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TITLE: Electrophysiological and Behavioral Evaluation of C-LTMR Plasticity Induced by Spinal Cord Injury: Transformation from Pleasure to Pain Afferents

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14. ABSTRACT

The overall objective of the study is to test the hypothesis that C-low threshold mechanoreceptors (C-LTMRs) are transformed into allodynia-encoding nociceptors after SCI. Because C-LTMRs can be selectively identified by their expression of tyrosine hydroxylase (TH), all studies were done in adult transgenic TH-Cre mice. During the year 1 funding period, we made significant progress with the proposed experiments.

Using the ex-vivo skin-nerve preparation, C-LTMR-evoked neural responses were recorded in dorsal cutaneous nerves following mechanical and optogenetic stimulation of the trunk skin. Serendipitously, we found that our TH-Cre mice also express TH in faster conducting, myelinated Aδ-LTMRs, afferents that innervate the same hair follicles as C-LTMRs. Preliminary studies further showed that receptive fields of C-LTMRs (*and Aδ-LTMRs*) are enhanced after SCI.

Simultaneous behavioral studies showed that SCI acutely increased respiratory rates (RR), and that brush stimulation induced a slight increase in RR at 21 days after SCI. However, as the studies were done in TH-Cre mice, we are unable to specifically associate these responses to C-LTMRs.

Due to the lack to specificity of our TH-Cre strain, future electrophysiological and behavioral studies will be undertaken in an inducible Cre strain (TH-Cre^{ER}). This will enable us to more selectively target C-LTMRs.

15. SUBJECT TERMS

Spinal cord injury; receptive field; tyrosine hydroxylase (TH); mechanical allodynia; pain; respiratory rate

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1. **INTRODUCTION:**

A sub-population of cutaneous afferents that innervate hairy skin and encode pleasant touch is known as C-low threshold mechanoreceptors (C-LTMRs). The overall objective of the study is to test the hypothesis that C-LTMRs are transformed into allodynia-encoding nociceptors after SCI. In rodents, C-LTMRs can be selectively identified by their expression of tyrosine hydroxylase (**TH**). Therefore, using transgenic TH-Cre mice, the proposal which comprises a series of electrophysiological, behavioral and cellular experiments will be undertaken to show that (i) C-LTMRs are both necessary and sufficient for at-level mechanical allodynia, (ii) pain is correlated with changes in sympathetic autonomic drive (assessed as an increase in respiratory rate), (iii) sympathetic activity modulates C-LTMR activity and the expression of neuropathic pain, and (iv) temporal parallels in the development truncal pain and conventional below-level neuropathic pain measures. Recently-obtained preliminary data align with our overall hypothesis. We have observed that afferent receptive field, including although not restricted to C-LTMRs are enhanced following SCI. Moreover, behavioral studies show that truncal mechanical stimulation acutely increases respiratory rates in SCI subject. Overall, the proposed experiments aim to identify a potential neural mechanism that underlies at-level neuropathic pain after SCI, which involves the transformation of touch afferents into pain-encoding afferents at dermatomal levels adjacent to the spinal cord injury.

2. **KEYWORDS:**

Pain; allodynia; low-threshold mechanoreceptors; C-LTMRs; brush stimulation; trunk; sympathetic; respiratory rate; heart rate; ex-vivo skin-nerve; TH-Cre; channelrhodopsin

3. **ACCOMPLISHMENTS:**

- **What were the major goals of the project?**

Table 1: Our original statement of work, including the projected milestones

Specific Aim 1: Investigate the stimulation forces and velocities that engage C-LTMRs:	**0-8 months**	**Garraway**	**Hochman**
Subtask 1 & 2: Optogenetic and mechanical characterization of C-LTMRs using the Skin-Nerve Electrophysiology	0-3		Hochman Watkins
Subtask 3 & 4: Electrophysiological recording after SCI and pharmacology	4-8	Garraway	Watkins
Other: Construction and optimization of programmable brush stimulator		Goolsby	
Milestone(s) Achieved: (i) Construction on mechanical brush and optimization of the preparation (2) Establish the electrophysiological characterization and parameters that engage C-LTMRs in naïve and SCI mice.			
Specific Aim 2: Investigate the behavioral effect of C-LTMR activation in intact and SCI mice:	**9-34 months**	**Garraway**	**Hochman**
Subtask 2.1, 2&4: Mechanical and optogenetic stimulation of the trunk skin in naïve and SCI mice Behavioral tests: At level and below level pain tests	9-24	Grad Stud Martin	Watkins
Subtask 2:3 Behavioral effects of decreased sympathetic activity with β-blocker, propranolol	18-34	Grad Stud Martin	Watkins
Other: Maintenance of animal colony and post-surgical care of mice		Martin	Sawchuk
Milestone(s) Achieved: Establish the specific effects C-LTMRs have on pain and responses after SCI, the relationship between changes in HR and RR on the expression of pain and the behavioral effect of decreased sympathetic activity.			
Specific Aim 3: Assess whether activation of C-LTMRs result in central sensitization and increase expression of pain genes:	**18-36 months**	**Garraway**	**Hochman**
Subtask: RNA, protein extraction and cellular assays	18-36	Grad Stud Martin	Sawchuk

Other: Maintenance of animal colony and post-surgical care of mice		Grad Stud Martin	Sawchuk

Milestone(s) Achieved:
Show the effect of C-LTMR plasticity after SCI has on the expression of nociceptive genes in the spinal cord.
Data presentation

- **What was accomplished under these goals?**

Findings related to SA1: Investigate the stimulation forces and velocities that engage C-LTMRs:

We have successfully developed the ex-vivo skin-nerve preparation for electrophysiological characterization of C-LTMRs in response to optogenetic and mechanical stimulation (**Figure 1**). Our first set of studies were undertaken in TH::CHR-YFP [TH-Cre transgenic mice (JAX#008601) mice crossed with the Ai32D-ChR2-YFP mice to express the light-gated cation channel channelrhodopsin (ChR2; JAX #012569) in TH expressing primary afferents]. We recorded neural responses in dorsal cutaneous nerves (DCN), while the trunk skin was mechanically and optogenetically stimulated. Action potentials were reliably recorded in the DCN after stimulating the same area of the receptive field with mechanical brush sweeps at 3 cm/s and blue light pulse. However, although the objective of the study was to identify TH-expressing C-LTMRs, we identified responses that were mediated by both myelinated and unmyelinated afferents. We found that due to early developmental expression of TH in other primary afferents, the strain of TH-Cre mice used here also had ectopic expression in a population of myelinated afferents which innervate the same hair follicles as C-LTMRs. This second population appears to be Aδ-LTMRs, which are small, myelinated touch encoding fibers. Therefore, we subsequently switched to an inducible strain of TH Cre (TH-Cre^{ER}) mice which can be used to selectively identify C-LTMRs. We are in the process of establishing colonies of TH-Cre^{ER} mice, and crosses with CHR2-YFP. Despite this potential setback, so far, our preliminary findings have illustrated the overall success of the ex-vivo skin-nerve approach (**Figure 1**).

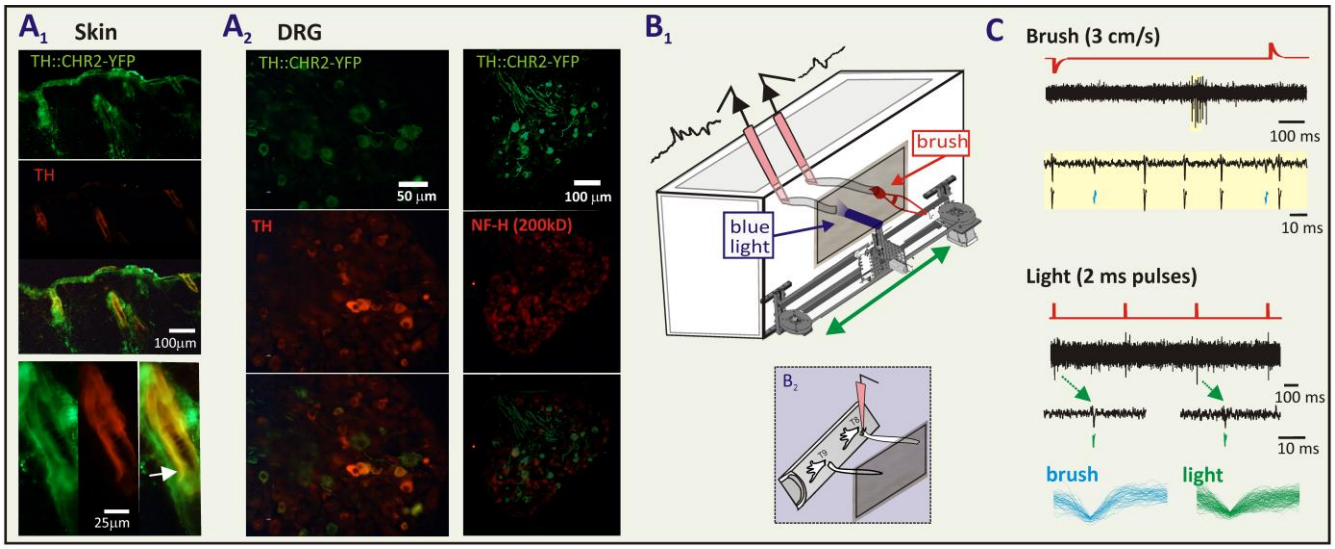

Figure 1. Development of a skin-nerve preparation to study recruitment and modulation of C-LTMRs. (**A$_1$**) In hairy skin associated with innervation of thoracic dorsal cutaneous nerves, the transgenic strain of TH::CHR2-YFP used show expression of CHR2-YFP in hair follicles that include axonal profiles with overlapping and non-overlapping expression with TH. (**A$_2$**) in DRG, CHR2-YFP cell bodies include smaller-diameter neurons that also express TH and large diameter neurons that are TH negative that co-express neurofilament-H (NF-H): known to be preferentially expressed in myelinated neurons (Li et al 2011). **(B$_1$)** Skin-nerve preparation with suction electrodes attached to segmentally-innervating cutaneous nerves. Preparation has robotic arm that sweeps optical or brush stimuli across afferent receptive fields at programmable rates. [**Bottom inset B$_2$**] Dorsal root attached preparation. Dorsal roots are cut near spinal entry zone and suction electrodes are applied to thin filaments to minimize number of recorded afferents (spinal cord is shown for reference but not present during experimentation). **(C)** Comparison of recordings from a dorsal cutaneous nerve in response to brushing of the skin **(top)** and blue light pulses **(middle)** applied to the same area of the receptive field. Red traces at top indicate when stimuli are applied. Spike sorted events identify an optogenetically recruited neuron (green) similar to those of one neuron recruited by brush stimuli (blue) (**bottom**).

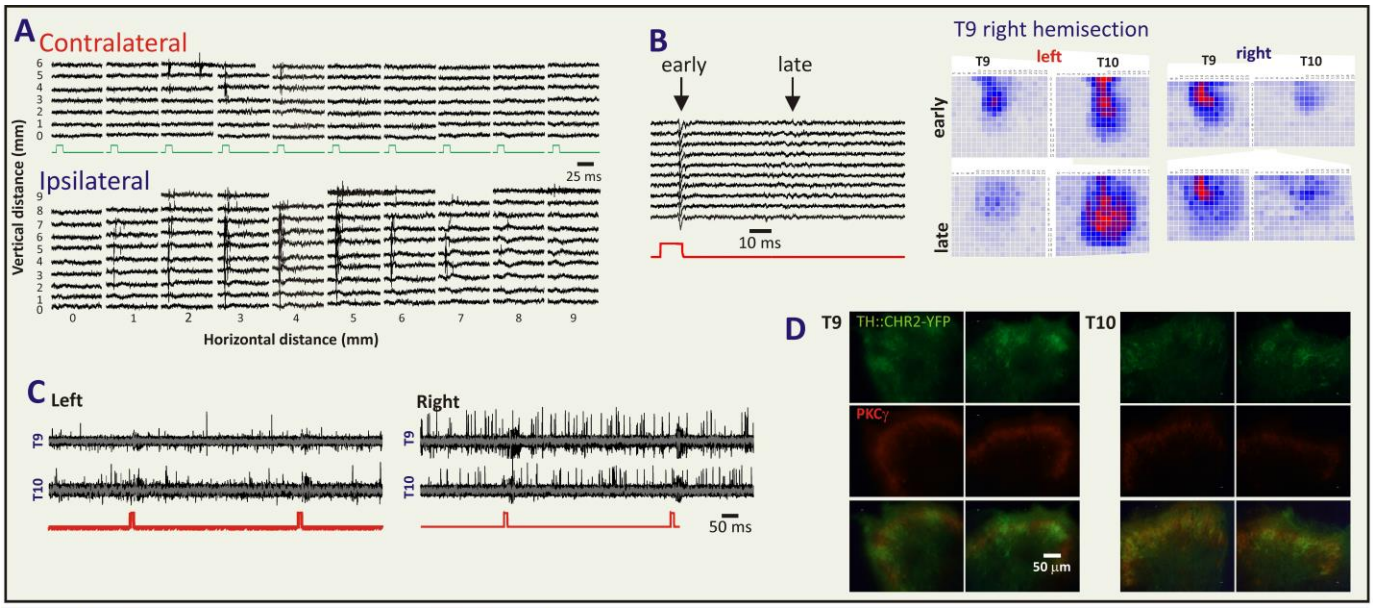

Figure 2. SCI-induced modulation of LTMRs. (A) Optogenetic receptive field mapping. Recordings from dorsal cutaneous nerves contralateral (**top**) and ipsilateral (**bottom**) to hemisect SCI, distributed horizontally and vertically to represent recording from each 1mm x 1mm point in a grid. **(B-D)** Optogenetic recruitment of Aδ and C fibers in dorsal cutaneous nerve recordings from left and right side in adult mouse two weeks after right T9 hemisection. **(B)** Myelinated and unmyelinated afferents can be identified based on differences in latency (**left**) and separated to generate independent receptive field maps (**right**; shown as heat maps) to separately evaluate receptive field plasticity of different afferent fiber types. **(C)** Apparent increase in background spontaneous activity on lesioned side. Shown are 40 superimposed episodes (top event is in gray)from simultaneous recordings from T9 and T10 dorsal cutaneous nerves in isolated skin-nerves recordings either from uninjured (left) or hemisected cord (right). Recordings preferentially at a specific skin site with receptive field overlap such that afferent recruitment is seen in both T9 and T10 nerves. Note that the apparent increase in background activity observed in both nerves on the lesioned compared to unlesioned sides. **(D)** TH::CHR2-YFP labelled afferents terminate in laminae II-III, consistent with known termination sites of C-LTMR and Aδ-LTMR afferents. In dorsal horn, immunolabelling for PKCγ identifies lamina II interneurons as a landmark for assessment of afferent fiber terminations.

Using the original TH-Cre strain, we also undertook studies that assessed changes in the receptive field of C-LTMRs. Again, because this strain identified TH expression in both myelinated and myelinated afferents, the preliminary findings are not selective to C-LTMRs. However, as illustrated in **Figure 2A-C**, afferent receptive fields, which include C-LTMRs, are indeed expanded after SCI. Receptive field of afferents representing Aδ-LTMRs are also expanded after SCI. **Figure 2D**, shows that the central terminals of TH+ afferents project to the superficial spinal cord dorsal horn. This observation is consistent with the projection profiles of C-LTMRs.

Findings related to SA2: Investigate the behavioral effect of C-LTMR activation in intact and SCI mice:

As was previously reported, we have obtained preliminary data that align with our overall hypothesis. In TH-Cre animals, we observed an acute increase in respiratory rates 1 day after SCI and a slight increase in respiratory rates in SCI mice following brush stimulation at 21 days (**Figure 3**). Unfortunately, at this stage, the results cannot be attributed to C-LTMR plasticity, because ❶ as stated above, the TH-Cre strain of mice used in these studies appear to also express TH in myelinated Aδ-LTMRs afferents. ❷ Further, the study only assessed truncal mechanical stimulation, which

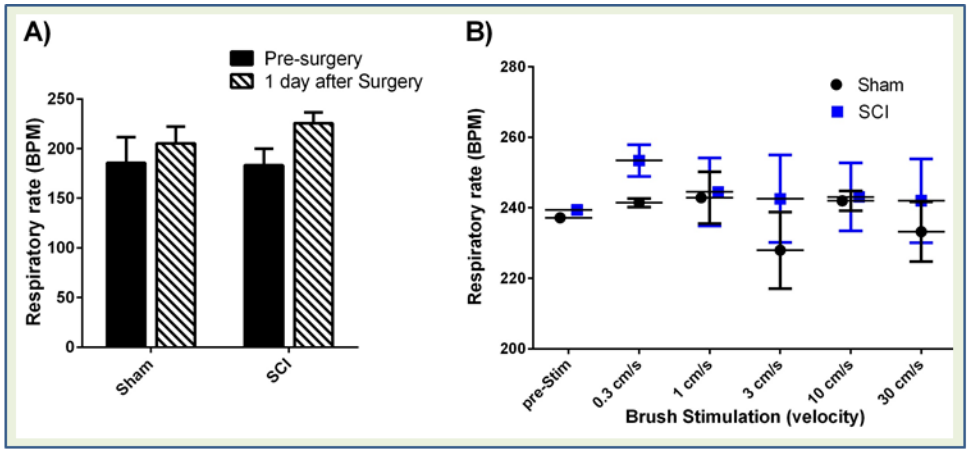

Figure 3. Respiratory rates are acutely increased by SCI and by brush stimulation. A) Respiratory rates are increased just 1 day after SCI at T10. B) At 21 days after injury, a time-point when chronic pain is typically evident, Brush stimulation to the trunk causes an increase in respiratory rates. The greatest effect is seen when stimulation is administered at a velocity of 0.3 cm/sec.

is expected to engage afferents other than C-LTMRs. ❸ There were no clear frequency-specific responses to mechanical stimulation, although the greatest effect was seen with stimulation administered at 0.3cm/sec. However, it should be noted that brush is routinely administered in a sequentially increasing stimulation velocities, starting with 0.3 cm/sec. Thus, the increase in respiratory rates seen during stimulation at 0.3cm/sec may be a physiological response to the initial contact with brush and not a specific stimulation velocity, *per se*. We had already added two additional cohorts of mice to the data set and will continue to analyze the newly acquired data. Meanwhile, we are establishing TH-Cre^{ER} crosses with CHR2-YFP to more selectively assess the behavioral responses elicited by C-LTMRs' activation.

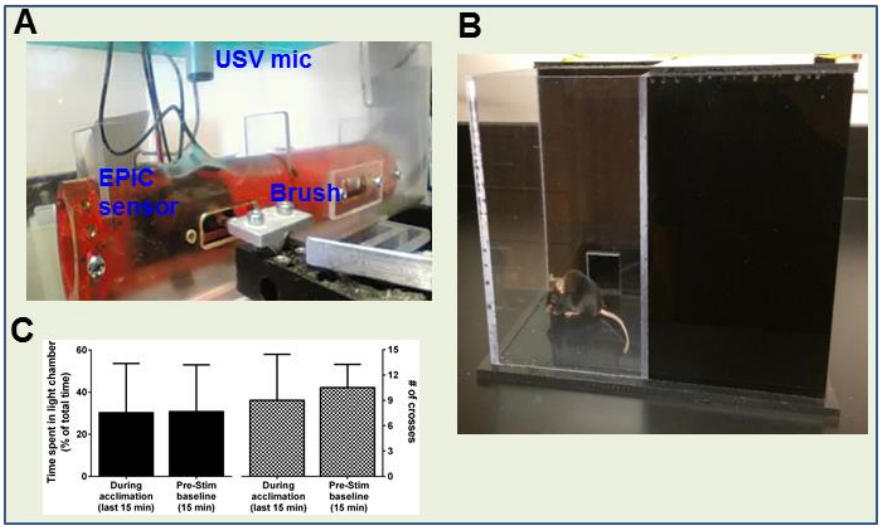

Figure 4. Modified PEAP devices for moderately-restrained (A) and for freely-behaving (B) mice. **(A)** Screenshot demonstrates the behavioral apparatus currently used. It shows placement of EPIC sensors for monitoring respiratory and heart rates, the microphone for USV recording and use of computerized robotic arm for mechanical brush and optical stimulation. Stimuli are swept through a window across the side of a semi-restrained mouse at different frequencies, to recruit unique populations of sensory afferents. **(B)** Shows a newly constructed dual chamber Place Escape/Avoidance paradigm, consisting one 'dark' compartment and an otherwise identical 'light' compartment. Site preference before and during stimulation of C-LTMRs will be monitored. (*note, we show 2 pairs of dual chambers*). (C) Preliminary testing of time spent and # of crosses between light and dark chambers reveals a greater preference to the dark chamber, an expected observation. During the later stages of acclimation (last 15 minutes) and 15 minutes of pre-stimulation baseline, mice spend approximately 30% of their time in the light chamber. However, because mice would show preference to the dark chamber, we will be able to administer light and mechanical stimulation with von Frey hairs, via a small window, while they are in the dark chamber. We will then measure changes in the out-put measures (time spent in light chamber and # of crosses) during and after truncal stimulation.

Recently, we modified our behavioral apparatus. Our current recording device has enabled us to successfully assess respiratory rates before and during brush stimulation in awake, resting animals in a semi-restrained context (**Figure 4A**). However, we have not been able to also assess 'preference' or time spent in the tube during stimulation. Thus, we recently constructed a two chamber modified Place Escape/Avoidance Paradigm (mPEAP), adapted from version described previously [LaBuda CJ, Fuchs PN (2000) A behavioral test paradigm to measure the aversive quality of inflammatory and neuropathic pain in rats. *Exp. Neurol. 163(2): 490-4*] (**Figure 4B**), which will enable us to assess C-LTMR-evoked responses in freely-behaving animals. Using Light and Infrared video recordings, we will monitor side preference, as well as animals' behavior in the 'dark' chamber, prior to, during and after mechanical and optogenetic stimulation in TH::CHR2-YFP mice. Video recordings will be analyzed using TopScan analysis software (CleverSys Inc; available in our core facility) with dependent variables being percentage or duration of time spent on light/dark side of chamber and the number of crossings between sides. Recently, we undertook basic studies to monitor mice's baseline preferences in the dual chambers during a 1 hour acclimation period and a follow-up pre-stimulation baseline period (15 minutes). Our analyses of the last 15 minute of the acclimation period and the subsequent 15 minute pre-stimulation baseline period showed that mice spend nearly 70% of the time in the dark chamber, a somewhat expected finding (**Figure 4C**). Thus, we will administer light or mechanical stimulation, with calibrated von Frey hairs, via a small window while the mice are in the dark chamber. Then, we will assess changes in output measures during and after stimulation. Although we have just begun preliminary testing with the mPEAP, we are confident that this modification will allow us to more reliably examine whether stimulation that engages C-LTMRs are indeed perceived as aversive or painful after SCI in freely-behaving mice.

What opportunities for training and professional development has the project provided?

Nothing to Report

- **How were the results disseminated to communities of interest?**

  Nothing to Report

- **What do you plan to do during the next reporting period to accomplish the goals?**

We expect that our TH-Cre^{ER} related colonies will be fully established in the near future. Hence, as the technical needs are already met, we expect the electrophysiological and behavioral studies to resume. We anticipate that during the next reporting period, we will have new data supporting our hypotheses. We will be able to selectively target C-LTMRs and will therefore report on the specific electrophysiological and behavioral responses evoked by C-LTMRs stimulation in intact mice and at varying time-points after SCI. We expect to show that (i) C-LTMRs receptive fields are indeed expanded after SCI and (ii) equate behavioral responses indicative of pain to engagement of C-LTMRs.

4. **IMPACT:**

   - **What was the impact on the development of the principal discipline(s) of the project?**

     Nothing to Report

   - **What was the impact on other disciplines?**

     Nothing to Report

   - **What was the impact on technology transfer?**

     Nothing to Report

   - **What was the impact on society beyond science and technology?**

     Nothing to Report

5. **CHANGES/PROBLEMS:**

   - **Changes in approach and reasons for change**

     Nothing to Report

   - **Actual or anticipated problems or delays and actions or plans to resolve them**

   As was mentioned above, we encountered a problem in with our current strain of TH-Cre mice, which lack specificity for C-LTMRs. We found that due to early developmental expression of TH in other primary afferents, the strain of TH-Cre mice used, also had ectopic expression in a population of myelinated afferents as TH is also expressed in small myelinated afferents that innervate hair follicles of hairy skin. These afferents appear to belong to the Aδ-LTMRs sub-population. Therefore, as we go forward, the experiments will be undertaken in TH-Cre^{ER} mice which we expect will allow us to selectively target C-LTMRs. Since our previous report, we have obtained the mice and are currently establishing colonies as well as crosses with CHR2-YFP. While this has been an unanticipated set-up, it has not significantly challenged or delayed the progression of the project.

   We have modified our behavioral paradigm, by introducing a modified Place Escape/Avoidance paradigm that would allow for better recording in freely-behaving mice. The mPEAP is already built in house and we have begun preliminary testing of 'preference' in adult mice. The addition of this

experimental approach is not expected to negatively impact our studies. Instead, it would greatly increase our understanding of the behavioral outcomes induced by C-LTMRs plasticity after SCI.

- **Changes that had a significant impact on expenditures**

  Nothing to Report

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

  Nothing to Report

- **Significant changes in use or care of human subjects**

- **Significant changes in use or care of vertebrate animals.**

- **Significant changes in use of biohazards and/or select agents**

6. **PRODUCTS:**

- **Publications, conference papers, and presentations**.

  - **Journal publications.** Nothing to Report

  - **Books or other non-periodical, one-time publications.** Nothing to Report

  - **Other publications, conference papers, and presentations**

    Noble DJ, Martin KK, Parvin S and Garraway SM. (2016) Development of a novel technique to investigate the role of autonomic dysfunction in mechanical allodynia following spinal cord injury. Soc Neurosci Abstr #2016-S-15198-SfN. Poster Presentation.

- **Website(s) or other Internet site(s)**
  Nothing to Report

- **Technologies or techniques**
  Nothing to Report

- **Inventions, patent applications, and/or licenses**
  Nothing to Report

- **Other Products**
  Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Table 2: List of Personnel who are currently working on the project

(1) PDs/PIs

Name:	**Sandra M Garraway**
Project Role:	PI
Nearest person month worked:	48/month
Contribution to the project:	I have been directly involved in many aspects of the experiments and have supervised the direction of all experiments performed to date.
Name:	**Shawn Hochman**
Project Role:	Co-I
Nearest person month worked:	24/month
Contribution to the project:	Dr. Hochman supervises all the electrophysiology-related components of the study.

(2) Other persons

Name:	**Donald Noble**
Project Role:	Postdoctoral Fellow (Garraway)
Nearest person month worked:	160/month
Contribution to the project:	Dr. Noble is responsible for the majority of behavioral studies to be undertaken. He also works closely with Mr. Goolsby on constructing the modified conditioned place preference chamber.
Name:	**Michael Sawchuk**
Project Role:	Lab Manager (Hochman)
Nearest person month worked:	32/month
Contribution to the project:	He works with Karmarcha Martin to establish the animal colonies. He will also perform histology.
Name:	**Karmarcha Martin**
Project Role:	Research Specialist (Garraway)
Nearest person month worked:	48/month
Contribution to the project:	She is working on establishing animal colonies, performs surgery and assists with behavioral studies. She performs general lab duties associated with the project.
Name:	**Mallika Halder** and **Makalele Gorsich** (substitute for Kevin Watkins)
Project Role:	Research Specialist (Hochman) Gorsich- Graduate student (Hochman/Garraway)
Nearest person month worked:	160/month
Contribution to the project:	Both have been training under Dr. Hochman's supervision to undertake the electrophysiological studies.
Name:	**William Goolsby**
Project Role:	Engineer
Nearest person month worked:	16 hrs/month
Contribution to the project:	Mr. Goolsby has built many of the apparati needed for the studies. These include the mechanical brush, diode lasers and CO2 incubation chamber. More recently, he has constructed the modified Place preference chamber for behavioral testing.
Name:	**Alycia Patton**
Project Role:	Research Specialist (Garraway and Hochman)
Nearest person month worked:	140hrs/month
Contribution to the project:	She recently joined our team and will be primarily responsible for maintaining the animal colonies. She will also assist Michael Sawchuk with histology and will assist with post-surgical care of the mice. Ms. Patton is jointly supervised by both Drs. Hochman and Garraway.

8. **SPECIAL REPORTING REQUIREMENTS**

- **COLLABORATIVE AWARDS**.
- **QUAD CHARTS**.

9. **APPENDICES:**

Development of a novel technique to investigate the role of autonomic dysfunction in mechanical allodynia following spinal cord injury

*D. NOBLE, K. K. MARTIN, S. PARVIN, S. M. GARRAWAY; Physiol., Emory Univ., Atlanta, GA

Spinal cord injury (SCI) commonly results in the development of maladaptive pain and autonomic dysfunction. Because we do not fully understand the mechanisms of chronic and maladaptive pain after SCI, there has been limited success in controlling this pernicious facet of injury and developing new therapeutic interventions for its alleviation. Allodynia, defined as a painful response to innocuous stimulation, is indicative of maladaptive pain in studies using animal models. Behavioral tests commonly only assess allodynic responses distal to the dermatomal site of injury at the expense of at-level changes. Here, we present pilot data obtained from a novel setup designed to monitor rodent ventilatory responses - potentially a key autonomic index of at-level allodynia - to clinically relevant mechanical stimulation, with the aim of developing a more complete profile of clinical SCI pain.

We focus on a specific fiber population, the C fiber low-threshold mechanoreceptors (C-LTMRs). C-LTMRs can undergo injury-induced changes to produce allodynia and are of especial interest since it is an active research question whether they are involved in allodynia and chronic pain after SCI. We stimulated adult mice (SCI with lateral T10 hemisection or sham) mechanically with a small brush at several frequencies within a range previously reported to activate C-LTMRs. At the same time, we used highly sensitive noncontact electric field sensors (EPIC, Plessey Semiconductors) to monitor the ventilatory response to stimulation.

Breathing rates were elevated from baseline in SCI mice one day after injury, prior to the development of mechanical allodynia (assessed with the von Frey test), and remained elevated at later time points. Furthermore, mechanical brush stimulation across the animal's side at frequencies ranging from 0.3-30 Hz, corresponding to the tuning properties of CLTMRs, revealed frequency-dependent changes in breathing rate. Together, these results suggest the possibility that pain is modulated by autonomic dysfunction following SCI and encourage further inquiry into the role of C-LTMRs. Ongoing studies are aimed at investigating breathing rate as a predictive autonomic physiological marker for below and at-level SCI-induced pain, and selectively recruiting or inhibiting CLTMRs using optogenetics to more precisely delineate their functional contribution to maladaptive pain following SCI.