Award Number:	W81XWH-18-1-0729
Log Number:	SC170046
Project Title:	Increasing sensory neuron regeneration in the injured spinal cord with acute intermittent hypoxia
Principal Investigator Name:	Valeria Cavalli
Organization	Washington University in St Louis St Louis
Report Date:	OCTOBER 2019
TYPE OF REPORT	ANNUAL

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

DISTRIBUTION STATEMENT: APPROVED FOR PUBLIC RELEASE; DISTRIBUTION UNLIMITED

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
OCTOBER 2019	ANNUAL	15SEP2018 - 14SEP2019

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)

Valeria Cavalli

E-Mail: cavalli@wustl.edu

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	11	19b. TELEPHONE NUMBER *(include area code)*

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 is to be completed between month 0 and 20. We are at 40% completion
  - **Major Task 2: Determine if AIH promotes regeneration of sensory axons in the injured spinal cord**
    This task is to be completed between month 18 and 36. We are 30% completion
  - **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. We are 0% completion

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

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

Major activities: In the current reporting period, we continued our work on Major Task 1, Subtask 1. The major goal is to determine if AIH mimics the epigenomic changes elicited by peripheral injury, even in the context of central projection injury.

Specific Objectives:

We have continued our bioinformatic analysis of the single cell RNAseq data to determine the effects of sciatic nerve crush (SNC), 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). We previously used Partek flow for gene expression and Metacore for GO pathway analysis. Comparisons were made for either all up- or all down-regulated genes in any given condition followed by cross-referencing enriched KEGG pathways across conditions (Figure 1-3, red and green text). We found that while each condition (SNC, SCI, AIH) induced up- and down-regulation of many genes in neurons, only SNC induces changes in large numbers of genes

in satellite glial cells and macrophages (Figure 1-3). This suggested a limited impact of AIH on non-neuronal cells within the DRG compared to neurons. Most notably, HIF1-alpha signaling pathway was enriched for genes upregulated in neurons after SNC and AIH, but not SCI. Another significant overlap between AIH and SNC was observed in genes downregulated in macrophages, which were related to IL-17,TNF, and NF-kappa B signaling; cytokine-cytokine receptor interaction pathways. Despite differences in the overall number and identity of the individual genes altered by SNC and AIH, gene ontology indicates many shared gene pathways. It is thus possible that AIH promotes axon regeneration by altering different genes than SNC, but that these genes are part of similar pathways that converge on common downstream targets to promote axon growth.

We have performed additional bioinformatic analysis to better understand the gene pathways uniquely altered by SNC, SCI, and AIH.

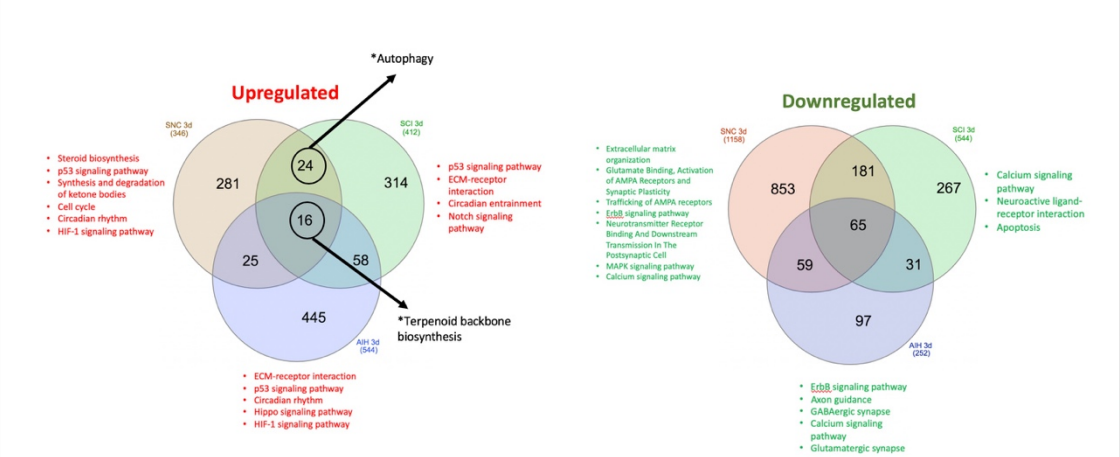

Figure 1: Gene expression analysis in Neurons

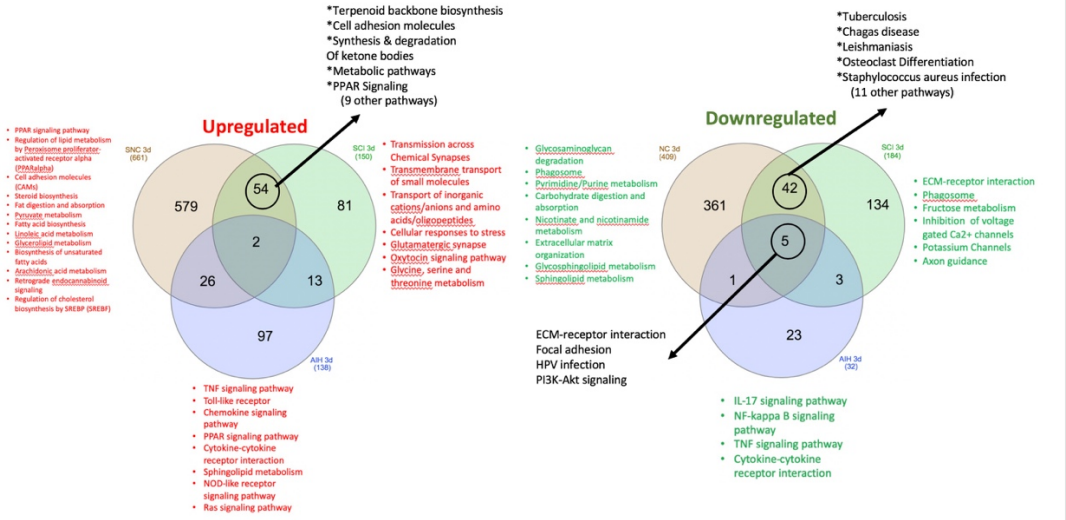

Figure 2: Gene expression analysis in Satellite Glial cells

To achieve this goal, we first clustered gene changes across all conditions (e.g., whether they are in 1, 2, or all 3 conditions for neurons, satellite glia, and macrophages), and then performing KEGG pathway analysis on the unique clusters and clusters shared between two conditions using the STRING software for genes having an FDR < 0.05 and at least a 50% increase or decrease in fold change from uninjured control (Figure 1-3). We used STRING because we no longer have access to Metacore. One of the most notable finding from this analysis is that SNC leads to many uniquely

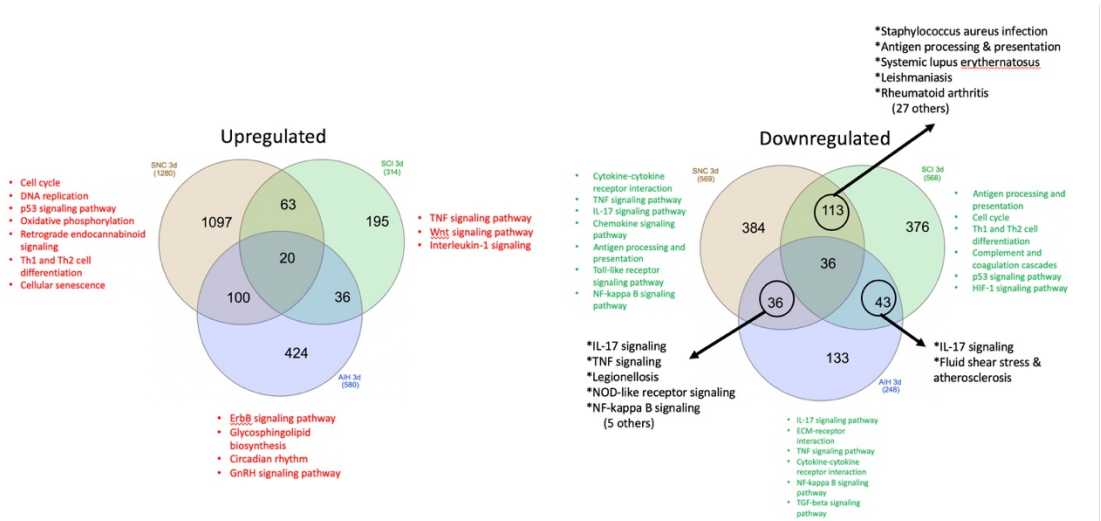

Figure 3: Gene expression analysis in macrophages

differentially expressed genes compared to SCI or AIH, and that for these specific genes many significantly enriched pathways exist for both up- and down-regulated genes in all cell types (neurons, satellite glia, macrophages). In contrast, genes changed uniquely by SCI alone lead to almost no uniquely enriched pathways, while genes unique to AIH are enriched primarily for genes downregulated in satellite glia and macrophages. This does not mean that SCI and AIH elicit no enriched pathways, as indeed we see that the pathways enriched by SCI and AIH share some genes that are also differentially expressed by SNC (Figure 1-3). Our previous analysis indicated Il-17, TNF, and NF-kappa B signaling in macrophages to be particularly shared between SNC and AIH and this finding holds true in this more stringent analysis. Given the known role of macrophages in axon regeneration, it will be interesting to investigate whether AIH enhances axon regeneration via macrophages.

One limitation of the single cell RNAseq analysis is the relatively low number of neurons recovered. We believe this is due to the large size of neurons which limits their capacity to be captured by the 10x Chromium platform. To address this problem we will perform single nuclei RNAseq to ask how SNC, AIH and SCI impact transcription in neurons specifically. We characterized a novel cre line (BAF-53b-cre) in the context of other ongoing projects in our laboratory. This mouse line drives cre-expression under the BAF-53b promoter, a pan-neuronal gene. We observed that when crossed with the Sun1:GFP mouse line, which express GFP in nuclei in a cre-dependent manner, nuclear GFP was specifically detected in neurons within the DRG (Figure 4). We will use this line to reveal the transcriptional changes induced by AIH in neurons and move on to subtask 2 to elucidate the state of chromatin condensation genome wide using ATAC-seq.

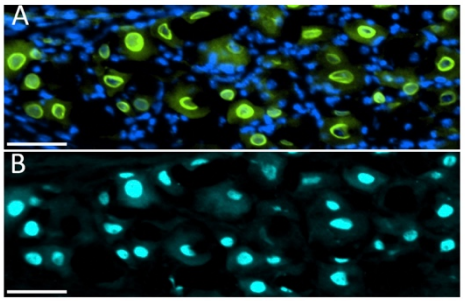

Figure 4: BAF-53b-Cre;Sun1:GFP mice express GFP specifically in neurons within the dorsal root ganglion (DRG). A,B) Representative image of L4 DRG in BAF-53b-Cre;Sun1:GFP mice. **A**) Nuclei of all cells are labeled with DAPI (blue) while those expressing GFP are shown in green. **B**) Nuclei labeled with the neuronal specific marker Islet-1. Note that GFP expression (**A**) is limited to neurons labeled with Islet-1 (**B**). Scale Bar = 50um

Description of the methodology:

Single-cell isolation for scRNAseq using the Chromium Single Cell Gene Expression Solution (10x Genomics): Freshly dissected L4 mouse DRG were isolated in Hanks' balanced salt solution (HBSS, Thermo Fisher) with 10 mM HEPES (HBSS-H). DRG were then digested with papain (15 U/ml, Worthington Biochemical) and collagenase (1.5 mg/ml, Sigma-Aldrich) at 37°C.Enzymatic digestion was halted by diluting the cell suspension with Hank's Balanced Salt Solution. DRG were dissociated by trituration, following centrifugation (800 rpm for 5 min) and removal of the supernatant. The cell pellet was resuspended in HBSS containing 0.01% BSA and passed through a 70 µm cell strainer (BD Biosciences). Hoechst 33258 was added in order to exclude dead cells. The Hoechst positive cells subsequently FACS purified using a MoFlo XDP cell sorter (Beckmann Coulter, Siteman Cancer center, WashU). Cells were then resuspended in PBS containing 0.01% BSA, counted with a hemocytometer, and the solution was adjusted to a final concentration of 500 cells/ml and run through the Chromium Single Cell Gene Expression Solution(10x Genomics, GTAC, WashU). Differentially expressed gene lists were generated using Partek Flow software. GO analysis was performed using Metacore software. For uniquely expressed genes, Venn Diagrams were generated and KEGG Pathway Analysis performed using STRING software for genes whose differential expression overlapped between treatment conditions. Genes having an FDR < 0.05 and at least a 50% increase or decrease in fold change from uninjured control were used for this analysis

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 instead are also typically influenced by SNC. SNC and AIH elicit similar response in macrophages, with downregulated genes related to Il-17, TNF, and NF-kappa B signaling. We also characterized a novel cre line that will allow us to study the neuronal responses to SNC, AIH and SCI.

Other achievements: n/a

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

Major activities: We found that AIH alone does not stimulate axon regeneration after SCI. Since combining approaches to both neutralize the inhibitory spinal environment with approaches to boost the intrinsic neuronal growth capacity provide greater regeneration, we tested if the combination of AIH with intrathecal ChABC injection enhances axon regeneration of ascending dorsal column DRG sensory neurons.

Specific Objectives: We determined if AIH can enhance axon regeneration after SCI, as described in Subtask 1. We found that AIH alone did not have significant effects in promoting axon regeneration 15 days post spinal cord injury. We performed a combinatorial approach and treated mice with AIH and chABC to reduce inhibition from the environment. We observed a decrease in axon retraction in the group treated with AIH and chABC compared to control, but no significantly enhanced regeneration beyond the injury site (Figure 5). We are currently performing additional histological assessments to confirm the effectiveness of ChABC to digest CSPG, and pursuing other possible endpoints beyond regeneration, including plasticity and sprouting of 5HT fibers, as this has been associated with functional improvements with other treatments.
Description of the methodology:

Mice received a T9 dorsal column hemisection SCI. ChABC was administered intrathecally via lumbar puncture immediately after SCI and every other day for 10 days. AIH (alternating 5 min of 8% oxygen with 21% oxygen for 2 hours) was administered 2 hours after SCI and daily for the next 7 days. On day 12 mice received an injection of 10% Dextran-Texas Red (2ul) into the sciatic nerve to trace ascending sensory axons in the dorsal column. Tissue was collected 3 days later (day 15) and histology performed to quantify distance of the longest axons from the injury site (+/-) by a blinded experimenter.

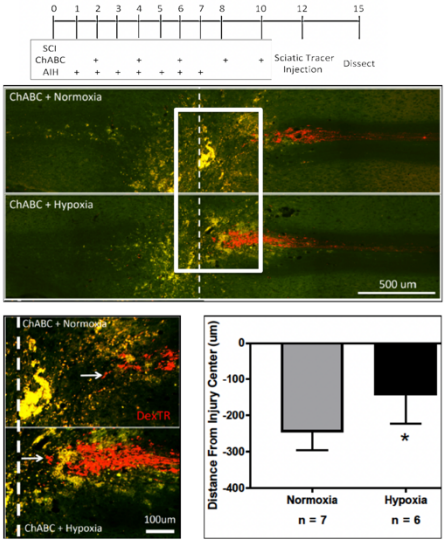

Figure 5: AIH in combination with ChABC reduces dorsal column axon retraction after SCI. We performed a combinatorial approach and treated mice with AIH and chABC to reduce inhibition in at the injuyr site. Mice received a T9 dorsal column hemisection SCI. ChABC was administered intrathecally via lumbar puncture immediately after SCI and every other day for 10 days. AIH (alternating 5 min of 8% oxygen with 21% oxygen for 2 hours) was administered 2 hours after SCI and daily for the next 7 days. On day 12 mice received an injection of 10% Dextran-Texas Red (2μl) into the sciatic nerve to trace ascending sensory axons in the dorsal column. Tissue was collected 3 days later (day 15) and histology performed to quantify distance of the longest axons from the injury site (+/-) by a blinded experimenter. We observed a decrease in axon retraction in the group treated with AIH and chABC compared to control, but no significantly enhanced regeneration beyond the injury site.

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?**

Major Task1: We will finalize 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, respond to AIH as compared to nerve injury and SCI. We have noticed that different software platform lead to different results with respect to GO pathway enrichment. We will use a third platform, (X2K) and focus on the pathways that are shared between the three analysis pipelines to improve rigor and robustness of the analysis. We will also expand the mice colony to generate sufficient Bfa53bcre;Sun1GFP mice to enrich neuronal nuclei via FACS and perform RNAseq as well as ATAC-seq. This will allow us to characterize the epigenomic response to SCI and AIH in all neuronal subtypes of DRG neurons and at sufficient numbers for appropriate statistical analysis.

Major Task 2: We found that AIH in combination with ChABC does not enhance axon regeneration of dorsal column axons. However, this does not exclude the possibility that AIH in combination with chABC had effects on the plasticity of other neuronal subtypes, as suggested by a previously published report. We will perform additional histological assessments to confirm the effectiveness of ChABC to digest CSPG, and pursue other possible endpoints beyond regeneration, including plasticity and sprouting of 5HT fibers, as this has been associated with functional improvements with other treatments.

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**
     - *Nothing to Report*
   - **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*

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?**

Name:	*Eric Ewan*
Project Role:	*Staff Scientist*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*7*

Name:	*Kathy Leahy*
Project Role:	*Lab Supervisor*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*2*

Name:	*Youngmi Oh*
Project Role:	*Staff Scientist*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*5*

Name:	*Marcus Mahar*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*0000-0003-3243-5052*
Nearest person month worked:	*5*

Name:	*Valeria Cavalli*
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	*0000-0001-9978-050X*
Nearest person month worked:	*2*

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

- *The following awards were listed as active, but are now closed:*

16-606 (Cavalli) 3/1/16-02/28/18 1.2 cm
University of Missouri System (SCIDRP) (anticipated direct costs over 2 years)
Using Acute Intermittent Hypoxia to Stimulate Axon Regeneration in the Injured Spinal Cord

R21 NS099603 (Cavallli) 7/15/2017-6/30/2019 0.6 cm
NIH-NINDS (anticipated direct cost over 2 years)
Elucidating the role of neuronal mTOR signaling in Schwann cell development

- *The following award was listed as pending, but is now active:*

R21EY029077 (Cavalli) 04/01/2018-3/31/2020 0.6 cm
National Institutes of Health (anticipated direct cost over 2 years)
Identifying epigenetic modifiers promoting optic nerve regeneration

- **What other organizations were involved as partners?**
  - *Nothing to Report*

7. **SPECIAL REPORTING REQUIREMENTS**
   - **COLLABORATIVE AWARDS:**
   - **QUAD CHARTS:**
8. **APPENDICES:**

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

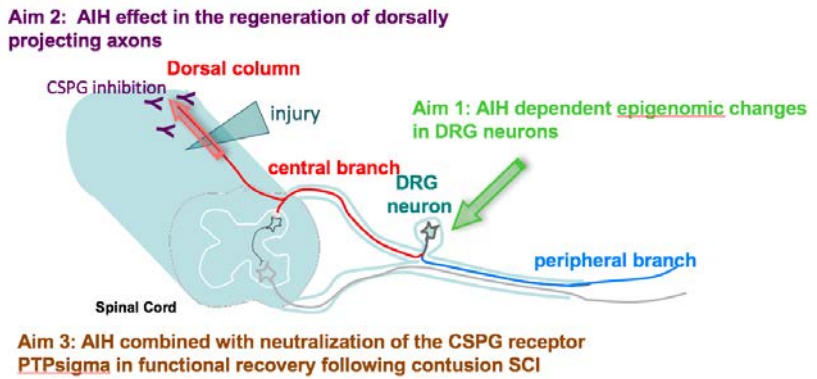

Figure representing the three aims to be tested using DRG neurons.

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: $166,666 (Direct)
Actual Expenditure: $166,666 (Direct)