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**TITLE:** Neuromodulation Promotes Tissue Regeneration Over Fibrosis

**PRINCIPAL INVESTIGATOR:** Dr. Jennifer Simkin

**CONTRACTING ORGANIZATION:** Louisiana State University Health Sciences Center

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Neuromodulation Promotes Tissue Regeneration Over Fibrosis

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**6. AUTHOR(S)**

Jennifer Simkin, Ashley Seifert

E-Mail: jsimki@lsuhsc.edu; awseifert@uky.edu

**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

Louisiana State University
Health Sciences Center,
New Orleans, LA 70124

University of Kentucky,
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**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

**Background:** Combat extremity injuries are typified by severe trauma to multiple musculoskeletal tissues. These injuries are known force-subtractors and source of long-term disability. ***This project directly addresses the FY20 PRORP ARA Focus Area of Retention Strategies by testing how neuromodulation can improve healing outcomes for multi-tissue extremity injuries, ultimately restoring tissue function and affecting return to duty.*** The outcomes of this research will evaluate clinically available therapeutics as potential modulators of nerve-mediated tissue regeneration and attempt to identify novel neuromodulation therapeutic targets.

**Objective and Hypothesis:** The unifying objective of the study is to regulate nerve signaling peptides that influence the tissue healing response, ultimately to discern how neuromodulation can facilitate tissue regeneration over fibrosis. We hypothesize that specific neuropeptides released from injured neurons may be regulated to (i) promote tissue growth, (ii) block cellular senescence and (iii) reduce collagen production following complex tissue injury.

**Specific Aims:** To test our hypothesis, an established animal model of tissue regeneration will be compared to typical fibrotic healing through three aims:

**Aim 1: Functionally test how afferent nerve-produced Calcitonin Gene Related Protein (CGRP) regulates tissue fibrosis.** The *working hypothesis* is that upregulation of CGRP in injured *Mus* tissue promotes excessive scar tissue.

**Aim 2: Functionally test how afferent nerve-produced tachykinins (TK) regulate tissue regeneration.** The *working hypothesis* is that high production of TK peptides in injured *Acomys* tissue promotes tissue regeneration.

**Aim 3: Identify other neuropeptides that regulate cytoprotective activity during tissue regeneration which may yield additional therapeutic targets.** The *working hypothesis* is that regenerating nerves temporally increase production of specific neuropeptides during regeneration (relative to scarring) and these neuropeptides promote cell proliferation over cellular senescence.

**Progress toward aims:** During this project period, the team carried out monthly meetings for progress reports among collaborative sites. Nerve denervation experiments were optimized for protein analysis studies (Aim 3). Evaluation of CGRP and Substance P signaling activity was carried out in vitro (Aim 1 and 2). Inhibitor experiments for CGRP were optimized for in vivo dosing (Aim 1). Supplementary assays (immunohistochemistry and ELISA) were conducted to confirm neuropeptide and inhibitor activity in vivo.

**15. SUBJECT TERMS**

Regeneration, fibrosis, volumetric muscle loss, neuropeptide, Substance P, CGRP

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
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| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | USAMRDC |
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## 1. INTRODUCTION:

This research has the potential to impact patients who sustain extremity injuries where the soft tissue is damaged and compromises functional outcomes. Specifically, muscle that is lost or damaged does not heal by forming new functional muscle. Instead, the injured muscle forms scar which does not contract like muscle and can result in pain and loss of motion. This research will test if currently available medications that target neuropeptide signaling could be feasibly applied in patients with extremity trauma to reduce scarring, increase functional tissue healing, and ultimately improve outcomes by modulating the types of signals injured nerves send to injured tissues. We will test two neuropeptides, Calcitonin Gene Related Peptide (CGRP) and Substance P for their role in regeneration and scar formation using a rodent model of complex tissue regeneration (African spiny mouse) and directly comparing this model to a rodent model of scar formation. We will test FDA-approved inhibitors and agonists of CGRP and Substance P to determine if these drugs can be used off-label to reduce fibrosis after traumatic injury (Figure 1). Finally, with these comparative rodent models, we will identify new molecules that can be targeted to reduce fibrosis.

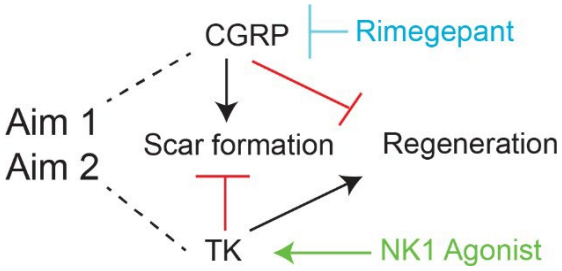


Figure 1. Graphical summary of experimental design to test the role of Substance P and CGRP in fibrosis.

## 2. KEYWORDS: Regeneration, volumetric muscle loss, neuropeptide, spiny mouse

## 3. ACCOMPLISHMENTS:

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

| **Major Task 1** : Project set-up | % of completion/ date |
|---|---|
| Subtask 1 – Seek IACUC/ACURO approval for proposed rodent studies. | 100%/ Q2 2022 |
| Subtask 2 - Hire and train research associate and graduate students on injection protocols. Order supplies. | 100%/ Q2-Q3 2022 |
| Subtask 3 – Run dose response tests to determine working CGRP antagonist(Rimegepant) concentrations for *Mus;* N=20 | 100%/Q2-Q3 2023 |

| | |
|---|---|
| Subtask 4 – Run dose response tests to determine working CGRP concentrations for *Acomys;* N=20 | 100%/ Q3-Q4 2023 |
| Subtask 5 – Run dose response tests to determine working Substance P concentrations for *Mus;* N=20 | 75% |
| Subtask 6 – Run dose response tests to determine working TK antagonist(Emend) concentrations for *Acomys;* N=20 | 50% |
| **Major Task 2 –** Test if inhibition of CGRP reduces scar tissue formation aftercomplex tissue injury | |
| Subtask 7 - Inhibit CGRP uptake in *Mus* ear tissue after 4mm circular punch. Inject antagonist, Rimegepant, subcutaneously following 4mm circular punch injury. Tissue will be collected for analysis at 4 time points after injury; N= 48 | In progress |
| Subtask 8 - Measure the effects of CGRP inhibition on cell proliferation,senescence using immunohistochemistry. | In progress |
| Subtask 9 – RNA analysis of *Mmp9, Mmp13, Col1a1 and Col3* to measure theeffects of CGRP inhibition on extracellular matrix production. | In progress |
| Subtask 10 - Measure the effects of CGRP inhibition on tissue patterning usinghistochemical stains for hair follicle and cartilage patterning, and immunohistochemistry for muscle (MHC1) and nerve patterning | In progress |
| **Major Task 3**: Determine the direct trophic effects of CGRP on cell proliferation, cellular senescence and collagen production | |
| Subtask 11- Grow and expand primary Acomys ear fibroblasts | 100% / Q4 2022 |
| Subtask 12 – Treat Acomys ear fibroblasts with graded concentrations of CGRP | 100% / Q4 2022 |
| Subtask 13 – Measure cell proliferation, senescence using immunohistochemistry. Measure effects on extracellular matrix production using RNA analysis for Col1a1, Col3, Mmp9, Mmp13. | In progress |

| | |
|---|---|
| **Major Task 4**: Test if upregulation of CGRP promotes excessive scar tissue aftercomplex tissue injury | |
| Subtask 14 – Inject exogenous CGRP subcutaneously in *Acomys* ears following4mm circular punch; N=48; colony established at UK | In progress |
| Subtask 15 - Measure the effects of CGRP on cell proliferation, senescence, andcollagen production using immunohistochemistry. | In progress |

| | |
|---|---|
| Subtask 16 – RNA analysis of *Mmp9, Mmp13, Col1a1 and Col3* to measure theeffects of CGRP agonist on extracellular matrix production. | In progress |
| Subtask 17 - Measure the effects of CGRP on tissue patterning usinghistochemical stains for hair follicle and cartilage patterning, and immunohistochemistry for muscle (MHC1) and nerve patterning | In progress |
| **Major Task 5**: determine the ability of the Tachykinin, Substance P, to promote regeneration and reduce scar formation | |
| Subtask 18: inject exogenous Substance P (SP) subcutaneously into Mus ears following 4mm circular punch injury; N=48 | upcoming |
| Subtask 19 - Measure the effects of SP on cell proliferation, senescence, and collagen production using immunohistochemistry. | upcoming |
| Subtask 20 – RNA analysis of Mmp9, Mmp13, Col1a1 and Col3 to measure the effects of SP on extracellular matrix production. | upcoming |
| Subtask 21 - Measure the effects of SP on tissue patterning using histochemical stains for hair follicle and cartilage patterning, and immunohistochemistry for muscle (MHC1) and nerve patterning | upcoming |
| **Major Task 6** – Determine the direct trophic effects of Substance P on cell proliferation, senescence and collagen production | |
| Subtask 22- Grow and expand primary Mus ear fibroblasts | 100% / Q3 2022 |
| Subtask 23 – Treat Mus ear fibroblasts with graded concentrations of exogenous Substance P | 100%/Q3 2023 |
| Subtask 24 – Measure effects on cell proliferation and senescence using immunohistochemistry. Measure effects on extracellular matrix production using RNA analysis for Mmp9, Mmp13, Col1a1, Col3 | upcoming |
| Subtask 26 - Measure the effects of NK1R inhibition on cell proliferation, senescence, and collagen production using immunohistochemistry. | upcoming |
| Subtask 27 – RNA analysis of Mmp9, Mmp13, Col1a1 and Col3 to measure the effects of NK1R inhibition on extracellular matrix production. | upcoming |
| Subtask 28 - Measure the effects of NK1R inhibition on tissue patterning using histochemical stains for hair follicle and cartilage patterning, and immunohistochemistry for muscle (MHC1) and nerve patterning | upcoming |

| | |
|---|---|
| Major Task 8: Identify signals specific to nerves of the regenerating ear | |
| Subtask 29: Denervate Acomys ears. Create circular punch wounds through denervated and innervated Acomys ears; N=16 | 100%/Q2-Q3 2023 |
| Subtask 30: Collect tissue during the proliferation phase of regeneration: D10, D20. Isolate proteins from tissue homogenates. | upcoming |
| Subtask 31: Run Discovery-based Mass Spectrometry on all samples.<br>● Annotate analytes<br>● Calculate concentration ratios (e.g. Day 10 concentration/Day 0 concentration)<br>● Determine proteins that are significantly more abundant in innervated ears compared to denervated ears<br>● Run two way ANOVA to measure main effects time and species, and time*species interactions | upcoming |
| Major Task 9: Define time course for nerve-specific signals in regenerating and scar-forming injuries | |
| Subtask 32: Collect tissue at D0, D5, D10, D15, D20, D40 from Acomys and Mus.Isolate total protein from tissue homogenates; N=30 animals / species | 75% |
| Subtask 33: Run targeted Liquid Chromatography/Mass Spectrometry for top 10 proteins identified in Subtask 31<br>● Calculate concentration ratios (e.g. D10/D0)<br>● Create timecourse for each protein based on concentration ratios<br>● Run comparisons, Two-way ANOVA with main effects time and species, and time*species comparison. | upcoming |

**What was accomplished under these goals?**

**Major Task 1 – Project Set up.** During this project period, doses for CGRP and Rimegepant were optimized for dosing in vivo. Varying concentrations of CGRP were injected into the ear of mice (n=20). We noted with CGRP injections, the ears twitch and blood vessels dilate which was not evident with PBS-vehicle injected control ears. cAMP concentrations increased after injection with …

**Major Task 2 – Test if inhibition of CGRP reduces scar tissue formation after complex tissue injury.** Dr. Simkin's group optimized injections of Rimegepant via IP injection versus local ear injections to test effects of CGRP inhibition across time. We found administration of CGRP

30pmol/kg into the ear of mice increases levels of cAMP after 5 minutes compared to PBS injections (Figure 1A). A single IP or local ear injection of 10 mg/kg Rimegepant inhibits this increase in cAMP (Figure 1B).

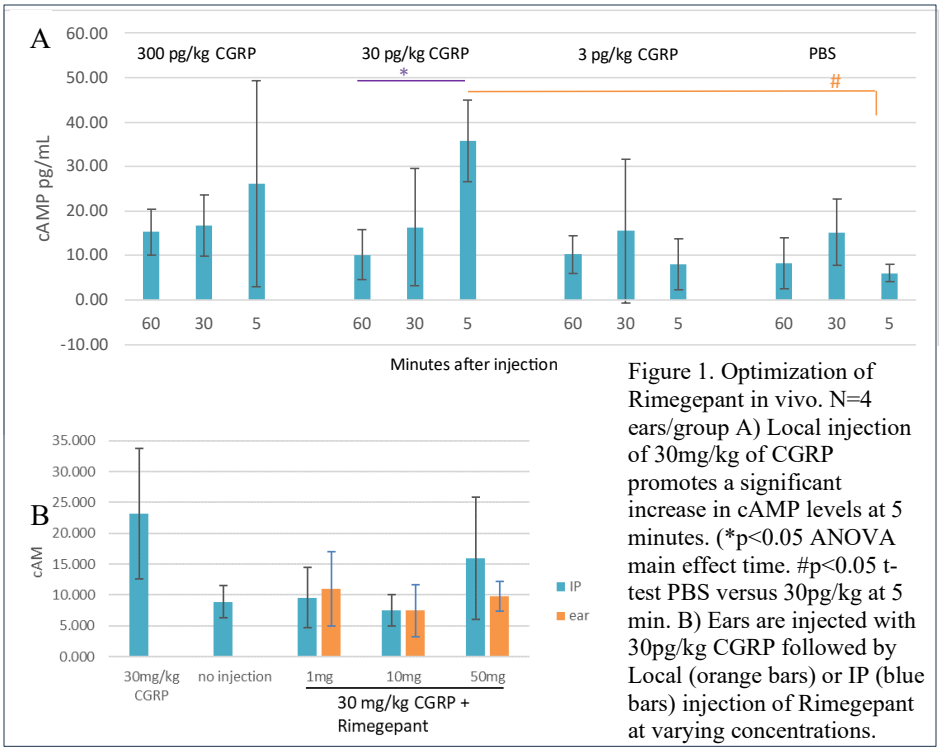


Figure 1. Optimization of Rimegepant in vivo. N=4 ears/group A) Local injection of 30mg/kg of CGRP promotes a significant increase in cAMP levels at 5 minutes. (*p<0.05 ANOVA main effect time. #p<0.05 t-test PBS versus 30pg/kg at 5 min. B) Ears are injected with 30pg/kg CGRP followed by Local (orange bars) or IP (blue bars) injection of Rimegepant at varying concentrations.

**Major Task 3:** Determine the direct trophic effects of CGRP on cell proliferation, cellular senescence and collagen production.

Dr. Simkin's group trained a new research associate on cell proliferation assays and expansion of ear cells. Cells were dosed with CGRP at 30pmol and assayed for cAMP response via ELISA and receptors for CGRP in fibroblasts were measured via Western Blot. We found fibroblasts express the CGRP receptor (CLR) but not RAMP1 at baseline conditions which suggests fibroblasts may not show a strong in vitro response to CGRP.

**Major Task 4**: Test if upregulation of CGRP promotes excessive scar tissue after complex tissue injury.

Dr. Seifert's group has initiated injections of 30pmol CGRP locally, into the ears of spiny mice. Tissue is being collected across timepoints for analysis of collagen production, cell proliferation, cellular senescence and new tissue growth.

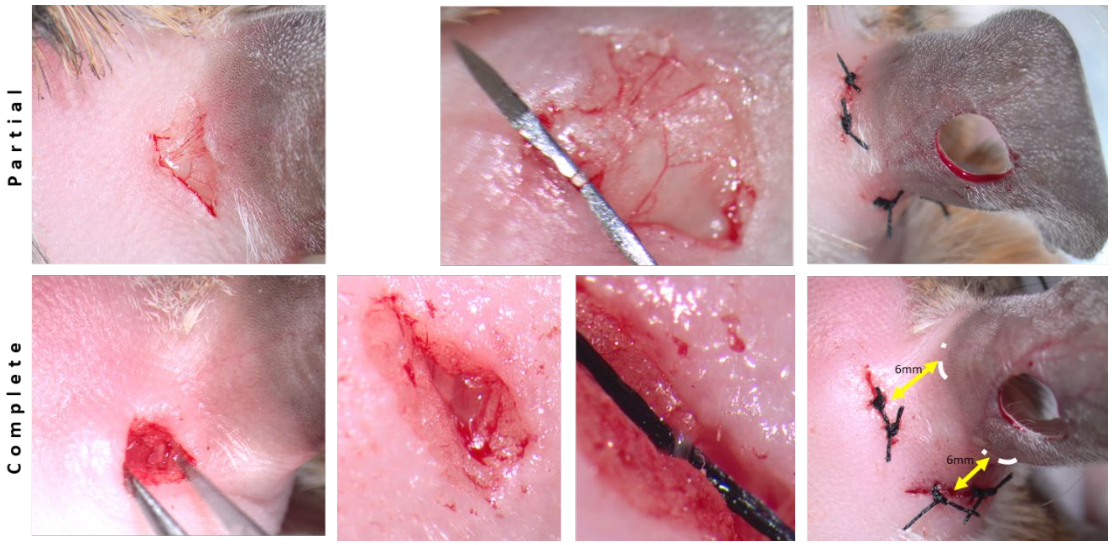


**Figure 2. Optimization of denervation surgeries on spiny mouse ears.** Cuts through nerves at the base of the ear demonstrate only a partial denervation with nerve regrowth during regeneration. Cuts through two major nerves at 6 mm proximal to the base of the ear allow for more complete denervation. Proximal denervation will be conducted moving forward.

**Major Task 5**: Determine the ability of the Tachykinin, Substance P, to promote regeneration and reduce scar formation.

Dr. Simkin's group has started optimization of Substance P in vivo using cAMP as a readout for response to Substance P. We have found that at 1 hour after dosing, tissue does not show an increase in cAMP levels. We are currently testing response at various time points after Substance P exposure to optimize proper Substance P dosing for experiments.

**Major Task 6:** Determine the direct trophic effects of Substance P on cell proliferation, senescence and collagen production.

In this project period, Dr. Simkin's group dosed cells with Substance P and collected cells for downstream RNA analysis.

**Major Task 8:** Identify signals specific to nerves of the regenerating ear.

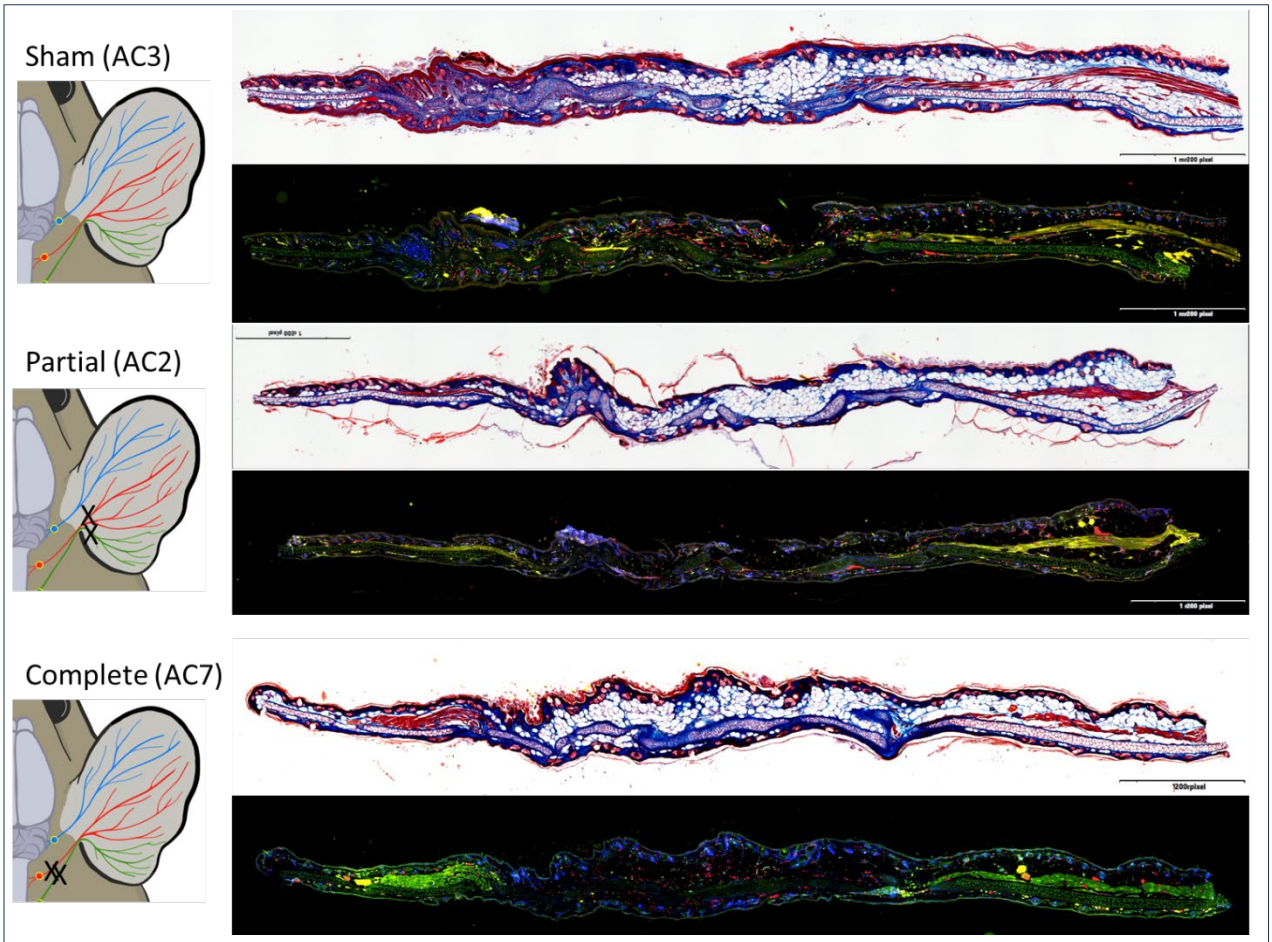


Figure 3. Mapping nerve regrowth after denervation to ensure complete denervation after injury. In the first surgical technique (partial denervation) nerves (red stain) regenerate alongside new tissue as the ear is healing. Complete denervation shows fewer new innervations (red stain) 55 days after injury compared to sham and partial denervation animals.

For this task, tissue will be collected from the ears after denervation. In this project period, Dr. Seifert's group optimized surgeries for denervation in the ear (Figure 2) and has started mapping out nerve regrowth after denervation to ensure proper animals are properly denervated (Fig. 3). Tissue has been collected for proteomic analysis to compare a regenerative response to a scar-forming response. Moving forward, proteomic analysis will be carried out at the LSUHSC proteomic core as denervation continues at University of Kentucky.

**Major Task 9:** Define time course for nerve-specific signals in regenerating and scar-forming injuries.

During this project period, Dr. Seifert's lab collected ear tissue stored for tissue for analysis by the LSUHSC proteomic core.

**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?**
During the next funding period, Dr. Simkin's group at LSUHSC will focus on major tasks 2, 5 and 9.

For major task 2, we will collect tissue for analysis of fibrosis at days 0 , 5, 10, 15, 20 after injury. We will perform histological and molecular analysis for collagen deposition, cell proliferation and cell senescence after treatment with Rimegepant for 20 days.

For major task 5, we will test optimized concentrations of Substance P in mouse ear. We will collect tissue for qPCR to measure changes in collagen and MMP9 expression and histological analysis to measure cartilage, muscle, and skin regeneration. We will additionally perform cell proliferation and senescence assays in vivo to determine the effects of Substance P on tissues after injury.

For major task 9, we will run mass spectrometry in conjunction with the LSUHSC proteomics core and analyze changes in protein levels in innervated and denervated spiny mouse ears.

We will additionally focus on the presentation of results at two major conferences.

Dr. Seifert's group at University of Kentucky will focus on major tasks 4, 7, and 9.

For major task 4, we will inject CGRP at the optimized concentration (30pmol/kg) into the spiny mouse ear. We will collect tissue at days 0, 5, 10, 15, 20 after injury. We will perform histological and molecular analysis for collagen deposition, cell proliferation and cell senescence.

For major task 7, we will quantify inhibition of Substance P after injection into spiny mouse ears. We will use cAMP assays as optimized by LSUHSC.

For major task 9, we will collect tissue samples from denervated ears over time and ship to LSUHSC for proteomic analysis.

## *4.* IMPACT:

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

During this project period the team focused on carrying out in vitro and in vivo experiments with commercially available, FDA approved antagonists to CGRP (Rimegepant) and collection of tissue to identify novel factors produced by nerves during tissue regeneration. We expect results from this study will provide novel therapies to shift fibrotic healing into regenerative healing after traumatic injury.

**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?**

During this project period, Dr. Simkin delivered presentations on the incidence and consequences of amputation injuries in military personnel. These presentations were delivered to medical and nursing students, raising awareness abut the severity of amputation injuries and also equipping future healthcare professionals with crucial insights to provide better care and support to wounded veterans.

CHANGES/PROBLEMS:

**Changes in approach and reasons for change**

During this project period, altered the delivery method for CGRP inhibitor. We originally planned for local delivery of Rimegepant and found that IP injections also showed a reduction in CGRP activity. Because IP injections require less technical accuracy than local delivery to the ear, we switched our delivery method for the 20 day injection experiments.

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

The LSUHSC proteomics core had a change in leadership. We are in the process of waiting for instrumentation to be optimized and on-boarding the new technical assistance for mass spectrometry. We expect the core to be able to run samples in Q2 of 2024.

**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**

Nothing to report

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

Nothing to report

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

Nothing to report

**5. PRODUCTS:**

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

  Nothing to report

  **Journal publications.**

  Nothing to report

- **Books or other non-periodical, one-time publications.**

  Nothing to report

- **Other publications, conference papers and presentations**.
  Nothing to report

- **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

## 6. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

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

| | |
|---|---|
| Name: | Jennifer Simkin |
| Project Role: | PI |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 6 |
| Contribution to Project: | Dr. Simkin performed project design, data analysis, student and RA training and headed monthly meetings between LSUHSC and University of Kentucky |

| | |
|---|---|
| Name: | Ashley Seifert |
| Project Role: | PI |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 6 |
| Contribution to Project: | Dr. Seifert performed project design, data analysis, student and RA training and oversaw all work at the University of Kentucky |

| | |
|---|---|
| Name: | Aysha Evans |
| Project Role: | Research Assistant |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 3 |
| Contribution to Project: | Ms. Evans performed in vivo CGRP and inhibitor studies including optimization of local ear injections over time |

| | |
|---|---|
| Name: | Jessica Rivera |
| Project Role: | co-I |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 3 |
| Contribution to Project: | Dr. Rivera performed project design and provided expert insight into pharmacology of CGRP and Substance P |

| | |
|---|---|
| Name: | Renee Donahue |
| Project Role: | Research Associate |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | 4 |

| | |
|---|---|
| Contribution to Project: | Ms. Donahue performed staining procedures and data analysis to optimize experiments for Aim 3 |

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to report

**What other organizations were involved as partners?**

Nothing to report

## 7. SPECIAL REPORTING REQUIREMENTS

**COLLABORATIVE AWARDS:** *N/A*

**QUAD CHARTS:** *N/A*

## 8. APPENDICES: *N/A*