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TITLE: Single Nucleus Expression Profiling of Human Sciatic Nerve After Traumatic Amputation: Predicting Pain and Functional Outcomes

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CONTRACTING ORGANIZATION: Duke University, Durham, NC

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14. ABSTRACT

A majority of service members who undergo traumatic amputation develop chronic phantom or chronic residual limb pain with 10-15% of these patients developing severe, disabling, long-term pain. 30-40% of traumatic amputees, however, have no clinically significant chronic pain. We believe this dichotomy of outcome is the key to understanding the development of chronic neuropathic pain after nerve injury. Preclinical studies using rodent models have provided some insights into the pathological sequelae of nerve injury, but this knowledge has not resulted in successful translation to the clinic. Recent evidence suggests that interspecies differences are a major barrier to successful translation, since rodent sensory neurons diverge considerably from their human counterparts. Accordingly, in order to better understand the pathological processes that lead to neuropathic pain after nerve injury, it is necessary to comprehensively study injured human nerves. Our colleagues at Walter Reed National Military Medical Center spent three years obtaining sciatic nerve samples from service members undergoing primary amputation revision surgery after suffering traumatic amputation on the battlefield. These unique samples allow, for the first time, study of nerve regeneration and neuroinflammation in humans during the days following traumatic amputation. Utilizing bulk tissue and single nuclei RNA-sequencing and unbiased global proteomics of the distal portion of sciatic nerve collected 1-14 days after initial traumatic amputation, we aim to establish the distinctive transcriptional, protein and glial/immune cell profile of injured sciatic nerve during injury and regeneration.

15. SUBJECT TERMS

Transcriptomics, single nuclei transcriptomics, proeteomics, neuroinflammation, neuropathic pain, nerve regeneration, phantom limb pain, residual limb pain.

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INTRODUCTION:

A majority of service members who undergo traumatic amputation develop chronic phantom or chronic residual limb pain with 10-15% of these patients developing severe, disabling, long-term pain. 30-40% of traumatic amputees, however, have no clinically significant chronic pain. We believe this dichotomy of outcome is the key to understanding the development of chronic neuropathic pain after nerve injury. Preclinical studies using rodent models have provided some insights into the pathological sequelae of nerve injury, but this knowledge has not resulted in successful translation to the clinic. Recent evidence suggests that interspecies differences are a major barrier to successful translation, since rodent sensory neurons diverge considerably from their human counterparts. Accordingly, in order to better understand the pathological processes that lead to neuropathic pain after nerve injury, it is necessary to comprehensively study injured human nerves. Our colleagues at Walter Reed National Military Medical Center spent three years obtaining sciatic nerve samples from service members undergoing primary amputation revision surgery after suffering traumatic amputation on the battlefield. These unique samples allow, for the first time, study of nerve regeneration and neuroinflammation in humans during the days following traumatic amputation. Utilizing bulk tissue and single nuclei RNA-sequencing and unbiased global proteomics of the distal portion of sciatic nerve collected 1-14 days after initial traumatic amputation, we aim to establish the distinctive transcriptional, protein and glial/immune cell profile of injured sciatic nerve during injury and regeneration.

KEYWORDS:

Transcriptomics, single nuclei transcriptomics, proteomics, neuroinflammation, neuropathic pain, nerve regeneration, phantom limb pain, residual limb pain.

ACCOMPLISHMENTS:

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

- **Aim 1 - Perform bulk tissue and single nuclei RNA-sequencing and unbiased global proteomics of the proximal and distal portion of sciatic nerve to establish the distinctive transcriptional, protein and glial/immune cell profile of injured sciatic nerve during injury and regeneration.**

- Major Task 1: Amend existing USUHS IRB and obtain approval for transcriptomic work on sciatic nerve samples. **100% complete (Performed at both Duke and USUHS/DVCIPM)**

  - IRB approval of the sequencing amendment was obtained.

- Major Task 2: Obtain USAMRMC Office of Research Protections HRPO approval for use of sciatic nerve samples. **100% complete (Performed at Duke under Dr. Vandeven)**

  - Approved

- Major Task 3: Renew IRB exemption for work on deidentified nerve samples at Duke. **100% complete (Performed at Duke under Dr. Vandeven)**

  - This exemption no longer has an expiration date

- Major Task 4: Process each nerve, collect nuclei and bulk RNA. **100% complete (Performed at Duke under Dr. Vandeven) This task is where we have spent most of our time this year and some of the alterations in research strategy and the progress we've made are detailed below:**
    - Tissue Processing
        - For each nerve, we cut 1 cm off the distal and 1 cm off the proximal ends and embedded them in OCT for precision sectioning on cryostat. We decided against using a manual razor blade cutting strategy for the more precise and reproducible cryostat which allows cutting while sample is frozen and allows the production of thin slices for staining and RNAscope (an additional technique not included in the original research plan).
            - First, we cut 75 um sections and collected in tube A for RNA extraction
            - Second, we cut 75 um sections and collected in tube A for proteomics
            - Third, we cut 10 um sections (x5), and placed on individual slides.
            - Slides: 25 slides per patient; each slide will have one distal, one proximal section.
- Major Task 5: Create RNA libraries and complete sequencing. **50% complete (Performed at Duke under Dr. Vandeven)**
    - One of the three main tasks in aim 1 of this project was to collect RNA from single nuclei and sequence the RNA to determine what cell populations are present at the injured tip of the sciatic nerve. As we said in the application, we had worries that the RNA in the nuclei in these relatively old samples would not be of sufficient quality to obtain usable sequence data. Unfortunately, nucleic and bulk RNA quality was poor (please see slide 8 of attached powerpoint file). However, we did find that the RNA was of sufficient quality to perform RNAscope on thin slices of the distal nerve endings. This means we can use the nerves themselves to validate the proteomic findings. For example if we find expression of a certain immune mediator is increased in the bulk RNA sequence of a patient with severe chronic pain we can go back and perform RNAscope to determine directly if that RNA is present in the nerve cross section and where it is present.
- Major Task 6: Complete proteomics. **100% complete (Performed at Duke under Dr. Vandeven)**
    - All proteomic experiments have been completed and initial analysis done. Please see slides 1-3 of the attached powerpoint. We found a number of proteins differentially expressed in the nerves of patients who went on to develop chronic pain (NR or non-resolvers) vs those who didn't (R or resolvers). A number of the differentially expressed proteins are targets that we have been interested in from separate studies we have performed. Please see slide 4 on the attached powerpoint for details but briefly we are investigating the TGF-beta signaling pathway (R > NR), MANF (R > NR) and Gasdermins (GSDMD & GSDME; R > NR)

- Major Task 7: Develop an expression and cell signature of nerve regeneration over the two weeks following traumatic amputation. **25% complete**

    - Since RNA quality was poor this major task will be completed using protein expression results and two new additional procedures – cytokine and chemokine profiling (to validate the pathways found to be important in major task 6) and immunohistochemistry of various macrophage markers in slices of the distal tip of the nerves. We found significant macrophage invasion at the distal nerve tips after injury and we are now in the process of staining for various markers of macrophage phenotype to determine if phenotype plays a role in subsequent chronic pain development. So far we have preliminary evidence from a subset of nerves that there is increased levels of M2 macrophages in patients whose pain does not resolve (please see slide 6 from attached powerpoint).

- **Aim 2 - Complete a pain and functional outcome database describing each patient enrolled in the SEXI trial and use those outcomes to identify immune cell populations, gene and protein expression changes around the time of amputation that correlate with positive outcomes of good function and minimal residual limb or phantom pain.**

- Major Task 1: Amend existing USUHS IRB to collect physical function and more recent pain and analgesic medication data on the patients who previously donated sciatic nerve samples. **100% complete (performed by the USUHS/DVCIPM team under Dr. Buckenmaier)**

- Major Task 2: Update current clinical outcomes database of SEXI patients for most recent pain scores and medication use (60 total patients) **100% complete (performed by the USUHS/DVCIPM team under Dr. Buckenmaier)**

    - This database of pain scores, medication use, and psychiatric comorbdities is complete.

- Major Task 3: Add functional outcomes data to database. **25% complete**

    - Our USUHS colleagues have attempted to acquire physical function outcomes data using physical therapy notes and by searching for physical function questionnaires in patient records (such as Oswestry and Promis PF short forms). Unfortunately, few of these patients have standardized PF outcomes in their medical records. Our USUHS colleagues are now working with the physical therapy department to determine if there are other ways of extracting this data. Currently only about 10% of the soldiers and veterans who donated nerve tissue have physical function outcomes data in our database.

- Major Task 4: Correlate outcomes with expression signature **0% complete**

    - We will be able to perform this analysis now that proteomics work is complete

- **Aim 3 - Correlate perioperative ketamine use with immune cell population and gene and protein expression profile.**

- Major Task 1: Determine whether ketamine treatment produces improved functional outcomes. **100% complete**

    - Please see slide 8 from the attached powerpoint. There was no significant difference in protein expression at the distal nerve tip between patients treated with ketamine and those without ketamine.

- Major Task 2: Identify the protein and RNA expression signature in sciatic nerve unique to patients treated with perioperative ketamine. **100% complete**

- **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 reporting period all major tasks will be completed including correlating clinical outcomes (other than pain resolvers vs nonresolvers) outcomes with expression profiles.

IMPACT:

- **What was the impact on the development of the principal discipline(s) of the project?**
  - Nothing to Report yet
- **What was the impact on other disciplines?**
  - Nothing to Report yet
- **What was the impact on technology transfer?**
  - Nothing to report
- **What was the impact on society beyond science and technology?**
  - Nothing to report

CHANGES/PROBLEMS:

- **Changes in approach and reasons for change**
  - The main changesin approach is an increased reliance on proteomics, shift to RNAscope and addition of inflammatory mediator profiling and immunostaining for macrophage markers. These experimental design changes should allow us to identifiy the molecules and pathways responsible for resolution of pain even though RNA quality was too poor for adequate sequencing.
- **Actual or anticipated problems or delays and actions or plans to resolve them**
  - No further delays are expected. We have a new highly qualified research technician hired who is helping to move the project forward quickly. Physical function data will likely remain difficult to collect.
- **Changes that had a significant impact on expenditures**
  - none
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

- We have obtained HRPO approval for the project and also IRB approval at USUHS.

- **Significant changes in use or care of human subjects**
- **Significant changes in use or care of vertebrate animals.**
- **Significant changes in use of biohazards and/or select agents**

PRODUCTS:

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

Journal publications.

Nothing to Report yet

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

Nothing to report

Other publications, conference papers, and presentations.

Nothing to report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

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

Name:	*Thomas Van de Ven*
Project Role:	*Principal Investigator*
Researcher Identifier (eRA Comm	*THOMAS.VANDEVEN*
Nearest person month worked:	*5*
Contribution to Project:	*Coordinates all aspects of the project and assumes overall responsibility for its success.*
Funding Support:	*No other support*

Name:	*Chester Buckenmaier*

Project Role:	*Site Prinicipal Investigator*
Researcher Identifier (eRA Comm	cbuckenmaier
Nearest person month worked:	*5*
Contribution to Project:	*Coordinates IRB approval of study activities and collection of clinical data on enrolled subjects*
Funding Support:	

- **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?**

- **Organization Name:** Defense and Veterans Center for Integrative Pain Management (DVCIPM) and Uniform Services University of the Health Sciences (USUHS)
- **Location of Organization:** Bethesda, Maryland
- **Partner's contribution to the projec**t

Collaboration Dr Buckenmaier and the research staff at DVCIPM are responsible for IRB approval of this study and for collection of the clinical data needed to tie molecular changes in the sciatic nerve samples to important clinical functional and pain outcomes

SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:**
- **QUAD CHARTS:**

APPENDICES: