any beneficial influence on locomotor drive [10].

3) To uncover the neural mechanisms driving recovery by quantifying projections of modulated afferents onto motor neurons and spinal interneurons, characterizing those interneurons, and testing for plasticity in key descending and propriospinal pathways. One advantage of our genetic approach is the ability to determine whether and how afferent modulation affects monosynaptic connections between Ia sensory and motor nerves by labeling for vGlut1 in transduced dorsal roots. Another is the ability to detect activated second order neurons;

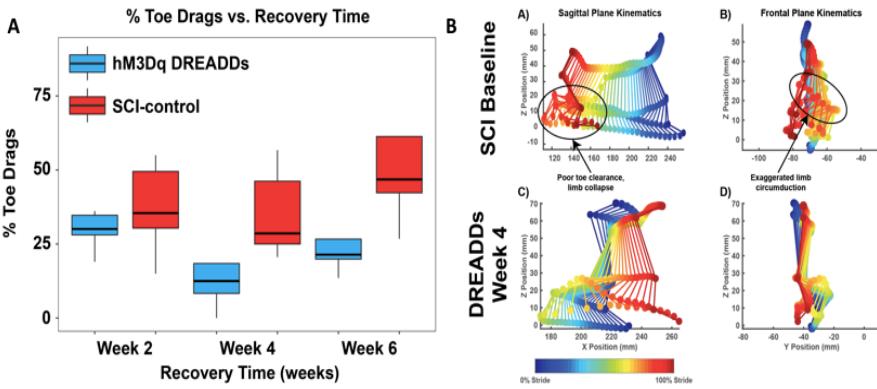


Figure 4: (A-left) Boxplot of percentage of steps initiated by dragging the plantar surface of the foot on the treadmill prior to the initiation of swing. Week 6 post hemi-section: Mann-Whitney U: p=0.055; n=5 animals per group. **(B-right)** Representative kinematic data for hM3Dq (excitatory DREADDs) animals immediately after hemisection **(A-B)**, and after 4 weeks of treadmill training with DREADDs activated **(C-D)**. **A)** Sagittal and **B)** frontal plane limb kinematics one week after spinal hemisection. **C)** Sagittal and **D)** frontal plane kinematics 4 weeks post-SCI. Circled areas in **A-B** show poor toe clearance and wide paw swing; these were not seen at week 4 with DREADDs (**C-D**).

we will use cFOS staining to find interneurons whose activity has been modulated, and then because we can anterogradely trace the affected afferent populations via the mCherry tag on our DREADDs construct, we will be more certain that these interneurons were indeed influenced the afferents we modulated. Using morphometric analysis and GAD67 immunohistochemistry (IHC), we will be able to further characterize whether these interneurons are excitatory or inhibitory. Lastly, we can determine whether key descending or propriospinal projections through the contusion site are sprouting in the lumbar cord. Comparison of between control, hyperactive, and hypoactive DRG neurons will exacerbate and highlight plasticity.

This study will test long-standing hypotheses about the role of specific neural populations in improving motor recovery. The use of DREADDs based approaches will allow us to identify which afferent classes (i.e. proprioceptive vs. cutaneous afferents) are necessary and sufficient for enhanced recovery, and to pinpoint the neural mechanisms that make it possible.

INNOVATION:

There are five key innovative aspects to this proposal:

1) Unlike electrical stimulation, DREADDs are genetically encoded meaning they can be used to manipulate specific subsets of neurons, even when those neurons have similar size and are in close proximity. Here, this allows us to interrogate the involvement of proprioceptors specifically, by restricting expression of the DREADDs to neurons expressing *Parvalbumin*, which includes those that innervate proprioceptive endings in muscle, but not those that innervate cutaneous sense organs. In the longer term, this may lead to further refinement of genetic approaches for targeting and manipulating sensory neurons in a way that optimizes recovery from SCI; as well as applications in other animal models where modulation of sensory information may be beneficial (e.g. stroke, aging, or spasticity).

2) DREADDs can either inhibit or excite neurons. While it is possible to block neuronal firing with high-frequency and other more sophisticated electrical stimuli [29], DREADDs allow direct inhibition of a selected population of neurons without off-target or unknown volumetric effects. Here we use this inhibition to strengthen our experimental design by looking for a reduction in recovery from SCI when we *inhibit afferent activity*. We are able to examine inhibited, normal, and excited conditions. If inhibited afferents cause a significant reduction in recovery then we have stronger, three-point evidence supporting the proposed mechanism, and we can further conclude that normal levels of activity post-injury are important to recovery. Comparison against inhibited conditions will be especially relevant if Hebbian mechanisms are in play.

3) All adeno-associated viruses used in experimental and control groups express a genetic tracer (mCherry) to identify transduced LDSA or proprioceptive DRG neurons and their projections onto spinal circuits. We will map affected spinal cord circuits via co-localization of afferent fibers with cFos staining to identify activated post-synaptic interneurons, by sacrificing animals immediately after strictly controlled treadmill training, in the hypo-, normal, and hyper-activated afferent conditions. We will also characterize the specific role of la sensory afferents in enhancing recovery by mapping their projections onto motor neurons and inhibitory interneurons identified by GAD67 IHC. Finally, the use of Hi-Ret technology to identify projections through the contusion site and whether they are sprouting in the lumbar spinal cord is at the cutting edge of neuronal tracing technology.

4) Chronic stimulation with chemogenetics has strengths when compared to optogenetic and electrical approaches. Optogenetic stimulation can only be administered for short time periods due to light induced thermal toxicity, though some bistable methods have been developed that may alleviate this [21]. Most methods typically require attachment of a head plug and tether, with associated measures to ensure the animal does not become

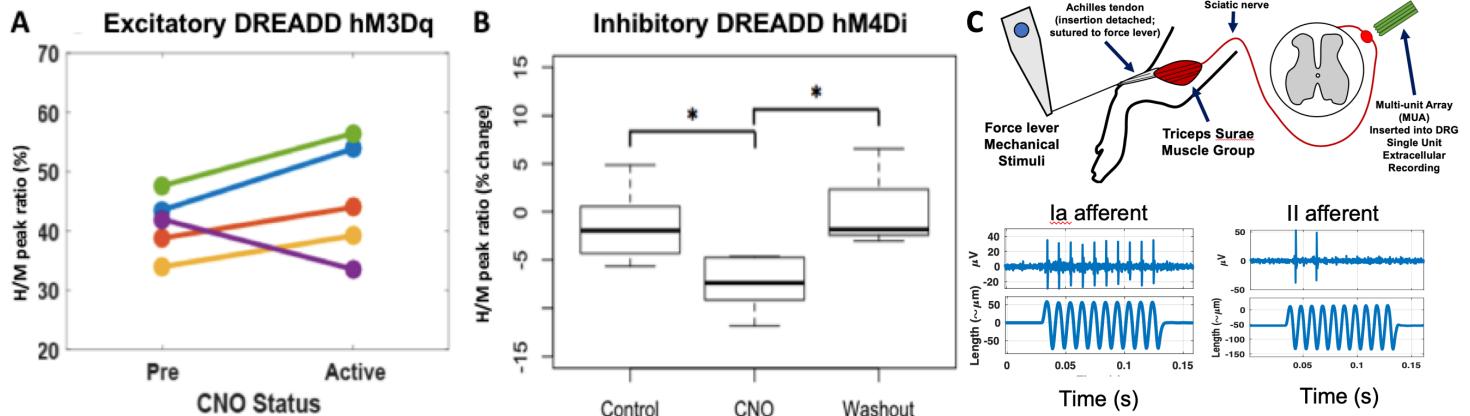


Figure 5: Modulation of H-reflex by DREADDs activation and feasibility of single unit recordings in rat DRG with extracellular multiunit arrays (MUAs). (A) Ratios of max H to max M (in %) for n=5 rats transduced with viral constructs expressing the excitatory DREADD hM3Dq, before and 30 minutes after CNO (2mg/kg) administration. 4 of 5 animals displayed increased excitability under these conditions. (B) Boxplot demonstrating significant reductions in the ratio H/M_{max} when CNO is active in hM4Di animals (n=8), and that the effect is reversible 2 hours post-CNO administration. For H-reflex testing, a bipolar stimulating electrode was inserted anterior to the Achilles tendon to stimulate the tibial nerve. Fine wire electrodes were inserted in the interosseous muscles of the foot pad, and range of stimulus currents were applied to elicit everything from subthreshold excitation to full recruitment of all motor nerves (typically 0.1-4mA). (C) Single unit extracellular recording from rat DRG with controlled force lever mechanical stimuli of the muscle tendon unit to identify afferents. Apparatus (above), and recordings from Ia afferent (below left) that can follow 100 Hz oscillations and spindle II afferent (below right) that cannot. Further ramp, twitch, and triangle stimuli (not shown) give positive identification including Ib afferents.

entangled and/or incur damage due to mechanical agitation at the site of implant when connected [30]. With drug induced chemogenetic modulation, the animal is unrestricted in movement and activity during modulation.

5) We will employ and develop cutting edge techniques from computer vision and non-linear dynamics to quantify changes in locomotor behavior. Rodents are notoriously difficult to perform motion capture on, and publications frequently have small samples of both individuals and strides. To overcome this, we will obtain rich, longitudinal kinematic data sets from over a hundred rats, using an open source Robot Operating System based closed loop treadmill system that we have developed [31] to increase yield, GPU enabled methods from Deep Learning (DeepLabCut [32]) to automatically track features on the animal, and tools from dynamical systems [33] theory to accurately estimate phase (*Phaser* [33]) and reduce the dimensionality of the data [31].

DREADDs-based approaches rely on chemical activation of synthetic receptors via injection or ingestion of CNO, which has a half-life of ~1.5 hours. This means that neuronal excitability can be sustained through the entire treadmill training period with relative ease. DREADDs have also been shown to function when activated continuously for days or even weeks [23], such that neuromodulation could theoretically be applied continuously and chronically in an un-tethered, freely moving animal, performing varied tasks. Finally, the systemic, drug induced mechanism of DREADDs means that manipulations at multiple locations across the body can be carried out simultaneously without implantation of multiple optical or electrical stimulators.

If we are successful, this proposal will constitute a significant contribution to the NINDS' mission to “seek fundamental knowledge about the brain and nervous system and to use that knowledge to reduce the burden of neurological disease.” **In the longer term, chemogenetic tools offer a fundamentally new take on targeted neuromodulation for SCI recovery because their expression can be genetically targeted, modulated neural populations can be identified, and changes in neural circuits can be characterized.**

APPROACH:

SPECIFIC AIM 1: To determine whether selective expression of DREADDs in large diameter (proprioceptive and tactile) neurons and their activation during treadmill training enhances recovery from SCI.

Hypothesis 1: We predict that the use of excitatory DREADDs post-SCI will result in enhanced recovery relative to controls using the following conventional metrics of gait: **A**) stance width, **B**), step length **C**) stance duration, **D**) step height, **E**) hip circumduction, **F**) slips/missed in ladder walking, and **G**) toe-drags in overground gait (**Figures 3 and 4**), and **H**) mean and range of hip, knee, and ankle joint angles; and concomitantly that inhibitory DREADDs will cause reduced recovery relative to controls. All computed kinematic variables will be further examined with a principal components analysis (**Figure 3A**). We further hypothesize that recovery is driven in

part by:

Hypothesis 2: Increased number and density of monosynaptic connections between sensory afferents and motor neurons, driving muscle contraction [9].

Hypothesis 3: Increased monosynaptic connections between sensory afferents and inhibitory interneurons that coordinate phasing of antagonist/agonist muscle pairs [34].

Hypothesis 4: Sprouting of descending reticulospinal neurons onto targets in the lumbar spinal cord [35].

Hypothesis 5: Sprouting of propriospinal neurons onto interneurons in the stimulated spinal segments [17].

Rationale: Previous human, animal, and modeling studies demonstrate that functional recovery following SCI is enhanced by exciting large diameter sensory neurons using an implantable electrical stimulation device. Using DREADDs to induce excitability in reflex pathways is likely to have a comparable effect. Furthermore, if Hebbian mechanisms of plasticity are induced, inhibitory DREADDs should cause an opposite effect, further isolating the mechanism. Because using DREADDs enables labeling of the exact neurons that were activated through co-expression of a fluorescent marker, it makes possible definitive mapping of the circuitry involved.

We have previously established our ability to modulate afferent activity with DREADDs using the Hoffman, or H-reflex, a standard electrophysiological method of assessing reflex function [36] (**Figure 5**). The H-reflex refers to the reflexive reaction of muscles in response to stimulation of sensory afferents [37], and plays a critical role in movement [11].

SPECIFIC AIM 2: To determine whether selective expression of DREADDs in proprioceptive neurons only and their activation during treadmill training enhances recovery from SCI.

Hypotheses 1-5: We hypothesize similar outcomes to those in **Specific Aim 1**.

Rationale: Epidural stimulation studies in rodents have hypothesized [38] that enhanced input from Ia proprioceptive afferents are the major driver behind functional recovery post-SCI, but direct measures of afferent sprouting or increased synaptic density are lacking. Because both LDSA and proprioceptor specific neuromodulation will target these neural populations, it stands to reason that behavioral and electrophysiological outcomes would be indistinguishable from studies where all LDSA are excited via chemogenetic stimulation with DREADDs. *If our hypothesis is supported, it will provide concrete evidence that proprioceptive afferents are the primary drivers of enhanced functional recovery with EES. If not, we will have discovered that cutaneous input is necessary for enhanced recovery, and will begin identification and characterization of the contributions of cutaneous afferents to SCI recovery.*

Experimental Design:

Aim 1: This study will require two experimental and six control groups (**Table 1**). For experimental groups, adeno-associated virus serotype 2 (AAV2) encoding either the excitatory DREADD hM3Dq or the inhibitory DREADD hM4Di will be injected into dorsal root ganglia (DRG; groups hM3Dq-SCI-CNO and hM4Di-SCI-CNO), and CNO will be administered during treadmill training. Control groups will be SCI only without CNO (NoDRGInject-SCI-NoCNO), SCI with a viral construct for expression of mCherry alone injected in place of DREADDs, both with and without CNO (mCherry-SCI-CNO and mCherry-SCI-NoCNO), SCI with expression of either excitatory or inhibitory DREADDs with no CNO (hM3Dq-SCI-NoCNO and hM4Di-SCI-NoCNO, respectively). NoDRGInject-SCI-NoCNO will serve as a ‘baseline’ dataset for the impact of the lesion and recovery with treadmill training. mCherry-SCI-CNO/NoCNO animals will have mCherry alone (without DREADDs) injected into the DRG. Comparing these with the NoDRGInject group will determine whether viral injections into DRG alone influence functional recovery. Comparison of these against DREADDs groups hM3Dq-SCI-NoCNO and hM4Di-SCI-NoCNO will determine whether expression of DREADDs alone influences recovery post-SCI. Comparison of mCherry-SCI-CNO with activated excitatory and inhibitory DREADDs groups (hM3Dq-SCI-CNO and hM4Di-SCI-CNO) will isolate the effect of DREADDs modulation in subsequent histological analyses for spinal circuit dissection and functional recovery assays. Comparison of the DREADDs groups with and without CNO will provide a further isolation of the effect of DREADDs modulation, controlling for CNO in the presence of DREADDs receptors. Comparison between the three pairs of CNO/NoCNO groups will determine whether CNO alone influences recovery, as there is evidence to suggest it may not be entirely inert in the absence of DREADDs [39]. Our preliminary data in contusion animals show no effect of CNO, however (**Figure 3A**). Three way comparison between activated inhibitory DREADDs (hM4Di-SCI-CNO), sham groups (mCherry-SCI-CNO, hM3Dq-SCI-NoCNO, hM4Di-SCI-NoCNO) and activated excitatory DREADDs groups (hM3Dq-SCI-CNO) will provide three points of data on functional recovery and spinal circuit plasticity. We do not include a no treadmill

training control group because extensive past research has found that treatments in this paradigm are only effective when used in conjunction with a rehabilitative activity [7].

Aim 2: This aim will require two experimental and one control group. Experimental groups will express excitatory or inhibitory DREADDs in *only* proprioceptive afferents. We will inject constructs for expression of DREADDs in the presence of Cre-recombinase (Cre) into the DRG of animals expressing Cre under the parvalbumin promoter (fPV), which is found exclusively in type Ia, Ib, and II proprioceptive neurons of the DRG [40, 41]. The NoDRGInject-SCI-NoCNO control for this group will allow us to identify confounding factors that may result from transgene insertion when compared with groups in **Aim 1**.

An overview of the timeline for procedures and experiments for both **Aim 1** and **2** can be found in **Table 2**. Three weeks prior to spinal cord contusion, we will inject the DRG with viral constructs for expression of DREADDs or mCherry only, or perform a laminectomy to expose the DRG without injection (NoDRGInject-SCI-NoCNO). The bilateral surgical procedure (described below) will transduce neurons in the DRG of segments L2-L5. These are the same roots stimulated in prior studies that have demonstrating enhanced recovery from SCI [17, 42]. DRG injections are carried out prior to spinal cord lesion so that expression levels of DREADDs have plateaued (~3 weeks) before initial physiological assessment. This will allow modulation of afferents at one week post lesion similar to past work [9, 42]. One week prior to spinal cord lesion we will gather a complete set of baseline data, consisting of ladder walk and limb/joint kinematics during treadmill locomotion. After a one week recovery, at the start of week 2 post lesion, ladder walk and treadmill kinematic data will be gathered again without CNO to serve as a baseline for functional recovery, and then with CNO in applicable groups to identify its immediate impact. Pain, ladder walk, and BBB [43] assays will further be conducted, as described in **Table 2**.

Long term treadmill training and data collection will consist of tri-weekly walking at speeds ranging from 8-32 cm/s in 8 cm/s increments. Animals will walk at each speed for 8 minutes. Each bout of walking will be interleaved with a 1-minute recovery period. This means a total of 32 minutes of treadmill time in each training session. High speed multi-camera motion capture data will be collected pre-SCI, twice in the second week post-SCI, and every two weeks following the initial collection throughout the recovery period. In each collection, three bouts of walking at each speed for each animal will be recorded. Training will be concluded after six weeks. Prior to euthanasia 8 of 18 rats per group will be injected with CNO (if applicable) and placed on a treadmill at a fixed speed and movement duration, to stimulate cFos expression. Five rats per group will be used for retrograde and anterograde tracing, and the remaining five rats in each group will be used to directly measure the excitability of functionally identified afferents and the H-reflex at the end of study. The former will be done via terminal single unit multi unit extracellular array (MUA) recordings from DRG during servomotor pulls of the Achilles tendon that distinguish afferent classes (**Fig. 5C**; [44]), with measurement of the H-reflex a straightforward addition to this experiment. For the seven SCI treatment subgroups of **Aim 1**, nine subsets of 14 rats will be studied at a time (two from each of the seven conditions), for 10 weeks with nine subsets * 10 weeks ≈ 23 months to complete data collection. Electrophysiological recording in animals dedicated to afferent stimulation validation/characterization will take place after data collection for **Aim 1**. For **Aim 2**, six sub-groups of nine rats (three from each of three conditions) will be studied at a time, for 10 weeks with six sub-groups * 10 weeks = 15 months to complete data collection.

Animal numbers were determined by power analysis extrapolated using previous data sets or published literature. For histological quantitation using a two-way ANOVA study and the Tukey (with control) multiple comparison test, the sample size necessary to be able to detect a minimum change of 25% between the means

SA1 Experiment	Type	Animals
NoDRGInject-SCI-NoCNO	Control	18
mCherry-SCI-NoCNO	Control	18
mCherry-SCI-CNO	Control	18
hM3Dq-SCI-CNO Excitatory DREADD AAV2-syn-hM3Dq-mCherry	Experimental	18
hM4Di-SCI-CNO Inhibitory DREADD AAV2-syn-hM4Di-mCherry	Experimental	18
hM3Dq-SCI-NoCNO Excitatory DREADD no CNO AAV2-syn-hM3Dq-mCherry	Control	18
hM4Di-SCI-NoCNO Inhibitory DREADD no CNO AAV2-syn-hM4Di-mCherry	Control	18
MUA Recordings (5x each hM3Dq, hM4Di, control)	Electrophys Control	15
SA2 Experiment	Type	Animals
TgPvalbiCre / NoDRGInject-SCI-NoCNO	Control	18
Pvalb-hM3Dq-SCI-CNO TgPvalbiCre / Excitatory DREADD AAV2-DIO-hSyn-hM3Dq-mCherry	Experimental	18
Pvalb-hM4Di-SCI-CNO TgPvalbiCre / Inhibitory DREADD AAV2-DIO-hSyn-hM4Di-mCherry	Experimental	18

Table 1: Experimental treatment groups and number of animal subjects required for **Specific Aims 1 and 2**.

with alpha of 0.05 and power of 95% was determined to be n=8 animals per group. However, for analysis of changes in kinematic parameters related to posture and locomotion, a power analysis for a mixed effects design (G*Power 3) shows that to detect a minimum 25% change (our preliminary data seen in **Figure 4** show effect sizes of 25% and 50% change in percentage of foot-drag at weeks 4 and 6, respectively; n=5 rats) with 12 measurements (strides) at a correlation between strides of 0.25 will require 15 rats per condition. To compensate for our observed ~20% loss of animals over the two recovery surgeries, we therefore require 18 rats per condition.

Experimental Methods:

Creation of DREADDs viral constructs:

Aim 1-The DREADD constructs pAAV-hSyn-HA-hM3D(Gq)-mCherry (Plasmid #50474) and pAAV-hSyn-HA-hM4D(Gi)-mCherry (Plasmid #50475) were obtained from Addgene. All vectors were sequenced to verify proper inserts, and all viral procedures were carried out in the dedicated viral core facility operated by Prof. Smith, at the appropriate biosafety level (BSL2+ for packaging work), and approved by Temple's Institutional Biosafety Committee and IACUC (see **Biohazards**). AAV virus was generated using our standard protocol [45] and tested for expression by immunohistology and Western blot analysis after transduction into DRG neurons. *In vivo* expression and physiological activation with addition of CNO was performed on DRG cultures and cortical slice assays using patch clamp technique (Smith, Kirby groups; data not shown).

Aim 2-We are using a construct for DREADDs expression where anti-sense DREADDs are floxed by loxp sites (AAV2-hSyn-DIO-hM3Dq-mCherry and AAV2-hSyn-DIO-hM4Di-mCherry, addgene plasmid # 44361 and 44362, respectively), so DREADDs express only in the presence of Cre-recombinase. We are using these constructs in transgenic animals expressing Cre under the fPV promoter which, in the periphery, is only expressed in proprioceptive neurons (Rat Resource & Research Center Strain #00773; Pvalb-iCre). We have a colony of these animals and have validated the presence of the transgene (see Authentication).

Injection of DREADDs into the dorsal root ganglion (DRG): To inject into DRGs, a laminectomy will be performed on the each side of the spinal column caudal to L2-L5, and posterior articular processes removed to expose the desired DRGs [46]. The rat will be attached to a spinal harness to prevent movement of the spinal column during injection. Using a glass micro-needle and micromanipulator, we will inject 1 – 2 μ l of AAV2 (1×10^{12} GC/ml) directly into the left and right L2, L3, L4, and L5 DRG over 10 minutes. **Our preliminary data show that AAV2 preferentially transduces large diameter cells within the DRG (Figure 8)** [45].

Electrophysiological assays of reflex modulation: To isolate the impact of the injury on afferent and reflex excitability we will further perform extracellular recordings from DRG neurons in A) five animals taken from the SCI-Control (NoDRGInject-SCI-NoCNO), Excitatory DREADD (hM3Dq-SCI-CNO), and Inhibitory DREADD (hM4Di-SCI-CNO) groups, alongside B) a separate group of Non-SCI DREADDs-transfected (L2-L5 DRGs; n=5 for hM3Dq and hM4Di) and control animals (n=5). Stimulation will be via a force lever attached to the Achilles tendon (**Fig. 5C**). Slow and fast ramp, vibratory (100 Hz), and muscle twitch stimuli will be used to identify recorded afferents. Afferent class will be identified by their responses to these stimuli [44]. We will analyze both the 1) single unit firing rates in response to stimulation, and the 2) magnitude of multiunit activity (MUA). Electrical sciatic nerve stimulation will be used to identify thresholds for recruiting c-fibers as well as large diameter afferents, and the H-reflex between tibial nerve and hind-paw interosseous muscle will be measured (**Fig. 5AB**). We will examine responses in pre- and post-CNO conditions for all treatments. We anticipate firing rates and MUA amplitudes for all large diameter afferents (spindle primaries, Golgi tendon organs (GTOs), large cutaneous mechanoreceptors) after CNO administration will increase in hM3Dq animals, decrease in hM4Di animals, and not change in controls. We expect no such increase for the responses at latencies associated with pain fibers in any of the treatment groups. For the Pvalb-iCre animals in **Aim 2**, we expect muscle spindles and GTOs will have affected firing rates, but not large cutaneous afferents. Dr. Lemay routinely uses MUAs in his research, and will supervise these experiments [47]. These results will provide direct evidence of how the excitability of identified afferents changes in response to controlled mechanical stimuli, measures of how reflex pathways

Week	Procedure	Experiments
-3	DRG injection	
-2		Treadmill training (3x)
-1		Treadmill training (3x), Healthy baseline MOCAP one day, pain sensitivity, ladder walk, BBB
0	Spinal contusion	
1		Recovery
2		Treadmill training (3x), baseline MOCAP, pain sensitivity, ladder walk, BBB
3		Treadmill training (3x)
4		Treadmill training (3x), MOCAP
5		Treadmill training (3x)
6		Treadmill training (3x), final MOCAP, pain sensitivity, ladder walk, BBB, electrophysiology
7	Perfusion	

Table 2. Timeline of procedures and experiments.

have been modulated, and further direct validation that the DREADDs receptors selectively affect large diameter afferents (**Aim 1**) and proprioceptors specifically (**Aim 2**). **Fig 5C** demonstrates capability of these recordings.

Spinal cord contusions: This study will employ a moderate contusion spinal cord injury model. A complete laminectomy will be performed at T7-T8 which exposes the T10 dorsal spinal cord. Contusions will be conducted by suspending the spinal cord below the pneumatic piston of an Infinite Horizon spinal cord impactor which will impact the spinal cord with 250 kilodynes of force. We chose 250 kDyn as it is considered a moderate contusion that preserves much of the ventral white matter. The extent of all lesions will be quantified using Nissl staining (**Fig 3C**) to calculate lesion volumes and verify spared tissue with methods adjusted for spinal cord diameter using the standard Cavalieri model.

Behavioral assays of sensory modulation: We will conduct standard and novel asymmetric ladder testing of motor function to determine whether modulation of sensory input influences performance in a precision motor task post-SCI. We will use our novel closed-loop treadmill system [31] with marker-less high-speed motion capture system that allows for 3D kinematic analyses without the use of retro-reflective markers known to agitate animal subjects (**Figures 3 & 4**).

Ladder testing: Behavioral assessment for changes in proprioceptive activation will be done by measuring the accuracy of paw placement on a grid runway as described by Kunkel-Baden et al. [48]. Before injections all rats will be trained using a food reward (Reese's puffs cereal) to walk across a 6-foot grid runway, with pegs placed randomly 2–5 cm apart. Before and after CNO injection (where appropriate), each rat will be videotaped making six complete crossings per trial. This will also be repeated after CNO effects are thought to have worn off (next day). Foot placement will be scored by analyzing the video in slow motion. Individuals conducting these experiments will always be blinded as to the treatment. Accurate placement of the hind-paw on the pegs will be scored as a positive (+) and misplacement (foot falls through the grid) will be scored as an error (-). The percentage of correct foot placements per trial will be calculated and averaged for the four trials. We have recently developed an asymmetric ladder in which the rungs can be angled, making the use of inter-leg coordination more difficult for the animal. This harder task gives higher resolving power on motor deficits (data not shown), and will be scored similarly to standard ladder walk.

Treadmill Walking: To capture 3D kinematic data, we will use our custom color multi-camera acquisition system capable of synchronous image capture at frame rates of up to 300Hz [49]. We will use a Sharpie marker to apply blue markings at the iliac crest, greater trochanter (i.e., hip rotation center), knee, ankle, and metatarsophalangeal joint (MTP, i.e. toe joint) of the desired (hind-right) limb. Markings will be reapplied prior to each motion capture session to ensure accurate tracking during analysis. We will use DeepLabCut [32] on an NVidia Titan Xp GPU to track features in raw video sequences at ~50 frames/sec, followed by our recently published open source 3D reconstruction techniques (**Figures 3 and 4**) [50-54].

Assays for Pain Sensitivity: One major concern with using any sort of excitatory stimulus on sensory systems is increased pain sensitivity. For this reason, we will conduct a standard battery of classical behavioral assays for thermal and tactile hyperalgesia including Von Frey and Hargreaves tests [55, 56]. We will perform this in all

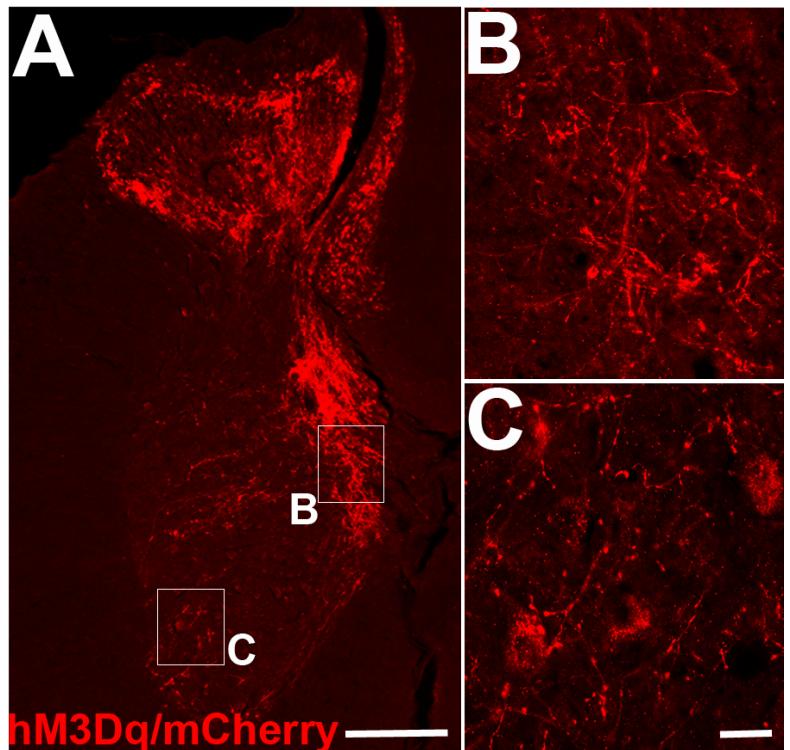


Figure 6. Eight weeks after injection of hM3dQ/mCherry into the DRGs we examined expression and distribution of mCherry positive axons. Under low magnification mCherry positive axons can be observed extending throughout the dorsal and ventral horn (A). Axons appear concentrated in the region of Clarke's nucleus, the site of proprioceptive neurons (B) and in the ventral horn (where motor pools reside) (C). Scale bars A: 200um, B & C: 50um

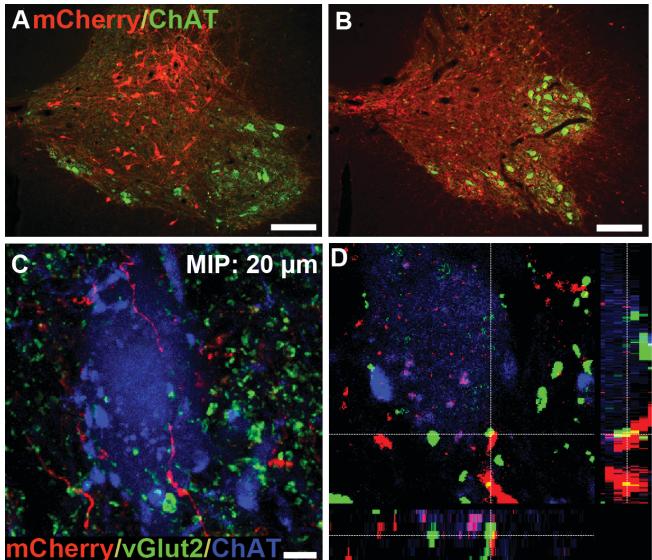


Figure 7. Imaging of synaptic connections between DREADDs transduced propriospinal neurons (PNs) and motoneurons. Expression of inhibitory DREADD hM4Di (mCherry/red) in C3-C4 propriospinal neurons within the upper cervical spinal cord (A) and their terminals extending among choline acetyltransferase (ChAT, green) positive motor neurons at C8 (B). Confocal images (C & D) of C3-C4 PNs mCherry terminals (red) co-labeled with vGlut2 (green) to identify synapses on ChAT positive C8 spinal motor neurons (blue). Z-stack shows associated hM4Di+/vGlut2+ PN axon terminals found on somas of ChAT+ MNs (D; yellow). Scale bars A & B: 200 um; C & D: 10 um.

expressing and activating hM4Di (inhibitory DREADD), hM3Dq (excitatory DREADD), the mCherry control for the DREADDs receptor alone (mCherry-SCI-NoCNO), the no DREADDs CNO control (mCherry-SCI-CNO), and the SCI-control (NoDRGInject-SCI-NoCNO) rats. We will also correlate the cFos positive neurons with the location of afferent terminal synapses, through triple-labeling for synapsin-1 or synaptophysin [45, 55].

It has been established that the vast majority of the Ia to motor neuron synapses label for vGlut1 [58], and these synapses are almost completely lost after dorsal root rhizotomy [59, 60]. With **H2** we hypothesize that recovery is driven in part by increased vGlut1 positive connections between sensory afferents and motor neurons, likely Ias. We will test **H2** by measuring changes in the density of vGlut1 synapses between afferents and motor neurons within the L2-L5 region of the spinal cord. This will be done through co-labelling of afferents, motoneurons, and vGlut1 positive synapses. In a cohort of 5 rats/group, we will inject the B-subunit of cholera toxin into the sciatic nerve at the end of the study, and extend euthanasia for these animals by 7 days. Though this will label both motor neurons and afferents, the afferents will be doubly labelled by the viral transduction, and thus discriminable. Ten evenly spaced frozen sections 300 microns apart through the L2-L5 region will be isolated and co-labeled for vGlut1, with the motor neurons identified using mouse anti-CTB and AMCA conjugated secondary antibody. Subsequently we will quantify the density of sensory afferents within the motor pools and of synapses onto motor neurons using confocal microscopy. The Smith lab routinely performs these types of labeling studies, and detailed explanations of procedures can be found in previous publications [45, 55, 56]. To test **H3**, that afferents will show increased synaptic density onto inhibitory spinal interneurons, we will co-label for cFos and GAD67, an antibody marker for GABAergic (inhibitory interneurons), and compare densities of connections to doubly positive neurons.

Retrograde tracing: To test **H4** and **H5** we need to determine whether neurons that project from either the reticular nuclei or from rostral spinal cord segments, past the lesion, to the lumbar spinal cord have formed new sprouts in the lumbar spinal cord. To do so, we will first use retrograde tracing to identify populations of neurons bypassing (projecting caudal to) the lesion, following by anterograde tracing to quantify sprouting within L2-L5. Retrograde tracing will employ HiRet-GFP to identify neurons with axons bypassing the lesion in experimental and control groups. To do this, musculature will be cleared from the T12-L1 vertebral bodies and bilateral laminectomy performed to expose the L2-L5 spinal cord, which lies directly under the T12 – L1 vertebrae [61].

experimental and control populations prior to SCI. Animals receiving CNO over the course of treatment will be tested both pre- and post-CNO administration. We will perform these same tests post SCI to insure hyperalgesia does not develop because of repeated CNO exposure. For all comparisons, the SCI only control group (NoDRGInject-SCI-NoCNO) will be considered the gold standard of normal pain sensitivity.

Histological analyses: One advantage of using chemogenetic stimulation of sensory afferents is the ability to identify the DRG population expressing the transgene and the location of their axons within the spinal cord. All AAVs generated for this project will co-express a protein marker mCherry (**Figures 6-8**). In all animals, we will examine the number and types of neurons expressing mCherry in the DRG. We will measure the cell body diameter and co-label neurons for parvalbumin (proprioceptive), CGRP and Isolectin B4 (nociceptive) (**Figure 8D-F**). Typically, we observe approximately 75% of large diameter neurons labeled after DRG injection.

We will further use mCherry expression to anterogradely label afferent sensory axons within the spinal cord. Combined with cFos labeling of second order neurons we will identify interneurons likely mediating enhanced recovery with afferent stimulation. cFos is an immediate early gene that is upregulated in neurons undergoing high levels of activation [57]. We will compare the locations of neurons expressing cFos between animals

expressing and activating hM4Di (inhibitory DREADD), hM3Dq (excitatory DREADD), the mCherry control for the DREADDs receptor alone (mCherry-SCI-NoCNO), the no DREADDs CNO control (mCherry-SCI-CNO), and the SCI-control (NoDRGInject-SCI-NoCNO) rats. We will also correlate the cFos positive neurons with the location of afferent terminal synapses, through triple-labeling for synapsin-1 or synaptophysin [45, 55].

It has been established that the vast majority of the Ia to motor neuron synapses label for vGlut1 [58], and these synapses are almost completely lost after dorsal root rhizotomy [59, 60]. With **H2** we hypothesize that recovery is driven in part by increased vGlut1 positive connections between sensory afferents and motor neurons, likely Ias. We will test **H2** by measuring changes in the density of vGlut1 synapses between afferents and motor neurons within the L2-L5 region of the spinal cord. This will be done through co-labelling of afferents, motoneurons, and vGlut1 positive synapses. In a cohort of 5 rats/group, we will inject the B-subunit of cholera toxin into the sciatic nerve at the end of the study, and extend euthanasia for these animals by 7 days. Though this will label both motor neurons and afferents, the afferents will be doubly labelled by the viral transduction, and thus discriminable. Ten evenly spaced frozen sections 300 microns apart through the L2-L5 region will be isolated and co-labeled for vGlut1, with the motor neurons identified using mouse anti-CTB and AMCA conjugated secondary antibody. Subsequently we will quantify the density of sensory afferents within the motor pools and of synapses onto motor neurons using confocal microscopy. The Smith lab routinely performs these types of labeling studies, and detailed explanations of procedures can be found in previous publications [45, 55, 56]. To test **H3**, that afferents will show increased synaptic density onto inhibitory spinal interneurons, we will co-label for cFos and GAD67, an antibody marker for GABAergic (inhibitory interneurons), and compare densities of connections to doubly positive neurons.

Retrograde tracing: To test **H4** and **H5** we need to determine whether neurons that project from either the reticular nuclei or from rostral spinal cord segments, past the lesion, to the lumbar spinal cord have formed new sprouts in the lumbar spinal cord. To do so, we will first use retrograde tracing to identify populations of neurons bypassing (projecting caudal to) the lesion, following by anterograde tracing to quantify sprouting within L2-L5. Retrograde tracing will employ HiRet-GFP to identify neurons with axons bypassing the lesion in experimental and control groups. To do this, musculature will be cleared from the T12-L1 vertebral bodies and bilateral laminectomy performed to expose the L2-L5 spinal cord, which lies directly under the T12 – L1 vertebrae [61].

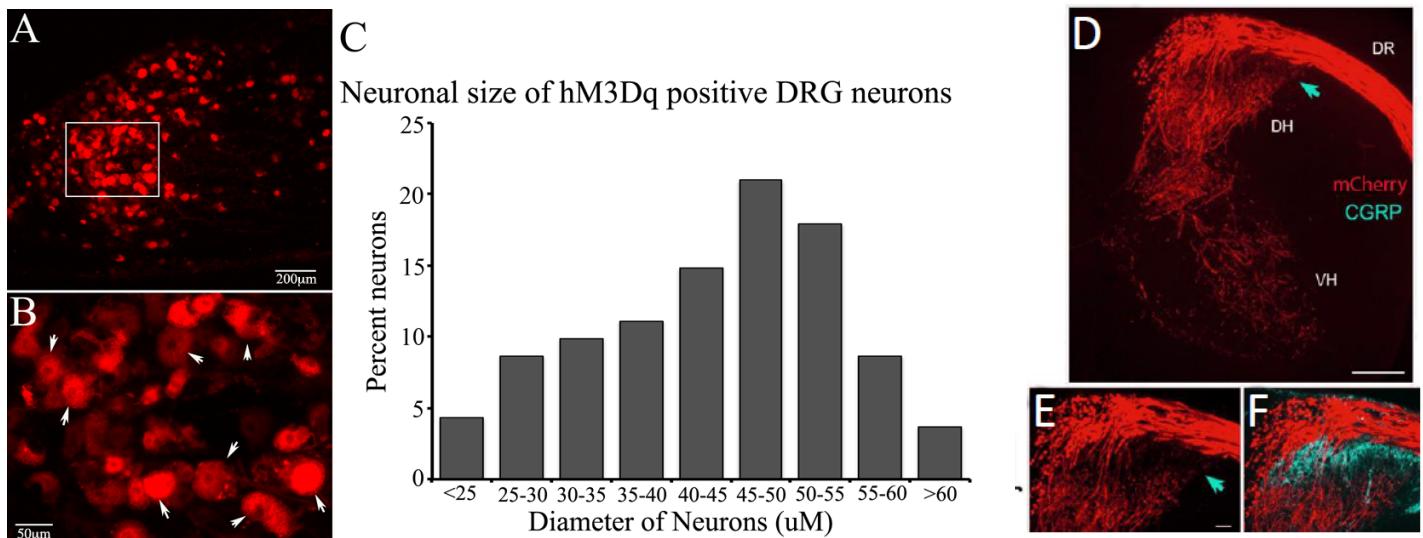


Figure 8. (A) Photograph of AAV/hM3Dq expression in DRG with majority of large diameter cells transduced. (B) Arrows indicating cells $> 50 \mu\text{m}$ in diameter, and (C) distribution of neurons transduced, note that most are $> 40 \mu\text{m}$ in diameter. (D) AAV2-GFP labels large diameter axons projecting into the deep dorsal and ventral horns and not nociceptive axons projecting to superficial DH laminae (E,F). GFP+ labeled sensory fibers in the DR project to the dorsal and ventral horns in the intact animal. GFP+ afferents do not occupy the most superficial laminae of the DH (E), corresponding to areas of CGRP+ small diameter axons (F). Cyan arrows indicate area of minimal GFP labeling. Scale bar: D = 200 μm , E,F = 50 μm . DR = dorsal root, DH =dorsal horn, VH = ventral horn.

HiRet-GFP will be injected with a beveled pulled glass needle with an aperture of 30-40 μm into 5 evenly spaced sites (1 mm) along this length on each side of the cord for a total of 10 injections. Virus will be injected at 3 separate depths of 0.5 mm, 1.0 mm, and 1.5 mm, for a total volume of 2 μl per site (0.7 μl at each depth). All injections will be done using a nanoliter injector (Nanoject, Drummond Scientific) attached to a micromanipulator (Narishige International) for precise measurements. After tracer injections, all animals will be maintained for an additional 4 weeks before euthanasia. We have previously used this virus to quantify reticulospinal and propriospinal axons bypassing a T10 contusion injury [62]. Stereology will be used to quantify the number of neurons expressing GFP within the brainstem, thoracic and cervical spinal cord to determine if experimental conditions increase the numbers of neurons synapsing within the L2-L5 spinal cord.

Anterograde tracing: Regions showing good neuronal labeling using the HiRet-GFP will be examined for axonal sprouting. BDA or AAV-GFP will be injected into select reticular nuclei or spinal cord regions to determine changes in axonal sprouting within the lumbar L2-L5 spinal cord as described in our previous publications [24, 63]. The extent of sprouting will be quantified as per our previous publications [56, 64-66].

Biohazards: Please see separate document uploaded under **Select Agent Research**.

Interpretation of Results.

Histological Analysis: We will compare cFos expressing neurons within the spinal cord for eight experimental groups, as described in our previous publications [45, 56]. Triple labeling of sections using anti-cFos, mCherry, and anti-synapsin-1 will allow us to better define the circuitry and interneurons stimulated by afferent activation and provide insight into the potential first and second order pathways involved. Excitatory/hM3Dq should show the highest numbers of cFos labeled neurons, inhibitory/hM4Di the fewest, and mCherry-SCI (sham) will serve as the baseline level, achieved with treadmill training alone. Comparisons between these animals will better identify which populations represent background excitability, and which are specific to afferent stimulation.

Ladder Walk Behavioral Assay: A reduction in slips/misses in experimental subjects treated with excitatory DREADDs during post-SCI training would be a strong indicator of increased precision foot placement ability. An increased occurrence of misses/slips in this behavioral assay is indicative of reduced ability to precisely control limb position. Videos will be scored anonymously and every video will be scored by at least two individuals.

Toe Drags in Overground Gait: We will use the percentage of steps initiated by dragging of the plantar surface of the foot as a simple method of analysis to gauge overall recovery post-SCI (**Figure 4**).

3D Kinematic Analysis: We will use kinematic data from pre-lesion collections as a gold standard for full recovery, and the first point post-lesion as a baseline from which functional recovery will be assessed (**Figure 4**). Previous analyses of post-SCI gait have found stance width, step length and stance duration symmetry between limbs, peak vertical toe clearance, and hip circumduction to be useful metrics for gauging functional

recovery [67-70]. In general, increased stance width indicates decreased lateral stability, and stance length/step time asymmetries indicate relative contributions of limbs to overall locomotion. We will use toe clearance as a general indicator of both push-off strength, and the ability to appropriately flex ankle/knee joints in swing. Reduced flexor activity and toe clearance is commonly compensated for with hip circumduction, or the tendency to swing a limb farther away from body midline in the frontal plane. Using our 3D motion capture system, we will determine to what degree this compensatory mechanism is contributing to overall toe clearance. Skin markers will be used to compute hip, knee and ankle joint angles, from which mean and range of these angles will be computed, and PCA analyses used to identify important variables (**Fig. 3AB**).

Pain Sensitivity: We will use force and time to withdrawal to assess pain sensitivity in Von Frey and Hargreaves tests, respectively. Reduced forces or time of exposure to heat stimulus post CNO administration within animals, or relative to SCI-controls between animals, will indicate development of hyperalgesia. If this is found to be the case, affected animals will be removed from the study.

BBB Hindlimb Locomotor Test: The hindlimb locomotor test is a measure of success and accuracy of gait in the hindlimbs while an animal moves across a flat plane. Gross measures such as the ability to step on the plantar surface of the paw, support the body weight and walk with a coordinated gait are taken. Fine motor skills such as stability of the trunk, position of the tail, lateral paw placement and ability to walk without dragging the toes are scored. Animals are assessed by two investigators trained in this method for a 4-minute time period, and a score is given on the 21-point BBB scale [43].

Statistical Analyses: For all normally distributed single time-point measurements, one-way ANOVA will be used to compare the experimental groups. When a significant overall difference is obtained on the outcome measure, Bonferroni's adjustment will be used for post hoc comparisons among the groups. Outcome measures collected from repeated measures collected over time will be analyzed using linear mixed effects models with the group variable as a fixed effect, animal as a random effect, and a variance structure for time that accounts for correlations between subsequent time points, determined by analyzing the data. Typically, in past analyses of gait data, data from subsequent steps has been adequately captured with at most first order autoregressive variance structure. We typically collect at least three bouts of several steps of running for each condition. We will test for an effect of bout by including it as an additional random effect and then comparing mixed effects models with and without a term for bout—if significant, it will be included. For outcome measures that are not normally distributed, transformation or non-parametric methods will be used in the analyses. A *p* value of <0.05 will be considered statistically significant.

Potential Problems and Alternative Approaches:

We do not anticipate difficulties with the performance of the experiments as the PIs have considerable expertise in their related areas of study in this proposal. We are confident that the inhibitory and excitatory DREADDs are functioning within the DRG neurons and will continue to monitor their activity electrophysiologically. We will also monitor nociceptive response since DREADD activation of even a few nociceptive axons could cause the animal discomfort and negatively affect training. Any animals showing severe pain during CNO application will be removed from the study. It is possible that certain treatment groups will have higher step-to-step or bout-to-bout variability in their treadmill locomotion and gait data. In that instance, we will extend kinematic data collection sessions to gather more bouts of animal running, to increase statistical power. It is also possible that some treatment groups will perform shorter or more sporadic bouts of running. Here again, treadmill data collections can be extended by up to 12 minutes to gather more strides.

Clinical Relevance: We hope that this study is a first step in the long road to developing interventions that maximize the recovery and motor function of people living with spinal cord injuries.

Scientific Rigor and Reproducibility: The experimental design is blinded, unbiased, randomized, well controlled, and transparent. For all experiments, both males and female rats at a ratio of 1:1 will be used. Other biological variables such as age, weight, and underlying health conditions are fully considered. Since lesion variability can have a profound effect on recovery, lesion completeness and size will be measured in every animal. All histological assessments will be performed blindly with evaluators not knowing the experimental groups. The joint effort between the laboratories of Dr. Spence, Dr. Lemay, and Dr. Smith will take advantage of each other's non-overlapping but highly synergistic expertise. The viral production, animal surgeries and histology will be performed in Dr. Smith's laboratory, treadmill training with and without CNO and gait analyses will be performed blindly by Dr. Spence's laboratory, and all electrophysiology will be performed in Dr. Lemay's laboratory. The P.I. and Co-I's interact frequently through collaborative visits between labs, shared mentoring of students and postdocs, and departmental seminars. The grant will be managed with bimonthly meetings, and the distribution of effort reflects the bulk of the work falling on animal treadmill training and kinematic analyses.

REFERENCES CITED

1. Thota, A.K., *Neuromechanical control of locomotion in intact and incomplete spinal cord injured rats.*, in *Biomedical Engineering*. 2004, University of Kentucky.
2. Minassian, K. and U.S. Hofstoetter, *Spinal cord stimulation and augmentative control strategies for leg movement after spinal paralysis in humans*. CNS neuroscience & therapeutics, 2016. **22**(4): p. 262-270.
3. Minassian, K., et al., *Targeting lumbar spinal neural circuitry by epidural stimulation to restore motor function after spinal cord injury*. Neurotherapeutics, 2016. **13**(2): p. 284-294.
4. Curt, A., et al., *Recovery from a spinal cord injury: significance of compensation, neural plasticity, and repair*. Journal of neurotrauma, 2008. **25**(6): p. 677-685.
5. Bareyre, F.M., et al., *The injured spinal cord spontaneously forms a new intraspinal circuit in adult rats*. Nature neuroscience, 2004. **7**(3): p. 269-277.
6. Courtine, G., et al., *Transformation of nonfunctional spinal circuits into functional states after the loss of brain input*. Nature neuroscience, 2009. **12**(10): p. 1333-1342.
7. Côté, M.-P., M. Murray, and M.A. Lemay, *Rehabilitation Strategies after Spinal Cord Injury: Inquiry into the Mechanisms of Success and Failure*. Journal of Neurotrauma, 2016. **34**(10): p. 1841-1857.
8. Hollis II, E.R., et al., *Remodelling of spared proprioceptive circuit involving a small number of neurons supports functional recovery*. Nature communications, 2015. **6**.
9. Takeoka, A., et al., *Muscle spindle feedback directs locomotor recovery and circuit reorganization after spinal cord injury*. Cell, 2014. **159**(7): p. 1626-1639.
10. Bouyer, L.J.G. and S. Rossignol, *The Contribution of Cutaneous Inputs to Locomotion in the Intact and the Spinal Cata*. Annals of the New York Academy of Sciences, 1998. **860**(1): p. 508-512.
11. Rossignol, S., R.J. Dubuc, and J.P. Gossard, *Dynamic sensorimotor interactions in locomotion*. Physiological Reviews, 2006. **86**(1): p. 89-154.
12. Shealy, C.N., J.T. Mortimer, and J.B. Reswick, *Electrical inhibition of pain by stimulation of the dorsal columns: preliminary clinical report*. Anesthesia & Analgesia, 1967. **46**(4): p. 489-491.
13. Barolat, G., J. Myklebust, and W. Wenninger, *Effects of spinal cord stimulation on spasticity and spasms secondary to myelopathy*. Stereotactic and Functional Neurosurgery, 1988. **51**(1): p. 29-44.
14. Dimitrijevic, M., et al., *Spinal cord stimulation for the control of spasticity in patients with chronic spinal cord injury: I. Clinical observations*. Central nervous system trauma, 1986. **3**(2): p. 129-143.
15. Harkema, S., et al., *Effect of epidural stimulation of the lumbosacral spinal cord on voluntary movement, standing, and assisted stepping after motor complete paraplegia: a case study*. The Lancet, 2011. **377**(9781): p. 1938-1947.
16. Angeli, C.A., et al., *Altering spinal cord excitability enables voluntary movements after chronic complete paralysis in humans*. Brain, 2014. **137**(5): p. 1394-1409.
17. Courtine, G., et al., *Transformation of nonfunctional spinal circuits into functional states after the loss of brain input*. Nat Neurosci, 2009. **12**(10): p. 1333-1342.
18. Verhaagen, J. and J. McDonald III, *Evidence-based therapy for recovery of function after spinal cord injury*. Spinal Cord Injury: Handbook of Clinical Neurology Series, 2012. **109**: p. 259.
19. Tourtellotte, W.G. and J. Milbrandt, *Sensory ataxia and muscle spindle agenesis in mice lacking the transcription factor Egr3*. Nat Genet, 1998. **20**(1): p. 87-91.
20. Capogrosso, M., et al., *A computational model for epidural electrical stimulation of spinal sensorimotor circuits*. J Neurosci, 2013. **33**(49): p. 19326-40.
21. Iyer, S.M., et al., *Optogenetic and chemogenetic strategies for sustained inhibition of pain*. Sci Rep, 2016. **6**: p. 30570.

22. Towne, C., et al., *Optogenetic control of targeted peripheral axons in freely moving animals*. PLoS One, 2013. **8**(8): p. e72691.
23. Sternson, S.M. and B.L. Roth, *Chemogenetic tools to interrogate brain functions*. Annual review of neuroscience, 2014. **37**: p. 387-407.
24. Liu, Y., et al., *Use of self-complementary adeno-associated virus serotype 2 as a tracer for labeling axons: implications for axon regeneration*. PloS one, 2014. **9**(2): p. e87447.
25. Gomez, J.L., et al., *Chemogenetics revealed: DREADD occupancy and activation via converted clozapine*. Science, 2017. **357**(6350): p. 503-507.
26. Boyden, E.S., et al., *Millisecond-timescale, genetically targeted optical control of neural activity*. Nature Neuroscience, 2005. **8**(9): p. 1263-1268.
27. Onishi, K., E. Hollis, and Y. Zou, *Axon guidance and injury—lessons from Wnts and Wnt signaling*. Current opinion in neurobiology, 2014. **27**: p. 232-240.
28. Edgerton, V.R. and S. Harkema, *Epidural stimulation of the spinal cord in spinal cord injury: current status and future challenges*. Expert Rev Neurother, 2011. **11**(10): p. 1351-3.
29. Holt, N., J. Wakeling, and A.A. Biewener, *The effect of fast and slow motor unit activation on whole-muscle mechanical performance: the size principle may not pose a mechanical paradox*. Proceedings of the Royal Society of London B: Biological Sciences, 2014. **281**(1783): p. 20140002.
30. Thelin, J., et al., *Implant size and fixation mode strongly influence tissue reactions in the CNS*. PloS one, 2011. **6**(1): p. e16267.
31. Vahedipour, A., et al., *Uncovering the structure of the mouse gait controller: mice respond to substrate perturbations with adaptations in gait on a continuum between trot and bound*. Journal of Biomechanics, 2018. **78**: p. 77-86.
32. Mathis, A., et al., *DeepLabCut: markerless pose estimation of user-defined body parts with deep learning*. Nature Neuroscience, 2018. **21**(9): p. 1281-1289.
33. Revzen, S. and J.M. Guckenheimer, *Estimating the phase of synchronized oscillators*. Physical Review E (Statistical, Nonlinear, and Soft Matter Physics), 2008. **78**(5): p. 051907-12.
34. Moraud, E.M., et al., *Mechanisms Underlying the Neuromodulation of Spinal Circuits for Correcting Gait and Balance Deficits after Spinal Cord Injury*. Neuron, 2016. **89**(4): p. 814-28.
35. Asboth, L., et al., *Cortico–reticulo–spinal circuit reorganization enables functional recovery after severe spinal cord contusion*. Nature Neuroscience, 2018. **21**(4): p. 576-588.
36. Ollivier-Lanvin, K., et al., *Proprioceptive neuropathy affects normalization of the H-reflex by exercise after spinal cord injury*. Experimental neurology, 2010. **221**(1): p. 198-205.
37. Hoffmann, P., *Beitrag zur Kenntnis der menschlichen Reflexe mit besonderer Berücksichtigung der elektrischen Erscheinungen*. Arch Anat Physiol, 1910. **1**: p. 223-246.
38. Moraud, E.M., et al., *Mechanisms Underlying the Neuromodulation of Spinal Circuits for Correcting Gait and Balance Deficits after Spinal Cord Injury*. Neuron, 2016. **89**(4): p. 814-828.
39. MacLaren, D.A., et al., *Clozapine-n-oxide administration produces behavioral effects in Long-Evans rats - implications for designing DREADD experiments*. eneuro, 2016.
40. Patel, T.D., et al., *Peripheral NT3 signaling is required for ETS protein expression and central patterning of proprioceptive sensory afferents*. Neuron, 2003. **38**(3): p. 403-16.
41. Wright, D.E., et al., *Introduction of a neurotrophin-3 transgene into muscle selectively rescues proprioceptive neurons in mice lacking endogenous neurotrophin-3*. Neuron, 1997. **19**(3): p. 503-17.
42. Lavrov, I., et al., *Facilitation of stepping with epidural stimulation in spinal rats: role of sensory input*. The Journal of Neuroscience, 2008. **28**(31): p. 7774-7780.
43. Basso, D., M. Beattie, and J. Bresnahan, *A sensitive and reliable locomotor rating scale for open field testing in rats*. Journal of Neurotrauma, 1995. **12**: p. 1 - 21.
44. Vincent, J.A., et al., *Muscle Proprioceptors in Adult Rat: Mechanosensory Signaling and Synapse Distribution in Spinal Cord*. Journal of Neurophysiology, 2017.
45. Lin, C.-L., et al., *Functional distinction between NGF-mediated plasticity and regeneration of nociceptive axons within the spinal cord*. Neuroscience, 2014. **272**: p. 76-87.

46. Fischer, G., et al., *Direct injection into the dorsal root ganglion: technical, behavioral, and histological observations*. Journal of neuroscience methods, 2011. **199**(1): p. 43-55.
47. AuYong, N., K. Ollivier-Lanvin, and M.A. Lemay, *Population spatiotemporal dynamics of spinal intermediate zone interneurons during air-stepping in adult spinal cats*. Journal of neurophysiology, 2011. **106**(4): p. 1943-1953.
48. Kunkel-Bagden, E., H.-N. Dai, and B.S. Bregman, *Methods to assess the development and recovery of locomotor function after spinal cord injury in rats*. Experimental neurology, 1993. **119**(2): p. 153-164.
49. Robertson, B.D., et al. *A computer vision controlled treadmill with high speed 3D motion capture and behaviorally triggered perturbation for use in rodents*. in *40th Annual Meeting of the American Society of Biomechanics*. . 2016. North Carolina State University
50. Maghsoudi, O.H., et al., *Application of Superpixels to Segment Several Landmarks in Running Rodents*. Pattern Recognition and Image Analysis, 2018. **28**(3): p. 468-482.
51. Haji Maghsoudi, O., A. Vahedipour, and A. Spence, *A novel method for robust markerless tracking of rodent paws in 3D*. EURASIP Journal on Image and Video Processing, 2019. **2019**(1): p. 79.
52. Haji Maghsoudi, O. and A. Spence, *Treadmill Training Effect on Kinematics: An Aging Study in Rats*. Journal of Medical and Biological Engineering, 2019.
53. Maghsoudi, O.H., et al., *Open-source Python software for analysis of 3D kinematics from quadrupedal animals*. Biomedical Signal Processing and Control, 2019. **51**: p. 364-373.
54. Haji Maghsoudi, O., A. Vahedipour, and A. Spence, *Three-dimensional-based landmark tracker employing a superpixels method for neuroscience, biomechanics, and biology studies*. International Journal of Imaging Systems and Technology. **0**(0).
55. Tang, X.-Q., et al., *Targeting sensory axon regeneration in adult spinal cord*. The Journal of neuroscience, 2007. **27**(22): p. 6068-6078.
56. Kelamangalath, L., et al., *Neurotrophin selectivity in organizing topographic regeneration of nociceptive afferents*. Experimental neurology, 2015. **271**: p. 262-278.
57. van den Brand, R., et al., *Restoring Voluntary Control of Locomotion after Paralyzing Spinal Cord Injury*. Science, 2012. **336**(6085): p. 1182-1185.
58. Rotterman, T.M., et al., *Normal distribution of VGLUT1 synapses on spinal motoneuron dendrites and their reorganization after nerve injury*. Journal of Neuroscience, 2014. **34**(10): p. 3475-3492.
59. Alvarez, F.J., et al., *Vesicular glutamate transporters in the spinal cord, with special reference to sensory primary afferent synapses*. Journal of Comparative Neurology, 2004. **472**(3): p. 257-280.
60. Alvarez, F.J., et al., *Permanent central synaptic disconnection of proprioceptors after nerve injury and regeneration. I. Loss of VGLUT1/IA synapses on motoneurons*. Journal of neurophysiology, 2011. **106**(5): p. 2450-2470.
61. Gelderd, J.B. and S.F. Chopin, *The vertebral level of origin of spinal nerves in the rat*. The Anatomical Record, 1977. **188**(1): p. 45-47.
62. Sheikh, I.S., et al., *Retrogradely transportable lentivirus tracers for mapping spinal cord locomotor circuits*. Frontiers in Neural Circuits, 2018 (in press).
63. Ohtake, Y., et al., *The effect of systemic PTEN antagonist peptides on axon growth and functional recovery after spinal cord injury*. Biomaterials, 2014. **35**: p. 4610-26.
64. Romero, M.I., et al., *Functional regeneration of chronically injured sensory afferents into adult spinal cord after neurotrophin gene therapy*. The Journal of Neuroscience, 2001. **21**(21): p. 8408-8416.
65. Tang, X.-Q., et al., *Targeting sensory axon regeneration in adult spinal cord*. Journal of Neuroscience, 2007. **27**(22): p. 6068-6078.
66. Tang, X.-Q., D.L. Tanelian, and G.M. Smith, *Semaphorin3A inhibits nerve growth factor-induced sprouting of nociceptive afferents in adult rat spinal cord*. The Journal of neuroscience, 2004. **24**(4): p. 819-827.

67. Maier, I.C., et al., *Differential effects of anti-Nogo-A antibody treatment and treadmill training in rats with incomplete spinal cord injury*. Brain, 2009: p. awp085.
68. Costa, L.M., et al., *Methods and protocols in peripheral nerve regeneration experimental research: part IV—kinematic gait analysis to quantify peripheral nerve regeneration in the rat*. International review of neurobiology, 2009. **87**: p. 127-139.
69. Côté, M.-P., et al., *Activity-dependent increase in neurotrophic factors is associated with an enhanced modulation of spinal reflexes after spinal cord injury*. Journal of neurotrauma, 2011. **28**(2): p. 299-309.
70. Ollivier-Lanvin, K., et al., *Either brain-derived neurotrophic factor or neurotrophin-3 only neurotrophin-producing grafts promote locomotor recovery in untrained spinalized cats*. Neurorehabilitation and neural repair, 2014: p. 1545968314532834.