AWARD NUMBER: W81XWH-18-1-0729

TITLE: Increasing Sensory Neuron Regeneration in the Injured Spinal Cord with Acute Intermittent Hypoxia

PRINCIPAL INVESTIGATOR: Valeria Cavalli

CONTRACTING ORGANIZATION: Washington University, St Louis, MO

REPORT DATE: October 2020

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
OCTOBER 2020	ANNUAL	15SEP2019 - 14SEP2020

4. TITLE AND SUBTITLE:

Increasing Sensory Neuron Regeneration in the Injured Spinal Cord with Acute Intermittent Hypoxia

5a. CONTRACT NUMBER
W81XWH-18-1-0729

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

Washington University
St. Louis, MO 63130

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT:
APPROVED FOR PUBLIC RELEASE; DISTRIBUTION UNLIMITED

13. SUPPLEMENTARY NOTES

14. **ABSTRACT: The poor intrinsic regenerative capacity of mature central nervous system (CNS) neurons combined with the barrier imposed by the inhibitory environment is a major contributor to regeneration failure and permanent disabilities following spinal cord injury (SCI). Targeting both neuron-intrinsic and -extrinsic factors has been shown to improve axon regeneration. My laboratory has focused on elucidating how peripheral neurons regenerate, with the goal to identify novel therapeutic strategies for the treatment of CNS injuries. We discovered that the transcription factor HIF-1α stimulates axon regeneration in sensory neurons via transcriptional and epigenetic mechanisms. We found that mice undergoing Acute Intermittent Hypoxia (AIH) treatment display enhanced axon regeneration in sensory and motor neurons in a HIF-1α dependent manner. We hypothesize that AIH stimulates the regenerative capacity of injured neurons in the spinal cord, via transcriptional and epigenetic mechanisms. Because combinatorial approaches to neutralize the inhibitory environment and to boost the intrinsic neuronal growth capacity provide greater recovery, we also hypothesize that AIH paired with approaches to relieve CSPG-mediated inhibition can stimulate functional recovery following contusive SCI. The scope of the research is to determine if AIH treatment mimics the epigenomic changes elicited by peripheral injury and stimulates axon regeneration of dorsally ascending sensory axons following SCI. We also propose to determine if AIH combined with modulation of the CSPG receptor PTPsigma have synergetic effects on functional recovery following SCI.**

15. SUBJECT TERMS; NONE LISTED

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	12	19b. TELEPHONE NUMBER *(include area code)*

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1. **INTRODUCTION:**

The poor intrinsic regenerative capacity of mature central nervous system (CNS) neurons combined with the barrier imposed by the inhibitory environment is a major contributor to regeneration failure and permanent disabilities following spinal cord injury (SCI). Targeting both neuron-intrinsic and -extrinsic factors has been shown to improve axon regeneration. My laboratory has focused on elucidating how peripheral neurons regenerate, with the goal to identify novel therapeutic strategies for the treatment of CNS injuries. We discovered that the transcription factor HIF-1α stimulates axon regeneration in sensory neurons via transcriptional and epigenetic mechanisms. We found that mice undergoing Acute Intermittent Hypoxia (AIH) treatment display enhanced axon regeneration in sensory and motor neurons in a HIF-1α dependent manner. We hypothesize that AIH stimulates the regenerative capacity of injured neurons in the spinal cord, via transcriptional and epigenetic mechanisms. Because combinatorial approaches to neutralize the inhibitory environment and to boost the intrinsic neuronal growth capacity provide greater recovery, we also hypothesize that AIH paired with approaches to relieve CSPG-mediated inhibition can stimulate functional recovery following contusive SCI. The scope of the research is to determine if AIH treatment mimics the epigenomic changes elicited by peripheral injury and stimulates axon regeneration of dorsally ascending sensory axons following SCI. We also propose to determine if AIH combined with modulation of the CSPG receptor PTPsigma have synergetic effects on functional recovery following SCI.

2. **KEYWORDS:** spinal cord injury, axon regeneration, transcription, HIF-1α, sensory neurons, dorsal column, acute intermittent hypoxia, CSPG inhibition, PTPsigma, functional recovery

3. **ACCOMPLISHMENTS:**

- **What were the major goals of the project?**
  - **Major Task 1: Determine the epigenomic profile elicited by AIH**
    This task was to be completed between month 0 and 20.
    Major Task 2: Determine if AIH promotes regeneration of sensory axons in the injured spinal cord
    This task was to be completed between month 18 and 36.
  - **Major Task 3: Determine if AIH combined with modulation of the CSPG receptor PTPsigma have synergetic effects on functional recovery following SCI**
    This task is to be completed between month 22 and 36.

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

The COVD19 pandemic has strongly affected our progress during year 2 of this award. In addition, Dr. Eric Ewan left the lab at the end of May 2020 to take a position in industry. I have hired a new lab member to replace Dr. Ewan. He is scheduled to start in December 2020, but the J1 visa transfer might delay his start date. It will also take some time for him to be acquainted with the new lab setting given the ongoing limitation in lab activity and limited in person lab interactions. The COVID19 pandemic has thus had a major effect on our progress. We are moving the project forward by performing additional analyses, but there is a delay in completion of Major Task 1 and Major Task 2.

Major Task 1: Determine the epigenomic profile elicited by AIH.

Major activities: We continued our bioinformatic analysis of the single cell RNAseq data to determine the effects of sciatic nerve crush injury (SNI), spinal cord injury (SCI) and acute intermittent hypoxia (AIH) on neurons, their surrounding satellite glial cells, and resident macrophages within the dorsal root ganglion (DRG). The preliminary analyses show that sciatic nerve injury (SNI) induces changes in many genes compared to spinal cord injury (SCI) and acute

intermittent hypoxia (AIH), and that the pathways associated with these genes mostly differ between these conditions.

Subtask 1: Generate the mice needed for epigenomic studies

Specific Objectives: To determine if AIH elicits transcriptional changes similar to peripheral nerve injury in DRG.
Major activities. We have continued our work related to the single cell data analysis that we have already collected. This will be submitted for publication in Experimental Neurology, which will have a special issue related to AIH. The due date is Jan 1 2021. **Figure 1** of this manuscript is ready (see below) and we are continuing our analysis, focusing, on the cell types indicated in Fig 1g. We have also plans to analyze specifically HIF1-alpha target genes and how they are affected by AIH or injury in different cell types.

Because in our single cell RNAseq approach, we recovered few, mostly small diameter neurons (nociceptors), we planned to isolate neuronal nuclei and perform snRNAseq. For this experiment we had generated Baf53b-cre:Sun1GFP mice, and verified expression of nuclear GFP specifically in all sensory neurons. This experimental plan has been paused, because the mouse colony had to be reduced and no new breeding were set up due to the COVID19 pandemic. Lab activity has resumed at the end of July, and it is now at 80% of the pre-COVID19 level. We have been ramping up the mouse line and verifying all genotypes. The sun1GFP mice are breeding well. However the Baf53-cre line had turned germ line and is no longer usable for this project. As an alternative, we will use AAV8-mediated expression of cre to drive nuclear GFP expression. The first step will be to quantify the proportion of neurons that can be labeled with AAV8 mediated cre expression. If at least 60 to 80% of the neurons are GFP labeled, we will then be able to purify neuronal nuclei and perform snRNAseq as proposed. The experiment will extend beyond the planned 20 months for this Subtask.

Subtask 2: Determine the state of chromatin condensation genome-wide elicited by AIH.

This task relies on purifying neuronal nuclei from Baf53b-cre:Sun1GFP mice, which has been halted due to the COVID19 pandemic and the reduced mouse colony as described above for subtask 1. If the AAV8-mediated sun1GFP expression in neurons is efficient, we will resume with these experimental plans, which will definitely exceed the estimated time line of 8-18 month. Our sequencing facility now offers to perform single nucleus ATACseq and RNAseq on the same samples, which will be very helpful for the proposed project.

Subtask 3: RNAseq and ATACseq data bioinformatics analysis

This task relies on combining ATACseq data and RNAseq data. As stated above, this task will extend beyond the estimated time line of 8-20 month due to the COVID19 pandemic and the Baf53b-cre mouse line turning germline.

Subtask 4: Test candidate gene expression for their dependence on neuronal HIF-1α.

The HIF1 floxed mouse line is being maintained. Because the Baf53-cre line has turned germ line, we will need to use an AAV8-cre approach to delete HIF1 in neurons. This subtask will not be achieved within the 16-20 months timeline due to the delay encountered with the COVID19 pandemic.

Significant results: We found that SNC produces the most robust changes in gene expression with many unique gene pathways either up or downregulated in all cells. SCI and AIH produce far less robust transcriptional changes, and when gene pathways are up- or down-regulated by these conditions, the genes in these pathways tend to not be unique to either condition per se, but are also typically influenced by SNC. Surprisingly, we found that AIH and SCI elicit robust gene expression changes in mesenchymal cells and T-cells, which will be analyzed in more details in the coming months.

Other achievements: n/a

Major Task 2, Determine if AIH stimulates axon regeneration following SCI

Subtask 1. Determine if AIH promotes regeneration of sensory axons in the injured spinal cord.

Major activities and Specific Objectives: We previously reported that a combinatorial approach in which mice are treated with AIH and chABC to reduce inhibition from the environment decreased axon retraction but did not significantly enhanced regeneration beyond the injury site.

Significant results: The additional histological assessments to assess the quality of CSPG digestion and possible sprouting and/or regeneration of descending spinal axons were halted due to the COVID19 pandemic. Resuming this task will require the new post-doctoral candidate to join the lab.

Significant results: We found that AIH alone or in combination with chABC does not enhance significant regeneration of dorsally ascending sensory axons passed the lesion site.

Other achievements: n/a

- **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?**

Due to the COVID19 pandemic, we have been limited to the analyses of existing data for preparation of a manuscript and recovery of our original mouse colony. Due to the Baf53-cre mouse line turning germ line, we are now planning to use an AAV8-cre approach to lead to GFP expression in neuronal nuclei. We will then be in the position to generate new data. Meanwhile, we will complete the analysis of the scRNAseq data to understand how neurons as well as other cell types relevant to axon regeneration, such as macrophages and satellite glial cells (SGC) respond to AIH as compared to SNI and SCI. We will examine known and predicted HIF1-alpha target genes and how AIH or injury affect their expression in different cell types. Our preliminary analyses, suggest that at AIH activates PPARalpha signaling pathway in SGC, similar to nerve injury. AIH also affects macrophages, and may reduce the expression of inflammatory cytokines. From other studies in the lab we know that this PPARalpha signaling in SGC is important to promote axon regeneration after central axon injury. This enhances the potential impact of AIH treatment and highlight the effect of AIH on non-neuronal cells. We are continuing our analysis for a manuscript that will report the findings from our existing single-cell dataset. This manuscript will be submitted on Jan 1 2021 to be part of a special AIH issue in the journal *Experimental Neurology*.

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*
   - **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:** *The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*
    - **Changes in approach and reasons for change**
        - *Nothing to Report*
    - **Actual or anticipated problems or delays and actions or plans to resolve them**

    As per my conversation with Dr. Sarah Fontaine on 7/15/2020, it was decided that a one year no cost extension will be requested in 2021, prior to the expiration of the award. This will allow us to effectively extend year 2 of the award to a 2 year period to complete Major Task1 and Major task 2. The subcontract with Dr. Phil Popovich at The Ohio State University described in Major Task 3 will thus be postponed to the fall of 2021. The situation will be reassessed in 2021 as the pandemic situation evolves.

    Dr. Eric Ewan left the lab at the end of May to take a position in industry. Dr. Ajeet Kumar is scheduled to join the lab as of 12/1 2020, but the visa transfer situation may delay this start date. It will also take longer than usual for Dr. Kumar to be acquainted to the new lab given the restrictions in lab activity and in person gathering. The COVID19 pandemic has thus had a major effect on our progress. We are moving the project forward by performing additional analyses, but there will be a delay in completion of Major Task 1 and Major Task 2. I have one staff scientist who is performing the analyses described. Kathy Leahy, lab supervisor, is taking care of the mouse colony.

    -
    - **Changes that had a significant impact on expenditures**
    - **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:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

    - **Publications, conference papers, and presentations**
      Report only the major publication(s) resulting from the work under this award.

        - **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
    - **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**
    - **What individuals have worked on the project?**

      Eric Ewan, Staff Scientist
      100% effort (through 5/30/2020)

      Kathy Leahy, Lab Supervisor
      10% effort (through furlough 4/27/2020 to 6/29/2020)

      Oshri Avraham, Staff Scientist
      10% effort effective 8/1 and 5% effort effective 9/1

      Rui Feng, Postdoctoral Research Associate
      10% effort effective 7/1 and 5% effort effective 9/1

      Valeria Cavalli, PI
      15% effort (2% effective 9/1/2020)
    - **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

        - Dr. Cavalli received a new NIH award R21NS115492. Updated active other support is attached.
    - **What other organizations were involved as partners?**

        - *Nothing to Report*

7. **SPECIAL REPORTING REQUIREMENTS**

    - **COLLABORATIVE AWARDS:** *For collaborative awards, independent reports are required from **BOTH** the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to https://ers.amedd.army.mil for each unique award.*

- **QUAD CHARTS:** *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

8. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report.* ***DO NOT RENUMBER PAGES IN THE APPENDICES.***

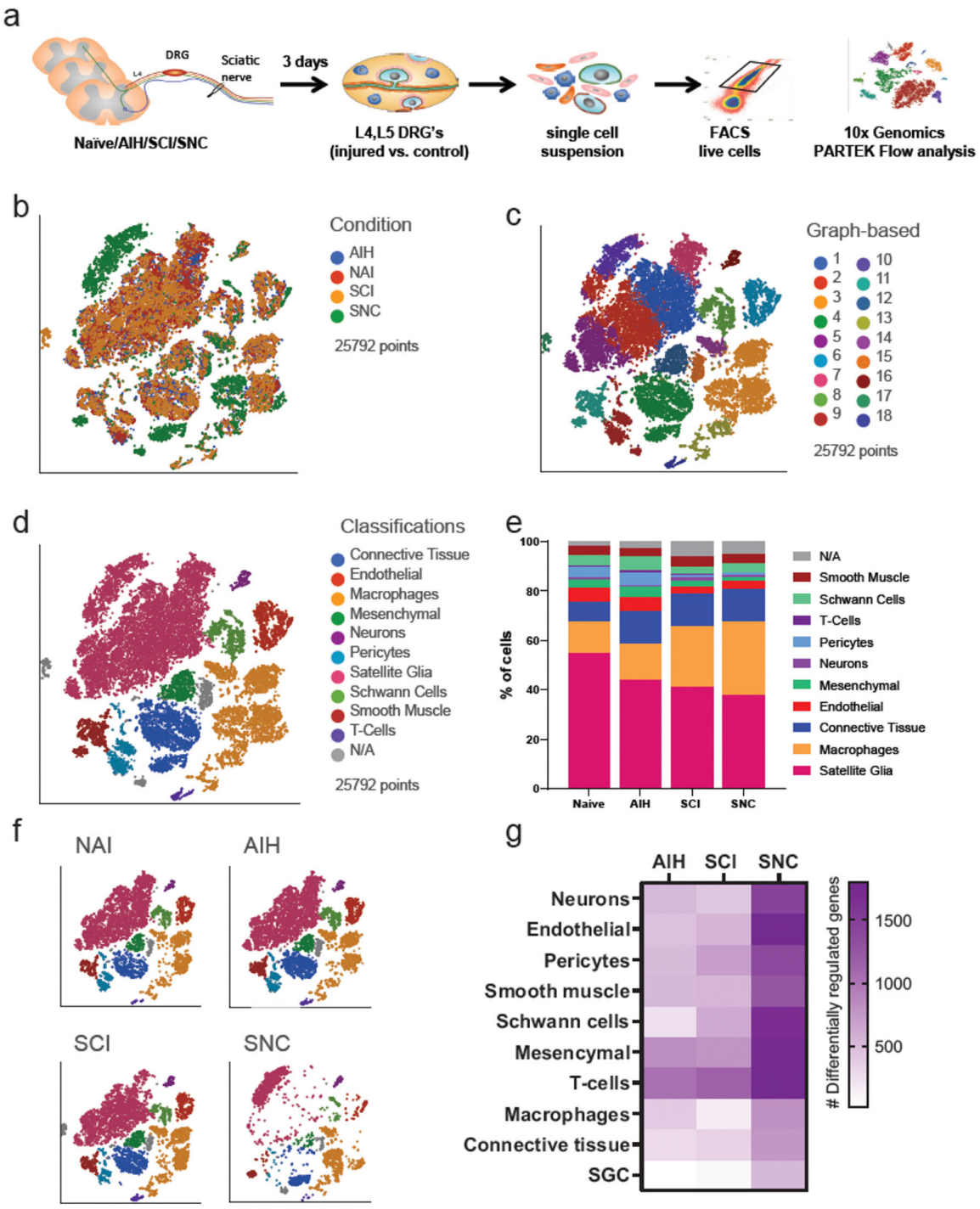

Figure 1. Characterization of cell populations in DRG after AIH, SCI and SNC. a Schematic of the experimental design for scRNAseq. **b,c,d** t-SNE plot of 25,792 cells from L4, L5 mouse dissociated DRG, with 18 distinct cell clusters. Classifications were assigned based on known marker genes or by unbiased, graph based clustering. **e** Fraction of each cell type within control and injury conditions. n = 2 biologically independent experiments. **f.** t-SNE plots of DRG cells separated by the different injury conditions, colored by cell type. **g.** Heatmap of the number of differentially regulated genes in each cell type and injury condition (p-value<.05, Fold change>2)

Cavalli, Valeria

Active Other Support

R01 NS096034 (Cavalli) 8/15/2016-5/31/2021 3
National Institutes of Health calendar
Mechanisms of Chromatin Remodeling Promoting Axon Regeneration
The major goal is to uncover the specific roles of hypoxia-inducible factor 1a in chromatin remodeling in injured neurons and test the hypothesis that acute intermittent hypoxia can recapitulate, at least in part, the changes in histone acetylation elicited by injury to activate a pro-regenerative program.

R01 NS111719 (Cavalli) 12/1/2019-11/30/2024 2.4
National Institutes of Health calendar
Functional role of satellite glial cells in axon regeneration
The major goal is to determining the molecular signature of satellite glial cells and to uncover the mechanisms by which they control nerve repair

not assigned (Cavalli) 1/1/2020-12/31/2022 0.6
Research to Prevent Blindness calendar
Identifying neuronal and glial mechanisms to promote RGC growth and survival
The major goal of this project is to identify compounds that increase survival and regeneration of RGC

R01 NS115960 (Wood) 4/1/2020-3/31/2025 0.48
National Institutes of Health calendar
T Cells Roles in Regeneration Across Nerve Graft Alternatives
The purpose of this project is to determine how T cells affect nerve regeneration across nerve autograft alternatives, such as acellular scaffolds, by identifying their role in inflammation and subsequent regeneration.

R21 NS115492 (Cavalli) 8/1/2020-7/31/2022 1.2
National Institutes of Health calendar
Unraveling the transcriptional response of sensory neurons to spinal cord injury at the single cell level
The major goal of this project is to unravel how different sensory neuron subtypes and non-neuronal cells respond to SCI, which may lead to new therapeutic targets to promote axon regeneration after SCI.

Overlap

None

Increasing sensory neuron regeneration in the injured spinal cord with acute intermittent hypoxia

Federal Domestic Assistance Number: 12.420; Applied Research Award

Funding Opportunity Number: W81XWH1810729

PI: Valeria Cavalli, PhD **Org:** Washington University (St. Louis, MO) **Award Amount:** $724,146 (total costs)

Study Aims

To determine if AIH in combination with strategies to neutralize the inhibitory environment can enhance sensory axon regeneration and functional recovery in rodents model of spinal cord injury

1.Determine if AIH mimics the epigenomic changes elicited by peripheral injury, even in the context of central projection injury

2.Determine if AIH stimulates axon regeneration following SCI

3. Determine if AIH combined with modulation of the CSPG receptor PTPsigma have synergetic effects on functional recovery following SCI

Approach

Using a mouse spinal cord injury model, the objective of our study is to determine if AIH can affect the epigenomic state of sensory neurons and determine the effectiveness of AIH combined with systemic modulation of the proteoglycan receptor PTPsigma with ISP improve functional recovery following contusive SCI, the most common type and extensively used model in SCI research

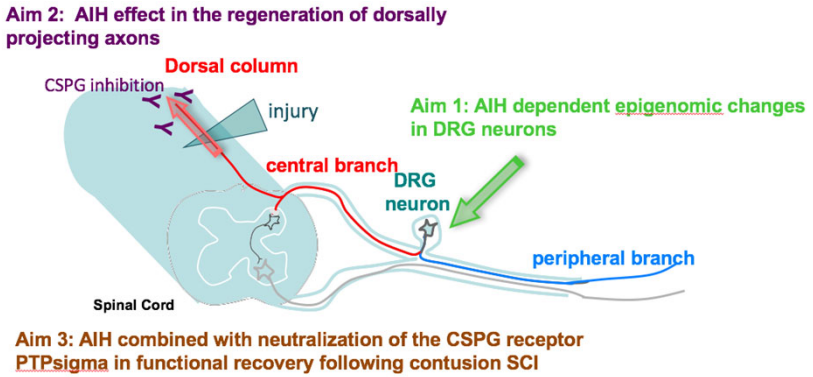

Figure representing the three aims to be tested using DRG neurons.

Accomplishments: We discovered that AIH encourage peripheral neurons to regenerate and that this depends on a factor called Hypoxia Induced Factor 1a (HIF-1α). In this proposal, we will test the genetic and epigenetic mechanisms by which this happens. We will also test whether AIH helps DRG neurons to regrow their axon in the injured spinal cord and improve recovery of function when paired with neutralization of the CSPG receptor PTPsigma with ISP peptide.

Timeline and Cost

Activities	Federal FY	19	20	21
Specific Aim 1				
Specific Aim 2				
Specific Aim 3				
Est. Budget (Direct)		**$166K**	**$166K**	**$166K**

Goals/Milestones

FY19 Goals

- ❑ Generate the mice needed for epigenomic studies:
- ❑ Determine the transcriptional profile elicited by AIH and peripheral nerve injury
- ❑ Determine the state of chromatin condensation genome wide elicited by AIH and nerve injury
- ❑ Test candidate gene expression for their dependence on neuronal HIF-1α

FY20 Goals

- ❑ Determine if AIH promotes regeneration of sensory axons in the injured spinal cord.
- ❑ Determine if promotes growth cone formation
- ❑ Ensure that AIH does not elicit adverse effects

FY21 Goals

- ❑ Determine if AIH combined with modulation of the CSPG receptor PTPsigma have synergetic effects on functional recovery following SCI usng the (BBB) locomotor rating scale
- ❑ Evaluate functional recovery on a horizontal ladder/grid walk task

❑ **Comments/Challenges/Issues/Concerns**

- None

Budget Expenditure to Date (Direct costs)

Projected Expenditure: $280,786.68 (Direct)

Actual Expenditure: $280,786.68 (Direct)