Sustained Peripheral Nerve Injury in Military Relevant Operational Environment: Role of Mitochondrial Function

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Introduction

- Traumatic peripheral nerve injuries (PNI) are caused by various injuries
  - Workplace accidents & car accidents
  - Combat-sustained injured[2,3]
- PNIs share a similar pathophysiological, inflammatory, and degenerative response[4].
- Due to concomitant injuries, PNIs are often overlooked in favor of treating life-threatening injuries, leading to a delay in diagnosis & worse outcomes[3].

Peripheral Nerve Anatomy

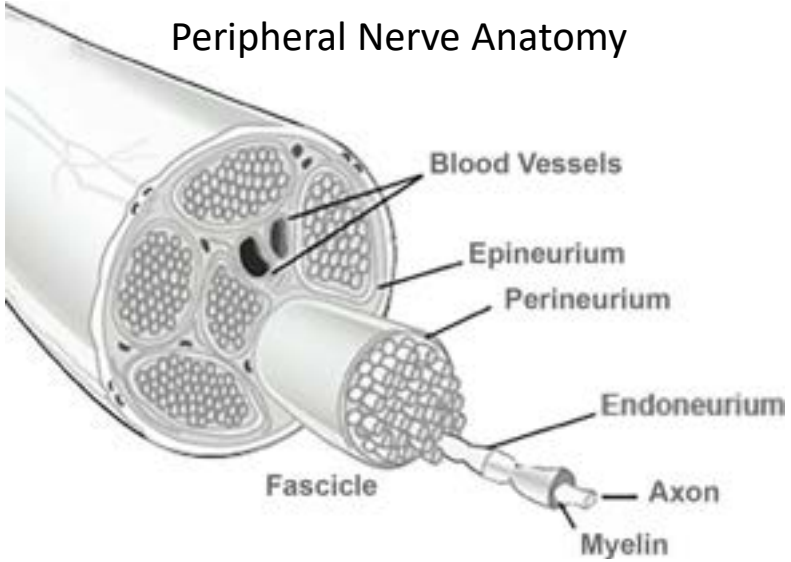
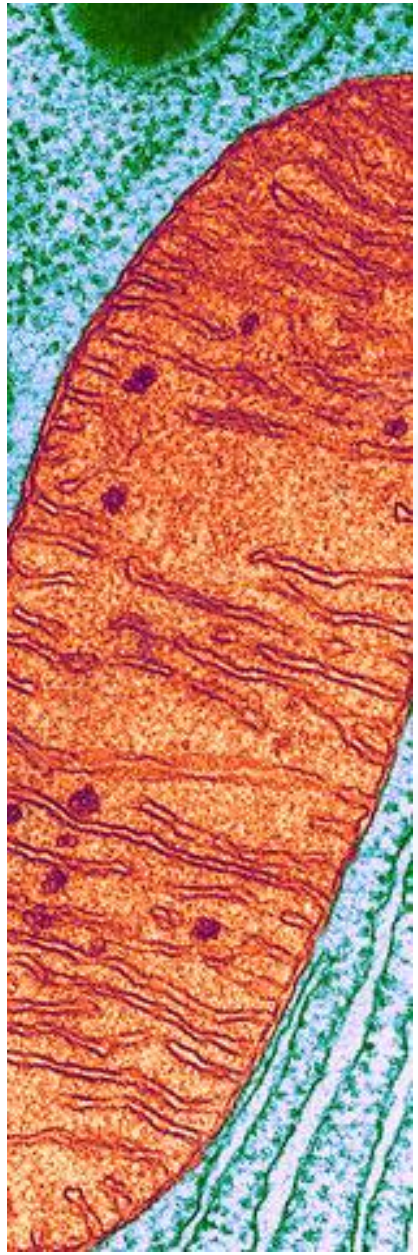


Figure 1: Adapted from Pedrosa et al., 2017.

- There are often deficits in sensory recovery, leading to impaired sensation and motor function:
  - Weakness, numbness, partial paralysis, sensorimotor deficits, and chronic neuropathic pain[5,6]
- Different types of PNI: nerve crush & nerve transection
  - Crush injury: acute compression of the nerve results in axon damage & intact endoneurium[1]
  - Transection injury: the complete severing of the nerve, including the axons, blood vessels, & connective tissues
- Ballistic & combat-sustained injuries often involve a combination of a crush and transection PNIs[7]
- Complete recovery requires reinnervation of muscle and other target tissues, coupled with synaptic connections in the spinal cord and proper spinal circuit function.
  - This process is often incomplete, preventing recovery of crucial reflexes[8]
- Previous studies have identified some aspects of recovery in peripheral nerves; however, knowledge regarding the cellular response of sensory neurons to injury and the mechanism of recovery and reinnervation are largely unknown.

Role of Mitochondria in PNI



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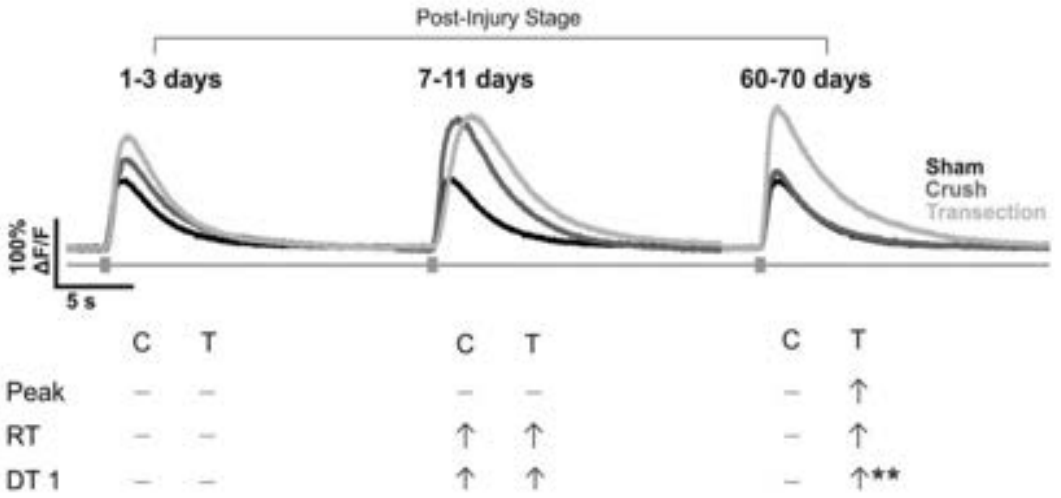
- Mitochondrial dysfunction has been observed in various peripheral neuropathies:
  - Trauma-induced painful neuropathy[9]
  - HIV-associated sensory neuropathy[10]
  - Diabetic peripheral neuropathy[11]
  - Neuropathy due to chemotherapy[12]
- Previous studies have hinted at the importance of mitochondrial dynamics in axonal integrity and regeneration in injured axons[13].
- Additionally, model mice with neurodegenerative diseases display irregular mitochondria shape and distribution in their axons[14-16].
- In addition to being the main source of energy production in the cell, mitochondria also play a major role in regulating intracellular Ca^{2+}. Therefore, it is likely that mitochondria play a role in sensory neuron recovery after injury.
  - Normally, mitochondria can modulate Ca^{2+} to fine-tune cellular energetics and buffer calcium released from the ER[17,18].
  - During mitochondrial dysfunction, there is a ↓ in ATP synthase activity, ↓ in ETC activity, and an ↑ in futile proton cycling[9].
    - Leads to a ↑ in ROS and cytosolic Ca^{2+} imbalances

Altered Calcium after PNI

Calcium transient: a rapid rise in intracellular calcium followed by a slow decay back to resting calcium levels.

- Researchers have previously identified changes to Ca^{2+} handling in DRG sensory neurons as a hallmark of PNI response.
  - Diminished resting intracellular Ca^{2+} levels[19,20]
  - Decreased Ca^{2+} transporter activity[21]
- Our lab has also identified differences in Ca^{2+} signaling after SNC and SNT injury[22].
  - 2 Days post-injury: Ca^{2+} transients from injured animals after crush and transection injury control (sham).
  - 10 Days post-injury: rise and decay times are significantly longer in the injured animals.
  - 60 Days post-injury: crush Ca^{2+} transients returned to normal, while transection calcium transients remained elevated.



Adapted from Walters et al., 2021. Summary of changes to Ca^{2+} transients after injury over time. Representative Ca^{2+} transients at three key postinjury time points for each injury type are shown at the top. Gray box on gray bar represents the electrical stimulus used to evoke transients. Transients from crush injury are similar to sham groups, whereas transients from mice with transection injury are larger than crush and sham groups. C, Crush; T, Transection; RT, rise time; DT1, decay time 1

- During episodes of elevated intracellular Ca^{2+}, calcium can activate multiple-second messenger cascades and alter gene transcription.
  - Any alterations in cellular Ca^{2+} homeostasis after injury can profoundly affect cellular function and recovery.
- However, what cellular changes contribute to this altered Ca^{2+} handling is unknown.
- Since mitochondria are a major contributor to Ca^{2+} homeostasis & energy production, it is likely that mitochondria play a significant role in sensory neuron recovery after injury.

Project Overview

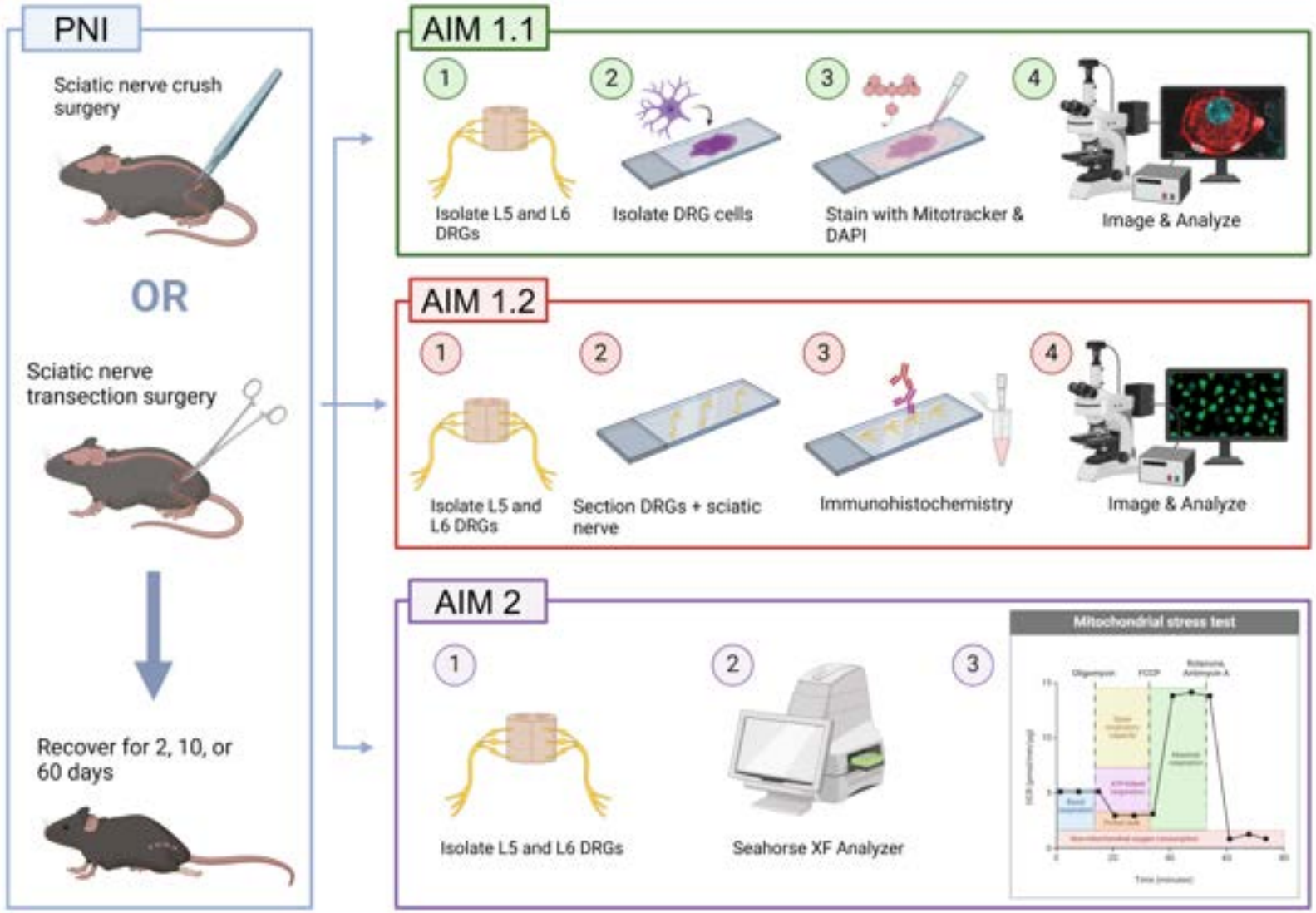


Figure 1: Summary of the experimental design. Created in https://BioRender.com

Objectives:

- Discover molecular mechanisms of mitochondrial dysfunction in the context of sensory neuron recovery after peripheral nerve injury.
  - Measure mitochondrial metabolism and characterize changes in mitochondria morphology indicative of metabolic dysfunction after PNI.
- Apply this data about mitochondrial function and use it to inform modeling studies of cellular responses to injury and other stressors such as fatigue or toxic exposures.

Central Hypothesis: Crush and transection PNI will result in altered mitochondria morphology and impaired mitochondrial function in DRG sensory neurons. In accordance with the observed differences in Ca^{2+} transients, we predict that the transection-injured neurons will display a larger degree of mitochondrial dysfunction and altered mitochondrial morphology than the crush-injured neurons.

PNI: SNT vs SNC and their effects on DRG neurons

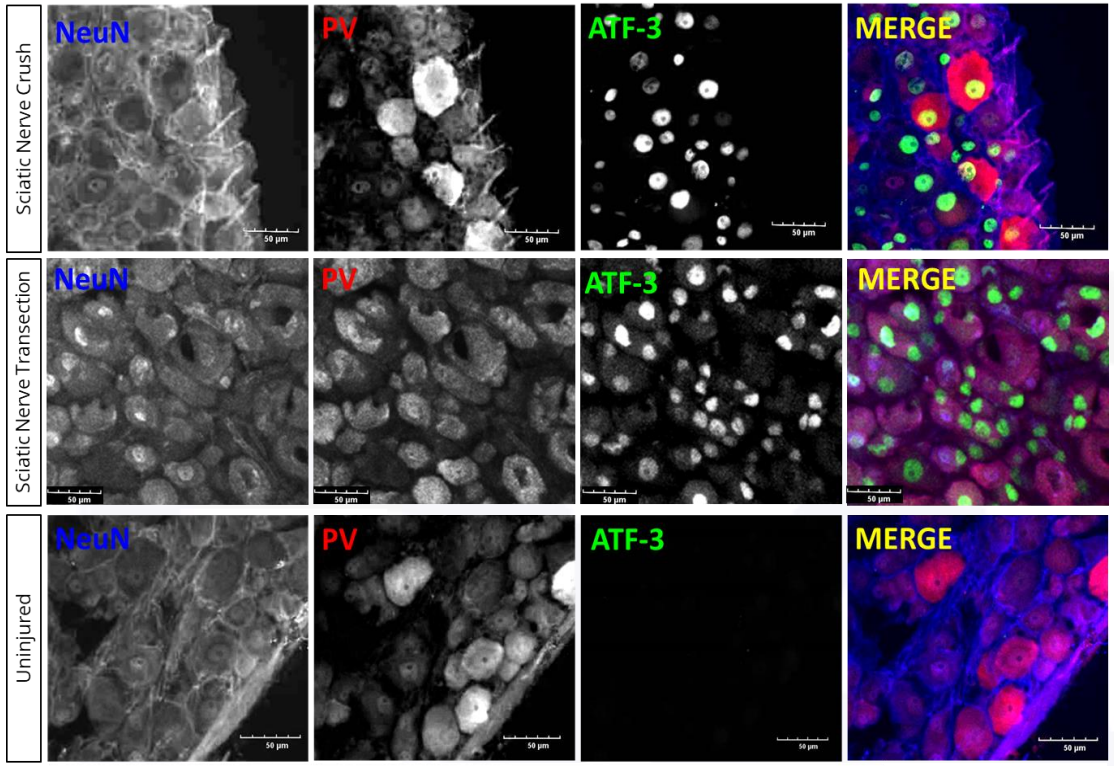


Figure 2: Top row: right L5 DRG 2 days post sciatic nerve crush. Middle row: L5 DRG 2 days post sciatic nerve transection. Bottom row: Uninjured L5 (control). Scale Bars = 50 µm. Property of the U.S. Airforce.

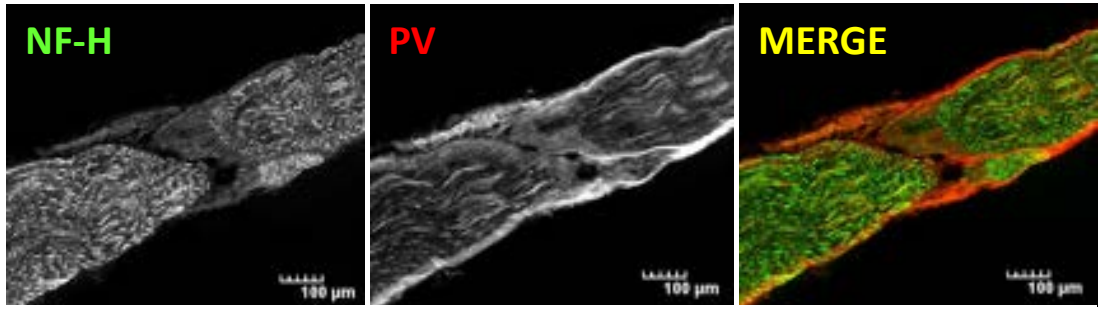


Figure 3: Injury site of the right sciatic nerve 2 days post sciatic crush PNI. Scale Bars = 100 µm. Property of the U.S. Airforce.

- To ensure neuronal damage after sciatic nerve crush injury, left and right L5 & L6 DRGs were sectioned and stained for ATF-3 (an early marker for neuronal damage), PV (a marker for proprioceptor neurons), and NeuN (a marker for neuronal nuclei).
  - Both SNC and SNT injured DRGs show increased neuronal damage due to an increase in ATF-3 expression (**Figure 2**).
- Sections of the sciatic nerve stained for NF-H (heavy chain neurofilament) and PV(parvalbumin) show the crush injury has disrupted the axons at the site of the injury (**Figure 3**).
- Recovery of DRG neurons after SNC (**Figure 4**):
  - Immunohistochemical stains on whole L5 DRGs 2 days and 10 days post-SNC confirm injury model.
  - DRGs undergo cellular changes after injury that continue & alter through 10 days after injury.

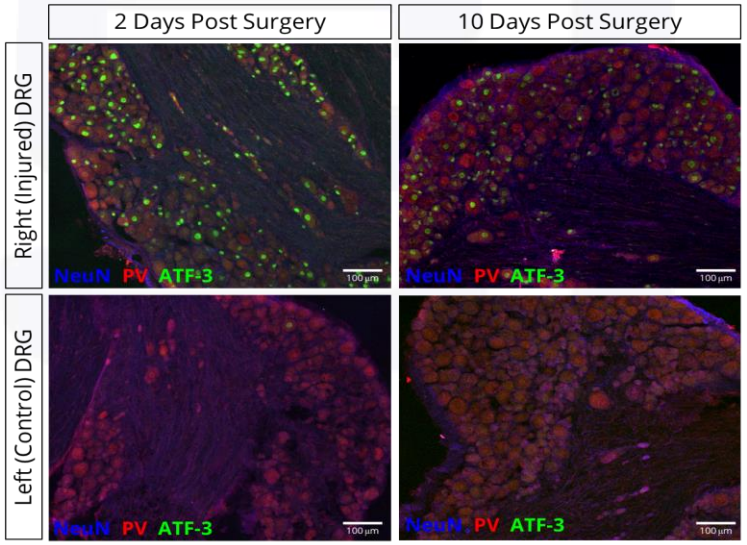


Figure 4: Injured (top) and uninjured (bottom) whole L5 DRG after a sciatic nerve crush (SNC) injury 2 days after surgery (left) or 10 days after surgery (right). Images were acquired using an FV3000 confocal microscope and stained for Parvalbumin (red), NeuN (blue), and ATF-3 (green). Scale Bars = 100 µm. Property of the U.S. Airforce.

Live DRG Cell Analysis

1. Wild type (C57BL/6NSD) mice of both sexes undergo either an SNC or an SNT surgery.

2. Mice recover for either 2 days, 10 days or 60 days before euthanasia.
   - Right and left L5 and L6 DRGs are isolated and cultured (live DRG cells are dissociated)

3. Stain DRG cells with:
   - MitoTracker Red FM (labels live mitochondria)
   - Hoechst dye (nucleic acid stain labels DNA – identify the nucleus)

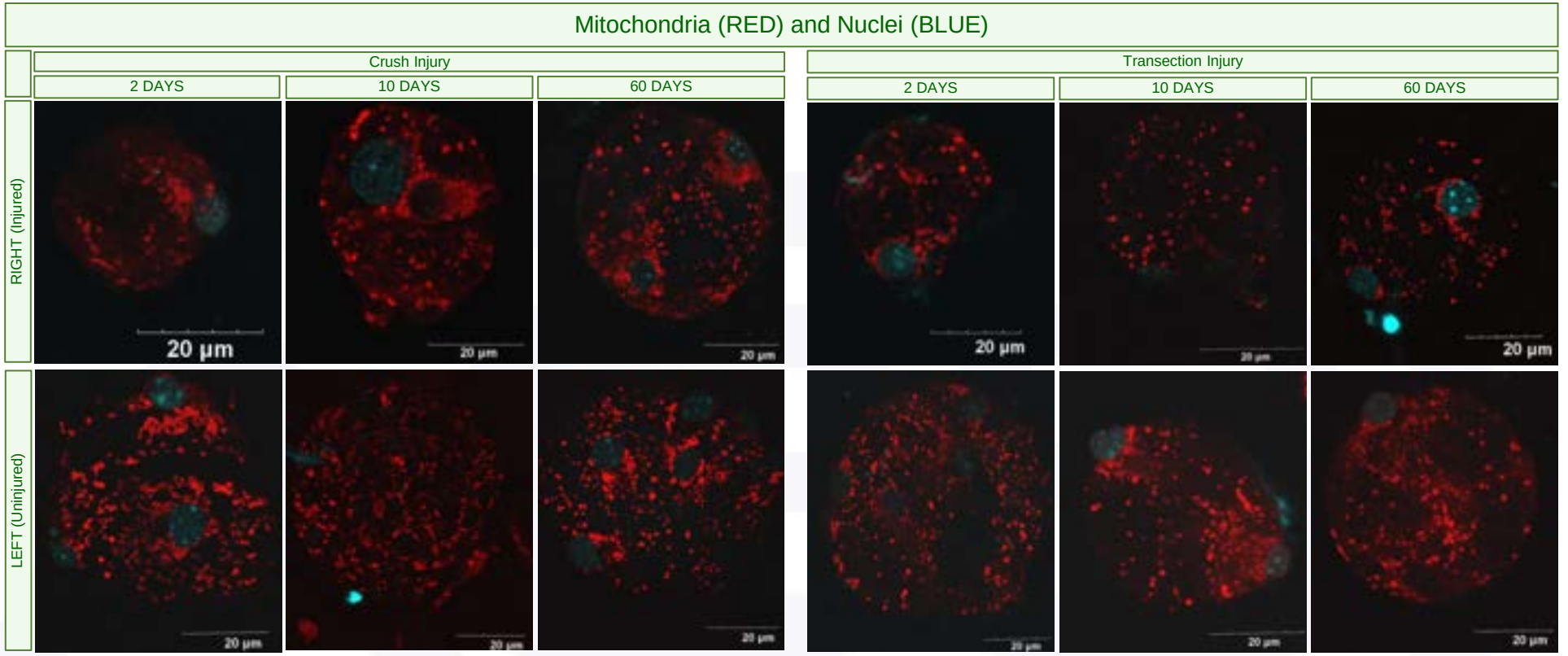


Figure 5: Injured and uninjured DRG cells after a sciatic nerve crush (SNC) injury or sciatic nerve transection (SNT) injury. Live cells were analyzed using FV3000 confocal microscope and stained with MitoTracker Red FM (red) and Hoechst dye (blue). Scale Bars = 10 µm. Property of the U.S. Airforce.

4. Confocal microscopy (FV3000) – 60X (**Figure 5**)

5. Images analyzed using Mitochondria Analyzer plug-in in ImageJ software.
   - 2D and 3D mitochondrial morphological analysis

Mitochondrial Morphological Alterations

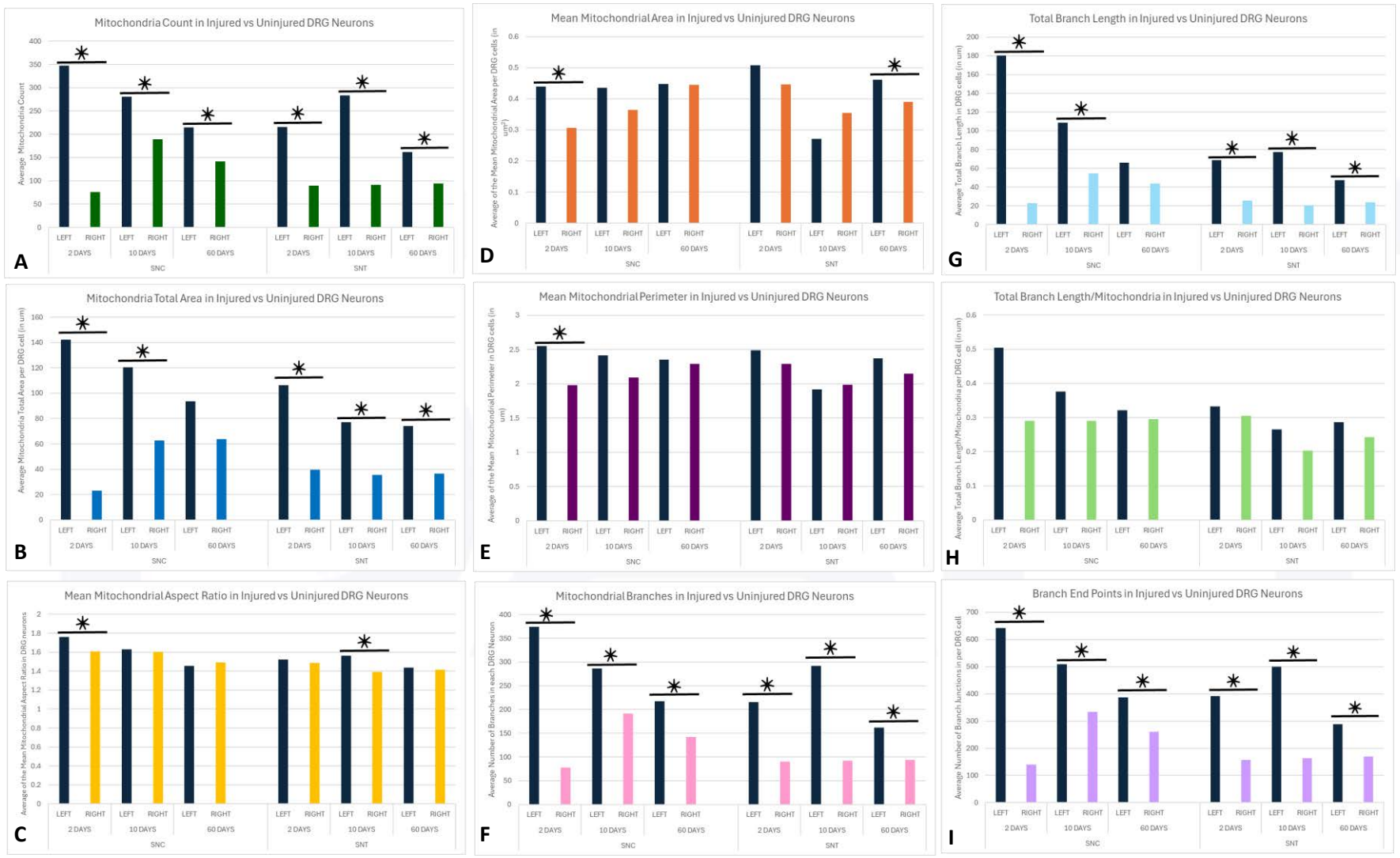


Figure 6: Mitochondrial morphological aspects in injured vs uninjured DRG neurons. * = statistical significance. Property of the U.S. Airforce.

- Nine different parameters are highlighted: mitochondrial count, total mitochondrial area, mean mitochondrial aspect ratio, mean mitochondria area, mean mitochondrial perimeter, mitochondrial branches, total branch length, total branch length/mitochondria, and branch end points (**Figure 6**).
  - Mitochondria in the right (injured) DRG cells are compared to mitochondria in the left (uninjured) DRG cells
- There were observed morphological differences in the mitochondria of injured vs uninjured DRG sensory neurons.
  - Time-point dependent (2 days, 10 days, or 60 days post-PNI)
  - Mitochondrial morphological alterations depend on type of injury (SNC vs SNT)

Conclusions & Future Steps

- Mitochondrial morphology is altered after PNI (both SNC & SNT)
  - Changes are dependent on the type of injury
  - Time-point dependent
- The functional experiments will reveal how mitochondria respond to injury and the role they play in recovery.
  - Gain insight into observed altered Ca^{2+} transients
- Post-hoc morphological study can elucidate mitochondria morphological changes in different cell types & along the sciatic nerve.
  - $Na_v1.8$ expressing cutaneous nociceptors
  - PV expressing proprioceptors
- Future experiments are necessary to uncover what is driving these observed changes in mitochondria.
  - If these alterations are necessary or inhibitory to the recovery process
  - If/how the promotion of mitochondrial morphological changes can aid in the recovery of sensory neurons after PNI
- Understanding the mechanisms that drive peripheral nerve recovery after injury is necessary for the development of **improved treatments and to prevent cognitive decline for service members with combat-sustained PNIs.**

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Disclaimer:

The study protocol was reviewed and approved by the Wright State University Institutional Animal Care and Use Committee and Air Force Medical Readiness Agency. Animals were handled, and studies were conducted, under a program of animal care accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and in accordance with the National Research Council's 2011 Guide for the Care and Use of Laboratory Animals (in compliance with Department of Defense Instruction 3216.1).

This study protocol was reviewed and approved by the Wright Patt Air Force Base Institute of Research Intuitional Animal Care and Use Committee (IACUC) and the U.S. Air Force Surgeon General's Office of Research Oversight and Compliance. The experiments in this report were conducted in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). All experiments were performed in compliance with the Animal Welfare Act and in accordance with the principles set forth in the "Guide for the Care and Use of Laboratory Animals".

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