Temple University

Animal Protocol form

Updated By: ANDREW SPENCE17-Aug-2023 02:29:05 AM

APPLICATION INFORMATION

Principal Investigator Name: SPENCE, ANDREW

Animal Protocol Title:
Chemogenetic afferent modulation to understand spinal cord circuit function and plasticity post injury

Animal Protocol Type: (Check all that apply):
☑ Research ☐ Teaching ☑ Breeding
☐ Euthanasia and Tissue Harvesting Only

* **Submission Type:** 3 Year Rewrite

* Previous Animal Protocol Number: 5003 * Provide Progress Report:

Funding Source

☐ Funded Internally ☑ Funded Externally

Please attach the Vertebrate Animal Section (VAS) for all NIH grants. For all other agencies, please include the animal use section of the grant, if applicable.

External Sources

* ERA Proposal Number:	* Sponsor Name:	Attach Vertebrate Animal Section (VAS)
265817	NATIONAL INSTITUTES OF HEALTH/DHHS	

2. LAY DESCRIPTION/RATIONALE FOR STUDY

When completing this page:

- Lay description should be written in lay language. Please avoid too much scientific vocabulary.
- Abbreviations need to be spelled out at first use.

* *Provide a summary of the objectives of this work in **LAY TERMS** understandable to a non-scientist with a high school level education (300 words or less). Information to provide includes:*

- *Brief background for the project;*
- *Hypothesis/goal/aims and how this work will benefit human and/or animal health or advance knowledge of the subject;*
- *Rationale for using animals, the species, and how they will be used.*

Spinal cord injury (SCI) causes life-long neurological impairment, and there is currently no effective treatment. The premise of this proposal is recent work demonstrating that stimulating sensory nerves while running on a treadmill can enhance the ability to stand, stepping, and control limbs after paralysis. The objective of this study is to identify which sets of sensory neurons are most important for recovery and how spinal cord circuits change when recovery is good. Our main hypothesis is that activation of large sensory nerves during treadmill training will enhance recovery, whereas turning them off will suppress recovery. Four questions will test how improved recovery is handled in the spinal cord. These are whether it's by increased connections between the sensory nerves and 1) motor neurons that drive muscles, or with 2) other intermediate neurons in the spinal cord where the sensory nerves come in; or by sprouting new connections to 3) neurons from the brain, or finally, 4) new connections with neurons that go to other parts of the spinal cord. The significance of this work lies figuring out which of these mechanisms of helpful changes in the spinal cord are improving a patients' recovery.

Unfortunately, computer models of spinal cord injury that accurately predict how a human will respond to a treatment do not yet exist. Furthermore, work in cell culture does not capture the complicated behavior of the changing nervous system and holistic behavior of an intact, moving organism well enough to aid in understanding, treatment, and measurement of recovery. Rats are the animal of choice because 1) spinal cord injuries in rats are well established both at Temple and at major spinal cord research centers nationally and internationally, 2) the database relevant to SCI that the Smith Lab has established in the past is based mainly on rats, 3) rats are cost effective in their use, 4) the way we are using the rats (injury model) has been used extensively in the past, making us able to draw stronger conclusions.

3. SPECIES INFORMATION

When completing this page:
- Please ensure that the total animal count correlates with the numbers justification.
- If you are using multiple strains/breeds of animals, you will need to add a new row in the strain/breed information table by selecting the plus sign icon

* **3.1 Species** Rat

Click here for Pain and Distress Classification.

Note: If this is a 3-year rewrite please be sure to account for the number of animals still in house under the expiring protocol in your total number being requested.

* 3.2 Total count of the animals for the protocol: 388

* 3.3 Highest Pain/Distress Category for the protocol II-D

☑ 3.3.1 Use of pain scoring charts. Templates for pain scoring charts are available on the IACUC web site. Note, these are provided as generic templates and it is expected that they will be modified to incorporate relevant model specific signs of pain and distress.

* 3.3.2 Attach pain scoring chart

3.4 Species/Strain/Genetic Nomenclature

Name	Long Evans
Sex	Both (Male and Female)
Vendor/Source:	Commerical Vendor
From Where:	Charles River (mice, rats)

Name	LE-Tg(Pvalb-iCre)2Ottc
Sex	Both (Male and Female)
Vendor/Source:	Non-Temple institution or facility (non-commercial)
Other Vendor:	NIDA / OTTC

Name	Sprague Dawley
Sex	Both (Male and Female)
Vendor/Source:	Commerical Vendor
From Where:	Charles River (mice, rats)

Name	LE-Tg(Pvalb-iCre)2Ottc
Sex	Both (Male and Female)
Vendor/Source:	Bred in House

3.5 Genetically Engineered Animals

Are you using genetically engineered animals on this study?
☑ Yes ☐ No

* 3.6 Does the phenotype result in a change in appearance and/or a functional deficit? No

4. JUSTIFICATION FOR ANIMAL NUMBERS

When completing this page:

- Group sizes must be justified.
- The number of experimental groups must be clearly detailed and must be consistent in throughout the sections of the ACUP.
- Please ensure that it is clear how the total numbers are calculated.
- An estimate of pups generated in a breeding protocol need to be accounted for in the totals. Please be sure this includes unwanted genotypes that are euthanized in the total number justification.

Provide an explanation for the numbers of animals requested. All groups experimental and control groups must be clearly explained and identified. Be sure to include the number of animals used for all breeding pairs/trios and an estimate of all offspring to be generated (e.g. those of both appropriate and inappropriate genotype).

* 4.1 Provide justification for the number of animals requested here.

Aim 1: This aim will require two experimental and six control groups (Supporting Attachment **Table 1**). For experimental groups, adeno-associated virus serotype 2 (AAV2) encoding either the excitatory designer receptor exclusively activated by designer drugs (DREADD) hM3Dq or the inhibitory DREADD hM4Di will be injected into dorsal root ganglia (DRG; groups hM3Dq-SCI-CNO and hM4Di-SCI-CNO), and clozapine-N-oxide (CNO), the agonist for DREADDs, 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 (MacLaren, 2016). Our preliminary data in contusion animals show no effect of CNO, however. 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 (Cote et al., 2016).

With 7 experimental groups of 18 animals plus 15 animals for multi-unit electrode array (MUA) recording experiments Aim 1 will require 141 experimental animals.

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 (Patel et al., 2003; Wright et al., 1997). 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**.

This aim will require 3 groups of 18 animals and thus 54 experimental animals.

The experimental animals used for Specific Aim 2 are transgenics that will be derived from a colony. 54 transgenic rats (Tg-Pvalb/iCre: Rat Resource & Research Center Strain #00773; LE background) are required. To provide these experimental rats we will establish a colony and require 153 rats total. We require 27 experimental rats/year for 2 years. The breeding protocol for the Parvalbumin-CreR rats recommends breeding hemizygote males with wildtype females, and utilizing hemizygote offspring, because the mutant females preferentially consume mutant offspring. Thus we need to use wildtype females. We will therefore purchase 3 mutant hemizygote males (on LE background) and 3 Long Evans wildtype females for breeding. At 8 pups per litter average, 3 litters/pair and 3 breeding pairs we have 9 litters total yielding 72 animals over a one year period sufficing for our study requirements (72 animals weaned at 50% hemizygotic yields 36 animals; study requires 27 per year, for two years). The male breeders will be replaced once for a total of six; wildtype females will be used from the breeding colony for mates when replaced. So we require a total of 78 Pvalb-iCre (72*2/2 = 72 bred + 6 transgenic male breeders) and 75 (72 bred + 3 initially purchased) Long Evans rats for a total of 153 rats. Year 1 will use 72 bred plus 3 purchased Tg breeders and 3 purchased WT breeder females, for total of 78 animals. Year 2 will use 72 bred plus 3 replacement purchased transgenic male breeders, for total 75 animals.

Thus, Specific Aim 2 will utilize 99 animals for breeding, 54 animals for experiments, and a total of 153 animals.

We request 32 Sprague Dewley rats to carry out dose response curves of H-reflex responses against CNO administration in animals that have DREADDs injected into their DRGs in order to understand whether this spinal reflex is modulated by DREADDs afferent modulation alone. This is an important understanding to interpret whether and how our treatment may be improving recovery from spinal cord injury.

Skin motion artifact is a well know problem in rodent kinematics (Bauman and Chang, 2009) that has been quantified with x-ray imaging. In rodents, the skin is very loosely attached to the bone, and thus moves around a large amount (several mm) relative to the bone, especially during locomotion. This makes kinematic data from the knee joint gathered with standard methods using markers on the skin very noisy. Therefore we are requesting to add 8 Long Evans rats to the protocol to determine whether we can fix these knee location errors in kinematic data by placing a small tattoo on the lateral aspect of the knee ligamenture with a short minimally invasive recovery surgery that we subsequently image with infra-red cameras and illumination. If we can show that one can image the knee joint directly, accurately, through the skin with inexpensive infra-red cameras after a minimally invasive surgery to place a marker on the knee itself, this could have a large

impact on how rodent behavioral data are gathered. We have carried out successful pilot experiments using store-bought chicken thighs to determine that imaging the marker through a layer of skin is feasible with our camera and illumination at frame rates useful for kinematics. We use the term motion capture with associated abbreviation MOCAP occasionally to refer to the process of recording video of animals while they move and then subsequently analysing the videos to produce kinematic data (joint angles, etc.).

In total, the protocol will use 235 animals for experimental aninals Aims 1 and 2 (see supporting docs) and 153 animals for the breeding giving a total of 388 animals.

Note: If you have any supporting documents (e.g. table, spreadsheet, outline, etc.) for animal number justification, please upload here:

4.2 How was the group size determined? (Check all that apply.)

☑ 4.2.1 Statistical sample size/power analyses.

* Describe the power analyses. Be sure to include details on what primary endpoint(s) the power analyses were based on, what were the null and alternative hypotheses, what were the type I and type II error rates used, what test statistic(s) were employed and what distribution assumptions, if any, were made.
Animal numbers were determined by power analysis extrapolated using previous data sets or published literature. For axonal quantitation using a two way analysis of variance (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 with alpha of 0.05 and power of 95% was determined to be n=8 animals per group. However, for analysis of changes in H-reflex excitability and kinematic parameters related to posture and locomotion, a power analysis for an repeated measures ANOVA design (G*Power 3) shows that to detect a minimum 25% change (effect size based on Spence lab previous work, Spence et al., J. Neurosci Methods 2013, and Takeoka et al., Cell 2014) with 10 measurements (strides) at a correlation between strides of 0.25 will require 18 animals per group.

For the H-reflex experiments, a power analysis of the sample size required to resolve the change in H-reflex that occurs with a transection injury (Ollivier-Lanvin et al, 2010) leads to a group size of 8. We are required to study four doses, leading to four groups and thus 32 animals.

For the infra-red imaging, we choose 8 animals as this is the minimum number required to demonstrate that animal-to-animal variability is not a problem. E.g., should we be able to image the knee in 8 animals, based on the binomial distribution we can rule out sampling error for the observed effect at the 0.05 level.

☐ 4.2.2 Previous experience with the model, and/or published work of others.

☐ 4.2.3 Tissue/cell requirements (yield per animal).

☐ 4.2.4 Other

☑ 4.2.5 Breeding (rodents/zebrafish)

Complete the following table
For each strain listed, be sure to use the same strain designations in this table as used in the animal number and pain/distress classification section.

Colony Designation (strain/transgene/KO/KI)	Tg-Pvalb/iCre: Rat Resource & Research Center Strain #00773; LE background
Adult Breeders (strain x strain)	Tg-Pvalb/iCre X WT
# of male breeders required (column a)	3
# of female breeders required (column b)	3
# of offspring needed for experiments (column c)	54
Approx. # of offspring estimated as unusable (column d)	90
Approx. # of offspring used to replace breeders (column e)	3

Expected total # of offspring generated (Sum of columns c+d+e)	147
Total Number of Animals Requested (Sum of all columns a through e)	153

4.3 Use of Surgically Altered Animals

* Will animals undergo one or more surgical procedures prior to being used on this project? (e.g. neutered, implanted catheters or telemetry devices, etc.)

☐ Yes ☑ No

4.4 Animal Acclimation and Quarantine

* If animals are transported between sites (e.g. between vendor or collection site and the site of use) will they be given **less than 72 hours** for physiologic, behavioral and nutritional acclimation before their use?

☐ Yes ☑ No

5. ANIMAL HOUSING AND PROCEDURE AREAS

5.1 Housing Location(s)

Check all animal facilities where the animals may be housed for the studies described in this Animal Protocol.

☐ Ambler ☑ Biolife ☐ Kresge ☑ MERB ☑ MRB ☐ MOB ☐ PAH ☐ Weiss

5.2 Satellite Housing (Housing of animals outside the ULAR facilities for more than 12 hours.)

* Are you requesting the use of Satellite Housing?
☐ Yes ☑ No

PROCEDURE AREAS:

- Procedure Location(s) are the locations that are outside of the ULAR facility and where any live animal procedures are conducted.
- Each procedure identified for use needs its own row in the procedure locations table. If the same procedure is performed in multiple locations, a new procedure location row must be entered for each location. If multiple procedures are performed in the same location, a new procedure location row needs to be entered for each procedure. To add multiple locations and/or procedures, select the plus sign icon.
- **NOTE:** If a location doubles as a Housing and Procedure location, please include the details below.

5.3 Procedure Location(s)

* Procedure:	Building Name:	Room Number:	Bio-Safety Level (if applicable BSL 2, BSL 2+)	Field/Wildlife Studies location:
DRG Injections	MERB	546		
Spinal cord lesions	MERB	546		
Ladder/grid walk testing	MERB	546		
Euthanasia	MERB	546		
Basso, Beattie, Bresnahan Locomotor Scoring (BBB; See Basso, et al, 1995)	MERB	546		
H-reflex testing	MERB	681A		
Euthanasia	MERB	681A		
Euthanasia	ENGR	825		
Treadmill training	ENGR	819		
Treadmill motion capture	ENGR	819		
H-reflex Testing	ENGR	819		
H-reflex Testing	ENGR	927		
Euthanasia	ENGR	927		
Spinal cord lesions	ENGR	927		
Sciatic Nerve Injections	ENGR	927		
Sciatic Nerve Injections	MERB	546		
Sciatic Nerve Injections	MERB	681A		
Pain and mechanical sensitivity assays	MERB	681A		
Pain and mechanical sensitivity assays	MERB	546		
Multi-unit Array (MUA) Recording	MRB	301		
Euthanasia	MRB	301		
H-reflex Testing	MRB	301		
	MERB	546		

Dying of fur, shaving, marking or tattoing skin, tattooing knee ligaments under skin, under anesthesia				
Dying of fur, shaving, marking or tattoing skin, tattooing knee ligaments under skin, under anesthesia	ENGR	927		
Tail clipping	MERB	546		
Ladder/grid walk testing	ENGR	819		
Multi-unit Array (MUA) Recording	MRB	300		
Euthanasia	MRB	300		
Spinal Cord Injections	MERB	546		
Spinal Cord Injections	ENGR	927		
Intrathecal Spinal Cord Injections	MERB	546		
Intrathecal Spinal Cord Injections	ENGR	927		

6. SPECIAL HUSBANDRY AND HOUSING REQUIREMENTS

Standard Requirements

When completing this page:
- Please ensure that any instances that include single housing, special food/water, non-standard environment are described/accounted for in the protocol.
- Please consider ways to mitigate some of the negative consequences of special arrangements. For example, single housed animals can be provided with extra enrichment.
- Some proposed procedures will require special services by ULAR (ex: exemptions from normal food/water/cage changing by ULAR staff).

Changes in standard husbandry requirements must be discussed with ULAR prior to submitting protocol.
Click here for the ULAR Standard Housing Conditions.

* 6.1 Are you requesting any exemptions to the standard conditions outlined in the ULAR Standard Housing Conditions? (e.g. altered light cycle, altered diet, enrichment, etc)
☑ Yes ☐ No

* 6.2 Are you requesting exemption(s) to the standard conditions for all animals on this protocol or for certain experimental groups?
Certain experimental groups:

* Identify experimental groups to be exempt:
Animals undergoing contusions (all animals except the 15 MUA animals) will be given soft diet gel post injury.

6.3 The following non-standard conditions are required: (Check all that apply)

- ☐ Non-Standard Housing
- ☐ Non-Standard Cage Changing/Run Sanitation
- ☑ Special Diet and/or Dietary Supplements
- ☐ Additives to Drinking Water (e.g. antibiotics, alcohol, chemicals, drugs, etc.)
- ☐ Food Regulation/Restriction (excluding pre-surgical fasting)
- ☐ Water Regulation/Restriction
- ☐ Non-Standard Light Cycle
- ☐ Non-Standard Room Temperature and/or Humidity
- ☐ Non-Standard Social Environment (Single Housing)
- ☐ Exemptions for Environmental Enrichment and/or Exercise
- ☐ Other Non-Standard Housing or Care

6.3.3 Special Diet and/or Dietary Supplements

* Diet/Supplement Name: DietGel

* Source: Commercial supplier - ClearH20 or similar

* Diet Form (e.g. pellet, liquid): Gel/liquid in plastic tub

* Storage Location (building/room): current animal housing suite location in MERB; 546 MERB

* Will the use of a special diet and/or dietary supplements result in nutritional deficiencies?
☐ Yes ☑ No

* Will food be formulated (mixed, produced, etc.) at a commercial vendor?
☑ Yes ☐ No

* Indicate the commercial vendor. ClearH20 or similar

* Will food be formulated (mixed, produced, etc.) or will medications, test materials, investigational agents, nutritional supplements, etc. be added to the food at Temple?
☐ Yes ☑ No

* Will food be provided in delivery devices other than those provided by ULAR?
☐ Yes ☑ No

* Explain why the non-standard diet/supplement will be provided based on project goals or animal needs:
In the acute phase of recovery from contusion injury animals may benefit from soft, enriched food.

6.4 Special Services Required from ULAR

* Will any special services be required from ULAR? (e.g., special food, special water, special bedding, BSL-2 or 2+ housing, anesthesia support, post procedural care.)
☐ Yes ☑ No

6.5 Cleaning, Disinfection, and Monitoring of Research Equipment

* Are you using equipment/items that come into contact with multiple animals that must be hand sanitized and/or cannot be autoclaved?
☑ Yes ☐ No

6.5.1 Cleaning, Disinfection, and Monitoring of Research Equipment

Equipment/items that come in contact with multiple animals that must be hand sanitized and/or cannot be autoclaved. Please see Policy on Cleaning **and** Guidelines for Validation.

* Describe the sanitation procedures including frequency of cleaning, etc.
After any animal is assayed using the ladder walk test, all animals waste is removed from the area and the ladder is cleaned with ethanol, and the log book entry completed.

After any animal is assayed using the Basso, Beattie, Bresnahan Locomotor Scoring test (BBB; See Basso, et al, 1995), all animals waste is removed from the kiddie pool and the equipment is cleaned with ethanol, and the log book entry completed.

After any animal is run on a treadmill, all animals waste is removed and the equipment is cleaned with ethanol and the log book entry completed.

After any animal is assayed using Von Frey filament aparatus, all animals waste is removed from the chamber and the equipment is cleaned with ethanol, and the log book entry completed.

After any animal is assayed using the Hargreaves thermal nociception assay, all animals waste is removed from the chamber and the equipment is cleaned with ethanol, and the log book entry completed.

After any animal is assayed using the ladder walk test, all animals waste is removed from the area and the ladder is cleaned with ethanol, and the log book entry completed.

* Will the verification of the sanitation procedure differ from the “Policy on Cleaning and Verification.”
☐ Yes ☑ No

7. EXPERIMENTAL DESIGN/PROCEDURES

Experimental Design

When completing this page:
- Please provide a clear timeline for each experimental group from entry to the facility to euthanasia with a minimum of experimental detail.
- For the procedures section, please provide all of the experimental detail for the ACUP that is not captured elsewhere. Please use subtitles to separate various procedures. Please exclude information that is captured elsewhere such as drug doses, anesthesia details, euthanasia details, etc).

* 7.1 Please provide a sequential description of all procedures to be conducted on this protocol. This should be a clear timeline of the work to be performed on animals from their entry into the facility until their euthanasia. This information should be presented in brief, bullet format or by attaching an outline, flowchart or table.
NOTE: Do NOT provide a detailed description of the procedures here. The details of the procedures MUST be included in the individual Procedures below; do not repeat procedural details in this section.

MOCAP=Motion Capture - recording videos of the behavior to later produce kinematics

For Aim 1, the timeline is:

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
7	Perfusion	

For Aim 2, the timeline is:

Week	Procedure	Experiments
-13 to -5	Breeding	Breeding to produce parv-cre tg rats
-5, -4	Genotyping	Genotyping and identification
-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

7	Perfusion	

The 32 Sprague Dawley rats for H-reflex experiments with CNO alone will be purchased, undergo the H-reflex measurement procedure at most twice on days separated by at least a week, and then will be euthanized by Fatal-Plus and thoracotomy.

The 8 Long Evans animals purchased for subcutaneous knee marker infra-red imaging will be purchased, acclimated, undergo the minimally invasive lateral knee ligament tattooing procedure under isoflurane anesthesia, recover until their wound clip(s) can be removed or have all naturally fallen off (typically at most 10-14 days) then will run on the treadmill with infra red video recording of their locomotion for 10 minutes on 3 separate days, and then will be euthanized by Fatal-Plus and thoracotomy.

* 7.2 Do you have any supporting documents (e.g. an outline, flowchart or table)? ☑ Yes ☐ No

* Please upload any supporting documents here:

Procedure Instructions

Please select all procedures from the list below that are planned for use on this IACUC protocol. Once the procedures are selected, complete all subsections related to each specific procedure. If applicable, the following details must be provided in the appropriate subsections below:

- Duration of the procedure with a clear definition of the experimental endpoint
- Monitoring frequency
- Post procedural monitoring details
- Number of times the procedure is done on an individual animal
- Criteria used to determine if the animal is in pain, distress or discomfort (e.g. loss of mobility, failure to groom, abnormal posture, weight loss, etc.)
- How pain and distress will be managed
- The point at which an animal will be removed from the study and/or euthanized
- Use of Anesthesia/Analgesia or other Therapeutic or Experimental substances
- Animal Handling and Restrain information
- Other details specific to the procedure being performed

7.3 Procedures List

☑ Identification and/or Genotyping

Click here for the IACUC Guideline on Identification and Genotyping.
Include the following in your detailed description of this procedure:

- Describe how and at what age, the selected method of identification and/or genotyping will be performed. If applicable, please include how hemostasis will be achieved.
- Note: Rodents that are 21 days or older require anesthesia for genotyping procedures involving tail biopsy and identification procedures involving tattooing. Rodents that are 7 days or older require anesthesia for genotyping and/or identification procedures involving toe clipping.

☑ Behavioral Assessment

- Describe the behavioral and/or observational procedure to be performed. Include maximum number of sessions, length of session, frequency, administration of anesthesia/other substances, and type of restraint.
- Will any animal undergo more than one behavioral assessment? If so, describe the testing order and the time between each procedure.
- Indicate the criteria used to remove the animal from the test (e.g. in the event of unexpected pain/distress and/or unable to acclimate/habituate to the test).
- How will animals be acclimated/habituated to the equipment or test? If animals will not be acclimated/habituated, provide justification based on project goals

☐ Non-Surgical Sample Collection (e.g. saliva, tears, urine, blood, fecal, etc.)

☐ Imaging

☐ Tumor Production

☑ Breeding

Click here for the IACUC Policies on Rodent Breeding Colonies and Rodent Breeding Cage Density.

- Describe breeding scheme, colony management, reason for breeding, fate of retired breeders and unusable offspring.
- If using genetically engineered animals, does the phenotype result in a change in appearance and/or a functional deficit? Include the strain(s) affected, a description of the appearance and/or functional deficits, how often the animals will be monitored and what actions will be taken to provide relief from pain and/or distress.

☑ Non-Survival Surgery/Terminal Procedure

Non-survival surgery is defined as any surgery in which the animal is euthanized prior to recovering from anesthesia. This includes terminal perfusions.
Click here for the IACUC Guideline for Non-Survival Surgery.

- Describe the terminal procedure/non-survival surgery.
- Include, administration of anesthesia/other substances, restraint, presurgical prep, aseptic technique, and sterilization of equipment.

☐ Irradiation

☐ Field/Wildlife Studies

☑ Other (Non-surgical survival procedures that do not fit into the above categories that may/may not involve the use of anesthesia (e.g. dietary studies, drug testing, locomotion studies, administration of chemical/biological/radioactive hazardous agents in non-surgical studies, etc.))

- Describe any other procedure(s) to be used with live animals here. Be sure to include all details applicable to these procedures as well as, anesthesia/other substance use, type of restraint, etc...

☑ Survival Surgery

Click here for the IACUC Policies and Guidelines; Conducting surgical Procedures on Laboratory Animals, Survival Surgery Involving Rodents and Survival Surgery Involving Large Animals

- Describe the surgical procedure(s). Include preoperative procedures, anesthetic use, monitoring and recovery, aseptic technique (surgical prep, sterilization of equipment).
- State whether these are major or minor surgical procedures.
- State if the animal will undergo multiple survival surgeries and provide justification and time between procedures.
- Describe how often will the animals be monitored post-operatively and duration of monitoring.

☑ Major Survival Surgery: Any surgical intervention that penetrates and exposes a body cavity, produces substantial impairment of physiological functions and/or involves extensive tissue dissection or transection

☑ Minor Survival Surgery: Surgical procedures that do not expose a body cavity and causes little to no physical impairment.

7.4 Procedure Details

* List all the Procedure and related details below:

Survival Surgeries

Items for all survival surgeries

Surgeons and assistants will wear the following personal protective equipment (PPE): clean scrubs or disposable gown, Sterile gloves, Closed-toe shoes, Appropriate face mask, Head Cover, and Shoe Covers. All surgeries will be carried out under aseptic conditions. Surgical tools are autoclaved before the start of any day that includes surgeries. For all surgeries except intrasciatic nerve injection, tools may be sterilized between animals and

reused at most once, using a hot bead sterilizer, before they will be autoclaved again. For intrasciatic nerve injection, tools maybe reused for up to five animals with hot bead sterilization. For dorsal root ganglion (DRG) and contusion survival surgeries, 1) pre-operative buprenorphine (extended release Ethiqa XR) will be administered 30 minutes prior to incision. Animals will be anesthetized via IP injection of ketamine/xylazine cocktail. Plain ophthalmic ointment is applied gently to the animals eyes pre-surgery. Hair/fur are removed from the surgical site using electric clippers. After shaving, the surgery site is prepared using a triplicate betadine scrub followed by alcohol rinse. To prevent hypothermia, commercial temperature controlled rodent electrical heat mats are placed under the animal, with a sterile wipe placed between the animal and head pad. To prevent dehydration, animals are given saline post-operatively (2.5 mL per side, subcutaneously). For closure, muscle and fascia layers are sutured with 4.0 chromic gut, followed by application of wound clips to the skin layer. Animals are given injectable meloxicam by subcutaneous injection post surgery, and triple antibiotic ointment (TAO) will be placed on stapled incisions. Post-surgical recovery is monitored continuously via respiratory rate and skin tugor until animals recover postural reflexes and are ambulatory.

Post-operative monitoring (for all survival surgeries)

For all surgeries except subcutaneous knee tattooing, animals will receive injectable meloxicam once daily for two days post surgery. Animals are monitored for general health once daily for three days. If pain or distress are observed (as assessed by IACUC provided pain scoring chart), the animal will be euthanized promptly. All wound clips will be removed 10-14 days after surgery. We utilize a password protected internally accessible only Google Sheet for post-surgical record keeping, and record all data listed on the IACUC Rodent Survival Form: Surgical Procedures. In the case of a surgical dehiscence, incision infection, or staple loss complication, the animal will be anesthetized with isoflurane, the wound debrided, the wound flushed with Dermachlor, wound clips re-applied, triple antibiotic ointment (TAO) applied. This repair will only be carried out once; if it re-occurs the animal will be euthanized. The animal will be monitored once daily for three days in the instance of dehiscence, and injectable meloxicam will be given once daily for two days following the repair. In the case of a subcutaneous fluid filled lesion, this will be manually drained, and TAO applied. The abdomen will be inspected for signs of injection site reactions and pressure ulcers as part of all animal checks. If pressure sores appear to be developing, pressure sore ointment (Terrasil) will be applied. Any suspected developing injection site reactions will result in Dermachlor wash and application of TAO to the site.

Criteria for, and interventions to be applied, upon signs of pain/distress

Upon a score of “1” in any of the categories listed on the attached IACUC provided pain scoring chart, we will ask for a veterinary consult, and; if the score of 1 was due to signs of mild/moderate pain on the physical or behavioral assessments but weight loss or gastrointestinal problems are not apparent, we will apply one dose of injectable buprenorphine analgesia once, and continue to monitor the animal for two further days. If the score of 1 was due to weight loss and/or gastrointestinal problems on the physical assessment, a single dietgel will be placed in the cage and one subcutaneous injection of 2mL of saline will be applied. The animal will be monitored for an additional two days from the date that this score was noted, and if still scoring a 1, then additional Dietgel will be provided. A score of 1 on the surgical incision category will result in a Dermachlor wash of

the incision and application of triple antibiotic ointment (TAO). Combinations of these problems will result in combinations of interventions being applied. If these have not improved after 2 days, we will ask for a further veterinary consult. A score of “2” in any category will result in immediate euthanasia.

Multiple Survival Surgeries:

This has been moved to the new text box below on the ERA system that seems to have been created for it.

Intraperitoneal injections (IP):

The animal is first calmed by gentle holding. A blue cloth towel is held in one hand, and the animal placed feet down on another surface. Using the cloth, the animal is then gently held around the forelimbs with the thumb and index fingers, and the rest of the fingers used to hold the hindlimbs/body. The animal is turned over while held, and a shallow injection is made using a 23 gauge needle in the lower right quadrant of the abdomen at a shallow angle.

Dorsal root ganglion (DRG) Injections

An approximately 1.0 cm long incision above vertebra L3, L4, and L5 will be made through the skin and the underlying spinal musculature. A partial laminectomy to remove part of the posterior articular process will be done at lumbar region L3, L4 & L5 to expose the DRGs. An ultraviolet-light sterilized micropipette will be inserted into the DRG to inject replication-defective Adeno-associated virus vectors encoding control labeling proteins or chemogenetic actuators. This will be done using a glass micropipette pulled to a diameter of 0.05mm and a micromanipulator. For each injection, the tip of the pipette will be inserted into the DRG 0.3 to 0.5mm. and virus will be injected over a 3 minute period. Two minutes after injection is completed the pipette is slowly removed over a 5 minute period. Two injections are made within each DRG. All viruses are in a sterile phostphate buffered saline (PBS) solution and will only be administered once. A small piece of gelfoam (1 x 4 mm) is placed above the spinal cord before closure. The process is first carried out on the right side, and then on the left side DRGs. These surgeries typically take three to four hours.

Spinal cord contusions

Animals will be anesthetized using ketamine/xylazine and analgesia provided by extended release buprenorphine. To access the spinal cord for contusion, it is exposed by a mid-dorsal skin incision and a laminectomy over spinal segments T7/T8. The skin is retracted to expose the underlying muscle. The exposed spinotrapezius muscle is cut along the midline with scissors then spread with an Alm retractor. The muscles are freed from the thoracic vertebrae with scissors and a bone scraper. Using a pair of micro-rongeur small pieces of the dorsal vertebral arch and the dorsal spinous process are carefully removed from vertebra T7/T8, so not to damage the spinal cord. This exposes dorsal spinal cord segment T10. 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 choose 250 kilodynes as it is considered a moderate contusion that preserves much of the ventral white matter. Post-operative care will include manual expression of the animals' bladder twice daily until no longer needed, typically 1-7 days post-op. The abdomen will be washed with soap and water and baby diaper rash cream (generic brand; eg CVS) will be applied to prevent urine scalding or other abdominal problems post contusion until bladder expression is ceased. Clinical signs that bladder expression can be ceased will be 1) bladder is empty during the twice a day check at least two times in a row, and 2) signs of wetting (wet/dark fur near bladder area) are absent. These surgeries typically take two hours.

Spinal cord hemi-sections

Animals will be anesthetized using ketamine/xylazine and analgesia provided with extended release buprenorphine. To access the spinal cord for unilateral lesion, it is exposed by a mid-dorsal skin incision and a laminectomy over spinal segment T10. The animal is prepared for surgery and draped, a 1.5 mm incision is made through the dorsal skin above vertebra T9 – T11. The skin is retracted to expose the underlying muscle. The exposed spinotrapezius muscle is cut along the midline with scissors then spread with an Alm retractor. The muscles are freed from the thoracic vertebrae with scissors and a bone scraper. Using a pair of micro-rongeur small pieces of the dorsal vertebral arch and the dorsal spinous process are carefully removed from vertebra T10, so not to damage the spinal cord. The hemi-section is made using a sliver of fine razor blade. These surgeries typically take one hour.

Spinal cord injections:

Animals will be anesthetized using ketamine/xylazine and analgesia applied via extended release buprenorphine. Injections are targeted at the ventral horn motor pools in the middle of the lumbar enlargement (spinal cord segments L3, L4, L5; underneath vertebrae T13 and L1). To access the spinal cord for injection, it is exposed by a mid-dorsal skin incision and a laminectomy. The animal is prepared for surgery and draped, a 1.5 mm incision is made through the dorsal skin above vertebra T11 – L3. The skin is retracted to expose the underlying muscle. The exposed spinotrapezius muscle is cut along the midline with scissors then spread with an Alm retractor. The muscles are freed from the thoracic vertebrae with scissors and a bone scraper. Using a pair of micro-rongeur small pieces of the dorsal vertebral arch and the dorsal spinous process are carefully removed from vertebra T11 and L1, so not to damage the spinal cord. The animal is mounted in a spinal holder, and the injection is made using micromanipulator coordinates zeroed at the spinal cord midline. Injections are made 1mm lateral from midline and 1.9 mm deep to target this

area. 3 injections of 5nL at a rate of 100MKN taking ~4 minutes are made spaced 1mm apart rostrocaudally. These surgeries typically take two hours.

Intrasciatic nerve injections

Two minor recovery surgeries in sequence are used to transduce neurons in the sciatic nerve. First, lysolethicin is injected to demyelinate the nerve. Then, five days later, a second injection with virus transduces the axons inside the nerve bundle. To access the nerve for both injections, the procedure is as follows. Anesthesia is provided by isoflurane whilst analgesia is applied via pre-operative injection of meloxicam. An incision is made just below the tuber coxa over the natural division between the gluteal muscles. Blunt dissection with forceps is then used to gently tease these muscle groups apart, and the sciatic nerve is then accessible. For lysolethicin injections, a 32 gauge Hamilton syringe is injected into the sciatic nerve. For viral construct injections, the viral construct is injected into the sciatic nerve, with a Hamilton syringe and 32 gauge needle. These surgeries typically take 30 minutes.

Intrathecal injections
Animals will be anesthetized using ketamine/xylazine and analgesia applied with pre-operative administration of meloxicam. The thoracolumbar area is shaved and cleaned aseptically. The rat is then positioned in sternal recumbency with pelvic limbs brought forward to arch the vertebral spine. A Hamilton syringe is inserted perpendicular to the spine between the L4 and L5 vertebrae. Successful IT location of the needle is confirmed by the presence of at least one of the following signs: twitch of the tail and/or presence of cerebrospinal fluid (CSF) in the needle hub. If none of these signs is, the needle is withdrawn and the operation repeated with another needle. If the needle is correctly located, 50nL of viral construct are injected over 3 minutes. As this is a minimally invasive procedure, analgesia will not be routinely applied after injection but animals are monitored for two days post injection and analgesia applied if any signs of distress are noted. These injections typically take 15 minutes.

H-reflex quantification of reflexes

This test does not open/expose any body cavities, and is minimally invasive. This protocol closely follows the methods of Ollivier-Lanvin et al., 2010. Anesthesia will be by IP injection of ketamine/xylazine and analgesia provided by pre-operative meloxicam. The tibial nerve is accessed by two small punctures through the skin with a fine needle that thread fine wire stimulating electrodes through a skin pouch that lies at the space between the achilles tendon and distal tibula/fibula bone. Little to no bleeding occurs after insertion. <1mm deinsulated portions of the wire remain inside the skin pouch, close the tibial nerve. Bipolar EMG electrodes are inserted subcutaneously in the digital interosseous muscles between the fourth and fifth metatarsals. The ground electrode is placed on the skin of the leg.

The reflex is tested with single pulses (100 μs duration) of varied amplitude to determine the threshold and the maximal response level for both the M-wave and the H-reflex. The level of stimulation that produces a maximum H-reflex response is then used for 2 series' of 16 consecutive stimulations, one at 0.3 Hz, the other at 10 Hz, with 2 min rest in between. These series characterize the reflex magnitude and frequency dependence. Stimuli are delivered via an isolated pulse stimulator (A-M Systems Model 2100, A-M Systems Inc,

Carlsborg, WA). EMG responses are recorded with a differential AC amplifier (A-M Systems Model 1700, A-M Systems Inc, Carlsborg, WA; Gain × 1000; pass band 10–5 K Hz). At the conclusion of the experiment, animals will be euthanized.

To activate the DREADDs constructs during either the above H-reflex or in gait analysis locomotion experiments, CNO will administered through I.P. injection. For H-reflex validation of DREADDs, the above protocol will be carried out before, and at 1-minute intervals post, administration of CNO, up to four hours, to determine the extent and time course of reflex inhibition or excitation by DREADDs. Thus the experiment can last up to four hours, the washout time required for DREADDs.

Lateral aspect knee ligament tattooing

Adult animals will be anesthetized under isoflurane and analgesia applied using pre-operative meloxicam. Anesthesia is induced with the animal in the induction box at 5% concentration, and then maintained with a nose cone at 2%. The fur on the hind right limb above the knee (patella) and on the lateral aspect of the leg in the knee area will be shaved. A 2 cm incision is made running over the inner thigh starting 5 mm above the knee and running medial to the knee joint to a point 2-3 mm below the knee. The right knee is exposed by sliding this incision up over the protruding knee joint. Small amounts of fascia and connective tissue are bluntly dissected to expose the surface of the musculature directly attached to the knee. Black pigment #242 (AIMS Inc.) is then tattooed on the knee area that covers the joint and connective tissue using the AIMS Animal Tattooing system. If we find this ink does not provide adequate contrast, we will attempt the procedure with Animal Tattoo Paste/Ink (Ketchum #327), and again if insufficient contrast with India Ink (Mckesson #1039754) (in different animals; not the same animal). The skin will be closed with wound clip(s).

Non-survival Surgeries / Experiments

Items for all non-survival surgeries

Hair/fur are removed from the surgical site using electric clippers. After shaving, the surgery site is

prepared using single alcohol rinse. Surgical tools will have been hand cleaned with water and 70% ethanol and then hot-bead sterilized or autoclaved.

Terminal Multiunit Array MUA recording experiments

For the animals used for terminal multi-unit electrode array (MUA) recording experiments, DRG injection will be carried out as above but unilaterally for right side vertebra L5 and L6 only, and after a minimum three-week recovery period, in a terminal experiment we will record either from these DRG directly or from the nucleus gracillus. For DRG recordings, these will be exposed using a similar protocol as the DRG injection surgery above, and a 16-channel multi-unit electrode array (MUA, 4x4 3mm 100-125-177, Neuronexus Technologies, Ann Arbor, MI) with 4 sites on each of 4 shanks, producing a rectangular

recording region with a height of 400 um and width of 500 um, will be inserted into the DRG with the sites covering the 600-200 um depth range. Anesthesia for DRG recording is induced with isoflurane and then transitioned to alpha-chlorolose. Anesthesia is induced with the animal in the induction box at 5% concentration, and then maintained with a nose cone at 2%. The reason for using alpha-chlorolose in these terminal experiments is that it preserves the function of the reflex pathways that we wish to record spikes from. Once stable isoflurane anesthesia has been achieved, an induction dose of 34 mg/kg alpha chlorolose is given IP; after 3 minutes the nose cone is removed and plane of anesthesia monitored with toe pinch and monitoring for whisking and eye-blinks; if appropriate, the cone is left removed; it is reapplied if not yet sufficient. Anesthesia is then checked every 10 minutes as above and when required (typically every two hours) a top up dose of 5mg/kg is given IP. For terminal recording from the nucleus gracillus, anesthetic induction and maintenance will be via ketamine xylazine cocktail, with the nucleus located via stereotaxy and exposed by removal of the occipital bone covering the cerebellum. These terminal recordings can take up to six hours.

Terminal Procedure Euthanasia via Commercial Euthanasia Solution.

*A*nimals will be euthanized via a commercial euthanasia and confirmed dead as indicated in section 10.2 of the form, the animal will then be perfused or a thoracotomy performed as secondary method when tissue are not required.

Procedures

Dyeing of fur under anesthesia.

Rats will be placed under isofluorane anesthesia so that we can dye specific locations of their fur in contrasting colors to make tracking of their body possible. Anesthesia is induced with the animal in the induction box at 5% concentration, and then maintained with a nose cone at 2%. The dye is non-toxic animal fur dye, and is applicable in 20 minutes. Because dying the fur of awake, restrained rats in accurate patterns is likely to distress the rats, we use isoflurane anesthesia. Animals recover well and the marked locations on the fur are permanent. Because this procedure is relatively brief, and may need to be repeated every few weeks, we propose to use isoflurane rather than ketamine/xylazine, to avoid repeated exposure to ketamine. Anesthetic recovery is monitored continuously via respiratory rate and skin tugor until animals recover postural reflexes and are ambulatory.

Shaving fur and marking points on the skin.

We will shave fur on the rats' legs such that joint centers can be marked on the skin for kinematic tracking of the leg segments. Fur will be shaved such that the iliac crest, hip, knee, ankle, and MTP joints can be marked with non-toxic skin permanent markers. The reason for this procedure is to prevent large errors in kinematic data that occur over time in a longitudinal spinal cord injury studies because marks made on the skin with a marker are often made in different locations depending on how the operator holds the animal during marking, because rodent skin is not tightly coupled to the underlying bones. To achieve the best possible accuracy, skin must be shaved to palpate and locate as best as possible the joint centers for kinematic tracking (MOCAP). Animals will be placed under isoflurane

anesthesia to avoid distressing restraint. This procedure typically takes 15 minutes. Anesthetic recovery is monitored continuously via respiratory rate and skin tugor until animals recover postural reflexes and are ambulatory.

Tail clipping for genotyping

We will perform distal tail clipping for genotyping of our transgenic parv-cre rats following the ULAR guidelines. Tail clipping will be carried out <21 days of age without anesthesia, or with isoflurane anesthesia and analgesics if 21 days or older. Analgesia will be provided by pre-operative injection of meloxicam. Animals will be scruffed during the procedure using a blue cloth towel as in the method described in IP injection to minimize distress. Sterile sharp scissors or a sterile scalpel blade (disinfected between uses by hot bead sterilization) will be used for the procedure. Only the distal 0.3 cm may be amputated.Hemostasis will be achieved with a silver nitrate stick, Quick Stop powder, cauterizing tool, or by applying a gauze square over the site with gentle pressure. Bleeding will be controlled before the animals are returned to normal housing. Animals will be checked the day after the procedure to ensure they have recovered well.

Micro-tattooing of the skin for kinematic markers

Adult animals will be anesthetized under isoflurane to enable precisely positioned tattoing of markers on their hind legs. Anesthesia is induced with the animal in the induction box at 5% concentration, and then maintained with a nose cone at 2%. The fur will be shaved, and five landmarks (toe, ankle, knee, hip, ISIS) tattooed on the skin, using the instructions provided with the AIMs Animal Tattooing System.

Behavioral experiments / assays

Items for all behavioral tests/assays

None of the assays are performed more than once per day. The attached experimental design table shows the time points of application. Pain, ladder, and mechanical tests are done three times over the 10 week study timeline for each animals, in weeks -1, 2, and 6. Treadmilll training or treadmill motion capture occur 3x/week for six weeks during the study, never more than once per day. The maximum frequency of assessment for any one animal would be treadmill walking 3x per week, once each on MWF, and ladder, thermal pain, and mechanical testing each once on Thursday. Animals are acclimated to all tests once before carrying out the test, on two separate days prior to the testing day, by being placed into the apparatus for 10 minutes and given BioServ treats. All apparati are cleaned and wiped with 70% ethanol between every use. The IACUC pain/distress scoring chart will be used to assess whether removal is necessary from a behavioral assay. In addition, animals that refuse to walk on the treadmill after acclimation and 10 minutes of treadmill belt movement will be removed from the treadmill. All kinematic video data are stored on a secured Spence lab storage server behind Temple firewalls, and data sets are not placed on public repositories. Ladder test videos on SD cards are transferred to this server as well, and the SD cards wiped.

Thermal Hargreaves pain assay:

This assay procedure is as follows: rats are placed in a clear plastic chamber with a glass floor and allowed to acclimate for 10 min. Animals are acclimated to the apparatus over two days prior to the first test by being placed in the apparatus for 10 minutes once per day and given BioServ treats. A halogen lamp beneath the glass surface is used to direct an intense light beam

onto the plantar surface of the hind paw. This intensity is chosen based on past studies in the Smith lab (Kelamangalath, 2015) to give baseline responses of approximately 10 s in normal animals. Paw withdrawal latency was detected automatically by a photocell and taken as a behavioral index of the pain threshold. Animals are removed from the test after 22 s if they have not withdrawn their paw at this point. This test takes around 30 minutes.

Von Frey mechanical test:

Sensitivity to mechanical stimuli will be tested using von Frey hair (VFH; North Coast Medical, Morgan Hill, CA) applied to the midplantar region of the hindpaws, following an up-and-down profile (Dixon, 1980; Chaplan et al., 1994). Animals are placed on a wire mesh floor and treats (BioServ Fruit-Topia) are provided. Animals are acclimated to the apparatus over two days prior to the first test by being placed in the apparatus for 10 minutes once per day and given BioServ treats. Measurements are taken only when the animals are eating cereals so as to rule out visual responses from seeing the presentation of monofilament (Touch Test, North Coast Medical, Gilroy, CA). Presentation of the monofilament starts at 4.93 (8 g) and ends at 6.10 (100 g) if there is no response. Each monofilament was applied in a smooth and steady manner over a two second period until it bends. The filament was then removed in a smooth and steady manner. This single touch constituted one single trial. A positive response was indicated by the quick lifting of the paw away from the monofilament before the monofilament was removed. If there was no response, the next higher monofilament was presented after 30 s to prevent adaptation. If there was a response, the next lower filament was used to determine a response. This up and down procedure for systematically applying higher or lower monofilaments based on positive or negative responses is used until 10 trials per paw are gathered. Animals that do not acclimate to the test chamber within 60 minutes will be removed from the test. This test takes around 20 minutes.

Ladder/gridwalk test

A custom made horizontal ladder with irregularly spaced rungs will be used to test for proprioception. Animals are trained to walk on the horizontal ladder before they are subjected to any surgery. The percent efficiency in the right hindlimb is evaluated as a measure of proprioception. Before surgery, typically trained animals show almost 100% efficiency in the right hindlimb (no errors/missed rungs). Loss of proprioception causes dramatic increase in number of ladder misses (Kelamangalath, 2015), and improvement in proprioception is expected to increase the placement efficiency. Animals are acclimated to the apparatus over two days prior to the first test by being placed in the apparatus for 10 minutes once per day and given BioServ treats. Animals that do not cross the ladder after 60 minutes of acclimation will be removed from the test. This test takes around 20 minutes.

Basso, Beattie, Bresnahan Locomotor Scoring (BBB; See Basso, et al, 1995)

Animals are placed into a small plastic tub ("kiddie pool") and 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 (Basso et al., 1995). Animals are acclimated to the apparatus over two days prior to the first test by being placed in the apparatus for 10 minutes once per day and given BioServ treats. Animals that do not acclimate to the "kiddie pool" within 60 minutes will be removed from the test. This test takes around 20 minutes.

Treadmill training and treadmill training with motion capture (MOCAP)

Long term treadmill training ("treadmill training") and data collection consists of 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. When we are recording videos of the treadmill locomotion for kinematic analysis, which we also refer to as motion capture (MOCAP) for short, we record three bouts of walking at each speed for each animal while they move in the normal manner on the treadmill. Animals are acclimated to the apparatus over two days prior to the first test by being placed in the apparatus for 10 minutes once per day and given BioServ treats. Animals that do not acclimate to the treadmill after 60 minutes will be removed from the treadmill.

* Will Multiple Survival Surgeries be conducted on this protocol?
☑ Yes ☐ No

* In the description below provide a list of the survival surgical procedures, scientific justification as to why these surgeries are necessary to achieve scientific goals, the sequence of surgeries, a timeline of the surgical procedures and additional monitoring required due to multiple surgeries.
Multiple survival surgeries

Naïve animals will receive dorsal root ganglion (DRG) injections, followed by a three week delay, followed by a spinal cord lesion, as per the uploaded experimental design table. Multiple surgeries are required because the viral vector we are using to trace and identify helpful changes in plasticity that facilitate improved recovery from injury must be directly surgically injected into sensory afferents innervating the spinal cord, and needs three weeks to express at maximum level. At present there is not another way to manipulate the activity of afferents and subsequently measure the putatively helpful changes in connectivity within the spinal cord. The monitoring procedures after DRG injection and spinal cord lesion are as described above.

☑ * I certify that I have read and understand the applicable above referenced IACUC Guidelines or Policies for the procedures being performed. If there are any planned procedural changes, I have accurately documented and provided justification in this form.

8. ANESTHESIA AND ADMINISTRATION OF OTHER SUBSTANCES

When completing this page:
- Please ensure that you include substances administered to animals that are described in your experimental design or procedures section of the protocol.

Click here for the IACUC Guideline Anesthetics, Analgesics and Sedatives by Species.

* 8.1 Are there any planned procedural changes to the above referenced IACUC guideline?
☐ Yes ☑ No

* 8.2 Are you using an Anesthetic Agent? ☑ Yes ☐ No

If you are administering multiple agents, please create a new row for each agent by selecting the plus sign icon.

- Anesthesia - any anesthetic agent being used (i.e; isoflurane, ketamine..)
- Analgesia - any substance used for pain relief (i.e. buprenorphine, meloxicam…)
- Therapeutic - antibiotics, fluid therapy, neuromuscular blocking agents or other substances used for supportive care
- Experimental - all administered experimental drugs, contrast agents, immune modulators, biologicals (including viruses, bacteria, toxins) hazardous chemicals (including, chemotherapeutics).
- Pharmaceutical Grade – A drug, biologic, or reagent that is approved by the FDA for use in humans or animals or for which a chemical purity standard has been established by the United States Pharmacopeia-National Formulary (USP-NF), or British Pharmacopeia (BP) as >99%.
- Nonpharmaceutical Grade - Chemicals or compounds that do not meet the USP/NF/BP requirements.
- Please refer to the FDA database listing approved commercial formulations for human drugs (Orange Book) and veterinary drugs (Green Book). For chemicals, a certificate of analysis is usually available upon request.
- If you are administering biologics, cells, viruses, etc., these substances are obviously nonpharmaceutical grade so please check the box in the chart and answer the last three questions. Other questions would be Not applicable (NA).
- Click here for the IACUC Policy on the Use of Non-Pharmaceutical Grade Agents or Mixtures of Pharmaceuticals.

8.2.1 Anesthetics/Analgesic/Therapeutic/Experimental Substances

* Procedure:	All survival surgeries
* Type of Drug	Anesthesia
* Name of Drug	Ketamine/Xylazine cocktail
* Pharmaceutical Grade	☑ Yes ☐ No
Dose	induction 75-95 mg/kg (K) + 5-10 mg/kg (X), 38-48 mg/kg(K) + 2.5 to 5 mg/kg (X) in longer ops
Route	Intraperitoneal (IP)
Frequency/Duration:	1x induction, maintenance typically 1x per hour after

* Procedure:	Marking and tattooing, subcutaneous knee ligament tatooing, tail clip >3 weeks, terminal MUA induction
* Type of Drug	Anesthesia
* Name of Drug	Isoflurane
* Pharmaceutical Grade	☑ Yes ☐ No
Dose	3-5% induction, 1-3% maintenance
Route	Inhalation
Frequency/Duration:	20 minutes

* Procedure:	All survival surgeries
* Type of Drug	Analgesia
* Name of Drug	Meloxicam
* Pharmaceutical Grade	☑Yes ☐ No
Dose	5 mg/kg
Route	Intraperitoneal (IP)
Frequency/Duration:	1 dose given pre-operatively; 1x day for 3 days post surgery

* Procedure:	Terminal MUA recording
* Type of Drug	Anesthesia
* Name of Drug	Alpha chloralose
* Pharmaceutical Grade	☐ Yes ☑No
Dose	55-65 mg/kg/hr
Route	Intravenous (IV)
Frequency/Duration:	6 hours
* Grade or purity	>= 80%
* Is this substance known to induce fever?	○Yes ◉No ○Unknown
* Scientific justification to utilize the nonpharmaceutical grade compound or why the pharmaceutical grade solution or USP powders product cannot be used. For novel substances, any information currently available for the optimization of this substance for animal use.	I'm not sure it can be obtained in USP powder. For the purposes of this terminal experiment, it has been used successfully as an anesthetic as described in Holzgrefe et al., Lab Animal Science, 1985, and Bhowmik et al., 2003, and D. Saint-Georges et al., 1997.
* Description of the reagents and procedures to be used to ensure sterility in preparing mixtures/solutions including testing for pH if possible.	The alpha chloralose will be prepared anew on the day of each usage. It will not be stored. It will be prepared in the following manner: 1. Mix 60 mg of the powder into 6 mL of saline, making solution at 10 mg/mL. 2. Dissolve this powder in the saline using an agitator and hot plate set to 5. Do not let it boil. 3. To avoid evaporation, use triangular flask and put cap on it. 4. Draw off the initial amount required into a vial and let it cool to body temperature ~36.5C. 5. Place a syringe filter on your injection syringe to filter as you inject. 6. Provide an induction bolus of 60mg/kg 10 minutes before removing the isoflurane mask 7. Supplement as necessary at dose equivalent 55-65 mg/kg/hr.
* Describe how substance will be labeled, stored, and the expiration date	The powder is stored in a secured cabinet, labeled, and given clear expiration date.

* Procedure:	DRG Injections and Spinal Cord Lesion Survival Surgeries
* Type of Drug	Therapeutic
* Name of Drug	Triple Antibiotic Ointment (TAO)
* Pharmaceutical Grade	☑Yes ☐ No
Dose	Topical
Route	Topical
Frequency/Duration:	1x post surgery; if required for wound healing

* Procedure:	DRG Injections
* Type of Drug	Experimental
* Name of Drug	AAV2-hSyn-hM3Dq-mCherry

* Pharmaceutical Grade	☐ Yes ☑ No
Dose	-1 x 10 transforming units/ul; 1ul per DRG
Route	
Frequency/Duration:	Route: direct surgical microinjection. 1x per DRG surgery per DRG.
* Grade or purity	NA
* Is this substance known to induce fever?	○ Yes ◉ No ○ Unknown
* Scientific justification to utilize the nonpharmaceutical grade compound or why the pharmaceutical grade solution or USP powders product cannot be used. For novel substances, any information currently available for the optimization of this substance for animal use.	Pharmaceutical grade AAVs are unavailable, as all the viral constructs were generated in our viral core lab. Scientific justification: the only way to transduce neurons to activate them and then trace them with histology in the manner we proposed to do in the grant is with these viral vectors. We got the grant because we proposed to use these cutting edge tools that do more than is possible with electrical stimulation and allow us to figure out how helpful plasticity occurs in the spinal cord with rehabilitation. The packaging protocol used and methods for creating these recombinant tools are continuously being optimised by the field and the Shriners Viral Core is continuously updating their methods with the latest methods published in the field (for a recent review of these tools, see, e.g., https://www.nature.com/articles/s41583-020-00382-z).
* Description of the reagents and procedures to be used to ensure sterility in preparing mixtures/solutions including testing for pH if possible.	The viral construct was generated in the BSL-2 lab following the sterile procedure, and aliquoted into small Eppendorfs for storage.
* Describe how substance will be labeled, stored, and the expiration date	The Shriners Viral Core uses an online database of constructs with associated labeling system and expiration dates, and the constructs are stored in a -80C freezer. AAVs are stored in 30uL aliquots at -80 degrees C. All tubes are properly labelled. Each tube is used once, we do not freeze and thaw the viral samples, and after use the remaining viral content and tubes are properly disposed of (deposited in container of bleach).

* Procedure:	DRG Injections
* Type of Drug	Experimental
* Name of Drug	AAV2-hSyn-hM4Di-mCherry
* Pharmaceutical Grade	☐ Yes ☑ No
Dose	-1 x 10 transforming units/ul; 1ul per DRG
Route	
Frequency/Duration:	Route: direct surgical microinjection. 1x per DRG surgery per DRG.
* Grade or purity	NA
* Is this substance known to induce fever?	○ Yes ◉ No ○ Unknown
* Scientific justification to utilize the nonpharmaceutical grade compound or why the pharmaceutical grade solution or USP powders product cannot be used. For novel substances, any information currently available for the optimization of this substance for animal use.	Pharmaceutical grade AAVs are unavailable, as all the viral constructs were generated in our viral core lab. Scientific justification: The only way to transduce neurons to activate them and then trace them with histology in the manner we proposed to do in the grant is with these viral vectors. We got the grant because we proposed to use these cutting edge tools that do more than is possible with electrical stimulation and allow us to figure out how helpful plasticity occurs in the spinal cord with rehabilitation. The packaging protocol used and

	methods for creating these recombinant tools are continuously being optimised by the field and the Shriners Viral Core is continuously updating their methods with the latest methods published in the field (for a recent review of these tools, see, e.g., https://www.nature.com/articles/s41583-020-00382-z).
* Description of the reagents and procedures to be used to ensure sterility in preparing mixtures/solutions including testing for pH if possible.	The viral construct was generated in the BSL-2 lab following the sterile procedure, and aliquoted into small Eppendorfs for storage.
* Describe how substance will be labeled, stored, and the expiration date	The Shriners Viral Core uses an online database of constructs with associated labeling system and expiration dates, and the constructs are stored in a -80C freezer. AAVs are stored in 30uL aliquots at -80 degrees C. All tubes are properly labelled. Each tube is used once, we do not freeze and thaw the viral samples, and after use the remaining viral content and tubes are properly disposed of (deposited in container of bleach).

* Procedure:	DRG injections and spinal cord contusion surgeries
* Type of Drug	Analgesia
* Name of Drug	Ethiqa XR - Extended Release Buprenorphine
* Pharmaceutical Grade	☑ Yes ☐ No
Dose	0.65mg/kg
Route	Subcutaneous (SC)
Frequency/Duration:	Pre-operatively, once (slow release formulation over 72 hours)

* Procedure:	All survival surgeries
* Type of Drug	Therapeutic
* Name of Drug	Opthalmic ointment
* Pharmaceutical Grade	☑ Yes ☐ No
Dose	
Route	Topical
Frequency/Duration:	1x after anesthesia, before surgery

* Procedure:	Intrasciatic Nerve Injections
* Type of Drug	Experimental
* Name of Drug	Lysolethicin
* Pharmaceutical Grade	☑ Yes ☐ No
Dose	0.5 ul of 1% Lysolecithin with 32 gauge Hamilton syringe
Route	
Frequency/Duration:	1x direct injection into sciatic nerve

* Procedure:	Treadmill training
* Type of Drug	Experimental
* Name of Drug	Clozapine-N-Oxide
* Pharmaceutical Grade	☑ Yes ☐ No
Dose	5 mg/kg
Route	Intraperitoneal (IP)
Frequency/Duration:	1x per treadmill training session (animals have 1 session per day, 3 days/week for 6 weeks)

* Procedure:	DRG Injection Survival Surgeries
* Type of Drug	Experimental
* Name of Drug	AAV2/hSyn/GFP
* Pharmaceutical Grade	☐ Yes ☑ No
Dose	-1 x 10 transforming units/ul; 1ul per DRG
Route	
Frequency/Duration:	Route: direct surgical microinjection. 1x per DRG surgery per DRG.
* Grade or purity	NA
* Is this substance known to induce fever?	○ Yes ◉ No ○ Unknown
* Scientific justification to utilize the nonpharmaceutical grade compound or why the pharmaceutical grade solution or USP powders product cannot be used. For novel substances, any information currently available for the optimization of this substance for animal use.	Pharmaceutical grade AAVs are unavailable, as all the viral constructs were generated in our viral core lab. Scientific justification: The only way to transduce neurons to activate them and then trace them with histology in the manner we proposed to do in the grant is with these viral vectors. We got the grant because we proposed to use these cutting edge tools that do more than is possible with electrical stimulation and allow us to figure out how helpful plasticity occurs in the spinal cord with rehabilitation. The packaging protocol used and methods for creating these recombinant tools are continuously being optimised by the field and the Shriners Viral Core is continuously updating their methods with the latest methods published in the field ((for a recent review of these tools, see, e.g., https://www.nature.com/articles/s41583-020-00382-z).
* Description of the reagents and procedures to be used to ensure sterility in preparing mixtures/solutions including testing for pH if possible.	The viral construct was generated in the BSL-2 lab following the sterile procedure, and aliquoted into small Eppendorfs for storage.
* Describe how substance will be labeled, stored, and the expiration date	The Shriners Viral Core uses an online database of constructs with associated labeling system and expiration dates, and the constructs are stored in a -80C freezer. AAVs are stored in 30uL aliquots at -80 degrees C. All tubes are properly labelled. Each tube is used once, we do not freeze and thaw the viral samples, and after use the remaining viral content and tubes are properly disposed of (deposited in container of bleach).

* Procedure:	DRG Injection Survival Surgeries
* Type of Drug	Experimental
* Name of Drug	AAV2/hSyn/mCherry
* Pharmaceutical Grade	☐ Yes ☑ No
Dose	-1 x 10 transforming units/ul; 1ul per DRG
Route	
Frequency/Duration:	Route: direct surgical microinjection. 1x per DRG surgery per DRG.
* Grade or purity	NA
* Is this substance known to induce fever?	○ Yes ◉ No ○ Unknown
* Scientific justification to utilize the nonpharmaceutical grade compound or why the pharmaceutical grade solution or USP powders product cannot be used. For novel substances,	Pharmaceutical grade AAVs are unavailable, as all the viral constructs were generated in our viral core lab. Scientific justification: The only way to transduce neurons to activate them and then trace them with

any information currently available for the optimization of this substance for animal use.	histology in the manner we proposed to do in the grant is with these viral vectors. We got the grant because we proposed to use these cutting edge tools that do more than is possible with electrical stimulation and allow us to figure out how helpful plasticity occurs in the spinal cord with rehabilitation. The packaging protocol used and methods for creating these recombinant tools are continuously being optimised by the field and the Shriners Viral Core is continuously updating their methods with the latest methods published in the field ((for a recent review of these tools, see, e.g., https://www.nature.com/articles/s41583-020-00382-z).
* Description of the reagents and procedures to be used to ensure sterility in preparing mixtures/solutions including testing for pH if possible.	The viral construct was generated in the BSL-2 lab following the sterile procedure, and aliquoted into small Eppendorfs for storage.
* Describe how substance will be labeled, stored, and the expiration date	The Shriners Viral Core uses an online database of constructs with associated labeling system and expiration dates, and the constructs are stored in a -80C freezer. AAVs are stored in 30uL aliquots at -80 degrees C. All tubes are properly labelled. Each tube is used once, we do not freeze and thaw the viral samples, and after use the remaining viral content and tubes are properly disposed of (deposited in container of bleach).

* Procedure:	DRG Injection Survival Surgeries
* Type of Drug	Experimental
* Name of Drug	AAV2/DIO/hM3Dq/mCherry
* Pharmaceutical Grade	☐ Yes ☑ No
Dose	-1 x 10 transforming units/ul; 1ul per DRG
Route	
Frequency/Duration:	Route: direct surgical microinjection. 1x per DRG surgery per DRG.
* Grade or purity	NA
* Is this substance known to induce fever?	○ Yes ◉ No ○ Unknown
* Scientific justification to utilize the nonpharmaceutical grade compound or why the pharmaceutical grade solution or USP powders product cannot be used. For novel substances, any information currently available for the optimization of this substance for animal use.	Pharmaceutical grade AAVs are unavailable, as all the viral constructs were generated in our viral core lab. Scientific justification: The only way to transduce neurons to activate them and then trace them with histology in the manner we proposed to do in the grant is with these viral vectors. We got the grant because we proposed to use these cutting edge tools that do more than is possible with electrical stimulation and allow us to figure out how helpful plasticity occurs in the spinal cord with rehabilitation. The packaging protocol used and methods for creating these recombinant tools are continuously being optimised by the field and the Shriners Viral Core is continuously updating their methods with the latest methods published in the field ((for a recent review of these tools, see, e.g., https://www.nature.com/articles/s41583-020-00382-z).
* Description of the reagents and procedures to be used to ensure sterility in preparing mixtures/solutions including testing for pH if possible.	The viral construct was generated in the BSL-2 lab following the sterile procedure, and aliquoted into small Eppendorfs for storage.
* Describe how substance will be labeled, stored, and the expiration date	The Shriners Viral Core uses an online database of constructs with associated labeling system and expiration dates, and the constructs are stored in a -80C freezer. AAVs are stored in 30uL aliquots at -80 degrees C. All tubes are properly labelled.

	Each tube is used once, we do not freeze and thaw the viral samples, and after use the remaining viral content and tubes are properly disposed of (deposited in container of bleach).

* Procedure:	DRG Injection Survival Surgeries
* Type of Drug	Experimental
* Name of Drug	AAV2/DIO/hM4Di/mCherry
* Pharmaceutical Grade	☐ Yes ☑ No
Dose	-1 x 10 transforming units/ul; 1ul per DRG
Route	
Frequency/Duration:	Route: direct surgical microinjection. 1x per DRG surgery per DRG.
* Grade or purity	NA
* Is this substance known to induce fever?	○ Yes ◉ No ○ Unknown
* Scientific justification to utilize the nonpharmaceutical grade compound or why the pharmaceutical grade solution or USP powders product cannot be used. For novel substances, any information currently available for the optimization of this substance for animal use.	Pharmaceutical grade AAVs are unavailable, as all the viral constructs were generated in our viral core lab. Scientific justification: The only way to transduce neurons to activate them and then trace them with histology in the manner we proposed to do in the grant is with these viral vectors. We got the grant because we proposed to use these cutting edge tools that do more than is possible with electrical stimulation and allow us to figure out how helpful plasticity occurs in the spinal cord with rehabilitation. The packaging protocol used and methods for creating these recombinant tools are continuously being optimised by the field and the Shriners Viral Core is continuously updating their methods with the latest methods published in the field ((for a recent review of these tools, see, e.g., https://www.nature.com/articles/s41583-020-00382-z).
* Description of the reagents and procedures to be used to ensure sterility in preparing mixtures/solutions including testing for pH if possible.	The viral construct was generated in the BSL-2 lab following the sterile procedure, and aliquoted into small Eppendorfs for storage.
* Describe how substance will be labeled, stored, and the expiration date	The Shriners Viral Core uses an online database of constructs with associated labeling system and expiration dates, and the constructs are stored in a -80C freezer. AAVs are stored in 30uL aliquots at -80 degrees C. All tubes are properly labelled. Each tube is used once, we do not freeze and thaw the viral samples, and after use the remaining viral content and tubes are properly disposed of (deposited in container of bleach).

* Procedure:	Tattooing of Lateral Knee Ligaments
* Type of Drug	Experimental
* Name of Drug	Black Animal Tattoo Paste/Ink (Ketchum #327)
* Pharmaceutical Grade	☑ Yes ☐ No
Dose	n/a
Route	
Frequency/Duration:	once

* Procedure:	Tattooing of Lateral Knee Ligaments
* Type of Drug	Experimental

* Name of Drug	India Ink (Mckesson #1039754)
* Pharmaceutical Grade	☑Yes ☐ No
Dose	n/a
Route	
Frequency/Duration:	once

* Procedure:	Tattooing of Lateral Knee Ligaments
* Type of Drug	Experimental
* Name of Drug	Black Tattoo Pigment (AIMS Company; #242)
* Pharmaceutical Grade	☑Yes ☐ No
Dose	n/a
Route	
Frequency/Duration:	Once

* Procedure:	Contusions post operative care
* Type of Drug	Therapeutic
* Name of Drug	Generic diaper rash cream (zinc oxide 40% eg CVS brand)
* Pharmaceutical Grade	☑Yes ☐ No
Dose	n/a
Route	Topical
Frequency/Duration:	Daily after contusions until bladder expression no longer necessary

* Procedure:	Surgery post-operative care
* Type of Drug	Therapeutic
* Name of Drug	Terrasil
* Pharmaceutical Grade	☑Yes ☐ No
Dose	n/a
Route	Topical
Frequency/Duration:	As necessary should pressure ulcers begin to develop

* Procedure:	Euthanasia
* Type of Drug	Experimental
* Name of Drug	Fatal Plus
* Pharmaceutical Grade	☑Yes ☐ No
Dose	150mg/kg
Route	Intraperitoneal (IP)
Frequency/Duration:	Once

* Procedure:	Tattooing of Lateral Knee Ligaments
* Type of Drug	Experimental
* Name of Drug	AIMs Animal Tattooing System
* Pharmaceutical Grade	☑Yes ☐ No
Dose	N/A a plastic box with power supply and foot pedal and animal tattoo machine designed for this
Route	Subcutaneous (SC)
Frequency/Duration:	

	once for an animal during the study; note this not a drug but an apparatus; a reviewer requested I add it here

8.2.2 Pre-Anesthetic Fasting

* Will animals be fasted prior to the use of anesthesia?
☐ Yes ☑ No

8.2.3 Anesthetic Depth and Monitoring

Check which parameters will be used to determine the depth of anesthesia: Check all that apply. A minimum of 3 parameters must be used.

- ☐ Blood Pressure
- ☐ ECG
- ☑ Heart Rate
- ☑ Palpebral (eyelid) Reflex
- ☑ Respiratory Rate/Pattern
- ☑ Response to Surgical Stimulation
- ☐ SpO_2
- ☑ Withdrawal Reflex (e.g. toe/tail pinch)
- ☐ Other

How frequently will the depth of anesthesia be monitored? Please check other if any of the checked parameters above will not be monitored every 15 minutes.

- ☑ Every 15 minutes or less.
- ☐ Other

Describe what actions will be taken, if needed, to adjust the depth of anesthesia during the procedure.
For ketamine/xylazine, we supplement as needed with a 1/3 of induction dose, which is ~25 mg/kg (K) + 2 mg/kg (X)

For isoflurane, anesthesia is adjusted by changing the percentage of isoflurane in the gas between 1-3%.

For alpha chlorolose, anesthesia is supplemented as necessary at dose equivalent 55-65 mg/kg/hr.

8.2.4 Sedation/Anesthesia Recovery for Non-Surgical Studies

Note: Complete this section for non-surgical studies (e.g. imaging, restraint, etc.). Do not include information for animals undergoing non-surgical manipulations under anesthesia together with survival surgery. This information will be captured in the Survival Surgery subsection of this form

* Will the animals recover from anesthesia?
☑ Yes ☐ No

Describe the monitoring parameters used during the immediate recovery time period (minutes-hours).
This applies to shaving fur and marking skin with permanent marker and tattooing skin, and for tail clipping in unusual cases where animals are >3 weeks old. Sternal recumbency and movement.

How frequently will the animals be monitored during the immediate recovery period?

- ☑ Every 15 minutes or less.
- ☐ Other

Describe the criteria used to indicate that the animals are fully recovered and can be returned to their home cage (e.g. sternal recumbency, mobile, eating/drinking).
Sternal recumbency and normal ambulation.

* Will ULAR Veterinary services participate in/perform anesthesia monitoring services?
☐ Yes ☑ No

☑ * I certify that I have read and understand the above referenced IACUC guideline. If there are any planned procedural changes, I have accurately documented and provided justification in this form.

8.3 Paralytic/Neuromuscular Blocking (NMB) Agents

Click here for the IACUC Policy on the Use of Paralytics.

* Will any of the substances identified in the sections above be Neuromuscular Blocking Agents (NMB) or paralytics to be administered?
☐ Yes ☑ No

* Are there any planned procedural changes to the above referenced IACUC policy?
☐ Yes ☑ No

8.4 Non-Pharmaceutical Grade Agents

* Do you have any additional comments about your pharmaceutical or non-pharmaceutical grade compounds or dosing that are not captured in the table above?
☑ Yes ☐ No

* Please provide any additional notes or comments in the box below:
Please see above information in 8.2.1 in the additional drop down fields. I have done my best to answer this. I know that the constructs we use are safe to inject into the nervous system in the manner used by everyone in the field.

9. ANIMAL HANDLING AND RESTRAINT

* 9.1 Will you be restraining the animals for any described procedures? ☑ Yes ☐ No

* Procedure:	* Restraint Method
Genotyping < 21 days	Manual (restraint by hand only)
DRG Injections	Chemical
Spinal cord lesions	Chemical
Intrasciatic Nerve Injections	Chemical
Non-survival MUA Recordings	Chemical
H-reflex testing	Chemical
Dyeing of fur	Chemical
Shaving of fur, marking of skin	Chemical
Micro-tattooing	Chemical
Genotyping >= 21 days	Chemical
Subcutaneous knee ligament tattooing	Chemical
Intrathecal Injections	Chemical
Spinal Cord Injections	Chemical
Treadmill	Manual (restraint by hand only)
Ladder walk assay	Manual (restraint by hand only)
Hargreaves test	Manual (restraint by hand only)
von Frey assay	Manual (restraint by hand only)
BBB scoring	Manual (restraint by hand only)

* 9.2 Will the animals be manually or physically restrained for greater than 5 minutes per procedure? ☐ Yes ☑ No

* 9.3 Will the animals undergo acclimation procedures related to the method(s) of restraint selected? ☑ Yes ☐ No

* Please describe the acclimation procedure. Include the frequency and duration of the acclimation procedures, what, if any, positive reinforcement will be used and criteria used to determine if an animal has failed to adapt to the restraint and/or should be removed from restraint to relieve any distress. If multiple procedures require restraint acclimation, please ensure that all details are captured for each procedure.
We gentle all animals with 5 or 10 minutes of holding, 3x/week, starting on arrival at facilities.

10. FINAL ANIMAL DISPOSITION AND ENDPOINTS

When completing this page:

- *Please ensure that the euthanasia chart does not omit any experimental group such as pups in a rodent breeding protocol.*
- *Humane endpoints for early euthanasia must be consistent with the details described in the pain scoring chart.*

Click here for the IACUC Guideline on the Euthanasia of Laboratory Animals.

10.1 Are all methods of euthanasia to be performed consistent with the latest edition of the AVMA Guidelines for the Euthanasia of Animals (2013)?

☑ Yes ☐ No

10.2 Select the final animal disposition(s) planned for this study:
Check all that apply.

☑ Euthanasia

☐ Transfer to another approved IACUC protocol

☐ Other

10.3 Euthanasia Chart for All Animals

Experimental Group	Ages of Animals	Method of Euthanasia	Secondary Confirmation of Death	Route/Dose (mg/kg)
All groups except MUA animals	14-16 weeks	Commercial Euthanasia Solution	bilaterial thoracotomy	Fatal Plus. 150 mg/kg IV
MUA animals	8-10 weeks	Commercial Euthanasia Solution	bilateral thoracotomy	Fatal Plus. 150 mg/kg IV
humane endpoints	16 weeks	Commercial Euthanasia Solution	bilateral thoracotamy	Fatal Plus. 150 mg/kg IV
humane endpoints	16 weeks	Carbon Dioxide (C02) inhalation	bilateral thoracotamy	
rat pups	<7 days	Decapitation (under anesthesia)	decapitation	

10.4 Experimental Endpoints

* The experimental endpoint of a study occurs when the scientific aims and objectives have been reached" (Guide page 27).

For each experimental and control group, describe what factor(s) determine(s) when the animals are routinely euthanized (e.g. length of time from surgery, reaching a certain tumor size, development of certain treatment outcome, etc.).

All animals except the MUA recording group reach their endpoint after studying their recovery from spinal cord injury for six weeks (see experimental design table). The MUA group reaches it's endpoint after the DREADDs vectors have had enough time to saturate expression (4 weeks post DRG injection). The 32 Sprague Dawley animals for H-reflex CNO controls will be euthanized after two H-reflex recordings (e.g. they will not undergo spinal cord injury; approximately 14-16 weeks of age). The 8 LE rats for infrared knee marker imaging will be euthanized after 3 bouts of kinematic recordings, approximately 10-12 weeks of age.

10.5 Humane Endpoints

* The humane endpoint is that point at which pain or distress in an experimental animal is prevented, terminated, or relieved" (Guide page 27).

Please describe the humane endpoint criteria for permanent removal of an animal from study before the scheduled or anticipated experimental endpoint.

After recovery from contusion, animals will be monitored for general health on a daily basis for at least 3 days after surgery. For contused animals, bladders are manually expressed twice daily typically for up to seven days, and as such these animals will likely

be monitored for longer than 3 days post-op. If a pain or distress score of "2" is observed as assessed by the Temple IACUC pain/distress scoring chart, the animal will be euthanized. After the experiment is complete, the animal will be euthanized. If pain assays show reduced forces or time of exposure to heat stimulus post CNO administration within animals, or relative to SCI-controls between animals, this would indicate development of hyperalgesia. If animals having spinal cord lesions exhibit self trauma of any bone exposure or cannibalization of more that 2 toes they will be euthanized. If this is found to be the case, affected animals will be removed from the study and euthanized.

☑ * I certify that myself and all approved personnel having direct animal contact who perform animal procedures, manipulations and/or observations, including all approved methods of euthanasia, described in the protocol are technically competent and have been properly trained to ensure that no unnecessary pain and/or distress will be caused as a result of the procedures and/or manipulations

☑ * I certify that all personnel listed on this protocol have completed all training/medical clearance requirements determined by the IACUC and ULAR.

11. JUSTIFICATIONS AND ALTERNATIVES

11.1 Justification for the Use of Animals

When completing this page:

- Two justifications are required: one for the use of animals as compared to cell lines/mathematical models/in vitro systems, the other for the use of a particular species.
- For consideration of the 3Rs, only those relevant to the protocol should be checked and described.
- Literature search words are sometimes incomplete, though this is normally identified by the IACUC's library representative.

* *Provide a justification as to why the use of live animals must be used to achieve the scientific goals of the planned work. Explain why cell lines, computer or mathematical models, and in vitro biological systems cannot be used instead.*

Successful movement is an integrated phenomenon: we are able to move because of the concerted effort of ion channels, neurons, neural networks, muscles, skeletons, bodies, and finally the outside world. While reductionist experiments that take apart each of these systems and examine them in isolation are incredibly important and have provided a wealth of information, more and more we are learning that when we examine intact, freely behaving animals we often find a different function is performed by each subsystem than in reduced preparations, and the function of the system as a whole is different from what we predict when we connect models of these subsystems together (Tytell, 2011, *Curr Opin. Neurobiol;* Dombeck and Reiser, 2011; *Curr. Opin. Neurobiol.)*. Studies using cell lines are useful and may inform neural developmental or basal circuit questions that address movement, but for understanding the control of movement ultimately we need to sense and perturb intact animals, or at least intact tissue systems/organs, to understand function in the biologically relevant context. Mathematical modeling of neuromechanical systems in locomotion is important (Pearson, 2006; *Trends in Neurosci*), and the Spence group employs it to make predictions about the structure of quadrupedal gait control (Wilshin et al., 2017*)* but not enough data exist to build sufficiently accurate models to model even relatively basic tasks like trotting locomotion. For example, we know that spinal locomotor neural circuits play a large role in establishing locomotor rhythm, and that the use of sensory feedback of by these networks is phase-dependent in walking; but for trotting locomotion, which is the most common gait used by mice, and a fundamental gait used by many species, we don't' know whether and how these circuits use sensory feedback. One we have information on which sense organs are used across multiple forms of locomotion (walking + trotting), and how these depend on phase, we can move to building integrative, general models of the neuromechanics of a moving animal.

For the additional H-reflex animals, at present in silico, in vitro, or purely mathematical models are not detailed enough to tell us whether the drug CNO will affect reflexes in a living rat.

For additional infra-red knee marker imaging animals, at present we still need kinematic data from live animals in order to quantify recovery from injury, and reduction of the skin motion artifact in these animals would benefit a large community.

11.2 Justification for the Species

* *Provide a justification as to why the species chosen is/are the most appropriate for this study.*

Experiments in this application will examine the questions concerning chemogenetic therapy for restoring body weight support and movement after spinal cord injury. The studies outlined propose to discover whether chronic, non-invasive activation of afferent neurons can produce better recovery of function after spinal cord injury than the current state of the art, epidural stimulation, applied only during movement. The capability for repeated, chronic stimulation is made possible by our proposed validation of the use in SCI of a new tool for neuromodulation, designer receptors exclusively activated by designer drugs (DREADDs). In these studies, we will furthermore carry out axonal tracing to determine how which neurons have been activated, and their connectivity, that may support better functional outcomes. The results from these experiments will be instrumental in understanding the mechanisms by which stimulation of afferents can improve recovery from SCI, and if successful, open up new paradigms for treatment (chronic, genetically targeted afferent excitation and/or inhibition).

Rats are the animal of choice in this study for the following reasons: 1) rats are specifically bred for research purposes, 2) spinal cord injuries in rats are well established by others and us (and the Smith Lab, especially), 3) the database relevant to SCI that the Smith Lab has established in the past is based mainly on rats, 4) the selection of rats is in keeping with its use as the experimental spinal cord injury model used in other major spinal cord research centers in this country, and 5) rats are cost effective in their use. There is considerable basic information concerning peripheral nerve and spinal cord injury, particularly the spinal cord injury method used for these experiments.

For the additional Sprague Dawley rats for H-reflex as well as for the additional Long Evans rats for infra-red marking, we choose these lines for the wealth of past spinal cord injury research on this animal, such that our study will aid the most investigators.

11.3 Duplication of Research

☑ I assure that the activities described within do not unnecessarily duplicate previous experiments.

☐ This is a teaching protocol; therefore the procedures may be similar but the students or trainees may change each year.

11.4 Consideration of the 3Rs (Replacement, Reduction and Refinement)

Select all of the applicable ways in which you have addressed the concept of the 3Rs. Provide a short description of how you have reduced, replaced and/or refined each method selected.

☑ Use of in vitro, computer or mathematical methods, cell culture to replace animals for part of the study.

* Describe:
We tested all of our DREADDs vectors in vitro before bringing in vivo.

☐ Improved techniques for tissue harvesting to increase the yield of tissue, cells, etc.

☐ Reduction in the need for control or sham operated animals.

☐ Improved medical or invasive techniques to reduce post-procedure pain, distress or mortality.

☑ Pre-emptive use of analgesics to reduce wind-up or residual pain (i.e. before first incision or painful event).

☐ Use of non-invasive methods to replace invasive procedures.

☐ Other

11.5 Select all of the principal investigator activities that help ensure awareness of current animal models and techniques, as well as awareness of possible alternatives, with respect to the animal model(s) proposed in this protocol:

☑ Attends scientific meetings

☑ Is a reviewer for scientific journal articles

☐ Is a member of an NIH study section or equivalent

☑ Consults a recognized expert in the proposed animal model(s)

* Provide the name, title, affiliation of the expert and the date of consultation
18 June, 2020: Veterinary Consult, Temple IACUC
1 July, 2020: Prof. Michel Lemay, Temple University

11.6 Literature Search for Alternatives

This section is required for protocols involving all USDA covered species and all non-USDA covered species in pain category D and/or E studies.

* Is a literature search for alternatives required as a part of this protocol? ☑ Yes ☐ No

The Animal Welfare Act (AWA) regulations require principal investigators to consider alternatives to procedures that may cause more than momentary or slight pain or distress to the animals and provide a written narrative of the methods used and sources consulted to determine the availability of alternatives, including refinements, reductions, and replacements. Alternatives to the use of animals and for procedures that may cause more than momentary pain and/or distress must be considered. Please see the USDA Policy 12 "Consideration of Alternative to Painful/Distressful Procedures" for more information.

The literature search for alternatives should include the following:

1. Names of Databases Searched – due to the variation in subject coverage and sources used, one database is seldom adequate; therefore, please search a minimum of 2 databases
2. Date the search was performed
3. Time period covered by the search (please list as month/year – month/year format)
4. Keywords searched (including scientifically relevant terminology)

To request that a librarian perform the literature search for protocols involving USDA species, see the Searching for Animal Alternatives research guide.

11.6.1 Table for Literature Search for Alternatives

Search Date	Time Period Searched	Keywords Searched	Database	If Other, please type the database name
17-May-2023	1966-present	spinal cord injury recovery; afferent stimulation; epidural electrical stimulation; rat locomotion; rat gait; DREADDs spinal cord injury. DREADDs DRG. DREADDs afferent. rat treadmill; lysolecithin; fur dyeing	Other	Google Scholar
17-May-2023	1966-present	spinal cord injury recovery; afferent stimulation; epidural electrical stimulation; rat locomotion; rat gait; DREADDs spinal cord injury. DREADDs DRG. DREADDs afferent. rat treadmill; lysolecithin; fur dyeing	Medline	

11.6.2 Were any alternatives/alternative methods/refinements identified in the search that could be used and still accomplish the goals of your protocol (mark all that apply)?

☐ Yes, alternatives were identified and have been included in the protocol.

☐ Yes, alternatives were identified, but cannot be used in this study

☑ Literature has been fully researched and no alternatives have been identified

12. SAFETY AND HAZARDS IN ANIMALS

Note: An IBC and/or EHRS risk assessment is required for all materials listed in this section.

When completing this page:
- Potentially hazardous agents that are listed in the protocol must be included in this section.
- Full names of all DNA plasmids directly administrated into animals or used for production of the viral particles administered into animals must be provided.

EHRS: Phone (215) 707-2520 / Email: ehrs@temple.edu
IBC: Phone (215) 707-9741 / Email: ibc@temple.edu

12.1 Biological Agents

(Examples include viruses, bacteria, yeast, fungi, parasitic agents, rickettsia, prions, toxins that are derived from a biological organism or human or primate cells, such as human cancer or epithelial cells.)

* Does this study involve the use of biological agents? ☑ Yes ☐ No

* List all biological agents (including biological toxins) used in the project and explain their use:
- This includes the administration of infectious or toxic agents, fungi, bacteria, viruses (natural or recombinant vectors), prions, select biological agents (SBA), human or primate cells (specify if genetically modified), human or primate subcellular components, or biological-containing nanoparticles to animals.
- Specify the agent(s) to be administered (including names of the DNA constructs, if applicable. Specify if viral-mediated gene delivery method will be utilized)
- Specify the method of administration, location of administration, administration frequency and dosage.
- State if the administered agent or metabolic products constitute a potential hazard to anyone handling the treated animal or bedding (excrement).

Viruses:

AAV2/hSyn/GFP: subcloned into an AAV2 vector cassette under the control of the human synapsin 1 promoter driving GFP expression (1.0 × 1012 viral particles/ µl). Viral mediated gene delivery is utilized. These are administered by direct micro-injection into the nervous system, once. This does not constitute a potential hazard to anyone handling or treating the animal or bedding (excrement).

AAV2/hSyn/mCherry: subcloned into an AAV2 vector cassette under the control of the human synapsin 1 promoter driving mCherry expression (1.0 × 1012 viral particles/ µl). Viral mediated gene delivery is utilized. These are administered by direct micro-injection into the nervous system, once. This does not constitute a potential hazard to anyone handling or treating the animal or bedding (excrement).

AAV2/hSyn/hM3Dq/mCherry: subcloned into an AAV2 vector cassette under the control of the human synapsin 1 promoter driving hM3Dq DREADD::mCherry expression (1.0 × 1012 viral particles/ µl). (Addgene plasmid 50474). Viral mediated gene delivery is utilized. These are administered by direct micro-injection into the nervous system, once. This does not constitute a potential hazard to anyone handling or treating the animal or bedding (excrement).

AAV2/hSyn/hM4Di/mCherry: subcloned into an AAV2 vector cassette under the control of the human synapsin 1 promoter driving hM4Dq DREADD::mCherry expression (1.0 × 1012 viral particles/ µl). (Addgene plasmid 50475). Viral mediated gene delivery is utilized. These are administered by direct micro-injection into the nervous system, once. This does not constitute a potential hazard to anyone handling or treating the animal or bedding (excrement).

AAV2/DIO/hM3Dq/mCherry: subcloned into an AAV2 vector cassette under the control of a double-floxed inverse ORF construct driving hM3Dq DREADD::mCherry expression (1.0 × 1012 viral particles/ µl). Viral mediated gene delivery is utilized. These are administered by direct micro-injection into the nervous system, once. This does not constitute a potential hazard to anyone handling or treating the animal or bedding (excrement).

AAV2/DIO/hM4Di/mCherry: subcloned into an AAV2 vector cassette under the control of a double-floxed inverse ORF construct driving hM4Dq DREADD::mCherry expression (1.0 × 1012 viral particles/ µl). Viral mediated gene delivery is utilized. These are administered by direct micro-injection into the nervous system, once. This does not constitute a potential hazard to anyone handling or treating the animal or bedding (excrement).

These are used either to label neurons or to express the chemogenetic DREADDs tools in neurons as well as to label them.

* Have any of these biological agents been passaged through other animals? ☐ Yes ☑ No

12.2 Radiation and Radioactive Materials

(Examples include radiograph, fluoroscopy, CT, electron microscope, irradiators, MRI, ultrasound, lasers, microwaves, isotopes, etc.)

* Does this study involve the use of ionizing radiation or radioactive materials? (Examples include radiograph, fluoroscopy, CT, electron microscope, irradiators, isotopes, etc.) ☐ Yes ☑ No

* Does this study involve the use of non-ionizing radiation? (Examples: microwaves, ultrasound, MRI, lasers, infrared radiation, etc) ☑ Yes ☐ No

* List all non-ionizing radiological procedures and isotopes in this project:
Low levels of infra-red light are used (simple IR home security type LED panels) are used too better see the tattoo under the skin in the IR kinematics project.

12.3 Recombinant DNA and Synthetic Nucleic Acids

(Examples of typical experiments that use these, such as viral gene delivery experiments)

* Does this study involve the use of recombinant DNA or synthetic nucleic acids? ☑ Yes ☐ No

* List all DNA plasmids and oligonucleotides used in the project:
Viruses:

AAV2/hSyn/GFP: subcloned into an AAV2 vector cassette under the control of the human synapsin 1 promoter driving GFP expression (1.0 × 1012 viral particles/ µl).

AAV2/hSyn/mCherry: subcloned into an AAV2 vector cassette under the control of the human synapsin 1 promoter driving mCherry expression (1.0 × 1012 viral particles/ µl).

AAV2/hSyn/hM3Dq/mCherry: subcloned into an AAV2 vector cassette under the control of the human synapsin 1 promoter driving hM3Dq DREADD::mCherry expression (1.0 × 1012 viral particles/ µl). (Addgene plasmid 50474).

AAV2/hSyn/hM4Di/mCherry: subcloned into an AAV2 vector cassette under the control of the human synapsin 1 promoter driving hM4Dq DREADD::mCherry expression (1.0 × 1012 viral particles/ µl). (Addgene plasmid 50475)

AAV2/DIO/hM3Dq/mCherry: subcloned into an AAV2 vector cassette under the control of a double-floxed inverse ORF construct driving hM3Dq DREADD::mCherry expression (1.0 × 1012 viral particles/ µl).

AAV2/DIO/hM4Di/mCherry: subcloned into an AAV2 vector cassette under the control of a double-floxed inverse ORF construct driving hM4Dq DREADD::mCherry expression (1.0 × 1012 viral particles/ µl).

12.4 Hazardous Chemicals, Drugs or Toxins

(Examples include tamoxifen, paraformaldehyde, etc (for reference see https://www.cdc.gov/niosh/docs/2016-161/pdfs/2016-161.pdf)

* Does this study involve the use of hazardous chemicals, drugs or toxins? ☑ Yes ☐ No

* List all hazardous chemicals and hazardous drugs used in the project:
  - This includes the administration of experimental compounds, hazardous drugs (as defined by NIOSH, https://www.cdc.gov/niosh/docs/2016-161/pdfs/2016-161.pdf), hazardous chemicals (refer to the vendor and product specific SDS, Section 2) or chemical-based nanoparticles to animals.
  - Specify the agent(s) to be administered.
  - Specify the method of administration, location of administration, administration frequency and dosage.
  - State if the administered agent or metabolic products constitute a potential hazard to anyone handling the treated animal or bedding (excrement).

Perfusion of animals at the end of the experiment uses formaldehyde. Administered once intracardially at the end point. Perfusion is carried out under full PPE in a fume hood.

Infectious agents: microinjection of adeno associated virus through standard methods represents a hazard of accidental injection of the construct which will be minimized through proper training, protocols, and procedures in microinjection in rodents.

12.5 Controlled Substances

(Examples include ketamine, sodium pentobarbital, buprenorphine, etc)

* Does this study involve the use of DEA controlled substances? ☑ Yes ☐ No

* Identify the name of the controlled substance(s) and the name of the DEA registrant.
Note:
a.) It is required that the DEA Registrant be listed as support personnel on the study.
b.) The DEA requires that any personnel administrating the controlled substance must be listed as an authorized user on the DEA Registrants License.
The Drug Enforcement Agency (DEA) registrant is Andrew J. Spence
Ketamine
Buprenorphine
Fatal Plus (pentobarbital sodium)

12.6 Use of Complete Freund's Adjuvant (CFA)

* Does this study involve the use of Complete Freund's Adjuvant (CFA)? ☐ Yes ☑ No

12,7 IBC/EHRS Documentation

For assistance, please contact the IBC Coordinator and/or EHRS.

* If applicable, have the appropriate information/documents been submitted to the IBC and/or EHRS? ☑ Yes ☐ No

* Is there an approved IBC protocol that covers this study? Yes

IBC Approvals
If there are multiple IBC protocols then please click the 'Add New' link to list them all.

* IBC Approval Number:	* Date of Approval:
874-1 (outdated?)	21-Jun-2017
10944	26-Jun-2023

13. REFERENCES

When completing this page:
- All references that appear in the protocol need to be fully described in this section.

Include ONLY those publications referenced in the previous sections of this Animal Protocol.

Bauman, J. M. and Y.-H. Chang (2009). "High-speed X-ray video demonstrates significant skin movement errors with standard optical kinematics during rat locomotion." Journal of Neuroscience Methods **186**(1): 18-24.

Basso, D., M. Beattie and J. Bresnahan (1995). "A sensitive and reliable locomotor rating scale for open field testing in rats." Journal of Neurotrauma **12**: 1 - 21.

Bhowmik, M., V. Boyce, G. McConnell, D. Joyce, W. Grill and M. Lemay (2003). Experimental and biomechanical model force fields produced by intraspinal microstimulation of the cat lumbar spinal cord. 2003 IEEE 29th Annual Proceedings of Bioengineering Conference, IEEE.

Chaplan, S. R., F. Bach, J. Pogrel, J. Chung and T. Yaksh (1994). "Quantitative assessment of tactile allodynia in the rat paw." Journal of neuroscience methods **53**(1): 55-63.

Côté, M.-P., M. Murray and M. A. Lemay (2016). "Rehabilitation Strategies after Spinal Cord Injury: Inquiry into the Mechanisms of Success and Failure." Journal of Neurotrauma **34**(10): 1841-1857.

Dixon, W. J. (1980). "Efficient analysis of experimental observations." Annual review of pharmacology and toxicology **20**(1): 441-462.

Dombeck, D. A. and M. B. Reiser (2012). "Real neuroscience in virtual worlds." Current Opinion in Neurobiology **22**(1): 3-10.

Holzgrefe, H., J. Everitt and E. Wright (1987). "Alpha-chloralose as a canine anesthetic." Laboratory animal science **37**(5): 587-595.

Kelamangalath, L., X. Tang, K. Bezik, N. Sterling, Y.-J. Son and G. M. Smith (2015). "Neurotrophin selectivity in organizing topographic regeneration of nociceptive afferents." Experimental neurology **271**: 262-278.

MacLaren, D. A., R. W. Browne, J. K. Shaw, S. Krishnan Radhakrishnan, P. Khare, R. A. España and S. D. Clark (2016). "Clozapine-n-oxide administration produces behavioral effects in Long-Evans rats - implications for designing DREADD experiments." eneuro.

Nectow, A. R. and E. J. Nestler (2020). "Viral tools for neuroscience." Nature Reviews Neuroscience **21**(12): 669-681.

Ollivier-Lanvin, K., B. E. Keeler, R. Siegfried, J. D. Houlé and M. A. Lemay (2010). "Proprioceptive neuropathy affects normalization of the H-reflex by exercise after spinal cord injury." Experimental neurology **221**(1): 198-205.

Patel, T. D., I. Kramer, J. Kucera, V. Niederkofler, T. M. Jessell, S. Arber and W. D. Snider (2003). "Peripheral NT3 signaling is required for ETS protein expression and central patterning of proprioceptive sensory afferents." Neuron **38**(3): 403-416.

Pearson, K., O. Ekeberg and A. Buschges (2006). "Assessing sensory function in locomotor systems using neuro-mechanical simulations." Trends in Neurosciences **29**(11): 625-631.

Silverman, J. and W. W. Muir III (1993). "Special topic overview: a review of laboratory animal anesthesia with chloral hydrate and chloralose." Lab Anim Sci **43**: 210-216.

Spence, A. J., G. Nicholson-Thomas and R. Lampe (2013). "Closing the loop in legged neuromechanics: An open-source computer vision controlled treadmill." Journal of Neuroscience Methods **215**(2): 164-169.

St-Georges, D., C. Matthews and S. Nattel (1997). "Continuous maintenance infusion technique for stable anesthesia in the dog, using α-chloralose." Journal of the American Association for Laboratory Animal Science **36**(2): 62-65.

Takeoka, A., I. Vollenweider, G. Courtine and S. Arber (2014). "Muscle spindle feedback directs locomotor recovery and circuit reorganization after spinal cord injury." Cell **159**(7): 1626-1639.

Tytell, E. D., P. Holmes and A. H. Cohen (2011). "Spikes alone do not behavior make: why neuroscience needs biomechanics." Current opinion in neurobiology **21**(5): 816-822.

Wilshin, S., M. A. Reeve, G. C. Haynes, S. Revzen, D. E. Koditschek and A. J. Spence (2017). "Longitudinal quasi-static stability predicts changes in dog gait on rough terrain." The Journal of Experimental Biology **220**(10): 1864-1874.

Wright, D. E., L. Zhou, J. Kucera and W. D. Snider (1997). "Introduction of a neurotrophin-3 transgene into muscle selectively rescues proprioceptive neurons in mice lacking endogenous neurotrophin-3." Neuron **19**(3): 503-517.

14. PI ASSURANCES

As the Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

☑ * I understand it is the responsibility of the Principal Investigator to ensure the safe and ethical conduct of all research conducted under this protocol, and to assure that all research is carried out following to abide by PHS Policy, USDA regulations, Temple University policies for the care and use of animals, the provisions of the current ILAR Guide to the Care and Use of Laboratory Animals, and all other federal, state, and local laws and regulations governing the use of animals in research.

☑ * I assure that discomfort and injury to animals will be limited to that which is unavoidable in the conduct of scientifically valuable research and that analgesic, anesthetic and/or tranquilizing drugs will be used where indicated and appropriate to minimize pain and/or distress to animals. I understand that any unanticipated pain or distress must be reported to a ULAR veterinarian.

☑ * I understand that emergency veterinary care will be administered to animals showing evidence of pain or illness, in addition to routine veterinary care as prescribed for individual species. I understand that it is my responsibility to provide current and updated emergency contact information for personnel who will be contacted in an animal emergency.

☑ * I assure that I have consulted a ULAR veterinarian in the preparation of this proposal, if it includes procedures that could cause unrelieved pain and distress to a vertebrate animal and/or USDA covered species.

☑ * I assure that the animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

☑ * I certify that all experiments involving live animals will be performed under my supervision or that of another qualified biomedical scientist listed on this protocol.

☑ * I certify that all personnel having direct animal contact, including myself, who perform animal procedures, manipulations and/or observations described in the protocol are technically competent and have been properly trained to ensure that no unnecessary pain and/or distress will be caused as a result of the procedures and/or manipulations. I also certify that all personnel listed on this protocol have completed all training/medical clearance requirements determined by the IACUC and ULAR.

☑ * I understand that the use of hazardous agents in animals may only be initiated after approval from IBC and/or EHRS.

☑ * I understand that should I use the project described in this application as a basis for a proposal for funding (either extramural or intramural), it is my responsibility to ensure that the description of animal use in such funding proposals are identical in principle to that contained in this application.

☑ * I understand that I must submit an amendment for any proposed changes to this protocol and wait for IACUC approval before beginning the work.

☑ * I certify that I will maintain complete, up-to-date and accessible records of procedures on animals as required by policy and regulation.

☑ * I declare that the information provided in this protocol is accurate to the best of my knowledge.

Appendix 1

EForm Name: Animal Protocol form
Page: 1. Application Information
Section:
Question: 1.2.2 Provide Progress Report:
File Name: Progress Report

Progress Report for 3-year Rewrite of ACUP 5003

We have made reasonable progress on the R01 associated with ACUP 5003. We published a solid paper on the work in Summer 2022 in a Special Topic issue of Frontiers in Molecular Neuroscience, and we developed a novel IR imaging method that could have a big impact on rodent kinematics, currently published as a preprint. We have large amounts of tissue and kinematic data to be processed and imaged for our next publication, and are looking forward to pushing that to a higher impact journal.

Appendix 2

EForm Name: Animal Protocol form
Page: 1. Application Information
Section: 1.3.1 External Sources
Question: Attach Vertebrate Animal Section (VAS)
File Name: nih_r01a1_nov5_2019_vertanimals_R2.docx

VERTEBRATE ANIMALS

All surgical interventions, pre- and post-surgical care will be provided in accordance with the PHS Policy on Humane Care and Use of laboratory Animals, *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council), and the guidelines provided by the Animal Care and Use Committee of Temple University School of Medicine. The protocol used for this project (IACUC#: 4675) was approved on August 6th, 2019.

1. **Provide a detailed description of the proposed use of the animals in the work outlined in the Research Design and Methods section. Identify the species, strains, ages, sex and numbers of animals to be used in the proposed work.**

ANIMALS NEEDED

YEAR	TYPE OF ANIMAL	SPECIFIC AIM 1	SPECIFIC AIM 2	TOTAL #
Year 1	Rats	42 (3 subsets of 14)		42
Year 2	Rats	56 (4 subsets)		56
Year 3	Rats	43 (2 subsets+15 MEA)		43
Year 4	Rats		78	78
Year 5	Rats		75	75
				294

Approximately 294 rats of both sexes will be housed in pairs, given food and water ad libitum and housed under a 12-hour light cycle. Equal numbers of male and female rats will be used. Specific Aim 1 will use 141 Long-Evans rats. We choose Long-Evans rats as the transgenic rats for Aim 2 are on a Long-Evans background, thus results will be comparable between aims. Rats between 250-300 grams will be housed in pairs, given food and water ad libitum and housed under a 12 hour light cycle. A summary of animal use for each proposed aim/experiment is illustrated in the table above. All animals will be housed in a licensed, fully staffed animal care facility in the University Laboratory for Animal Research (ULAR) in the Lewis Katz School of Medicine at Temple University, or in the BioLife Sciences ULAR facility on Temple's main campus.

Specific Aim 2 will require 54 transgenic rats for experiments (Tg-Pvalb/iCre: Rat Resource & Research Center Strain #00773; LE background) over two years. To provide these experimental rats we will establish a colony and require 153 rats total. 78 are transgenic rats (Pvalb-iCre purchased from RRRC), and 75 are Long-Evans (LE). These rats, and the rats in Aim 1, will undergo two survival surgeries, DRG injection and spinal cord contusion, and then have treadmill training, pain assays, and locomotion behavioral assays.

The animal numbers calculation for Specific Aim 2 is as follows. We require 27 experimental rats/year for 2 years. The breeding protocol for the Parvalbumin-CreR rats recommends breeding hemizygote males with wildtype females, and utilizing hemizygote offspring, because the mutant females preferentially consume mutant offspring. Thus we need to use wildtype females. We will therefore purchase 3 mutant hemizygote males (on LE background) and 3 Long Evans wildtype females for breeding. If this grant is awarded, and we are maintaining our current colongy of these animals at the time of starting Aim 2, then we will not need to purchase these founders. At 8 pups per litter average, 3 litters/pair and 3 breeding pairs we have 9 litters total yielding 72 animals over a one year period sufficing for our study requirements (72 animals weaned at 50% hemizygotic yields 36 animals; study requires 27 per year, for two years). The male breeders will be replaced once for a total of six; wildtype females will be used from the breeding colony for mates when replaced. So we require a total of 78 Pvalb-iCre (72*2/2 = 72 bred + 6 transgenic male breeders) and 75 (72 bred + 3 initially purchased) Long Evans rats for a total of 153 rats. Year 1 will use 72 bred plus 3 purchased Tg breeders and 3 purchased WT breeder females, for total of 78 animals. Year 2 will use 72 bred plus 3 replacement purchased transgenic male breeders, for total 75 animals.

Description of Procedures:

Spinal cord injections: Animals will be anesthetized with a ketamine (67 mg/kg)/xylazine (6.7 mg/kg, i.p.) mixture. Under this anesthesia, adult rats will receive injections into the dorsal root ganglia (DRG) followed by a three-week recovery period, and then a spinal cord contusion. To inject into DRGs, a laminectomy will be performed on both sides of the spinal column caudal to L2 – L5, and posterior articular processes removed from L2, L3, L4, and L5 to expose the DRGs. The rat will be attached to a spinal harness to partially suspend the rat, preventing movement of the spinal column during injection. Using a glass micro-needle and micromanipulator, we will inject 1 – 2 μl of AAV directly into the L2, L3, L4, and L5 DRG over a period of 10 minutes.

Spinal cord lesions: For spinal cord contusions, a complete laminectomy will be performed at T7/T8 vertebrae to expose dorsal spinal cord segment T10. 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 choose 250 kilodynes as it is considered a moderate contusion that preserves much of the ventral white matter. The spinal musculature will be closed by suturing and the skin stapled closed. Neosporin ointment will be placed over wound to help prevent any infection. Following all procedures, animals receive analgesics (Rimadyl 2 mg/tablet; Bio Serv, Flemington, NJ) and antibiotics (cefazolin 10 mg/mL, s.c.). Saline (0.9% NaCl) is administered (s.c.) to prevent dehydration post-surgery. After recovery, animals will be monitored for general health on a daily basis within the first week after surgery. If pain or distress are observed (assessed by our IACUC approved pain scoring chart), the animal will be euthanized promptly. After the experiment is complete, the animal will be euthanized. Euthanized rats will be sacrificed by cardiac perfusion with aldehyde fixatives 8 – 10 weeks after surgery. For the 15 animals used for terminal multielectrode array (MEA) recording experiments, DRG injection will be carried out as above, and after a minimum three-week recovery period, in a terminal experiment these DRG will be exposed using a similar protocol, and a 16-channel multi-electrode array (MEA, 4x4 3mm 100-125-177, Neuronexus Technologies, Ann Arbor, MI) with 4 sites on each of 4 shanks, producing a rectangular recording region with a height of 400 um and width of 500 um, will be inserted into the DRG with the sites covering the 600-200 um depth range.

2. Justify the use of animals the choice of species and the numbers to be used. If animals are in short supply, costly, or to be used in large numbers, provide an additional rationale for their selection and numbers.

Experiments in this application will examine the questions concerning how spinal cord circuitry is influenced by afferent stimulation to produce enhanced recovery after spinal cord injury. We seek to determine whether chemogenetic afferent activation can produce similar enhancement to existing work using electrical stimulation, because it will allow us to determine which afferents are responsible for recovery and to trace the mechanisms responsible inside the spinal cord. In the longer term, it is also possible that our gene-therapuetic, drug actuated, chemogenetic approach could be used in concert with or in place of electrical epidural stimulation, which would constitute a genetically targeted, non-invasive method of afferent stimulation. However, the goal of this study is purely basic research to understanding plasticity supporting enhanced recovery. In the present studies, we will carry out axonal tracing to determine which neurons have been activated locally, and their connectivity to some surpraspinal and propriospinal pathways, that may support better functional outcomes. The results from these experiments will be instrumental in understanding the mechanisms by which stimulation of afferents can improve recovery from SCI, and if successful, open up new paradigms for basic inquiry and perhaps eventually, treatment (chronic, genetically targeted afferent excitation and/or inhibition).

Rats are the animal of choice in this study for the following reasons: 1) rats are specifically bred for research purposes, 2) spinal cord injuries in rats are well established by others and us (and the Smith Lab, especially), 3) the database relevant to SCI that the Smith Lab has established in the past is based mainly on rats, 4) the selection of rats is in keeping with its use as the experimental spinal cord injury model used in other major spinal cord research centers in this country, and 5) rats are cost effective in their use. There is considerable basic information concerning peripheral nerve and spinal cord injury, particularly the spinal cord injury method used for these experiments.

Animal numbers were determined by power analysis extrapolated using previous data sets or published literature. For axonal 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 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 an repeated measures ANOVA design (G*Power 3) shows that to detect a minimum 25% change (effect size based on Spence lab previous work, Spence et al., J. Neurosci Methods 2013, and Takeoka et al., Cell 2014) with 10 measurements (strides) at a correlation between strides of 0.25 will require 18 animals per group. PCA analysis was carried out using the PCA object from the Python package *scikit-learn*. The sample size calculated is slightly over-powered for the group as a whole; it should be adequate to reveal trends amongst males and females. If differences arise, we will add more males or female subjects within the study where they occur.

3. **Provide information on the veterinary care of the animals involved.**
All animals will be housed in the fully staffed facility managed according to PHS guidelines by the University Laboratory Animal Research Division, Temple University College of Medicine. This is an AAALAC-approved facility and is maintained and operated in compliance with the Guide for the Care and Use of Laboratory Animals. All surgical procedures will be carried out in a BL-2+ surgical suite dedicated for recombinant viral experimental procedures in a pathogen free environment with sterile instrumentation. All animals will be housed in a BL-2 rat housing room.

4. **Describe the procedures for ensuring that discomfort, distress, pain, and injury will be limited to that which is unavoidable in the conduct of scientifically sound research. Describe the use of analgesic, anesthetic, and tranquilizing drugs and/or comfortable restraining devices, where appropriate, to minimize discomfort, distress, pain and injury.**
Analgesics are used for all surgical procedures. Post-surgical buprenorphine will be administered SC at a dose of 0.02mg/kg, starting immediately upon sternal recumbency and continuing twice daily for the first 2 days. Laboratory personnel will monitor pain and distress (IACUC approved pain scoring chart) in the animals, and if the animals exhibit signs of pain or distress after the second day, buprenorphine will be administered. If pain remains unabated, a veterinary consult will be obtained to determine the best course of action, either medical management or euthanasia.
Following SCI, animals will experience pain and distress, although with our lesion paradigm this should be minimal. We will objectively and reproducibly evaluate animals for signs of pain and distress following a scoring system suggested by the Veterinary Reviewer of this application:

Clinical signs	0	1	2
Body condition score (BCS)	BCS3	BCS2	BCS1
Dehydration	normal	Slight (1 sec skin tenting)	Moderate to severe (2+ skin tent)
Coat condition	normal	Slightly ruffled	Moderately to severely ruffled
Eye squinted	No squint	Mild squint	Eyes mostly closed
Ability to walk	No impairment	reduced	paralyzed
porphyrin	none	mild	Moderate to severe

Score 0-2: no additional analgesia
Score 3-6: additional analgesia required
Score 7+: recommend vet consult and/or euthanasia

5. **Describe any method of euthanasia to be used and the reasons for its selection. State whether this method is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. If not, present a justification for not following the recommendations.**
At the conclusion of experiments, all animals will be euthanized using Fatal-plus or similar commercially available euthanasia solution. Secondary confirmation will be by either creating a pneumothorax or by thoracotomy. Pneumothorax will be accomplished by making an incision through the musculature immediately caudal to the sternum, cutting the lower ribs bilaterally, and then completely cutting the diaphragm. This method has been chosen as it allows us to perform transcardiac perfusion

procedures after euthanasia by Fatal-plus injection. This method is consistent with the recommendations of the Panel on Euthanasia of the American Medical Association.

Appendix 3

EForm Name: Animal Protocol form
Page: 3. Species Information
Section:
Question: 3.3.2 Attach pain scoring chart
File Name: rodent_pain_scoring_chart_general_surgical.docx

TEMPLE UNIVERSITY
Office of the Vice President for Research
Research Compliance
Student Faculty Center
3340 N. Broad Street, Suite 427
Philadelphia, PA 19140
Institutional Animal Care and Use Committee (IACUC)
Phone: (215) 707-7263
Email: iacuc@temple.edu

Rodent Pain Scoring Chart – General + Surgical Observations

The following is a method to help quantify pain and distress in rodents by using a pain scoring system recommended by the IACUC and ULAR veterinarians. If there are species or model-specific signs of pain and/or distress, the PI must modify the chart to incorporate these into the monitoring plan. The form should be attached to the protocol for IACUC review and approval. The frequency of monitoring must be detailed in the protocol based on the animal model and the procedures to be performed. An example of a Pain and Distress monitoring chart can be found below the scoring system. This chart can be used as is or modified as needed and should also be attached to the protocol at the time of submission.

Physical Assessment		
Normal	Alert, normal posture, normal fur coat	**0**
Mild/Moderate pain	Shifting position to get comfortable; mildly ruffled fur; mild to moderate dehydration; mild diarrhea; mild to moderate change in appetite, drinking, urination/defecation; eyes partially closed (squinting)	**1**
Severe pain	Not moving or reluctant to move unless touched, lateral recumbency or severely hunched, erect or matted fur, labored breathing, severe diarrhea/dehydration, eyes closed; lack of food and water intake	**2**
Behavioral Assessment		
Normal	Moving around cage, exploratory behavior	**0**
Mild/Moderate pain	Quieter than usual, decreased movement, decreased alertness	**1**
Severe pain	Agitated, hiding, severe lethargy, self-mutilation, reluctant to move even when stimulated	**2**
Weight Loss		
Normal	No weight loss apparent	**0**
Mild/Moderate loss	Greater than 5% but less than15%	**1**
Major loss	Greater than or equal to 15%	**2**
Surgical Incision		
Normal	Closed, no swelling or discharge, no response to palpation	**0**
Mild/Moderate state	Mild, clear discharge; slightly open but not full skin thickness; mild crusting; mild erythema (redness) and/or inflammation	**1**
Severe state	Open wound, closure not intact; severe erythema (redness), purulent discharge; vocalizing on palpation	**2**
A score of "1" in any category will require veterinary consult and/or medical intervention as described in the protocol. A score of "2" in any category will require immediate euthanasia.		

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Pain and Distress Monitoring Chart – Surgical Observations

Parameter	Score	Date/Time	Date/Time	Date/Time	Date/Time	Date/Time
Physical Assessment	0					
	1					
	2					
Behavioral Assessment	0					
	1					
	2					
Weight Loss	0					
	1					
	2					
Surgical Incision	0					
	1					
	2					
Total Score						

Date of Euthanasia: _____ Initials: _____

Appendix 4

EForm Name:	Animal Protocol form
Page:	4. Justification for Animal Numbers
Section:	
Question:	Note: If you have any supporting documents (e.g. table, spreadsheet, outline, etc.) for animal number justification, please upload here:
File Name:	animal_number_table

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
H-reflex Validation	Electrophys Control	32
Infra-red Bone Imaging	Data Collection Refinement	8

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**, and ancillary support experiments.

Appendix 5

EForm Name: Animal Protocol form
Page: 7. Experimental Design/Procedures
Section: Experimental Design
Question: Please upload any supporting documents here:
File Name: timeline_aim1and2_revised.png

For IACUC

All experimental and control groups are on the same timeline both studies

Aim 1 in WT Long Evans Rats (below):

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
7	Perfusion	

Aim 2 in Transgenic PV-iCre rats (below):

Week	Procedure	Experiments
-13 to -5	Breeding	Breeding to produce parv-cre tg rats
-5, -4	Genotyping	Genotyping and identification
-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
7	Perfusion	