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TITLE: Neuromodulation Promotes Tissue Regeneration Over Fibrosis

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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 optimized new assays and trained new personnel on standard operating procedures. Additionally, the team set up and carried out biweekly meetings for progress reports among collaborative sites. All animals were successfully bred and set aside for each aim. Initial evaluation of CGRP and Substance P signaling activity was carried out in vitro to identify proper timing and concentrations of treatments. Primers, antibodies, and assays were optimized and are ready for experimental use. This work resulted in one poster presentation at LSUHSC during the student summer research symposium.

15. SUBJECT TERMS

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

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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.

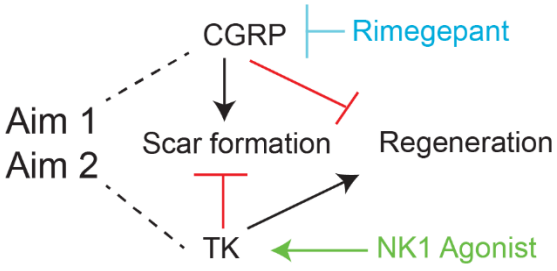

Figure 1. Graphical summary of experimental design to test the role of Substance P and CGRP in fibrosis.

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 injectionprotocols. Order supplies.	100%/ Q2-Q3 2022
Subtask 3 – Run dose response tests to determine working CGRP antagonist(Rimegepant) concentrations for *Mus;* N=20	50%

Subtask 4 – Run dose response tests to determine working CGRP concentrations for *Acomys;* N=20	75%
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	upcoming
Subtask 8 - Measure the effects of CGRP inhibition on cell proliferation,senescence using immunohistochemistry.	upcoming
Subtask 9 – RNA analysis of *Mmp9, Mmp13, Col1a1 and Col3* to measure theeffects of CGRP inhibition on extracellular matrix production.	upcoming
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	upcoming
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.	upcoming

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	upcoming
Subtask 15 - Measure the effects of CGRP on cell proliferation, senescence, andcollagen production using immunohistochemistry.	upcoming

Subtask 16 – RNA analysis of *Mmp9, Mmp13, Col1a1 and Col3* to measure theeffects of CGRP agonist on extracellular matrix production.	upcoming
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	upcoming
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	25%
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	50%
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. ● Annotate analytes ● Calculate concentration ratios (e.g. Day 10 concentration/Day 0 concentration) ● Determine proteins that are significantly more abundant in innervated ears compared to denervated ears ● 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	25%
Subtask 33: Run targeted Liquid Chromatography/Mass Spectrometry for top 10 proteins identified in Subtask 31 ● Calculate concentration ratios (e.g. D10/D0) ● Create timecourse for each protein based on concentration ratios ● 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, the team set up weekly meetings to discuss progress within each site and biweekly meetings to discuss progress between sites. Both sites hired and trained new personnel dedicated to the project, acquired IACUC and ACURO approval and optimized new assays.

Major Task 2 – Test if inhibition of CGRP reduces scar tissue formation after complex tissue injury.

Dr. Simkin's group optimized antibodies for fibrotic and regenerative output and collected tissue across wound healing time points (days 0, 3, 5, 10, 15, 20, 40) to measure baseline activity of CGRP, cell proliferation and collagen deposition.
We localized CGRP receptors within the ear tissue and optimized assays to validate the inhibition of CGRP activity on cells (Figure 2) and within mouse ear tissues. These assays include measurements of downstream signaling proteins, cAMP (via ELISA) and phosphorylated CREB (via Western blot). With these experiments in place, we will quantify the extent and duration of inhibitor and agonist activity in vivo.
Additionally, dose response tests were conducted to determine the best concentration of inhibitors and agonists. This work resulted in a winning poster presentation at LSUHSC by a summer trainee.

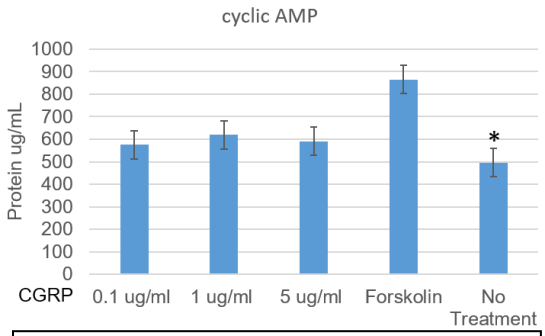

Figure 2. Measurement of cyclic AMP using ELISA to determine CGRP activity in cells and identify optimal concentration for experiments. 3 concentrations of CGRP were tested on mouse fibroblasts against a positive control for cyclic AMP (Forskolin) and a negative control (no treatment). All three CGRP concentrations showed an increase in cAMP activity compared to no treatment.

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

Dr. Simkin's group trained new students on cell senescence assays. Primers for quantitative PCR were designed and optimized. *Acomys* and *Mus* ear fibroblasts were isolated and expanded for cell culture analysis. With these supplies in place and with the optimization of CGRP concentration (Major task 2), we will perform in vitro assays in the upcoming project period.

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

At University of Kentucky, Dr. Seifert's group completed breeding of the animals required for this task and all subtasks. The group additionally acquired necessary pharmacology for these tasks and trained new research associate on local injections in vivo and output assays (quantitative PCR and immunohistochemistry).

Major Task 6:
In this project period, Dr. Simkin's group isolated and created mouse fibroblast cell lines necessary for this task. We have trained research personnel in primary cell culture techniques and preserved enough cell lines to complete this aim. We have additionally dosed cells with graded concentrations of Substance P to determine the optimal working concentration.

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

For this task, tissue will be collected from the ears after denervation. In this project period, Dr. Seifert's group completed baseline characterization to quantify nerves within the ear prior to and after denervation (Figure 3). A new research associate was trained on denervation techniques where

three major nerves are severed at the base of the skull. Moving forward, nerves will be quantified after denervation to ensure proper technique. Tissue will be collected for proteomic analysis to compare a regenerative response with and without neural contribution.

Major Task 9: Define time course for nerve-specific signals in regenerating and scar-forming injuries.
During this project period, we bred and set aside all the animals necessary to complete major task 9. We have trained personnel on ear punch and tissue collection methods and have begun collection of tissue necessary for this aim.

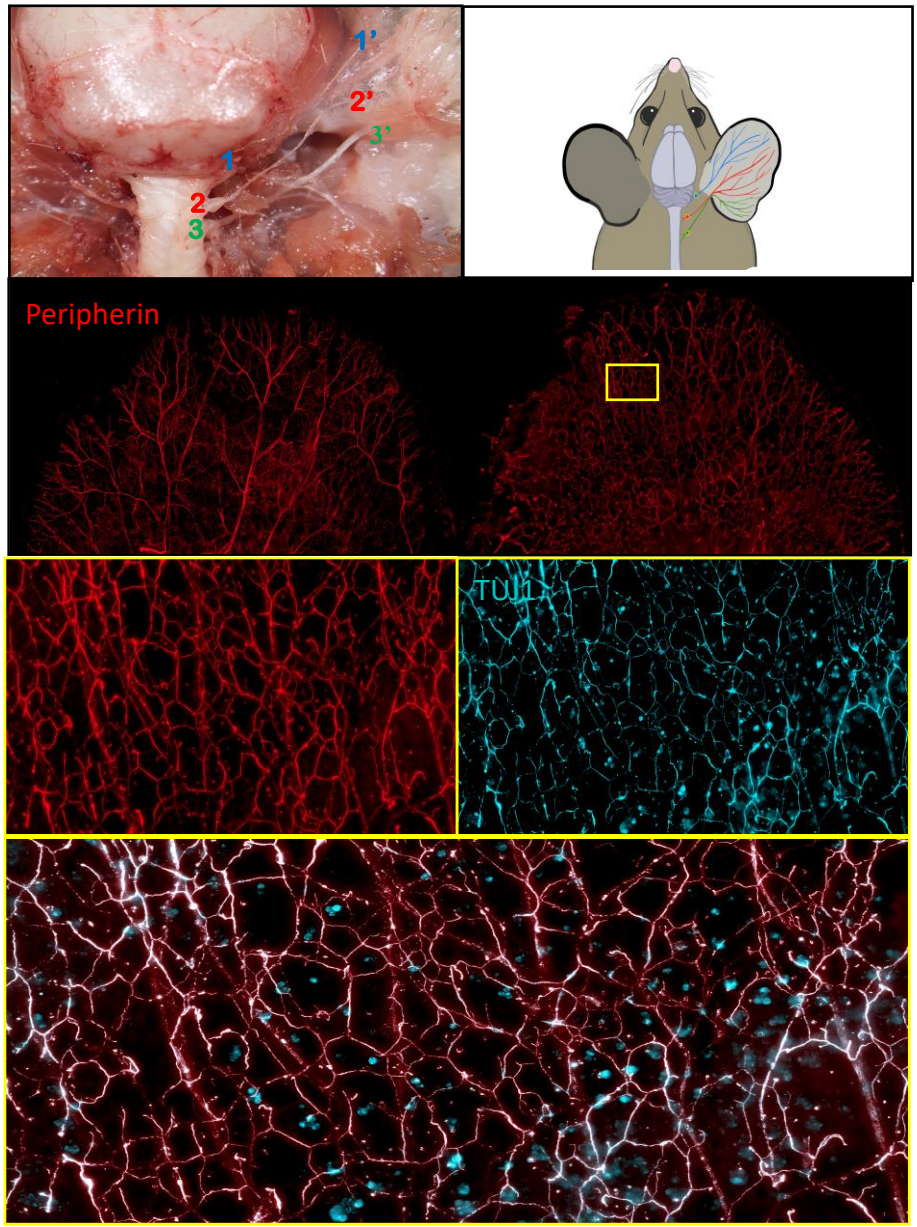

Figure 3. Whole mount staining of ear of Spiny mouse to quantify nerves (red = peripherin for sensory nerves; blue = TUJ1 as a pan-neuronal marker).

What opportunities for training and professional development has the project provided?
Nothing to report

How were the results disseminated to communities of interest?
During this period, there was one poster presentation at LSUHSC for high school and undergraduate research. The high school student presented data on assay optimization and won first place in the poster competition. Alphonese, G., Williams, A., and Simkin, J. "Testing the role of Calcitonin Gene Related Peptide (CGRP) in scar formation after multiple tissue injury" (2022) Poster Presentation. LSUHSC Undergraduate Research Education symposium.

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 6.

For major task 2, we will quantify the inhibition of CGRP after injection into the mouse ears. We will measure inhibition using optimized cAMP and phosphoCREB assays. 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.

For major task 5, we will test optimized concentrations of Substance P in mouse ear. We will 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 6, we will treat Mus cells with Substance P and collect for protein and RNA analysis to quantify changes in fibrotic output. We will also determine changes to cell proliferation and senescence in vitro.

Dr. Seifert's group at University of Kentucky will focus on major tasks 3, 4, and 7.

For major task 3, we will dose Acomys cells with the optimized concentration of CGRP. We will collect cells for protein and RNA analysis to look at changes in cell proliferation, senescence and fibrotic output.

For major task 4, we will inject CGRP at the optimized concentration 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 and phophoCREB assays as optimized by LSUHSC.

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 optimization of new assays, training of personnel, and set up of supplies/equipment. 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, two high school students were trained in techniques used by the lab. Both students are from under-represented populations in the sciences. Exposure to lab techniques and people at the university is designed to cultivate interest in biomedical sciences and prepare the students for a career in clinical or basic sciences.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change
During this project period, we optimized new methods to assess neuropeptide activity. Previously, we used a visible mark of neuropeptide activity (swelling and erythema). To more accurate quantify inhibition of CGRP and Substance P activity, we switched to molecular readouts. These include Western blot for phosphorylated CREB, a major signaling protein in the pathway downstream of CGRP and Substance P and ELISA for cyclic AMP, a second major signaling protein in the CGRP and Substance P pathways. These assays will be used to validate in vivo inhibition of the signaling pathways.

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

Ordering supplies has been delayed due to nationwide shortages in supplies and delays in nationwide mailing services. These delays often required us to find and optimize assay kits that were different from our standard operating procedures. Thus for Q1 of 2022 we spend time creating new standard operating procedures with available reagents.

Changes that had a significant impact on expenditures

Lab personnel turnover increased during 2022 and hiring of new personnel was delayed by the institutions. We have resolved these issues by increasing offered compensation. This required combining resources dedicated for a graduate research assistant to offer higher compensation for a research assistant**.**

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

6. 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**.
  Alphonese, G., Williams, A., and Simkin, J. "Testing the role of Calcitonin Gene Related Peptide (CGRP) in scar formation after multiple tissue injury" (2022) Poster Presentation. LSUHSC Undergraduate Research Education symposium.

- **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?

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:	Tierra Strange
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	Ms. Strange performed tissue collection, optimization of qPCR, and oversaw student workers on the project for Aim 1

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:	Ashlee Williams

Project Role:	Student worker
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2
Contribution to Project:	Ms. Williams performed cell line expansion and tissue isolation. She optimized protein dosing assays in vitro for Aims 1 & 2
Funding Support:	LSUHSC Department of Orthopedic Surgery

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

Name:	Gabrielle Alphonese
Project Role:	Stuent worker
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	Ms Alphonese helped optimize cAMP ELISA assays
Funding Support:	LSUHSC summer research program

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

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *N/A*

QUAD CHARTS: *N/A*

9. APPENDICES: *N/A*