

APRA OVERVIEW AND COORDINATION

AWARD NUMBER: MR157005

TITLE: Applied Pain Research: Discovering Answers and Delivering Solutions for Service Members from the Battlefield through Recovery

CO-PRINCIPAL INVESTIGATORS: Robert Gibbons, Robert Christy, PhD; Kathy Ryan, PhD; MAJ Crimmins, PhD; Rasha Hammamieh, PhD

RECIPIENT: US Army Institute of Surgical Research

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

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14. ABSTRACT Combat injuries can result in severe acute pain, and options for pain control on the battlefield are currently limited. There is a need for improved pain control not only at point of injury but also at higher echelons of care. Initial pain control can increase patient comfort and aid in evacuation from the point of injury. The Battlefield Pain Management Research Task Area and United States Army Institute of Surgical Research (USAISR) is uniquely positioned within the Department of Defense (DoD) to accomplish the goals and objectives of this award. The diverse scientific expertise, extensive laboratories, clinical specialization, institutional resources and patient populations all combined make USAISR the place to center DoD Pain Research. This proposal, "Applied Pain Research: Discovering Answers and Delivering Solutions for Service Members from the Battlefield through Recovery," is led by the coordinating PI, COL Robert V. Gibbons, MD, MPH, FACP and is comprised of three primary projects and one alternate project that, when combined, address each of the focus areas of the Applied Pain Research Award. Completion of these projects will significantly advance the treatment of pain for the United States Service Member. The projects proposed will provide requirements-driven solutions for pain treatment in Service Members throughout the entire continuum of care and will serve as a capability resource for applied pain research for the DoD.					
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Final Report:

The Battlefield Pain Management Program at the US Army Institute of Surgical Research (USAISR) received APRA funding at the beginning of FY16. Thanks to this generous award, a number of advances have been made in the organization of the program that have allowed for significant progress toward meeting the goals of the APRA Grant. This program is currently led by the coordinating co-PIs, Robert Christy, PhD, MAJ Stephen Crimmins, PhD, and Kathy Ryan PhD. It is comprised of three primary projects and one programmatically complementary project that, when combined, address each of the focus areas listed within the original program announcement. Attached to this coordination report is a report from each of the 4 projects which details the progress that each has made during FY16-18.

At the USAISR, the APRA Grant effort is broken into 3 groups, each led by a PI who coordinates the effort for their project. Due to the departure of Dr. Fowler from ISR, MAJ Crimmins led Project #1, “Assessing the Effects of Novel Analgesics and Drug Delivery Mechanisms on Acute Pain.” Project #2, “Factors that Influence Long-Term Outcomes and Pain Chronicity,” is led by Dr. Bopaiah Cheppudira. Both of these projects are being performed under the auspices of the Battlefield Pain Task Area (BPTA), which is led by MAJ Stephen Crimmins. Project #3, “Effect of Pain and Analgesia on Physiological Compensatory Responses to Hemorrhage,” is led by Dr. Carmen Hinojosa-Laborde who is assigned to the Tactical Combat Casualty Care Research (TCCCR) Task Area managed by Dr. Kathy Ryan. The fourth project contained within the APRA grant “Molecular Mechanisms of Pain Perception Involving Key Brain Regions and Potential Surrogate Signatures in Blood: An Integrated –Omics Study” is led by Dr. Rasha Hammamieh from the US Army Center for Environmental Health Research (USACEHR). Drs. Christy, Crimmins, Ryan, and Hammamieh have long-standing collegial and industry relationships and are coordinating the efforts of their investigators. During this reporting period, each of the 4 groups has developed a working team to perform the research within each project, as detailed below:

- Project #1: MAJ Stephen Crimmins, PhD (PI)
Misty Malamakal, PhD (Postdoctoral Fellow)
David Trapolsi (Former Research Associate)
Roger Chavez (Research Associate)
Thomas Garza (Research Associate)
Alex Trevino (Research Associate)
- Project #2: Bopaiah Cheppudira, PhD (PI)
Natasha Sosanya, PhD (Postdoctoral Fellow)
Alex Trevino (Research Associate)
Sirima Tongkhuya (Former Research Associate)
- Project #3: Carmen Hinojosa-Laborde, PhD (PI)
Harold Klemcke, PhD (Senior ORISE Fellow)
Lusha Xiang, MD (ORISE Fellow)
Martha Avila (Research Associate)
Alfredo Calderon (Research Associate)
Mariam Calderon (Research Associate)

Project #4: Rasha Hammamieh, PhD (PI)
John Clifford, PhD
Raina Kumar, MS

In addition to these efforts, there are 2 lines of complementary efforts that are funded by the Task Areas involved. First, BPTA independently funds preclinical projects “Analgesic and Wound Healing Effects of Peripherally Administered Opioids,” and “Development of Biocompatible Dressings for the Delivery of Analgesics to Burn Wounds” (SBIR with KeraNetics). These efforts seek to explore the use of novel analgesics in preclinical models of pain and therefore complement the APRA-funded projects. The BPTA has additional personnel working on these non-APRA projects. Second, the TCCCR Task Area is independently funding efforts to understand issues of pain management on the battlefield (“Battlefield Analgesia: Adherence to Tactical Combat Casualty Care Guidelines”) and clinical studies to assess analgesics (“Intranasal Ketamine + Fentanyl Versus Morphine for Acute Pain: A Randomized, Controlled Trial”). These efforts are led by clinical staff within the TCCCR Task Area, including MAJ Steven Schauer, DO, CPT Derek Brown, MD, and Christian Magby, RN. Use of this resource going forward could potentially allow APRA Grant PIs the ability to translate their laboratory results to the clinical setting. In sum, the non-APRA funded projects synergize with the APRA-funded projects to provide a development pipeline of novel analgesics developed and tested in preclinical models, with the ability to leverage the resources of a translational medicine group into future clinical studies.

Abstracts/Posters Presented FY18:

- Sosanya, N.M., Tongkhuya, S., Christy, R.J., Crimmins S.L., Cheppudira, B.P. (Aug 2018). Effect of Chronic Stress on Anti-nociceptive Tolerance in a Thermal Injury Rat Model. Poster. MHSRS. FL.
- Sosanya, N.M., Clifford, J., Kumar R., Workman E., Chavez R., Dmitrov, G., Srinivasan, S., Yang R., Williams, J., Walsh, A., Gautam, A., Cheppudira, B.P., Crimmins, S., Christy, R.J. Hammameih, R. (Aug 2018). Gene Expression in Key Pain Processing Brain Regions During the Development of Chronic Neuropathic Pain. Poster. MHSRS. FL.
- Sosanya, N.M., Kumar R., Clifford, J., Chavez R., Trevino, A.V., Dmitrov, G., Srinivasan, S., Gautam, A., Trevino, A.V., Williams, M., Cheppudira, B.P., Hammameih, R., Crimmins, S., Christy, R.J. (Aug 2018). Identifying Plasma Derived Exosome contained Biomarkers in the Development of Chronic Neuropathic Pain. Poster. MHSRS. FL.
- Sosanya, N.M., Kumar R., Clifford, J., Chavez R., Trevino, A.V., Dmitrov, G., Srinivasan, S., Gautam, A., Trevino, A.V., Williams, M., Cheppudira, B.P., Hammameih, R., Crimmins, S., Christy, R.J. (March 2018). Investigating the Role of Exosomal microRNA in the Development of Chronic Neuropathic Pain. Oral Presentation at Circulating Biomarkers World Congress. MA.
- Clifford J.L., Kumar R., Srinivasan S., Dmitrov G., Gautam A., Walsh A., Workman E., Chavez R., Sosanya N., Christy R., and Hammamieh R. Gene Expression in the Rat Thalamus following Chronic Neuropathic Pain Development: mRNA sequencing analysis. Experimental Biology annual conference, 2018.

- Clifford JL, Kumar R, Srinivasan S, Dmitrov G, Gautam A, Yang R, Williams J, Workman E, Chavez R, Sosanya N, Cheppudira B, Christy R, and Hammamieh R. Gene Expression in Multiple Regions of the Rat Brain During Chronic Neuropathic Pain Development: mRNA Sequencing Analysis. Neurotrauma 2018.
- Sosanya NM, Kumar R, Clifford JL, Workman E, Srinivasan S, Dmitrov G, Gautam A, Walsh A, Chavez R, Alrufaiee O, Trevino AV, Cheppudira BP, Christy RJ, Crimmins S, Hammamieh R. Gene Expression in Key Pain Processing Brain Regions During the Development of Chronic Neuropathic Pain. Military Health System Research Symposium (MHSRS), 2018.*
- Kumar, R, Campbell R, Gautam A, George Dimitrov G, Srinivasan S, Hamad D, Sosanya NM, Cheppudira BP, Christy RJ, Crimmins S, Clifford JL, Hammamieh, R. Data Agnostic Knowledge Exploration and Integration for the Identification of Chronic Neuropathic Pain Gene Networks from the Transcriptome Profile of Brain Regions. . Military Health System Research Symposium (MHSRS), 2018.*
- Clifford JL, Kumar R, Srinivasan S, Dmitrov G, Gautam A, Yang R, Williams J, Workman E, Chavez R, Sosanya N, Cheppudira B, Christy R, and Hammamieh R. Global Transcriptomic Analysis of the Rat Brain During Chronic Neuropathic Pain Development. Society for Neuroscience Annual Meeting, 2018.*
- Klemcke HG, Calderon ML, Calderon AS, Crimmins SL, Hinojosa-Laborde C. Effects of ketamine on cardiovascular and respiratory responses to severe traumatic hemorrhage in rats. Poster presentation at American Pain Society Meeting, Anaheim, CA, Mar 2018.
- Xiang L, Klemcke HG, Ryan KL, Hinojosa-Laborde C. Analgesic effects of intravenous morphine, fentanyl, sufentanil, and ketamine in a novel rat model of extremity trauma. Poster presentation at Experimental Biology 2018, San Diego, CA, Apr 2018.
- Klemcke HG, Calderon ML, Calderon AS, Crimmins SL, Hinojosa-Laborde C. Effects of ketamine on cardiovascular and respiratory responses to a moderate 40% hemorrhage in rats. Poster presentation at Experimental Biology 2018, San Diego, CA, Apr 2018; podium presentation at MHSRS, Kissimmee, FL, Aug 2018.

Abstracts/Posters Presented FY17:

- Xiang L, Avila MM, Ryan KL, Hinojosa-Laborde C. A novel rat model of orthopedic trauma for prehospital pain studies. Poster presentation, Experimental Biology 2017, Chicago, IL and MHSRS 2017. *
- Xiang L, Avila MA, Ryan KL, Fernandez JD, Hinojosa-Laborde C. Analgesic effects of intravenous morphine, fentanyl, and ketamine in a rat model of orthopedic trauma. Poster presentation, MHSRS 2017.*
- Sosanya, N.M., Kumar R., Clifford, J., Chavez R., Trevino, AV., Dmitrov, G., Srinivasan, S., Gautam, A., Cheppudira, BP., Hammameih, R., Christy, RJ. (Aug 2017). Investigating the Role of Exosomal microRNA in the Development of Chronic Neuropathic Pain. Poster at Military Health System Research Symposium (MHSRS). FL.
- Sosanya, N.M., Garza, T., Stacey, W., Trevino, AV., Christy, RJ., Cheppudira, BP. Prior chronic stress induces aggravated allodynia and hyperalgesia behaviors in thermal injured rats: Involvement of BDNF and CRF signaling mechanisms. (Aug. 2017). Oral presentation at MHSRS. FL.

- Sosanya, N.M., Garza, T., Christy, R.J., Cheppudira, B.P. Development and Characterization of a Preclinical Model Recapitulating Battlefield Stress. (June 2017). (San Antonio Military Health System and Universities Research Forum (SURF)). San Antonio, TX. Poster.
- Sosanya, N.M, Stacey, W, Garza, T, Trevino, AV, Christy, R.J, Cheppudira, B.P. (May 2017). Altered tropomyosin receptor kinase B (TrkB) protein expression in the hypothalamus links to prolonged stress-induced enduring mechanical allodynia in naïve and thermal injured rats. (American Pain Society (APS)). Pittsburg, PA. Poster.

Abstracts/Posters Presented FY16:

- Xu M, Norton K, Peckham D, Posakony J, Tarcha E, Bartron J, Brown M, Lustig K, Manelli LDC, Ghelardini C, Fowler M, Christensen S, McIntos JM, Iadonato SP, Mercado J. A Novel, Non-opioid, Conesnail Peptide-based Analgesic as a Therapeutic Alternative for the Treatment of Chronic Pain. American Pain Society, 2016, Austin TX.
- Sosanya NM, Trevino AV, Chavez RL, Christy RJ, Cheppudira BP. Sound Stress Activates Corticotropin-releasing Factor Receptor-2 (CRFR2) in the Lumbar Spinal Cord of Rats with Thermal Injury. Society for Neuroscience Annual Meeting. November 12-16, 2016. San Diego, CA.
- Sosanya NM, Chavez RL, Trevino AV, Christy RJ, Cheppudira BP. Sound Stress-induced Changes in Pain Behaviors in Rats with and without Thermal Injury. Military Health System Research Symposium (MHSRS), August 15-17, 2016. Orlando, FL.
- Sosanya NM, Trevino AV, Chavez RL, Cheppudira BP, Christy RJ. Alterations in MicroRNA Expression in a Rat Model of Spinal Nerve Ligation. Military Health System Research Symposium (MHSRS), August 15-17, 2016. Orlando, FL.
- Workman E, Fowler M. Characterization of Interaction between Complex Regional Pain Syndrome and Post-Traumatic Stress Disorder. Military Health System Research Symposium (MHSRS), August 15-17, 2016. Orlando, FL.
- Fowler, M., Cheppudira, B., Chavez, R., Mercado, J. Evaluation of the Analgesic Effects of Alpha Conotoxins and Alpha Conotoxin Analogs. Military Health System Research Symposium (MHSRS), August 15-17, 2016. Orlando, FL.
- Fowler, M, Neelagiri, Venugopal rao. Sucholeiki, I. Analgesic and Anti-inflammatory Effects of the Dual MMP2/MMP9 Inhibitor Aqu-118 in a Rat Model of Thermal Injury Pain. Military Health System Research Symposium (MHSRS), August 15-17, 2016. Orlando, FL.

Publications

- Kumar, R, Campbell R, Gautam A, George Dimitrov G, Srinivasan S, Hamad D, Sosanya NM, Cheppudira BP, Christy RJ, Crimmins S, Clifford JL, Hammamieh, R. Data Agnostic Knowledge Exploration and Integration for the Identification of Chronic Neuropathic Pain Gene Networks from the Transcriptome Profile of Brain Regions. In preparation.
- Clifford JL, Kumar R, Srinivasan S, Dmitrov G, Gautam A, Yang R, Williams J, Workman E, Chavez R, Sosanya N, Cheppudira B, Christy R, and Hammamieh R. Global Transcriptomic Analysis of the Rat Brain During Chronic Neuropathic Pain Development. In preparation.
- Xiang L, Calderon AS, Hinojosa-Laborde C, Ryan KL. Fentanyl impairs but ketamine preserves the microcirculatory responses to hemorrhage---a novel approach to evaluate hemodynamic impacts of analgesics. *Anesth Analg*, under review.
- Sosanya, N. M., Kumar, R., Clifford, J., Chavez, R., Dmitrov, G., Srinivasan, S., Gautam, A., Trevino, A. V., Williams, M., Hammameih, R., Cheppudira, B. P., Christy, R. J., Crimmins, S. L. Identifying Plasma Derived Extracellular Vesicle (EV) Contained Biomarkers in the Development of Chronic Neuropathic Pain. *J Pain*. 2019. Manuscript resubmitted.
- Sosanya, N. M., Garza, T., Stacey, W., Christy, R. J., & Cheppudira, B. P. (2019). Brain-Derived Neurotrophic Factor Contributes to Chronic Stress-induced Enhanced Pain in a Thermal Injured Rat Model. *BMC Neurosci*. 2019 Apr 24;20(1):17.
- Xiang L, Klemcke HG, Wienandt NA, Ryan KL, Hinojosa-Laborde C. A novel rat model of extremity trauma for prehospital pain management research. *J Trauma Acute Care Surg* 85(1S Suppl 2): S49-S56, 2018.
- Cheppudira BP, Trevino AV, Petz LN, Christy RJ, Clifford JL. Anti-nerve growth factor antibody attenuates chronic morphine treatment-induced tolerance in the rat. *BMC Anesthesiology*. 2016 Sep 5; 16(1):73.
- Fowler, M., McGhee, LL. Pain Management: Maintaining the Force. *Small Wars Journal*. Mad Scientist Megacities/ Dense Urban Areas Special Report. <http://smallwarsjournal.com/jrnl/art/pain-management-maintaining-the-force> <https://community.apan.org/wg/tradoc-g2/mad-scientist/m/mdua/156587> 1
- Sosanya, N. M., Trevino, A., Chavez, R., Christy, R., & Cheppudira, B. P. (2017). Involvement of spinal corticotropin-releasing factor receptor-2 (CRFR2) in sound stress-induced enhanced thermal hyperalgesia in a rat model of burn injury. *J Pain Res*. 2017 Sep 1; 10:2135-2145. doi: 10.2147/JPR.S144055.

PROJECT 1

AWARD NUMBER: MR157005C

TITLE: Assessing the Effects of Analgesics and Drug Delivery Mechanisms on Acute Pain

PRINCIPAL INVESTIGATOR: MAJ Stephen Crimmins, PhD

RECIPIENT: US Army Institute of Surgical Research

REPORT DATE: 05/02/2019

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

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14. ABSTRACT Opioids are the foremost prescription drugs for the current treatment of severe acute and chronic pain. These drugs are at high risk for abuse and addiction, as well as many other potential adverse effects. These include nausea, immunosuppression, respiratory depression, hemodynamic effects as well as cognitive and motor coordination decline. Therefore, pre-clinical testing of novel non-opioid pharmaceutical therapies for both safety and efficacy is of the utmost importance and the key goal of this project. Here, we have identified several potential safe alternatives to opioids for the treatment of pain. These include Aqu-118 (Aquilus), CSP-4 (Kineta), D-112 (Kalyra), and SQEXPAN-21 (Expanesthetics). These compounds attenuate either thermal hyperalgesia, mechanical allodynia, or both without sedation or motor deficits. These industrial partners are utilizing the preclinical data obtained in this project to file for the FDA Investigational New Drug (IND) program to obtain permission to start human clinical trials. In conclusion, the collaboration between the USAISR and its industry partners has strengthened the possibility that new and better prescription drugs for severe acute and chronic pain will be available in the near future.		

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1. INTRODUCTION:

Current treatment for severe acute and chronic pain often includes opioids, including morphine, fentanyl, and similar drugs. However, these drugs induce multiple side effects, including nausea, immunosuppression, respiratory depression, hemodynamic effects as well as cognitive and motor coordination decline. Opioid therapies are also subject to concerns related to addiction potential and the possibility of drug diversion. The overall purpose of this project is to evaluate novel drugs for the treatment of pain using animal models and molecular methods. In Specific Aims 1 and 2, we use two rat models of pain, the thermal injury model (TI) and the spinal nerve ligation (SNL) model. These models represent different types of pain, each of which may respond differently to drugs with discrete mechanisms; the molecular pathways that are active in these models likely also differ.

2. KEYWORDS:

Acute pain, analgesic, mechanical allodynia, thermal hyperalgesia, burn, matrix metalloprotease, conotoxin, acetaminophen

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Major Task 1: Test analgesic efficacy of novel compounds in rat models of pain.

Subtask: Obtain IACUC Approval. (100% completed)

Subtask: Evaluate acute, repeated, and prophylactic dosing of novel compounds on pain behaviors and molecular markers. (100% completed)

Major Task 2: Determine the effects of novel analgesics on opioid requirements and tolerance by assessing pain behaviors and molecular markers.

Subtask: Obtain IACUC approval. (100% completed)

Subtask: Evaluate effects of combination analgesic therapy. (100% completed)

Major Task 3: Analysis of the analgesic and neurotoxic effects of continuous low dose ketamine infusion.

Subtask: Obtain IACUC approval. (100% completed)

Subtask: Establish low dose intrathecal ketamine animal model. (25% completed)

Major Task 4: Develop enhanced analgesia using timed-release anesthetics and analgesics.

Subtask: Obtain IACUC approval: (100% completed)

Subtask: Mechanical and thermal testing following anesthetic IV dosing. (100% completed)

What was accomplished under these goals?

Major activities:

- (1) Obtained IACUC approval for studies.
- (2) Performed in vivo experiments to evaluate efficacy of novel analgesics. Evaluate acute, repeated, and prophylactic dosing of novel compounds on pain behaviors and molecular markers.
- (3) Conducted in vitro experiments to understand underlying mechanisms of pain and analgesia.

Specific objectives:

Identify non-opioid analgesics that are good candidates for clinical testing by determining their efficacy and safety in pre-clinical animal models of pain.

Methods:

All the experiments were performed on male Sprague Dawley rats. Animals were anesthetized prior to the thermal injury with 4% isoflurane in 100% oxygen. Injury was induced on the plantar surface of the right hindpaw using a temperature-controlled super soldering station equipped with a slanted soldering tip (RX-80HRT-5.4D, Goot, Hiroshima, Japan) at 100°C for 30 seconds. To examine the potential efficacy and potency of therapeutics on thermal injury-evoked pain, pain behaviors were examined using tests for thermal hyperalgesia, mechanical allodynia, and sedation/motor function. To test for mechanical allodynia, rats were individually placed in clear Plexiglas chambers (non-restrictive) on an elevated grid platform. Using an electric anesthesiometer (Ugo Basile), a blunt mechanical stimulus was applied to the plantar surface of the hindpaw with slowly increasing force until the rat voluntarily withdrew the paw. The force (in grams) required to elicit voluntary withdrawal of the hindpaw, or paw withdrawal threshold (PWT), was used as the measure of mechanical allodynia. Each animal was tested in triplicate at each time point. To test for thermal hyperalgesia, rats were individually placed on a glass surface maintained at 30°C in clear plastic chambers (non-restrictive) and a radiant visible light beam was aimed at the plantar surface of the right hindpaw using a Paw Thermal Stimulator (University of California-San Diego). The light beam goes through the glass and heats the animal's paw, causing the animal to remove its paw due to discomfort (hyperalgesia). The time it takes for the animal to remove its paw is recorded as paw withdrawal latency (PWL). The intensity of the beam was set to produce baseline PWLs of approximately 12-15 sec. A maximal latency of 20 sec was used to prevent tissue damage due to repeated application of the thermal stimulus. To examine the effect of drugs on motor coordination/sedation, rats were trained to run on the rotarod (Med Associates Inc., St. Albans, VT, USA). On the first two days of training, the rats underwent three 5 min training sessions at speeds of 8, 12, and 16 revolutions per minute (rpm). On the third day of training, rats received one 5 min refresher session at 16 rpm and two sessions with acceleration from 4 to 40 rpm over 5 min. The following day, the number of seconds each rat remained on the rotarod as it accelerates from 4 to 40 rpm over 5 min was recorded as their baseline number. Each rat was tested twice. If a rat failed to stay on the rotarod for at least 60 seconds, it was removed from the study. Following behavioral experiments, the animals were euthanized either by exsanguination following perfusion (to collect fixed tissues) or by decapitation (for the harvesting of fresh tissues). Immunohistochemical analyses were performed on the dorsal horn sections of the spinal cord for analysis of IL-1B expression.

Results

Experiment 1: Examine the effective dose of Aqu-118 on reducing mechanical allodynia and thermal hyperalgesia.

Aqu-118 is an effective, novel analgesic in a rat model of thermal injury pain (Figure 1-3). The analgesic effects of Aqu-118 are dose dependent and Aqu-118 does not cause sedation or inhibition of motor function (Figure 4). The analgesic properties of Aqu-118 are likely partly due to the effect of inhibition of matrix metalloproteinase (MMP) activity on the cleavage of IL-1 β to its active, pro-inflammatory form (Figure 5).

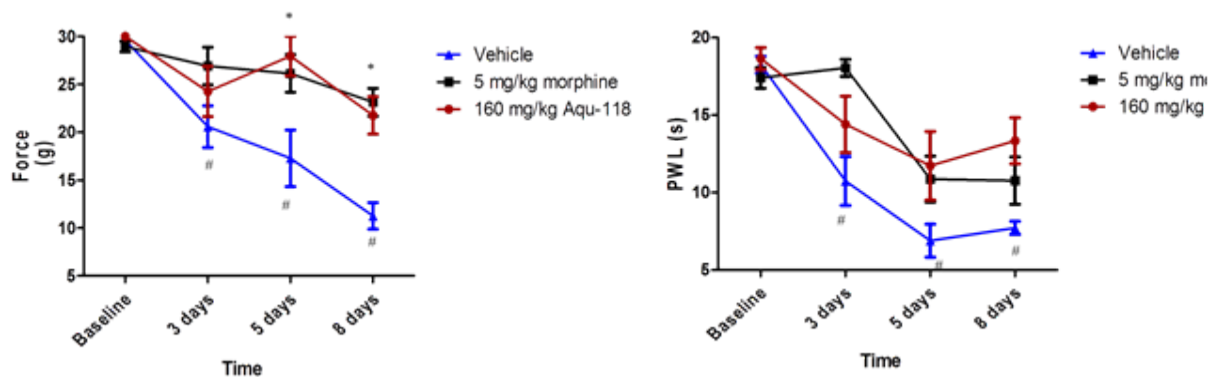


Figure 1: Aqu-118 attenuates mechanical allodynia and thermal hyperalgesia. Significant mechanical allodynia developed within 3 days, and persisted for at least 8 days, in the rat TI model ($\#p < 0.01$). Animals treated daily with either 5 mg/kg morphine s.c. or 160 mg/kg Aqu-118 p.o. exhibited decreased mechanical allodynia, as compared to vehicle treated rats at 5 days and 8 days following thermal injury ($*p < 0.05$). For thermal hyperalgesia, the analgesic effects were significant for morphine at day 3 only, and for Aqu-118 at day 8 only ($*p < 0.05$).

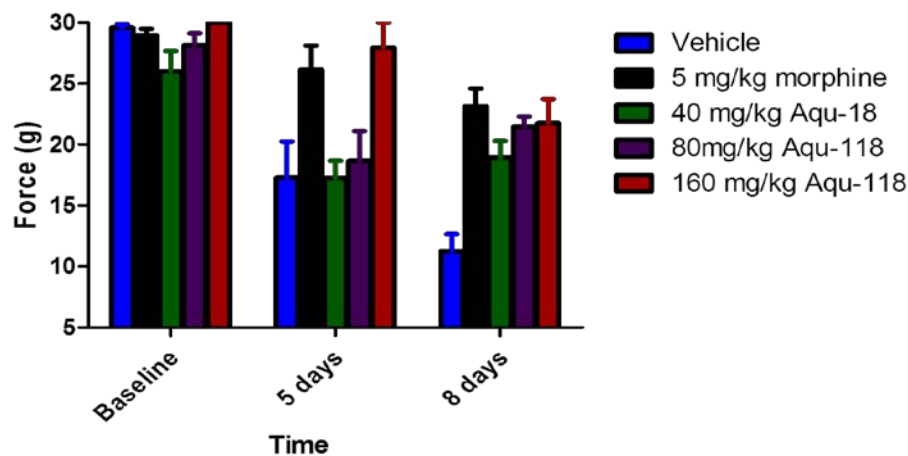


Figure 2: Aqu-118 attenuates mechanical allodynia dose-dependently. There is a significant dose-dependent relief of mechanical allodynia at day 5 following TI (Aqu-118 160 mg/kg vs. vehicle). At day 8, there is a trend towards dose dependent Aqu-118 mediated analgesia ($p>0.05$).

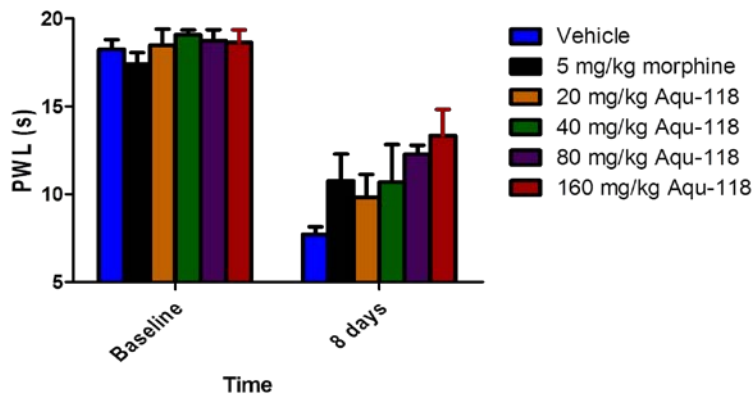


Figure 3: Aqu-118 attenuates thermal hyperalgesia dose-dependently. There is a trend towards dose-dependent relief of thermal hyperalgesia at day 8 following thermal injury.

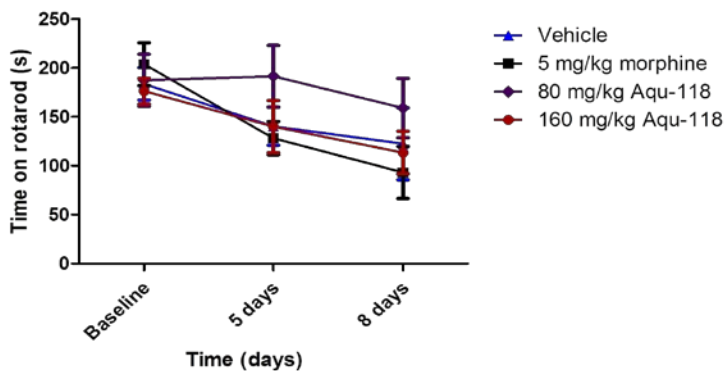


Figure 4: Aqu-118 is not sedative and does not affect motor coordination. Rats were trained to walk on a rotarod for three days prior to baseline measurements. The rotarod was set at 4-40 rpm, with a total ramp time of 30 seconds. The number of seconds a rat stayed on the rotarod was recorded. Animals received drug treatment daily (5 mg/kg morphine s.c., vehicle, 80 mg/kg Aqu-118 p.o., or 160 mg/kg p.o). On testing days, the animals received the drug one hour prior to behavioral testing. There are no significant differences between the experimental groups (Aqu-118) and controls (vehicle and morphine) at any timepoint tested ($p>0.05$).

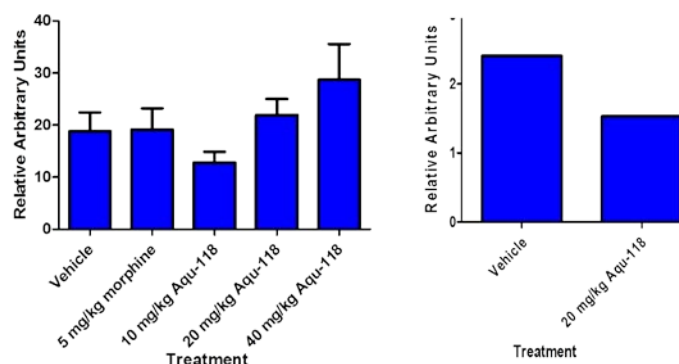
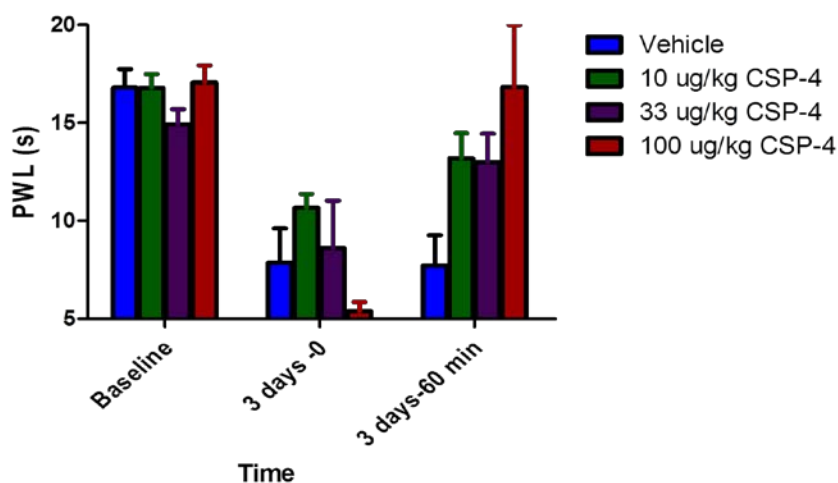


Figure 5. Treatment with Aqu-118 alters IL-1 β protein levels and the ratio of full-length IL-1 β to cleaved IL-1 β in the dorsal horn. Left: Tissue from the dorsal horn of rats treated with Aqu-118, morphine, or vehicle was fixed, mounted on slides, and stained with an antibody raised against full length IL-1 β . A biotin-conjugated secondary antibody was added, and signal was visualized using brightfield microscopy. Data was quantified using densitometry and is reported in relative arbitrary units. Right: Tissue, as above, was for either full length IL-1 β or the cleaved, active IL-1 β . A biotin-conjugated secondary antibody was added, and signal was visualized using brightfield microscopy. Data was quantified using densitometry, a ratio of full-length to cleaved IL-1 β was calculated, and relative arbitrary units are reported.

Experiment 2: Examine the effective dose of CSP-4 on reducing mechanical allodynia and thermal hyperalgesia.

CSP-4 is a novel, peripherally-acting $\alpha 9\alpha 10$ nicotinic acetylcholine receptor (nAChR) antagonist that is effective in reducing mechanical allodynia and thermal hyperalgesia in a rat model of burn pain (Figure 7 and 6, respectively). CSP-4 was effective through multiple routes of administration and was not sedative (Figure 8). CSP-4 showed a cumulative effect over time, exhibiting no tolerance. Data suggest an involvement of $\alpha 9\alpha 10$ nAChRs in the pathophysiology of burn pain.



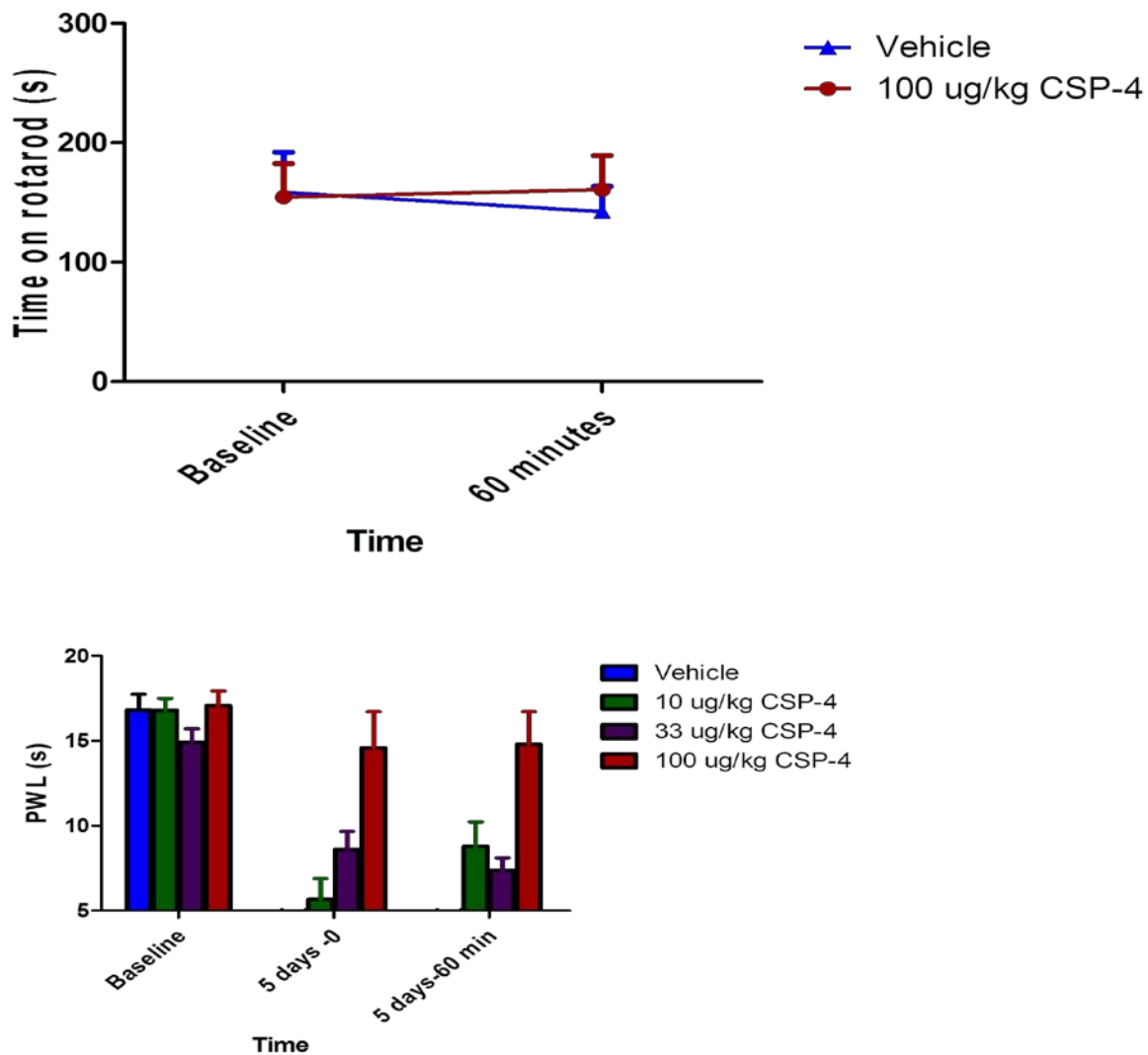


Figure 6: CSP-4 attenuates thermal hyperalgesia at 3 days. Significant thermal hyperalgesia developed within 3 days, and persisted for at least 8 days, in the rat TI model ($\#p < 0.01$). Animals treated daily with subcutaneous CSP-4 exhibited thermal hyperalgesia following their daily injection on the test day (3 days-60 minutes) ($p < 0.05$), but did not show significant analgesia prior to dose ($p > 0.05$).

Figure 7: CSP-4 attenuates thermal hyperalgesia at 5 days. Significant thermal hyperalgesia developed within 3 days, and persisted for at least 8 days, in the rat TI model ($\#p < 0.01$). Animals treated daily with subcutaneous CSP-4 decreased thermal hyperalgesia both prior to (0) and following their daily injection on the test day (5 days-60 minutes) ($p < 0.01$) at day 5.

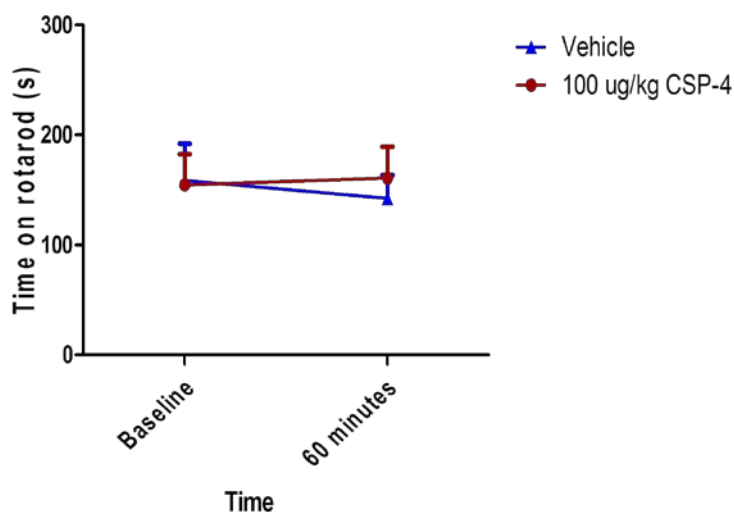
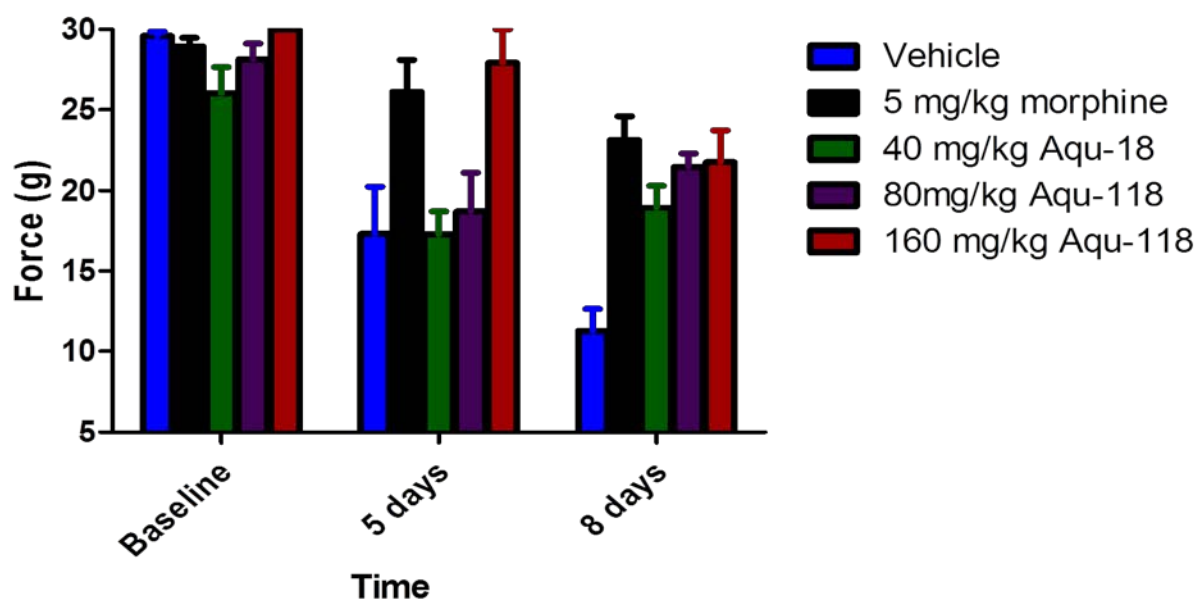


Figure 8: CSP-4 is not a sedative and does not affect motor coordination. Rats were trained to walk on a rotarod for three days prior to baseline measurements. The rotarod was set at 4-40 rpm, with a total ramp time of 300 seconds. The number of seconds a rat stayed on the rotarod was recorded. Animals received drug as indicated (100 ug/kg CSP4 or vehicle). On testing days, the animals received the drug one hour prior to behavioral testing. There are no significant differences between the experimental group (CSP-4) and control (vehicle) ($p > 0.05$).



Experiment 3: Examine the effective dose of D-112 on reducing mechanical allodynia and thermal hyperalgesia. The acetaminophen (APAP) analogue D-112 (PO) is efficacious and alleviates both mechanical allodynia and thermal hyperalgesia for at least four hours after dosing (figure 9). D-112 is as effective as and longer lasting than morphine (5 mg/kg, SC) and more potent and longer lasting than APAP (100 mg/kg, PO).

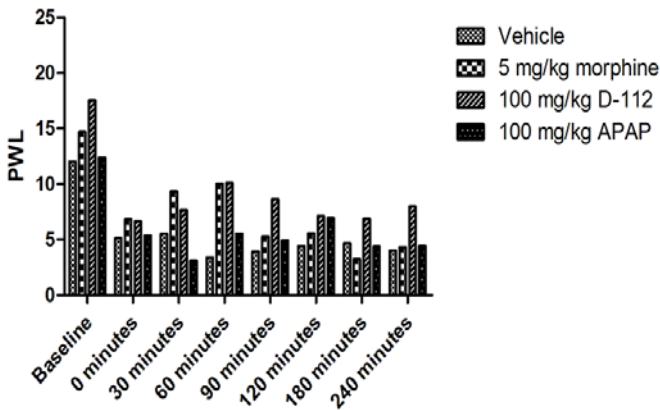


Figure 9: D-112 (PO) alleviates thermal hyperalgesia in a rat model of burn pain. Seven days after thermal injury, animals received either vehicle (PO), 100 mg/kg D-112 (PO), 100 mg/kg APAP (PO), or 5 mg/kg morphine (SC). Measurements of paw withdrawal latency were obtained for up to four hours following dosing with drugs or vehicles.

Experiment 4: Examine the effective dose of SQEXPAN-21 on reducing mechanical allodynia and thermal hyperalgesia. The volatile anesthetic, SQEXPAN-21 produced a transient thermal hyperalgesia at one hour (Figure 10). Other time points were examined out to 72 hours, however no significant changes were seen. Its analgesic effect of SQEXPAN-21 was not as robust as morphine (pink line) and it did not block mechanical allodynia (Figure 11).

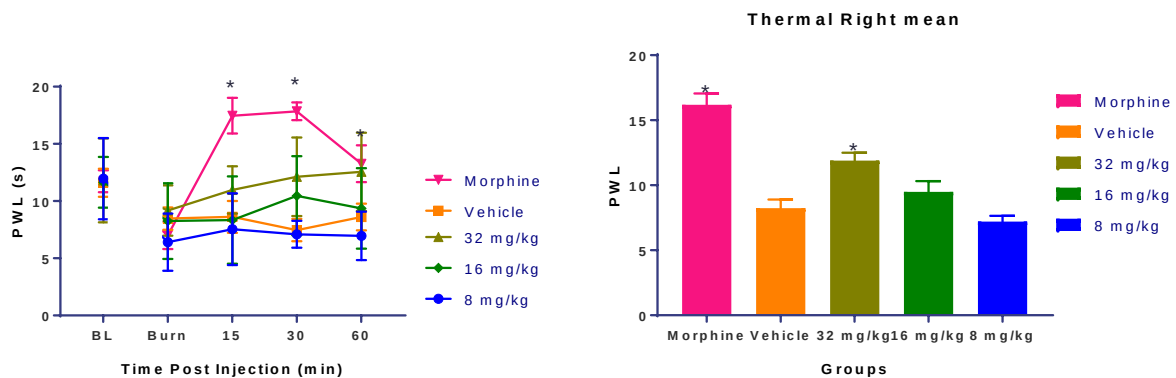


Figure 10: SQEXPAN-21 (IV) given at 32 mg/kg alleviates thermal hyperalgesia at one hour in a rat model of burn pain. Seven days after thermal injury, animals received either vehicle (IV), 8 mg/kg SQEXPAN-21 (IV), 16 mg/kg SQEXPAN-21 (IV), 32 mg/kg SQEXPAN-21 (IV), or 2 mg/kg morphine (IV). Measurements of paw withdrawal latency were obtained for up to one hour following dosing with drugs or vehicles.

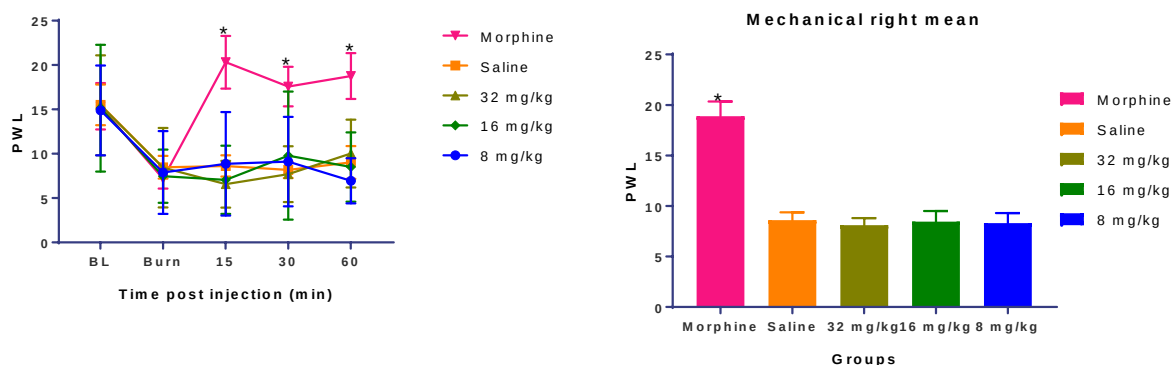


Figure 11. SQEXPAN-21 (IV) does not reduce mechanical allodynia in a rat model of burn pain. Seven days after thermal injury, animals received either vehicle (IV), 8 mg/kg SQEXPAN-21 (IV), 16 mg/kg SQEXPAN-21 (IV), 32 mg/kg SQEXPAN-21 (IV), or 2 mg/kg morphine (IV). Measurements of paw withdrawal latency were obtained for up to one hour following dosing with drugs or vehicles.

Training activities & professional development:

Dr. Misty Malamakal joined the laboratory as postdoctoral fellow under contract with Leading Edge. Under this grant, she learned to perform pain testing in rodents and gained experience working with drug companies. She is currently working on publishing data from project 1 concerning the use of SQEXPAN-21. She is also working on the intrathecal ketamine project.

Dr. Emily Workman joined our laboratory as an ORISE postdoctoral fellow. Under this grant, she has received training in animal pain model development and pain behaviors, and she contributed to both project 1 activities (this project) as well as project 4 activities. She presented a poster at MHSRS using data mining and bioinformatics techniques. Dr. Workman will be an author on the manuscripts from project 1 and 4. Dr. Workman currently works as a Data Scientist at Hypergiant Space Age Solutions.

Dr. Winfred Stacy joined the laboratory as an ORISE postdoctoral fellow and received training under this grant. She learned small animal surgical and behavioral techniques and developed in vitro tissue culture assays to aid in mechanistic understanding of the novel analgesics. She also contributed to Project 2 and is an author on a manuscript. Dr. Stacey currently works as a Data Scientist in the Department of Clinical Investigation at the Brooke Army Medical Center.

Mr. Alex V. Trevino, Mr. Roger L Chavez and Mr. Thomas Garza are technicians who are working on this project. They received training in animal surgery, behavior testing, molecular biology and in routine laboratory procedures. The training has advanced their technical skills and expertise in the areas of pain and basic molecular/cell biology.

Publication of Results

Published the following invited article:

Fowler, M., McGhee, LL. Pain Management: Maintaining the Force. Small Wars Journal. Mad Scientist Megacities/ Dense Urban Areas Special Report.
<http://smallwarsjournal.com/jrnl/art/pain-management-maintaining-the-force>
https://community.apan.org/wg/tradoc-g2/mad-scientist/m/mdua/156587_1

This article, Pain Management: Maintaining the Force article from the Small Wars Journal, was summarized and presented in the “Good News” briefing to the Commanding General of MEDCOM for the 3rd quarter FY16.

Plan to do during the next reporting period.

As stated in the SOW, we have completed all experiments under Specific Aims 1-2 and the majority of experiments from Specific Aims 3-4. Briefly, we are currently studying the effects of low dose intrathecal ketamine administration. There were several technique hurdles associated with this particular experiment that are currently being solved. .

4. IMPACT:

These studies show that novel analgesics in three classes tested are able to alleviate thermal injury pain in an animal model. These data provide support for moving these products through development and provide supporting documentation for the submission of multiple INDs to the FDA.

Impact on other disciplines?

Nothing to report

Impact on technology transfer

Aquilus, Inc., anticipates filing an IND for Aqu-118 within the next year, seeking an indication for neuropathic pain.

Impact on society beyond science and technology

Nothing to report

5. CHANGES/PROBLEMS:

Changes in approach

Nothing to Report

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Actual or anticipated problems or delays

The IACUC protocol has already been approved to begin examining intrathecal ketamine after a spinal nerve ligation. During our initial pilot (n=12 rats), we discovered that more training was needed to ensure the spinal cord would not become damaged during surgery. Additionally there were some issues concerning the connection between the intrathecal tubing and the osmotic pumps. Thus, we are currently training technical staff and ordering different connector for the osmotic pumps. The project should be restarting June 2019.

Changes that had a significant impact on expenditures

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**Journal publications**

Fowler, M., McGhee, LL. Pain Management: Maintaining the Force. Small Wars Journal. Mad Scientist Megacities/ Dense Urban Areas Special Report. <http://smallwarsjournal.com/jrnl/art/pain-management-maintaining-the-force>
<https://community.apan.org/wg/tradoc-g2/mad-scientist/m/mdua/156587>

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**
Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to Report

- **Inventions, patent applications, and/or licenses**

Nothing to Report

- **Other Products**

Nothing to Report

7. What individuals have worked on the project?

Name: MAJ Crimmins, PhD.

Project role: PI

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 12

Contribution to Project: MAJ Crimmins is managing this project by planning, designing and executing all the experiments outlined in the proposal. He is communicating with collaborators and companies about the availability and function of novel analgesics. He is responsible for reporting data in the form of abstracts, manuscripts, presentations, and reports. MAJ Crimmins oversees all aspects of this project and works with the other project PIs to provide synergy and leverage a variety of skills to produce required results.

Name: Misty Malamakal, PhD.

Project role: Postdoctoral Fellow

Researcher Identifier (e.g. ORCID ID): 0000-0002-7264-7087

Nearest person month worked: 12

Contribution to Project: Dr. Malamakal conducts experiments under the direction of the PI. She writes animal protocols, collects data, and presents findings.

Name: Mr. Alex V. Trevino, MS.

Project role: Technician

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 12

Contribution to Project: Mr. Trevino aids in performing the experiments and in managing laboratory needs.

Name: Mr. Roger Chavez

Project role: Technician

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 12

Contribution to Project: Mr. Chavez aids in performing the experiments and in managing laboratory needs.

Name: Mr. Thomas Garza, BS

Project role: Technician

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 12

Contribution to Project: Mr. Garza aids in performing the experiments and in managing laboratory needs.

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: Kineta, Inc

Location of Organization: Seattle, WA

Partner's contribution to the project:

Financial support

In-kind support

Collaboration:

Organization Name: Aquilus, Inc

Location of Organization: Seattle, WA

Partner's contribution to the project:

Financial support

In-kind support

Collaboration:

Organization Name: Kalyra

Location of Organization: San Diego, CA

Partner's contribution to the project:

In-kind support

Collaboration

Organization Name: Expanesthetics

Location of Organization:

Partner's contribution to the project:

In-kind support

Collaboration

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Nothing to Report

QUAD CHARTS: Nothing to report

9. APPENDICES: Nothing to report

Project 2

AWARD NUMBER: MR157005C

TITLE: Factors that influence long-term outcomes and pain chronicity

PRINCIPAL INVESTIGATOR: Bopaiah Cheppudira, PhD

CONTRACTING ORGANIZATION: US Army Institute of Surgical Research

REPORT DATE: 5/2/2019

TYPE OF REPORT: Final report

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

DISTRIBUTION STATEMENT: Choose Distribution Statement A or B. (Reference https://mrmc.amedd.army.mil/index.cfm?pageid=researcher_resources.technical_reporting for additional information.)

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				5b. GRANT NUMBER MR157005C	
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6. AUTHOR(S) Bopaiah Cheppudira Bopaiah.p.cheppudira.ctr@mail.mil				5d. PROJECT NUMBER	
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9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S.Army Medical Research and Material Command				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
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13. SUPPLEMENTARY NOTES					
14. ABSTRACT Service Members are often exposed to various combat stressors on the battlefield, including both physical and psychological stress. Additionally, combat injuries can result in severe acute and chronic pain. Based upon the interaction between stress and pain, it is becoming increasingly clear that combat stress may enhance pain and compromise pain management. The mechanisms linking combat stress and pain are unclear. Furthermore, due to lack of appropriate animal models of battlefield stress and pain, it is difficult to understand the pathophysiological mechanisms of prior stress-induced effects on pain and also to evaluate novel analgesics. This study has two main purposes: (1) to characterize an animal model of battlefield stress that influences thermal and neuropathic pain and (2) to investigate combat stress-mediated neuroplasticity and glial hyperactivity in pain relevant areas of the brain and in the spinal cord of rats with thermal and neuropathic pain. We have developed animal models that mimic battlefield acute and chronic stress state and also have investigate interaction between stress and pain. We have also examined the role of corticotrophin-releasing factor and brain-derived nerve growth factor systems in stress-induced exacerbated pain mechanisms.					
15. SUBJECT TERMS chronic intermittent stress, pain, corticotrophin-releasing factor, corticosterone, stress-induced analgesia and stress-induced hyperalgesia, Brain-derived nerve growth factor, nerve growth factor, morphine tolerance					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES 21	19a. NAME OF RESPONSIBLE PERSON
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (include area code)

4. INTRODUCTION:

Service Members are often exposed to various combat stressors on the battlefield, including both physical and psychological stress. Additionally, combat injuries can result in severe acute and chronic pain. Based upon the interaction between stress and pain, it is becoming increasingly clear that combat stress may enhance pain and compromise pain management. The mechanisms linking combat stress and pain are unclear. Furthermore, due to lack of appropriate animal models of battlefield stress and pain, it is difficult to understand the pathophysiological mechanisms of prior stress-induced effects on pain and also to evaluate novel analgesics. This study has two main purposes: (1) to characterize an animal model of battlefield stress that influences thermal and neuropathic pain and (2) to investigate combat stress-mediated neuroplasticity and glial hyperactivity in pain relevant areas of the brain and in the spinal cord of rats with thermal and neuropathic pain. The findings from this study will provide insights about how stress events in the warzone contribute to exacerbating and enduring pain that result from thermal injury state.

5. KEYWORDS:

Sound stress, chronic intermittent stress, mechanical allodynia, thermal hyperalgesia, corticotropin-releasing factor, corticosterone, stress-induced analgesia and stress-induced hyperalgesia, Brain-derived nerve growth factor, nerve growth factor, morphine tolerance

6. ACCOMPLISHMENTS:

What were the major goals of the project?

Goal 1: Development of an Animal Model of Battlefield Stress

Subtask 1a: Test the effects of various stress model on pain following TI

(Predicted: 12-15 months, 100 % completed).

Subtask 1b: Determine underlying mechanisms (naltrexone and duloxetine)

(Predicted: 12 months, 75 % completed).

Subtask 1c: Investigate the effects of stress on neuropathic pain (SNI model)

(Predicted: 6-8 months, 75% completed).

Subtask 1d: Determine effects of stress on analgesic efficacy (morphine and tramadol)

(Predicted: 6-8 months, 75% completed).

Goal 2: Neuronal and Glial Adaptations in Chronic Pain

Subtask 2a: Investigate neural adaptations resulting in the chronification of pain (Predicted: 12 -16 months, 25% completed).

Subtask 2b: Determine the role of central microglial activation on pain chronicity.

(Predicted: 8-12 months, 0% completed).

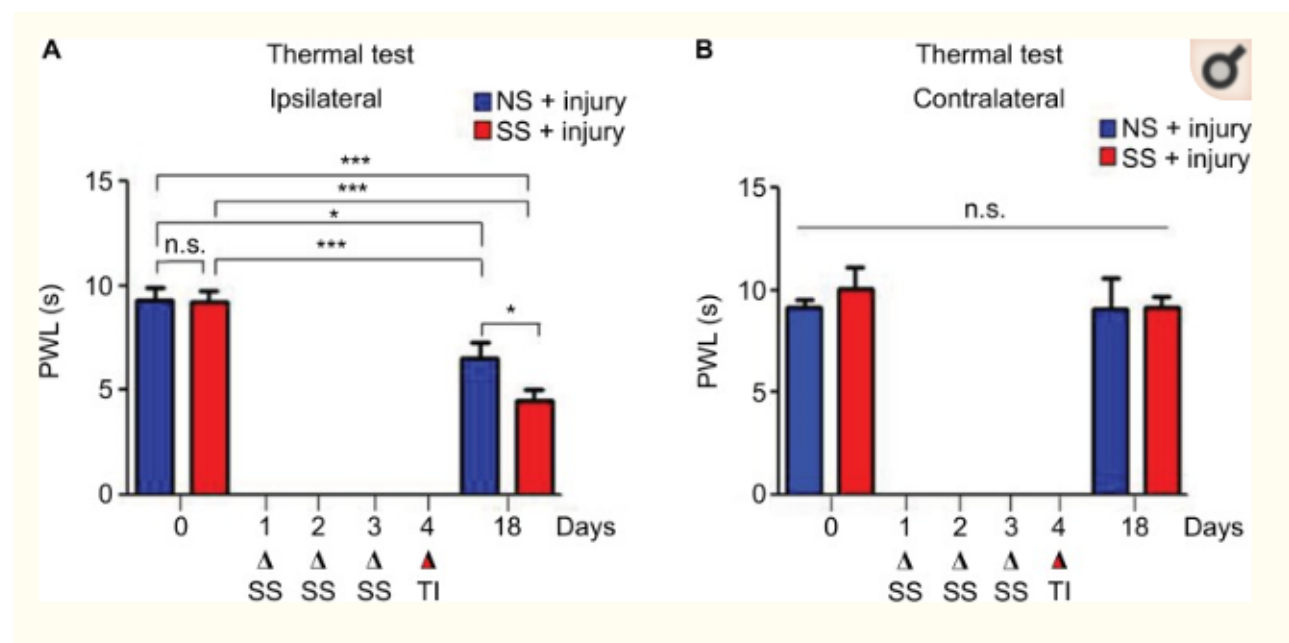
Subtask 2c: Investigate interventions that enhance analgesia. (Predicted: 6-8 months, 75% completed).

Major accomplishments/Major activities/Specific objectives/Significant results:

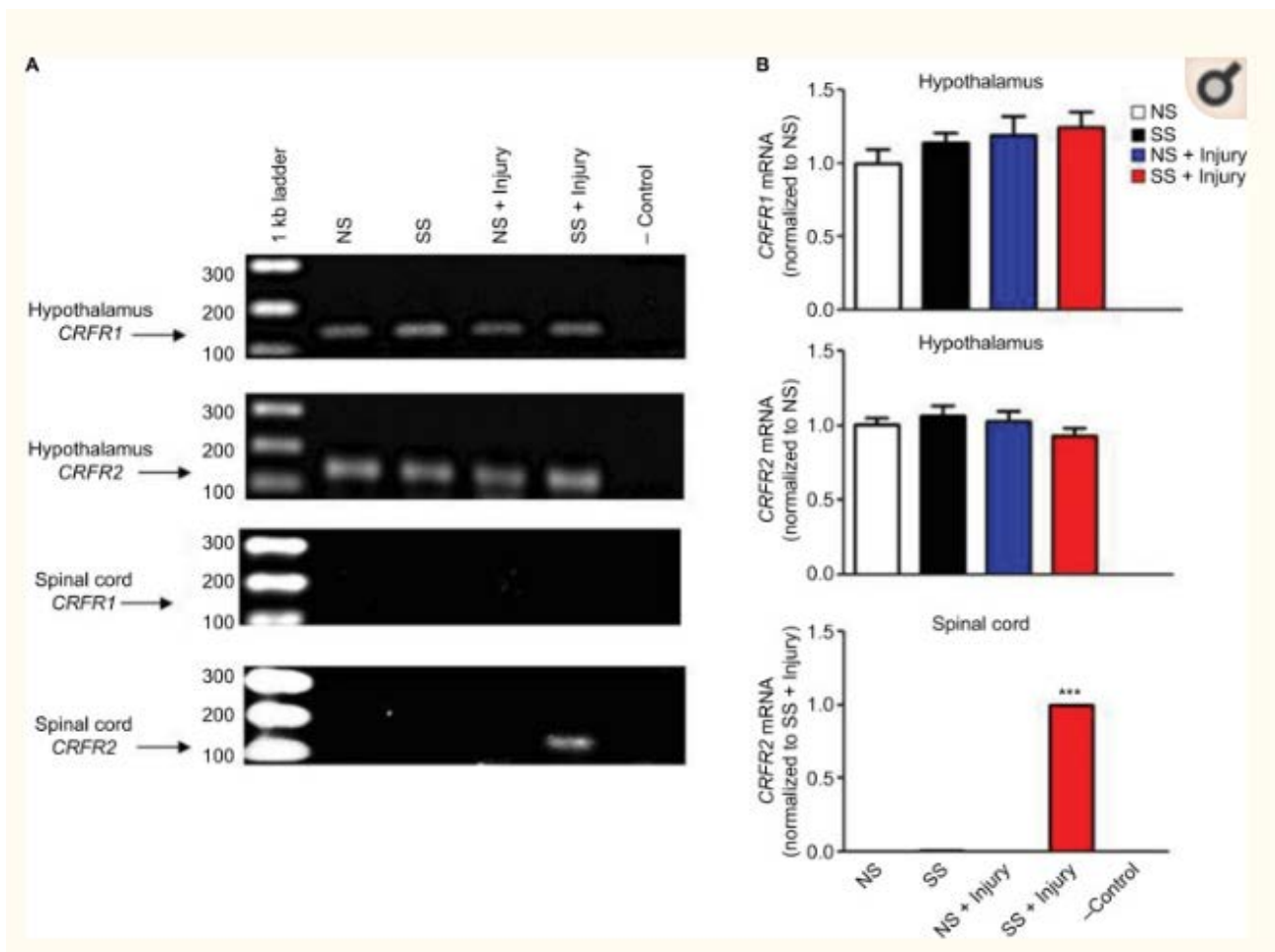
1. Effect of Sound Stress on Burn Pain Mechanism (100% completed). The impact of sound stress on post-injury pain mechanisms was unknown. The aim was to develop a rat model to study the interaction between sound stress and burn pain. Sound stress on rats was induced by exposing them to 105 dB tone over several different frequencies ranging from 11 to 19 kHz, each lasting for 5 - 10 s within a 30 min period, over 3 consecutive days. Sound stressed rats were subjected to unilateral partial-thickness thermal injury by placing a pre-heated (100° C) soldering tip on the mid-plantar surface of the right hind paw for 30 sec. The development of nociceptive behaviors (hyperalgesia and allodynia) were assessed at multiple time points (1, 4, 7, and 14 days) using Hargreaves' thermal and von Frey tests, respectively.

Significant Results: Sound stress is capable of exacerbating post-burn thermal pain (Fig 1A and 1B). Additionally, the corticotrophin-releasing factor (CRF) system appears to play a key role in sound stress-mediated enhanced post-burn pain behaviors (Fig. 2). This rat model can be used to develop novel anti-stress and analgesics drugs to mitigate sound stress associated pain symptoms. Please see the publication by Sosanya et al. 2017 for additional data.

Fig.1



Abbreviations: n.s., nonsignificant; NS, no stress; PWL, paw withdrawal latency; SEM, standard error of the mean; SS, sound stress; TI, thermal injury.



Abbreviations: *CRFR1*, corticotropin-releasing factor receptor-1; *CRFR2*, corticotropin-releasing factor receptor-2; NS, no stress; RT-PCR, reverse transcription polymerase chain reaction; SEM, standard error of the mean; SS, sound stress.

2. Development and Characterization of a Preclinical Model Recapitulating Battlefield Stress (100 % completed). Sound, physical restraint, vigorous physical activities and extreme temperature are some of the common environmental stimuli to which Service Members are often exposed to on the battlefield. However, the effect of battlefield stressors on pain mechanisms is unclear. One reason was due to lack of an appropriate animal model to study interaction between chronic stress and pain. Now we have developed a preclinical model that mimics battlefield setting-induced stress disorders and also its impact on post-burn. We also examined the effects of battlefield stress on brain-derived nerve growth factor (BDNF) in the brain regions that are sensitive to stress and pain.

Significant findings: Stressed rats with thermal injury displayed persistent exacerbated mechanical allodynia (Fig. 1A). Increased expression of BDNF mRNA in the prefrontal cortex, and elevated TrkB and p-TrkB protein levels in the hypothalamus were observed in stressed rats with thermal injury but not in stressed or thermally injured rats alone (Fig. 2a,c,d). Furthermore, administration of TrkB antagonist, CTX-B, significantly reduced stress-induced exacerbated mechanical allodynia in thermally injured rats (Fig.1B). Taken together, a rat model to study the effect of battlefield stressors on post-burn pain mechanisms is established. TrkB receptor antagonist appears to be a novel agent to attenuate stressed-mediated exacerbated burn pain. Please see our publication (Sosanya *et al.* 2019) for additional data.

Fig. 1

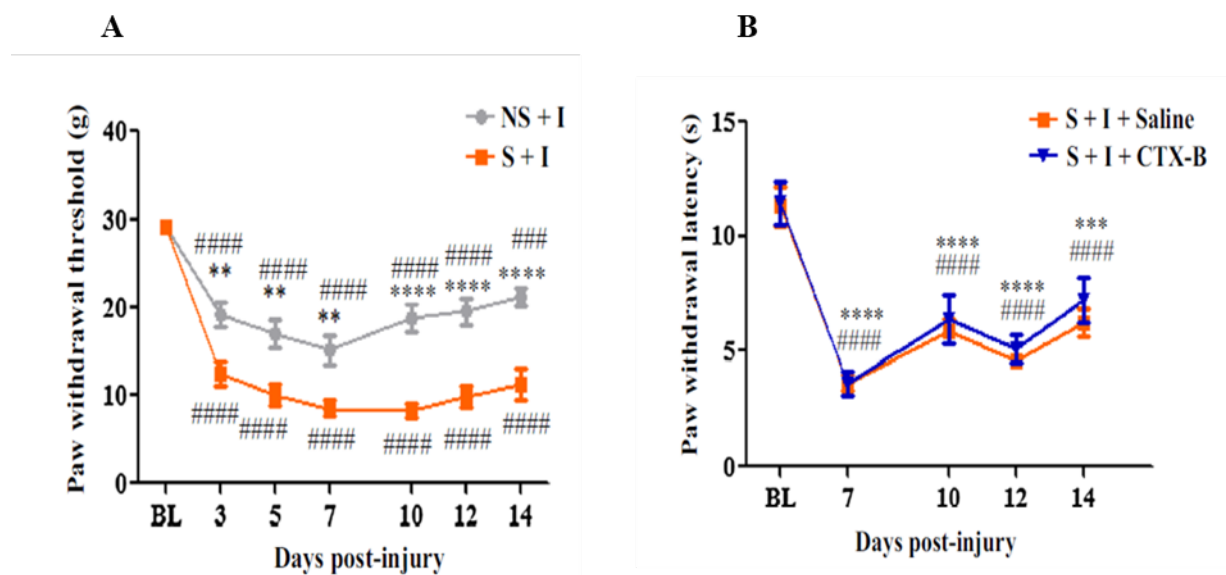
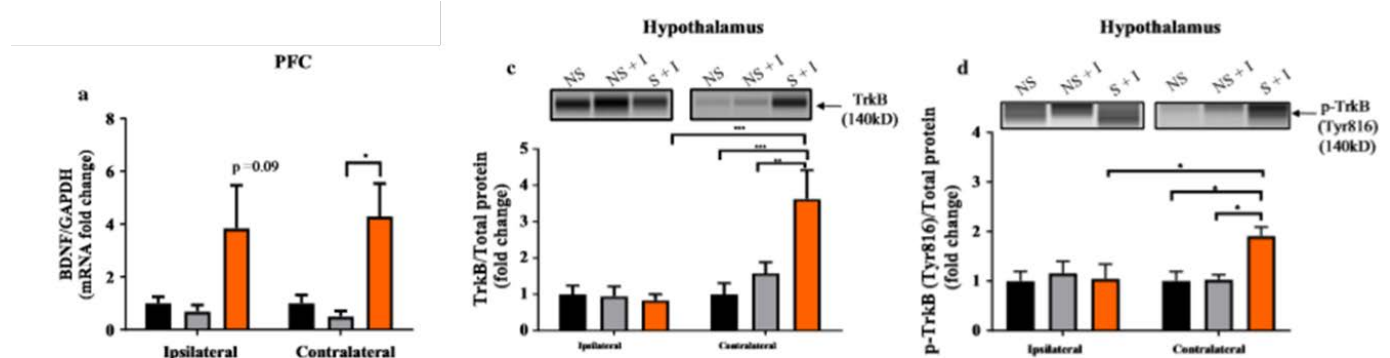


Fig. 2



BL, Stress + injury; NS, Right hind paw; PFC, Prefrontal cortex; BDNF: Brain-derived Nerve growth Factor

3. Standardization of spared nerve injury (SNI) neuropathic pain (75% completed).

The objective was to study the effect of chronic stress on neuropathic pain. We established SNI model in our laboratory. SNI was induced in rats by exposing and ligating peroneal and tibial

nerves while sparing sural nerve. This procedure produced mechanical and cold allodynia (Fig 1A & B) that lasted for more than 21 days.

Significant results: We examined SNI-induced changes in neurochemicals in the pain relevant brain regions. For example, SNI effects vascular endothelial growth factor (VEGF) system, Fig. 2. Additionally, this model has been extensively used in project 4 of this grant application to study effect of neuropathic pain on gene expression in multiple areas of the brain using systems biology approach. Next step was to study the effect of battlefield stressors on neuropathic pain mechanisms. However, there was no time and manpower to complete this objective as one of our postdoctoral fellow left our lab.

Fig.1

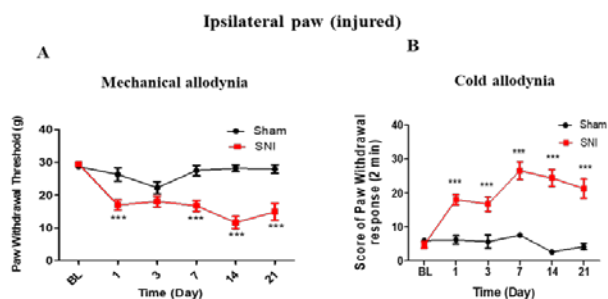
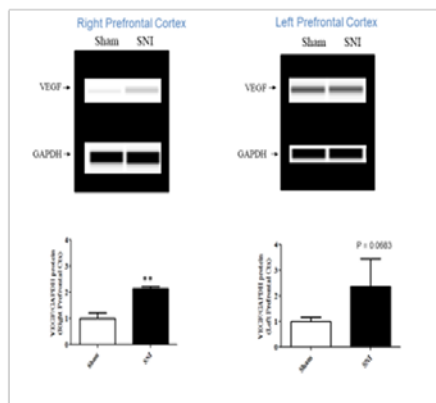


Fig. 2



Abbreviations:
injury; VEGF:
endothelial growth

SNI: spared nerve
vascular
factor

4. Effect of battlefield stressors on analgesic efficacy (100% completed). The objective was to determine the effects of stress on morphine analgesia and tolerance. First we established morphine-induced analgesic tolerance in a rat model. Rats received chronic morphine injections (twice/day for 6 days). The development of analgesic tolerance was assessed by behavioral thermal test. We also investigated the role of nerve growth factor (NGF), substance P and calcitonin gene-related peptide (CGRP) using ELISA technique in morphine-mediated tolerance state.

Significant findings: We have established this model in our laboratory (Fig. 1). NGF level significantly decreases at the spinal level. We also observed that anti-NGF treatment delays the analgesic tolerance. We have published this data in the journal BMC Anesthesiology (Cheppudira et al. 2016).

Fig. 1

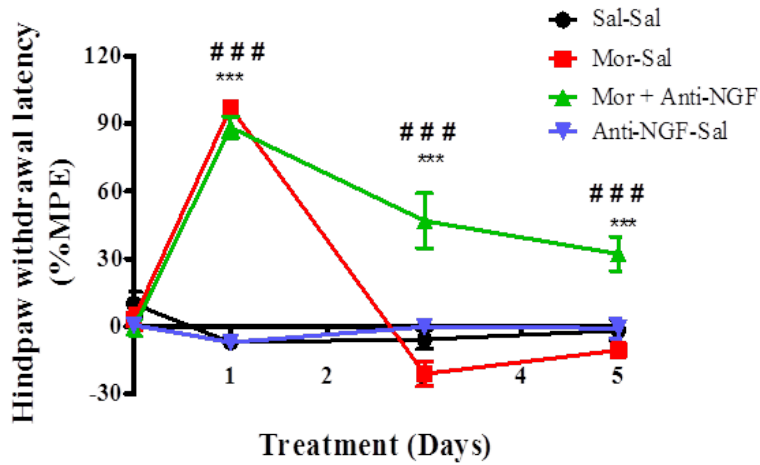
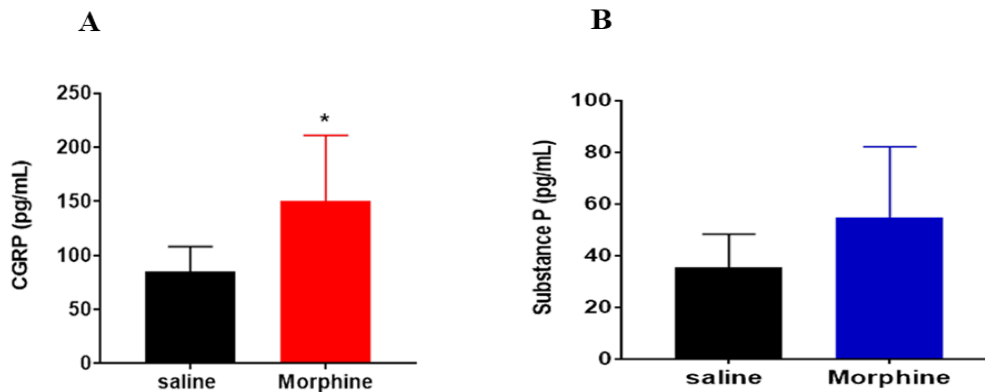


Fig. 2

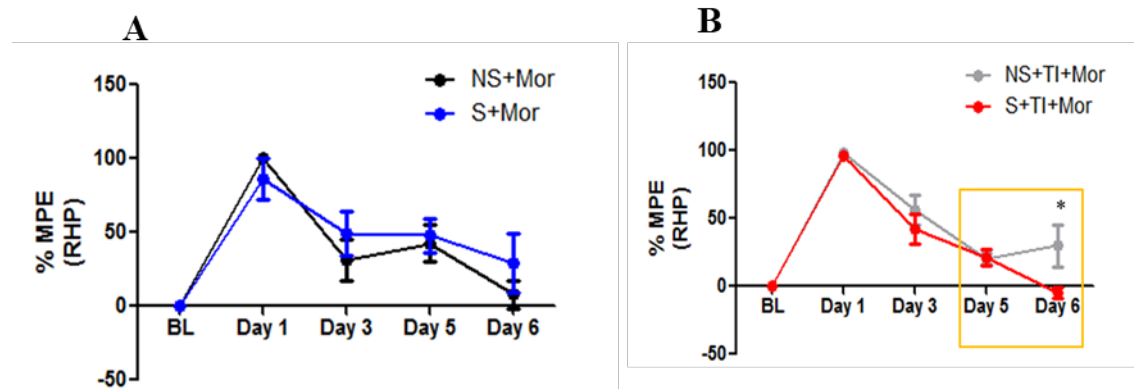
Our data also showed significant increase in the spinal CGRP level in morphine tolerant rats (Fig. 2a) but not substance P (Fig. 2b).



Next we performed experiments to study the effect of battlefield stressors on morphine analgesic tolerance in rats with and without thermal injury.

Significant findings: Our data showed that exposure to chronic stress prior to morphine treatments has no significant effect on morphine-mediated analgesic tolerance in uninjured rats (**Fig. 3a**). However, chronic stress appears to increase pain sensitivity to mechanical stimulus in morphine tolerant rats on day 6 (**Fig. 3b**).

Fig 3.



Abbreviations: NS: No stress; S: Stress; Mor: Morphine; TI: Thermal injury

5. Investigate interventions that enhance analgesia (75% completed). The objective was to use a combination of drugs to enhance analgesia and also to study the effect of stress on enhanced analgesia. Till date, we have examined the combined effects of midazolam and morphine on analgesia, tolerance, and respiration rate in a rat model. Rats received subcutaneous midazolam (2.5 mg/kg) or morphine (10 mg/kg) or midazolam (2.5 mg/kg) + morphine (10 mg/kg) or saline (S; 0.5 ml), twice per day for four days and once on the fifth day. Analgesia was tested by paw withdrawal from a heat stimulus as reported previously (4).

Respiratory parameters by whole body plethysmography were recorded as well. After the final behavioral experiments, animals were euthanized; trunk blood and L4-L6 region of the spinal cord were harvested and analyzed for calcitonin gene-related peptide (CGRP) and substance P (SP) proteins expression using ELISA technique.

Significant results: Under our experimental conditions, midazolam has no antinociceptive activity. A non-analgesic dose of midazolam (2.5 mg/kg) potentiates morphine-induced antinociceptive activity and reduces morphine-mediated tolerance behavior (Fig.1A and 1B). Midazolam and morphine contributes to respiratory depression (Fig. 1C). Chronic treatment of midazolam, morphine, midazolam and morphine has no significant effect on liver enzyme activity (Table 1). Repeated administration of midazolam, morphine, and midazolam plus morphine increases CGRP protein expression but no SP (Fig 1D and 1F). However, because of lack of time we could not study a combination of other potential analgesic drugs.

Fig. 1

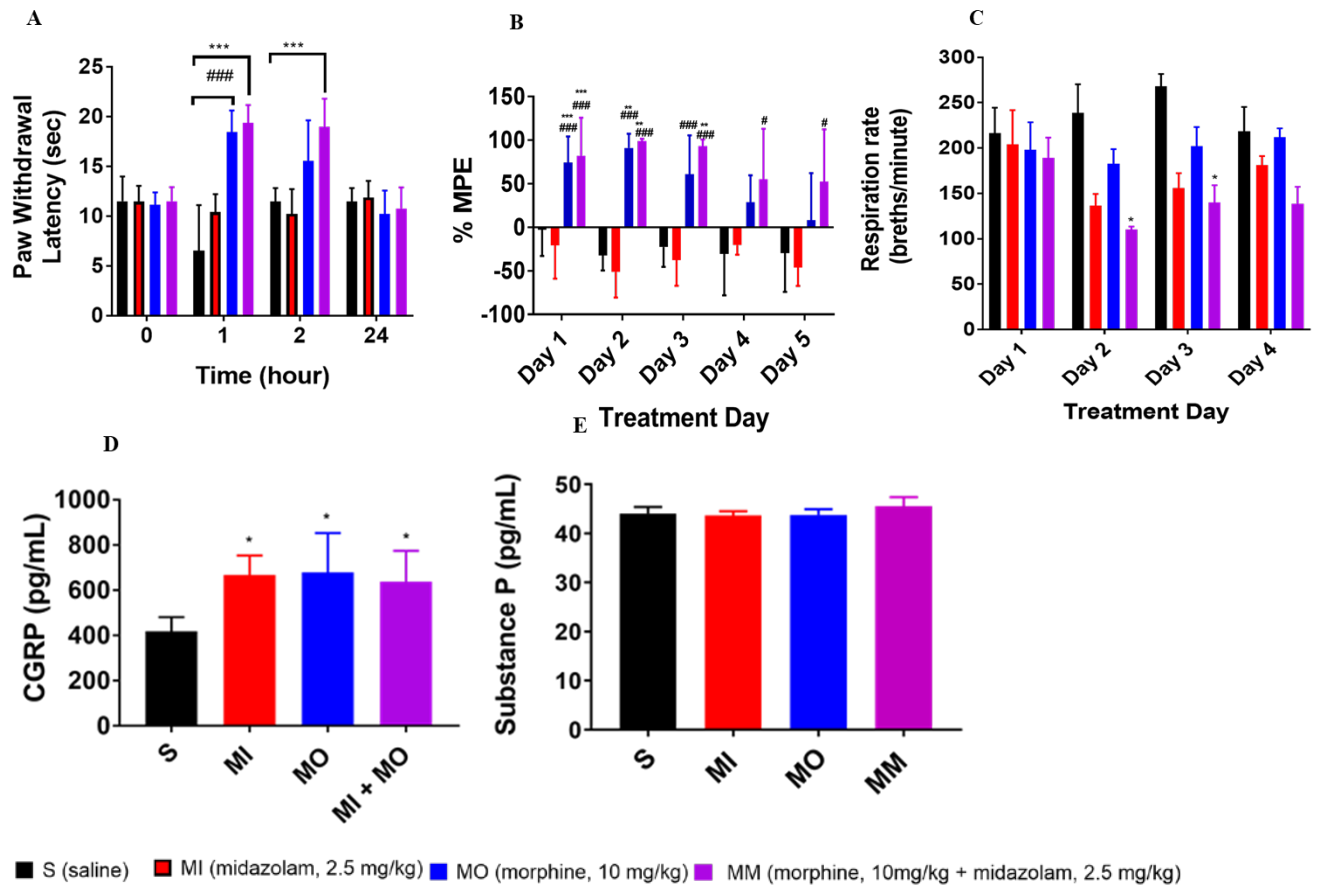


Table 1

	ALP (U/L)	ALT (U/L)	GGT (U/L)	BA (μmol/L)	TBIL (mg/dL)	ALB (g/dL)	BUN (mg/dL)	CHOL (mg/dL)
S	27.57	27.42	<5	3.142	0.257	2.24	6	32.85
MI	27	32.6	<5	11.2	0.22	2.42	6.2	39.2
MO	32.625	30.37	<5	4.3	0.212	2.36	8	37.625
MM	40	31.42	<5	8.71	0.22	2.57	8.28	39.57

Alkaline Phosphatase (ALP), Alanine Aminotransferase (ALT), Gamma Glutamyl Transferase (GGT), Bile Acids (BA), Total Bilirubin (TBIL), Albumin (ALB), Blood Urea Nitrogen (BUN), Total Cholesterol (CHOL).

What opportunities for training and professional development has the project provided?

Dr. Natasha M. Sosanya was working as a postdoctoral fellow in this project. She received extensive training in the areas of pain mechanisms and stress neurobiology. Already, she has demonstrated her expertise by performing experiments related to this project. She has presented the data in our lab meetings and also in the form of posters at national and international scientific meetings. She also contributed in manuscript writings. Recently, she was promoted as Staff Scientist.

Dr. Emily Workman, a postdoctoral fellow, obtained training in developing and standardizing an animal model of neuropathic pain and also in pain behavioral methods. She completed her training within three months and later moved to Project 4 related to this grant application. She performed the animal experiments for Project 4 by utilizing these techniques.

Mr. Alex V. Trevino, Mr. Roger L Chavez and Mr. Thomas Garza are technicians who are working (40% -50% of their time) for this project. They received training in animal surgery, behavior testing, and molecular biology and in routine laboratory procedures. The training advanced their technical skills and expertise in the areas of pain and stress research fields.

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Some of the data from this project is published.

Neurobiology of battlefield stress mechanisms research area was established at US Army Institute of Surgical Research which has gained interest from other task areas within our institute. This research line has also provided a research training opportunity for students and visiting scientists interested in battlefield stress and pain management.

What do you plan to do during the next reporting period to accomplish the goals?

As stated under objectives, we have collected several data set. Manuscripts will be written based on those data and will be submitted for publications.

5. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Exposure to sound stress is common in the battlefield. However, the effect of sound stress on post-injury pain was unknown. Our data showed that sound stress can profoundly influence the intensity of thermal pain in post-injury state. Corticotrophin-releasing factor (CRF), a hormone whose levels are altered in the blood plasma and in the various areas of the brain in stress state, was found to be associated with sound stress mediated exacerbation of pain response in thermal injured rats. The data from this study has allowed us to consider CRF system as a novel drug target to treat stress-mediated enhancement of thermal pain. Please refer to the publication Sosanya et al., 2017 for additional information.

The interaction and underlying mechanism between battlefield stress and pain is not fully understood. One reason was due to lack of an appropriate animal model. Now we have developed an animal model that mimics battlefield stressors and its impact on post-thermal pain. We have also investigated the combined effect of stressors and thermal pain on brain-derived nerve growth (BDNF) factor which may help to consider BDNF as a novel target to treat stress associated pain. The findings are published in the journal BMC Neuroscience (Sosanya et al., 2019).

Development of analgesic tolerance is a major problem in the treatment of chronic pain. We have examined the role of nerve growth factor (NGF) in morphine-mediated analgesic tolerance mechanism. NGF can be a novel target to minimize opioid-induced analgesic tolerance. The outcome of this study is published in BMC Anesthesiology (Cheppudira et al. 2016).

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

10. CHANGES/PROBLEMS:

Nothing to Report

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

It took 5-6 months to standardize and perform Wes Protein Simple and ELISA techniques. This delayed to complete some of the experiments under specific aim 2. Also, two postdoctoral fellows left our lab during this period.

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

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals

No significant changes to animal protocols occurred during the project.
All studies conducted in the project were approved by the USAISR IACUC:

Protocol A-16-039 “Chronic Unpredictable Stress-induced Changes in Pain Sensitivity in a Rat Thermal Injury” approved” approved May 31st, 2016.

Protocol A-14-027 “The Role of Nerve Growth Factor (NGF) in Antinociceptive Tolerance of Morphine in a Rat Model” approved June 18th, 2014.

Significant changes in use of biohazards and/or select agents

Nothing to Report

11. PRODUCTS:

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

Journal publications.

Sosanya NM, Garza TH, Stacey W, Crimmins SL, Christy RJ, Cheppudira BP. Involvement of brain-derived neurotrophic factor (BDNF) in chronic intermittent stress-induced enhanced mechanical allodynia in a rat model of burn pain. BMC Neurosci. 2019 Apr 24; 20(1):17.

Sosanya NM, Trevino AV, Chavez RL, Christy RJ, Cheppudira BP. Sound-stress-induced altered nociceptive behaviors are associated with increased spinal CRFR2 gene expression in a rat model of burn injury. J Pain Res. 2017 Sep 1; 10:2135-2145.

Cheppudira BP, Trevino AV, Petz LN, Christy RJ, Clifford JL. Anti-nerve growth factor antibody attenuates chronic morphine treatment-induced tolerance in the rat. BMC Anesthesiol. 2016 Sep 5;16(1):73.

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

Abstracts:

1. Cheppudira BP, Trevino A, Petz LN, Gibbons R, Clifford J. Anti-nerve growth factor antibody alters morphine tolerance in rats with and without thermal injury. American Pain Society Scientific Meeting, May 13-16, 2015. Palm Springs, CA.*
2. Sosanya NM, Trevino AV, Chavez RL, Christy RJ, Cheppudira BP. Sound Stress-induced Changes in Pain Behaviors in Rats with and without Thermal Injury. Military Health System Research Symposium (MHSRS), August 15-17, 2016. Orlando, FL.*
3. Sosanya NM, Trevino AV, Chavez RL, Christy RJ, Cheppudira BP. Sound Stress Activates Corticotropin-releasing Factor Receptor-2 (CRFR2) in the Lumbar Spinal Cord of Rats with Thermal Injury. Society for Neuroscience Annual Meeting. November 12-16, 2016. San Diego, CA.*
4. Sosanya NM, Stacey W, Garza T, Trevino AV, Christy RJ, Cheppudira BP. Altered tropomyosin receptor kinase B (TrkB) protein expression in the hypothalamus links to prolonged stress-induced enduring mechanical allodynia in naïve and thermal injured rats. American Pain Society Scientific Meeting. May 17-20, 2017. Pittsburgh, PA.*
5. Sosanya, N.M., Garza, T., Stacey, W., Trevino, AV., Christy, RJ., Cheppudira, BP. Prior chronic stress induces aggravated allodynia and hyperalgesia behaviors in thermal injured rats: Involvement of BDNF and CRF signaling mechanisms. (Aug. 2017). Oral presentation at MHSRS. FL.
6. Sosanya, N.M., Garza, T., Christy, RJ., Cheppudira, BP. Development and Characterization of a Preclinical Model Recapitulating Battlefield Stress. (June 2017). (San Antonio Military Health System and Universities Research Forum (SURF)). San Antonio, TX. Poster.
7. Cheppudira BP, Trevino AV, Alrufaiee OA, Sosanya NM, Crimmins SL, Christy RJ. Role of nerve growth factor/tyrosine kinase receptor A signaling in the plasma of morphine tolerant rat. American Pain Society Scientific Meeting. March 4-6, 2018. Anaheim, CA.
8. Sosanya NM, Tongkhuya S, Christy RJ, Crimmins SL, Cheppudira BP. Effect of Chronic Stress on Antinociceptive Tolerance in a Thermal Injury Rat Model. Military Health System Research Symposium (MHSRS), August 20-23, 2018. Orlando, FL.
9. Cheppudira BP, Trevino AV, Christy RJ, Crimmins SL. Relative Role of Calcitonin Gene-related Peptide (CGRP) and Substance P (SP) in Morphine-mediated Antinociceptive Tolerance mechanism. Military Health System Research Symposium (MHSRS), August 20-23, 2018. Orlando, FL.
10. Linsenbardt HR, Sosanya NM, Tongkhuya S, Christy RJ, Crimmins SL, Cheppudira BP. Effect of Chronic Stress and Morphine Treatments on Spinal Nerve Growth Factor in a Rat Model. This poster will be presented at Military Health System Research Symposium (MHSRS), August 20-23, 2018. Orlando, FL.

Other publications, conference papers and presentations.

Sosanya, N.M. (April 2019). Battlefield Pain and the Future Fight. Oral presentation. USAISR Science Seminar. TX.

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

We have established several techniques in our laboratory that are related to this project.
Animal models of stress: Restraint stress model, forced swim stress model, cold stress model and sound stress model.
Pain models: Spared nerve injury (SNI) model.
Analgesic tolerance model: Morphine-induced tolerance model.
Biochemical techniques: Wes Protein Simple, ELISA, RT-PCR

- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

- We have developed a novel rat model to study battlefield stress-induced effects on pain mechanisms. This model can be used to study stress-pain interaction and also to investigate new analgesics and anti-stress agents.
- We have identified nerve growth factor (NGF) system as a new drug target to reduce morphine-induced analgesic tolerance.

12. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Bopaiah Cheppudira, PhD., Principal Investigator: No Change
Natasha M. Sosanya, PhD., Postdoctoral Fellow: No Change
Alex V. Trevino, BS., Research Technician: No Change
Thomas Garza, BS., Senior Biological Technician: No Change

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

13. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Nothing to report

QUAD CHARTS: Nothing to report

14. APPENDICES: Nothing to report

AWARD NUMBER: MR157005

TITLE: Effect of Pain and Analgesia on Physiological Compensatory Responses to Hemorrhage

PRINCIPAL INVESTIGATOR: Carmen Hinojosa-Laborde, PhD.

CONTRACTING ORGANIZATION: U.S. Army Institute of Surgical Research

REPORT DATE: April 30, 2019

TYPE OF REPORT: Final Annual Report

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

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				5b. GRANT NUMBER MR157005	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Carmen Hinojosa-Laborde, PhD E-Mail: carmen.hinojosa-laborde.civ@mail.mil				5d. PROJECT NUMBER	
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12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Currently multiple drugs are being used on the battlefield to ameliorate pain, but effects of these medications on the physiological responses to hemorrhage have not been systematically evaluated. To address this deficiency in our knowledge, the goals of this project are 1) to determine how unrelieved pain and pain relief with different analgesics affect the physiological compensatory responses and survival time to compensated and decompensated hemorrhagic shock, 2) to identify the drugs which have non-depressive effects on the compensatory responses and survival time to hemorrhage, and 3) to evaluate the use of non-depressive analgesics during hemorrhage in swine. The central hypothesis of this application is that pain relieving analgesics used as standard of care on the battlefield have varying effects on physiological compensatory responses and survival characteristics to compensated and decompensated hemorrhagic shock. Opioid analgesics (morphine, fentanyl, and sufentanil) are expected to cause greater suppression of compensatory responses and decrease survival time to hemorrhage compared to non-opioid analgesics (ketamine). This project will evaluate analgesic drugs currently used on the battlefield to provided evidence to support the goals and guidelines for pre-hospital pain management.					
15. SUBJECT TERMS prehospital, analgesia, pain, trauma, hemorrhage, blood pressure, heart rate, hemodynamic compensation, respiration, cardiorespiratory, opioid, morphine, ketamine, fentanyl, sufentanil, shock					
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	17	19b. TELEPHONE NUMBER (include area code)

7. INTRODUCTION:

Currently multiple drugs are being used on the battlefield to ameliorate pain, but effects of these medications on the physiological responses to hemorrhage have not been systematically evaluated. To address this deficiency in our knowledge, the goals of this project are 1) to determine how unrelieved pain and pain relief with different analgesics affect the physiological compensatory responses and survival time to compensated and decompensated hemorrhagic shock, 2) to identify the drugs which have non-depressive effects on the compensatory responses and survival time to hemorrhage, and 3) to evaluate the use of non-depressive analgesics during hemorrhage in swine. The central hypothesis of this application is that pain relieving analgesics used as standard of care on the battlefield have varying effects on physiological compensatory responses and survival characteristics to compensated and decompensated hemorrhagic shock. Opioid analgesics (morphine, fentanyl, and sufentanil) are expected to cause greater suppression of compensatory responses and decrease survival time to hemorrhage compared to non-opioid analgesics (ketamine). This project will evaluate analgesic drugs currently used on the battlefield to provide evidence to support the goals and guidelines for pre-hospital pain management.

8. KEYWORDS:

prehospital, analgesia, pain, trauma, hemorrhage, blood pressure, heart rate, hemodynamic compensation, respiration, cardiorespiratory, opioid, morphine, ketamine, fentanyl, sufentanil, shock

9. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: Determine the effect of pain and analgesia on physiological compensatory responses to compensated hemorrhagic shock in rats

Major Task 1: Assess the effect of pain on the compensatory responses to hemorrhage.

Subtask 1: Define hemorrhage volume for moderate and severe hemorrhage, develop orthopedic trauma pain rat model, and dose response curves for morphine, ketamine, fentanyl, and sufentanil. (Predicted: 1-12 months, Actual: 6-15 months, 100% completed)

Subtask 2: Determine the effects of unrelieved pain on physiological compensatory responses to compensated hemorrhagic shock in rats. (Predicted: 2-18 months, Actual: 15-24 months, 100% completed)

Subtask 3: Determine the effects of morphine, ketamine, fentanyl, and sufentanil on physiological compensatory responses to compensated hemorrhagic shock in rats. (Predicted: 4-14 months, Actual: 24-36 months, 25% completed)

Specific Aim 2: Determine the effect of pain and analgesia on survival time after decompensated hemorrhagic shock in rats

Major Task 2: Assess the effect of pain and analgesia on decompensated hemorrhage

Subtask 1: Determine the effects of unrelieved pain on survival time after decompensated hemorrhagic shock in rats. (Predicted: 18-36 months, Actual: 15-24 months, 100% completed)

Subtask 2: Determine the effects of morphine, ketamine, fentanyl, and sufentanil on survival time after decompensated hemorrhagic shock in rats (Predicted: 18-36 months, Actual: 24-36 months, 25% completed)

Specific Aim 3: Identify non-depressive analgesics in rats and test in swine models of hemorrhage

Major Task 3: Assess if analgesics that demonstrate minimal effects on hemorrhage responses in rats will provide the same results in swine.

Subtask 1: Effect of non-depressive analgesia on compensatory responses to compensated hemorrhage in swine. (Predicted: 18-24 months, Actual: 0% completed)

Subtask 2: Effect of non-depressive analgesia on survival after decompensated hemorrhage in swine. (Predicted: 18-36 months, Actual: 0% completed)

What was accomplished under these goals?

Major Activities/Specific Objectives/Significant Results for this Reporting Period

1. Orthopedic Trauma Model Development (100% completed): The objective was to establish a novel animal model of trauma which mimics pre-hospital traumatic injury. The orthopedic trauma (OT) model includes soft tissue injury and fracture but does not require fixation surgery. Soft tissue injury was induced by clamping the muscle groups adjacent to both the femur and fibula for 30 seconds with an angled Kelly clamp. Fibula fracture was induced through penetrating the skin and fascia with a 15-gauge needle between the tibia and fibula. Using the tibia as a fulcrum, the needle was twisted to apply sufficient pressure on the fibula to cause fibula fracture. Significant Results: This novel animal trauma model produced a unique trauma model that mimics pre-hospital traumatic injury and develops prompt hyperalgesia.

Major Activities/Specific Objectives/Significant Results (continued)

2. Comparison between Orthopedic Trauma and Thermal Injury Trauma models (100% completed): The objective was to characterize and compare the acute pain responses to orthopedic trauma and thermal injury. Animals were tested for mechanical allodynia, thermal hyperalgesia, weight bearing, and tail flick responses at 15, 30, 60, 90, and 120 minutes after injury. Significant results: The orthopedic trauma model produced an early-onset pain profile which better represented pre-hospital pain than the thermal injury model (Figure 1).

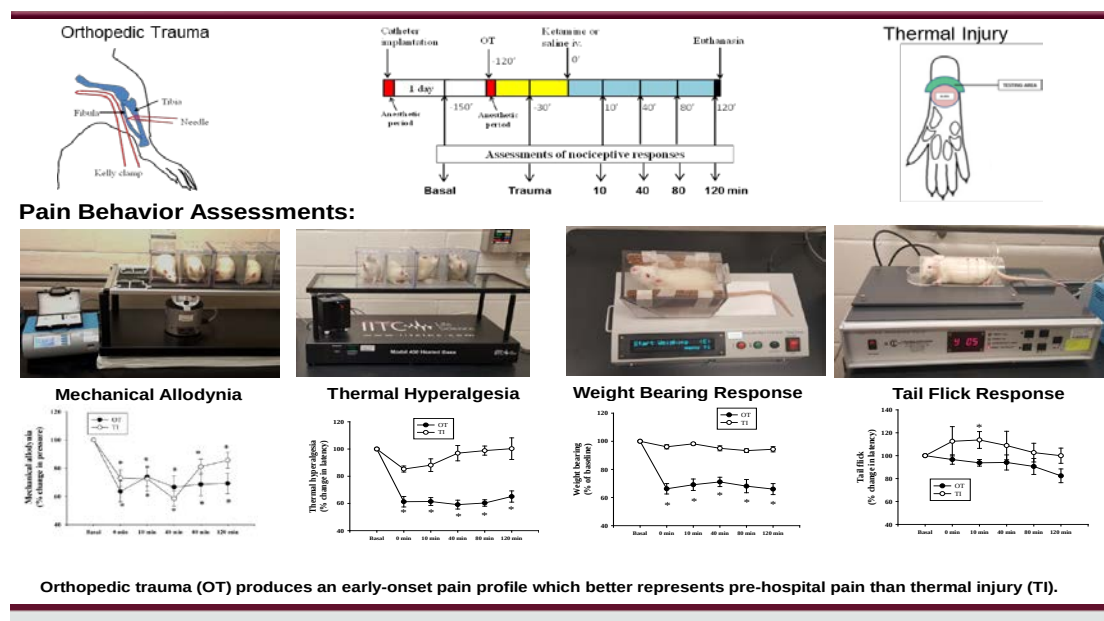
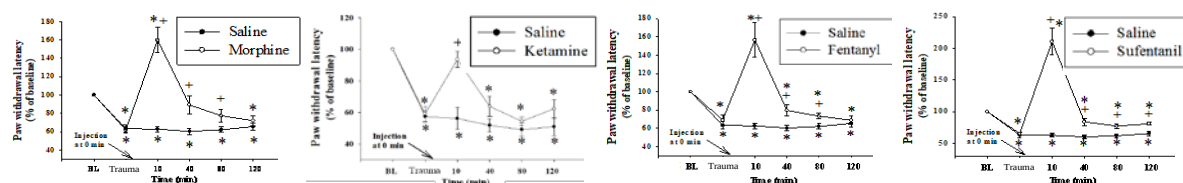


Figure 1.

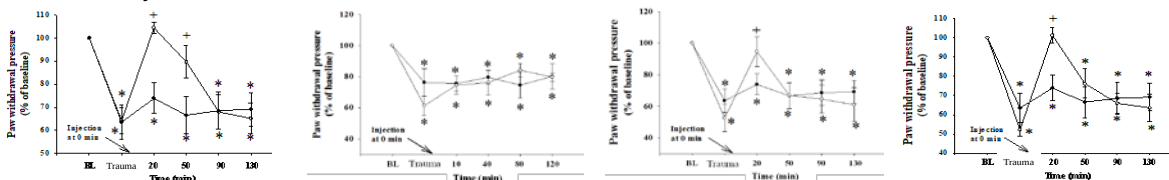
3. Ketamine, Morphine, Fentanyl, and Sufentanil Dose Response in OT model (100% completed): The objective was to determine the analgesic dose-response relationship for ketamine to identify the optimal dose of ketamine which provides significant analgesia with minimal sedation in the OT model of trauma. Three doses of ketamine (1, 5, 10 mg/kg), morphine (1, 2, 5 mg/kg), fentanyl (5, 10, 50 ug/kg), and sufentanil (1, 5 ug/kg) were administered via intravenous injection. Animals will be tested for mechanical allodynia, thermal hyperalgesia, weight bearing, and tail flick responses for each dose at 15, 30, 60, 90, and 120 minutes after dosing. Significant results: The optimal doses of ketamine (5 mg/kg), morphine (2 mg/kg), fentanyl (10 ug/kg), and sufentanil (1ug/kg) were identified to use in subsequent OT model and hemorrhage studies (Figure 2).

Major Activities/Specific Objectives/Significant Results (continued)

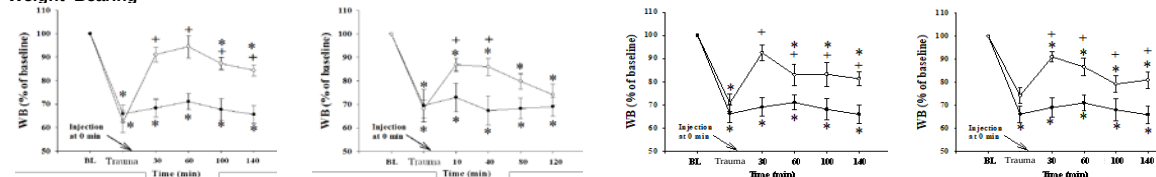
Thermal Hyperalgesia



Mechanical Allodynia



Weight Bearing



All four analgesics will be tested in the orthopedic trauma model in rats after moderate and severe hemorrhage.

Figure 2.

4. Conscious Rat Hemorrhage Model Development (100% completed): The objective was to establish the conscious rat hemorrhage protocol in which all analgesics will be tested. Procedures were refined to conduct a conscious, controlled hemorrhage of rats implanted with telemetry blood pressure recording devices and housed in a whole body Plethysmography chamber for respiration measurements during hemorrhage (Figure 1A). Significant results: Hemodynamic, respiratory, and metabolic measurements can be simultaneously recorded in a conscious animal exposed to trauma and hemorrhage.

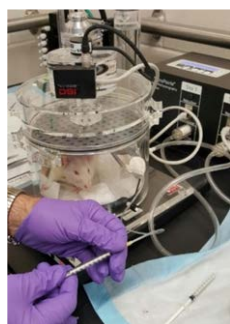


Figure 2A

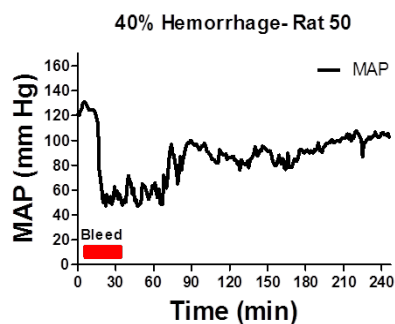


Figure 2 B

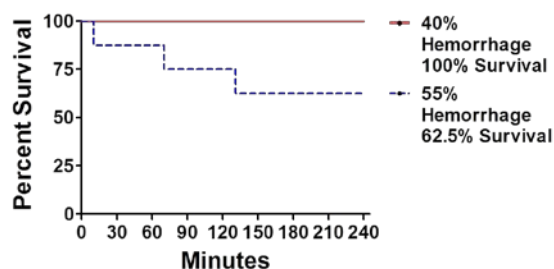


Figure 2C

Major Activities/Specific Objectives/Significant Results (continued)

5. Hemorrhage Severity Model Development (100% completed): The objective was to define the volume of blood removal to consistently mimic compensated and decompensated hemorrhage in rats. Seven hemorrhage volumes were tested while measuring cardiovascular (heart rate, mean arterial pressure), respiratory (respiration rate, tidal volume, minute volume), and metabolic (base deficit, blood lactate, blood [K⁺], body temperature) changes. Mortality was also recorded. Significant results: Compensated hemorrhage was produced by removal of 40% of total blood volume (Figure 2B). Decompensated hemorrhage was produced by removal of 55% of total blood volume (Figure 2C). These two hemorrhage models (40% and 55%) will be used for future tests of analgesic effects on responses to hemorrhage.

6. Effects of Trauma with Pain on Compensatory Responses and Survival to Hemorrhage (100% completed): Trauma with unrelieved pain was induced by orthopedic trauma, while control animals were not exposed to trauma. All animals were hemorrhaged by 40% or 55% blood volume. Significant Results: Trauma with pain did not affect the blood pressure compensatory response to 40% hemorrhage (Figure 3A). Trauma with pain significantly reduced the blood pressure compensatory response to 55% hemorrhage (Figure 3B). Trauma with pain significantly decreased survival to 55% hemorrhage (Figure 3C). These results support the requirement to include pain and trauma in the animal model used to test analgesic effects after hemorrhage.

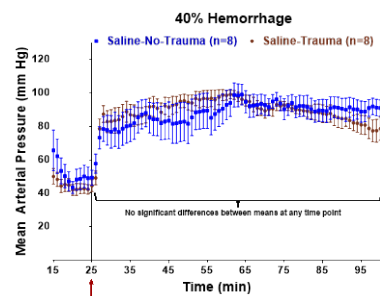


Figure 3A

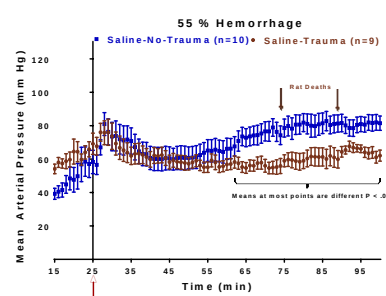


Figure 3B

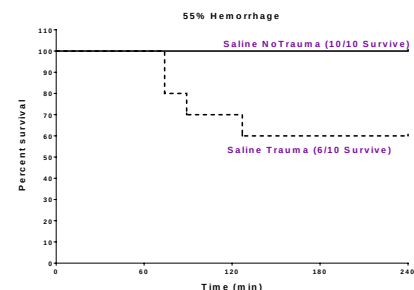


Figure 3C

7. Effects of Ketamine on Compensatory Responses to 40% hemorrhage (100% complete): All animals were exposed to trauma with associated pain followed by hemorrhage of 40% blood volume. Ketamine was administered at the end of hemorrhage. The control group was treated with saline. Significant Results: Analgesic doses of ketamine given immediately after hemorrhage did not affect the blood pressure (Figure 4A) or respiratory responses (Figure 4B) to moderate compensated 40% hemorrhage.

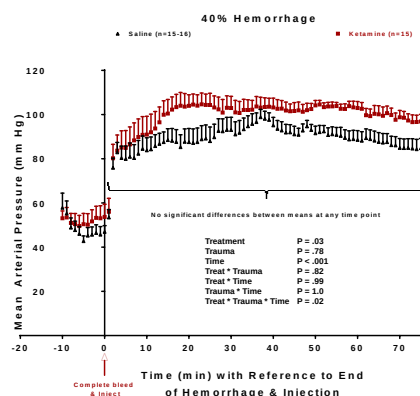


Figure 4A

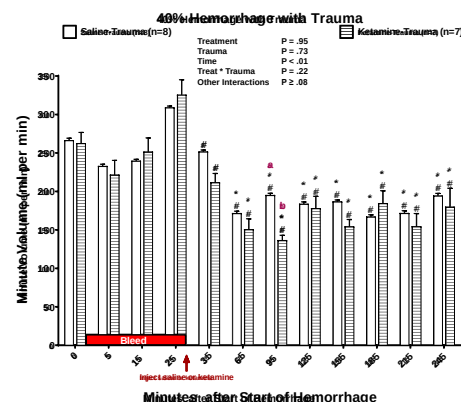


Figure 4B

Major Activities/Specific Objectives/Significant Results (continued)

8. Effects of Ketamine on Compensatory Responses and Survival to 55% hemorrhage (100% complete): All animals were exposed to trauma with associated pain followed by hemorrhage of 55% blood volume. Ketamine was administered at the end of hemorrhage. The control group was treated with saline. Significant Results: Analgesic doses of ketamine given immediately after hemorrhage did not affect the blood pressure (Figure 5A), respiratory responses (Figure 5B), or survival (Figure 5C) after severe decompensated 55% hemorrhage.

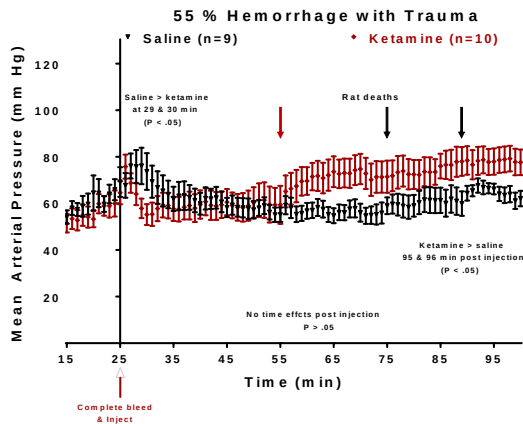


Figure 5A

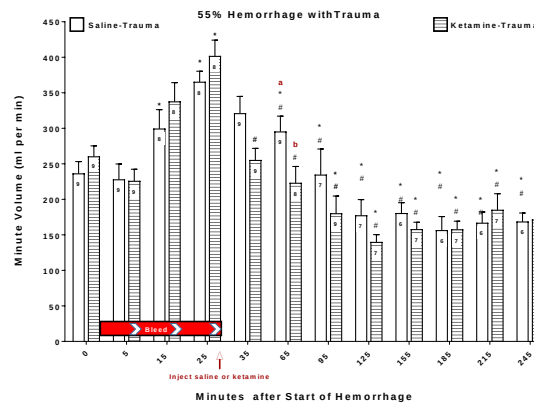


Figure 5B

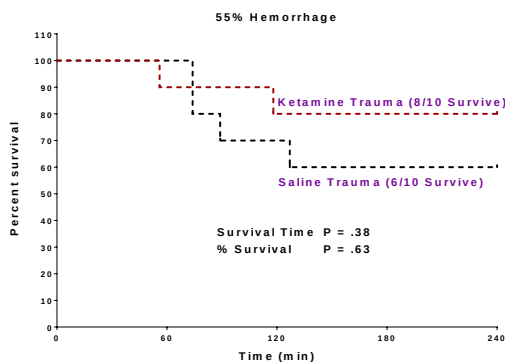


Figure 5C

9. Stated Goals Not Met:

a. Effect of Opioid Analgesics on 40% and 50% hemorrhage. The effects of morphine, fentanyl, and sufentanil were not tested during the 36 months of funding due to the unexpected delay resulting from losses of experimental animals after instrumentation surgery. However, this project has been extended via Army Core funds, and experiments on the opioid drugs are currently being conducted. These experiments will be completed by June 2019.

b. Assessment of selected analgesics in swine after compensated (moderate) and decompensated (severe) hemorrhage. The proposed experiment to test a non-depressive analgesic in a conscious swine model of hemorrhage without trauma was not initiated. Results obtained from rat studies at the end of 24 months indicated that trauma and the associated pain contributed significantly to the hemodynamic responses and survival to severe hemorrhage. Since the swine model did not include trauma and pain, it would be necessary to develop a new swine model to include trauma and pain. During the final 12 months of the project, efforts were focused on testing ketamine during hemorrhage in rats, and no time was available to develop the appropriate pain and hemorrhage swine model.

What opportunities for training and professional development has the project provided?

Project investigators attended the following pain-management conferences
2016 Annual Pain Society Meeting, Austin, TX, May 11-13, 2016

2018 Annual Pain Society Meeting, Anaheim, CA, March 5-7, 2018

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?

Final Report: Nothing to Report

6. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

We have developed a battlefield-relevant rat model of extremity trauma and conscious hemorrhage that provides a means to evaluate the effects of analgesics on cardiorespiratory responses during pre-hospital care. We have shown that ketamine at an analgesic dose did not produce profound adverse effects on cardio-respiratory function following extremity trauma and moderate (40%) hemorrhage. In addition, ketamine did not produce profound adverse effects on survival following extremity trauma and severe (55%) hemorrhage. After we complete the ongoing studies in opioids, we will be able to determine if opioids alter survival and cardiorespiratory response after hemorrhage.

What was the impact on other disciplines?

The results of this project are impacting the development and design of experiments in humans by Dr. Craig Crandall at Univ. of Texas Southwestern Medical Center. Dr. Crandall is currently evaluating the cardiovascular and autonomic nervous system effects of analgesic doses of ketamine, morphine and fentanyl in a human model of hemorrhage.

What was the impact on technology transfer?

This project does not impact “technology transfer” as this produced knowledge regarding an FDA-approved drug already in use on the battlefield.

What was the impact on society beyond science and technology?

The knowledge that analgesic doses of ketamine did not affect the compensatory response to hemorrhage are likely to make an impact on the use of ketamine as an analgesic. Currently, ketamine is FDA-approved as a sole anesthetic (not analgesic). Results from this project would support the change to include analgesia as an FDA-approved use of ketamine, or increase the acceptability of off-label use of ketamine as an analgesic.

15. CHANGES/PROBLEMS:

The proposed experiment in Specific Aim 3 was to test a non-depressive analgesic in a conscious swine model of hemorrhage without trauma. Results obtained from rat studies at the end of 24 months indicated that trauma and the associated pain contributed significantly to the hemodynamic responses and survival to severe hemorrhage. Since the swine model did not include trauma and pain, it would be necessary to develop a new swine model to include trauma and pain. During the final 12 months of the project, efforts were focused on testing ketamine during hemorrhage in rats, and no time was available to develop the appropriate swine model. As a result, the experiment in Specific Aim 3 was not executed.

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

Early delays in the project (1-8 months) were due to the prolonged time needed for equipment procurement and training of staff to use this new equipment. During mid-project (8-12 months), additional delays were due to high mortality rate in experimental animals due to multiple survival surgeries. Issues with animals not surviving surgeries was mitigated by acquiring expert training on how to improve surgical success. No significant delays were encountered after the first year of the project.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Delays and problems encountered during the project did not significantly impact expenditure. As reported, the experiment in Specific Aim 3 was not executed. However, the funds budgeted for this experiment were used to cover expenses for additional animals needed for experiments in Specific Aims 1 and 2. These additional animals were required because of the high mortality rate encountered during surgical instrumentation of the animals.

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

Significant changes in use or care of human subjects

Not applicable

Significant changes in use or care of vertebrate animals

No significant changes to animal protocols occurred during the project.
All studies conducted in the project were approved by the USAISR IACUC:

Protocol A-16-015 “The analgesic doses of ketamine in thermal injured rats-a model development protocol” approved Jan. 20, 2016

Protocol A-16-016 “Effects of Analgesics on Physiological Responses to Moderate or Severe Hemorrhage in Rats: Development of Two Controlled Hemorrhage Models”, approved Jan. 5, 2016

Protocol A-16-031 “The Analgesic Effects of Ketamine Following Orthopedic Trauma in Rats”, approved March 30, 2016.

Protocol A-17-001 “The Analgesic Effects of Morphine, Fentanyl, and Sufentanil Following Orthopedic Trauma in Rats”, approved Oct. 19, 2016.

Protocol A-17-007 “Effects of Ketamine on Physiological Response to Moderate and Severe Hemorrhage in Rats”, approved Nov. 16, 2016

Significant changes in use of biohazards and/or select agents

None

16. PRODUCTS:

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

Journal publications.

1. Xiang L, Klemcke HG, Wienandt NA, Ryan KL, Hinojosa-Laborde C. A novel rat model of extremity trauma for prehospital pain management research. *J Trauma Acute Care Surg* 85(1S Suppl 2): S49-S56, 2018.
2. Xiang L, Calderon AS, Hinojosa-Laborde C, Ryan KL. Fentanyl impairs but ketamine preserves the microcirculatory responses to hemorrhage---a novel approach to evaluate hemodynamic impacts of analgesics. *Anesth Analg*, under review.

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

Nothing to Report

Other publications, conference papers and presentations.

1. Xiang L, Avila MM, Ryan KL, Hinojosa-Laborde C. A novel rat model of orthopedic trauma for prehospital pain studies. Poster presentation, Experimental Biology 2017, Chicago, IL and MHSRS 2017. *
2. Xiang L, Avila MA, Ryan KL, Fernandez JD, Hinojosa-Laborde C. Analgesic effects of intravenous morphine, fentanyl, and ketamine in a rat model of orthopedic trauma. Poster presentation, MHSRS 2017.*
3. Klemcke HG, Calderon ML, Calderon AS, Crimmins SL, Hinojosa-Laborde C. Effects of ketamine on cardiovascular and respiratory responses to severe traumatic hemorrhage in rats. Poster presentation at American Pain Society Meeting, Anaheim, CA, Mar 2018.
4. Xiang L, Klemcke HG, Ryan KL, Hinojosa-Laborde C. Analgesic effects of intravenous morphine, fentanyl, sufentanil, and ketamine in a novel rat model of extremity trauma. Poster presentation at Experimental Biology 2018, San Diego, CA, Apr 2018.
5. Klemcke HG, Calderon ML, Calderon AS, Crimmins SL, Hinojosa-Laborde C. Effects of ketamine on cardiovascular and respiratory responses to a moderate 40% hemorrhage in rats. Poster presentation at Experimental Biology 2018, San Diego, CA, Apr 2018; podium presentation at MHSRS, Kissimmee, FL, Aug 2018.
6. Klemcke HG, Calderon ML, Rodriguez CM, Connor BS, Hinojosa-Laborde C. Effects of opioid analgesics (morphine, fentanyl, and sufentanil) and trauma on survival time after severe conscious hemorrhage in rats. Poster presentation at Experimental Biology 2019, Orlando, FL, 2019; abstract submitted to MHSRS 2019, Kissimmee, FL, Aug 2019.
7. Xiang L, et al., Impact of analgesics on macro- and microcirculatory responses to hemorrhage. Poster presentation at Experimental Biology 2019, Orlando, FL, 2019.*
8. Xiang L, et al. Fentanyl impairs but ketamine preserves the microcirculatory response to hemorrhage ---a novel approach to evaluate hemodynamic impacts of analgesics. Abstract submitted to MHSRS 2019, Kissimmee, FL, Aug 2019.

- **Website(s) or other Internet site(s)**

- **Technologies or techniques**

Nothing to Report

- **Inventions, patent applications, and/or licenses**

Nothing to Report

- **Other Products**

We have developed a battlefield-relevant rat model of extremity trauma and conscious hemorrhage that provides a means to evaluate the effects of analgesics on cardiorespiratory responses administered after hemorrhage. This model will be used in future studies to evaluate novel analgesics identified for potential use on the battlefield.

17. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Carmen Hinojosa-Laborde, PhD., Principal Investigator: No Change
Harold Klemcke, PhD., Research Scientist: No Change
Lusha Xiang, MD, Research Scientist: No Change
Mariam Calderon, Research Technician: No Change
Martha Avila, Research Technician: No Change

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

18. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Nothing to report.

QUAD CHARTS: Nothing to report.

19. APPENDICES: Nothing to report.

Project 4

AWARD NUMBER: MR157005

TITLE: Molecular Mechanism of Pain Perception Involving Key Brain Regions and Potential Surrogate Signatures in Blood: An Integrated-Omics Study

PRINCIPAL INVESTIGATOR: Rasha Hammamieh

CONTRACTING ORGANIZATION: US Army Center for Environmental Health Research

REPORT DATE: 30APR2019

TYPE OF REPORT: Closing/Final Report

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

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1. REPORT DATE 02/05/2019		2. REPORT TYPE Final		3. DATES COVERED 01/01/2016 - 12/31/2018
4. TITLE AND SUBTITLE Molecular Mechanism of Pain Perception Involving Key Brain Regions and Potential Surrogate Signatures in Blood: An Integrated-Omics Study			5a. CONTRACT NUMBER	
			5b. GRANT NUMBER MR157005	
			5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) John Clifford E-Mail: john.l.clifford11.civ@mail.mil			5d. PROJECT NUMBER	
			5e. TASK NUMBER	
			5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) US Army Center for Environmental Health Research 568 Doughton Dr. Ft. Detrick, MD 21704			8. PERFORMING ORGANIZATION REPORT NUMBER US Army Institute of Surgical Research 3698 Chambers Pass Bldg 3611 JBSA Ft. Sam Houston, TX 78234	
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)	
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12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				
13. SUPPLEMENTARY NOTES				
14. ABSTRACT Chronic pain, along with the overuse of opioids, carries a high cost for the military, both economically and in terms of military readiness. Much chronic pain is the result of injury to the nervous system itself (neuropathic pain). The neuroplastic changes associated with the onset and progression of peripheral neuropathic pain and associated nociceptive behaviors remain unclear. The purpose of this study was to identify the underlying molecular mechanisms of pain perception and processing in the brain, and in particular the temporal gene transcription changes in key pain processing brain regions during the onset and progression of chronic neuropathic pain. We also attempted to identify pain biomarkers in the blood. We have used state-of-the-art mRNA sequencing technology to measure gene expression in five key pain processing regions of the brain during the development of chronic neuropathic pain, using the rat spared nerve injury (SNI) model. We have also applied similar RNA sequencing technology towards identifying the complement of circulating exosomal miRNAs that are differentially expressed during chronic pain development in the spinal nerve ligation (SNL) model. Global visualization of the spatiotemporal transcriptome alterations in the SNI model reveals specific high impact times and brain regions where differential gene expression is greatest. We also showed that Circulating exosomal miRNAs were differentially expressed between animals with chronic pain and sham controls in the SNL model.				
15. SUBJECT TERMS neuropathic pain, chronic pain, mechanical allodynia, transcriptome, pain circuit, systems biology				
16. SECURITY CLASSIFICATION OF:		17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC

a. REPORT	b. ABSTRACT	c. THIS PAGE			
Unclassified	Unclassified	Unclassified	Unclassified	11	19b. TELEPHONE NUMBER <i>(include area code)</i>

INTRODUCTION:

Studies indicate that approximately 40% of Service Members deployed in OEF and OIF, and an astounding 80% of OIF/OEF veterans, report chronic pain. Chronic pain, along with the overuse of opioids, carries a high cost for the military, both economically and in terms of military readiness. Much chronic pain is the result of injury to the nervous system itself (neuropathic pain). The neuroplastic changes associated with the onset and progression of peripheral neuropathic pain and associated nociceptive behaviors remain unclear.

The purpose of this study was to identify the underlying molecular mechanisms of pain perception and processing in the brain, and in particular the temporal gene transcription changes in key pain processing brain regions during the onset and progression of chronic neuropathic pain. We also attempted to identify pain biomarkers in the blood.

We have used state-of-the-art mRNA sequencing technology to measure gene expression in five key pain processing regions of the brain (thalamus, cingulate cortex, insular cortex, somatosensory cortex, amygdala) during the development of chronic neuropathic pain, using the rat spared nerve injury (SNI) model. We have also applied similar RNA sequencing technology towards identifying the complement of circulating exosomal miRNAs that are differentially expressed during chronic pain development in the spinal nerve ligation (SNL) model.

10. KEYWORDS:

neuropathic pain, chronic pain, mechanical allodynia, transcriptome, pain circuit, systems biology

11. ACCOMPLISHMENTS:

What were the major goals of the project?

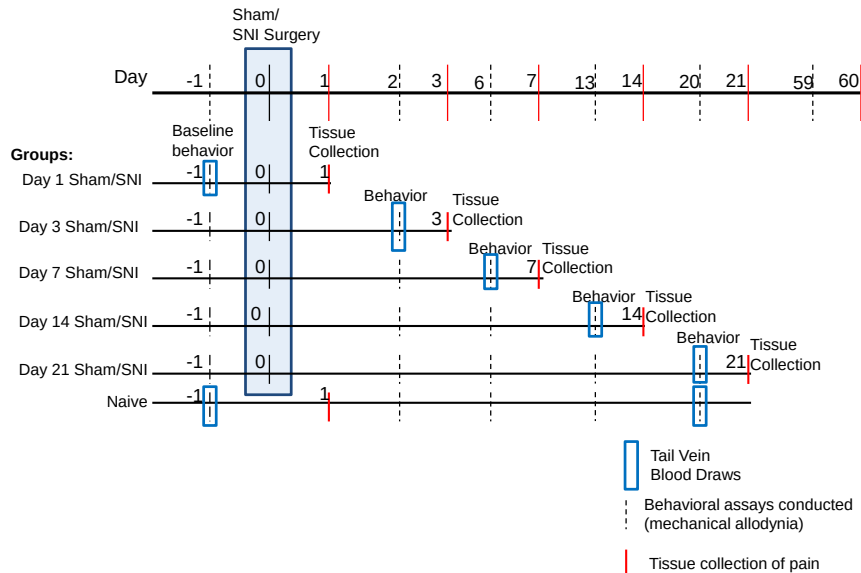
This project was initially proposed as an alternate project (Alternate Project 1) to the multi-investigator Applied Pain Research Award, and had three specific aims. Subsequent to programmatic and scientific review, the project was restructured in order to both conform to budget constraints and address the reviewer concerns. As a result we have focused the project to meet a modified version of the original first and third specific aims, which consist of the following major goals: 1.) Identify the underlying molecular mechanisms of pain perception and processing in the brain, and in particular the temporal gene transcription changes in key pain processing brain regions during the onset and progression of chronic neuropathic pain.; and 2.) Characterize the molecular signatures/ pathways/ networks in key brain regions and in the blood, in order to identify the underlying molecular mechanisms of chronic pain development, as well as to identify biomarkers for chronic pain.

What was accomplished under these goals?

Major Activities

1. Performed the initial time course experiment using the SNI surgical technique (Figure 1).

Figure 1: Experimental design



2. Isolated tissue from brain regions, spinal cord, DRGs, and blood and shipped tissue samples from USAISR to USACEHR, where RNA purification, and mRNA sequencing was conducted.
3. Computational/bioinformatic analysis of all mRNAseq data at USACEHR.
4. Conducted the SNL experiment for identification of pain biomarkers in the blood (USAISR).
5. Computational/bioinformatic analysis of miRNAseq data from SNL experiment (USACEHR).

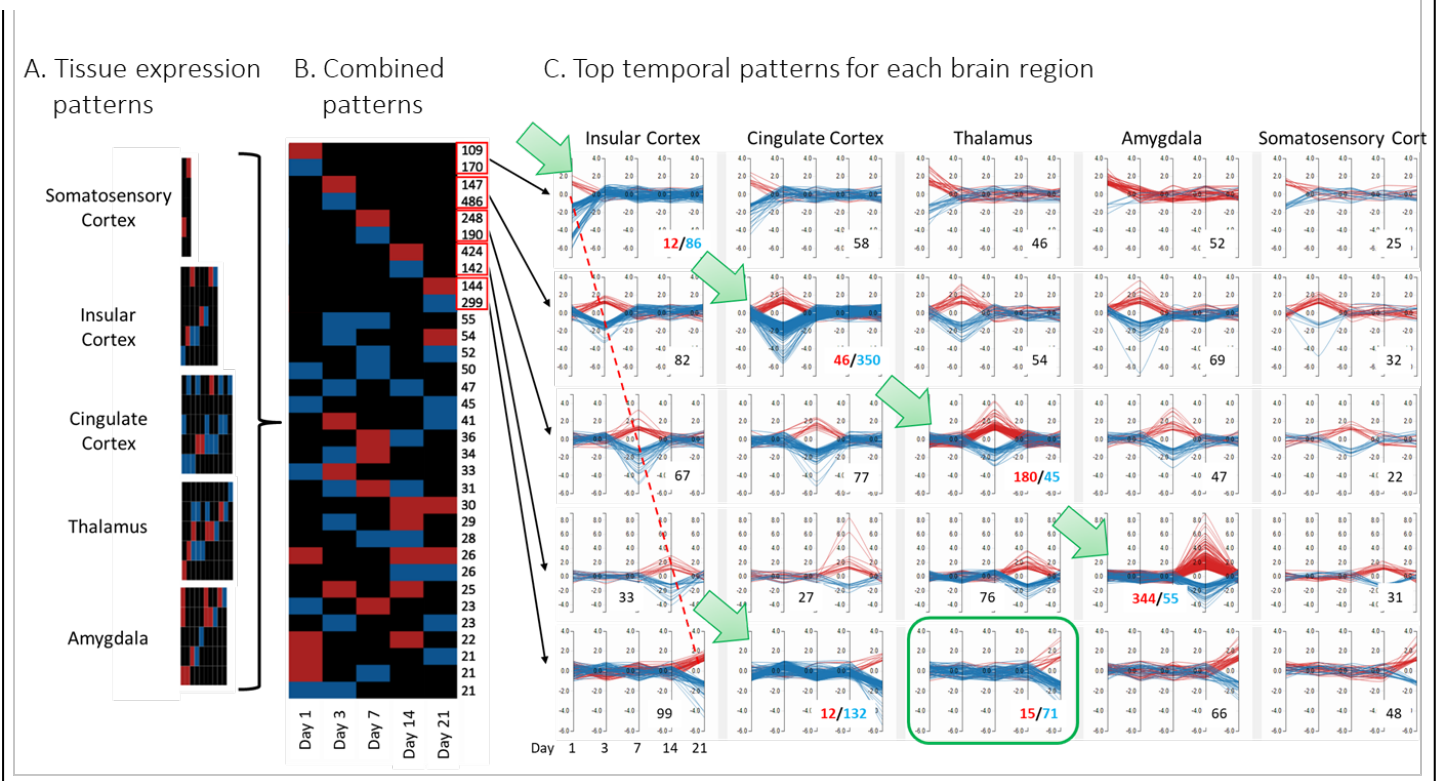
Specific Objectives

- 1.) Identify the underlying molecular mechanisms of pain perception and processing in the brain, and in particular the temporal gene transcription changes in key pain processing brain regions during the onset and progression of chronic neuropathic pain.
- 2.) Characterize the molecular signatures/ pathways/ networks in key brain regions and in the blood, in order to identify the underlying molecular mechanisms of chronic pain development.
- 3.) Identify biomarkers for chronic pain in the blood.

The significant results are summarized as follows:

1. Global visualization of the spatiotemporal transcriptome alterations in the SNI model reveals specific high impact times and brain regions where differential gene expression is greatest (Figure 2).
 - Gene expression consistent with desensitization is most evident in the thalamus at day 21, which is after chronic pain has been established.
2. A single 30 minute administration of bupivacaine given immediately prior to spinal nerve ligation, completely blocked the development of chronic neuropathic pain.
3. Circulating exosomal miRNAs were differentially expressed between animals with chronic pain and sham controls in the SNL model.
 - Identification of the putative mRNA targets for these miRNAs revealed genes implicated in mitochondrial dysfunction, inflammation, synaptic plasticity, and nervous system development and function.

Figure 2 Visual Assessment of Dominant Gene Expression Patterns During Chronic Pain Development



A) Panel indicates expression patterns for groups of DEGs ($n > 20/\text{group}$; $P < 0.05$ at one or more time points, $|FC| > 2.0$), which are represented by a single row, over the time course for each brain region. Red and blue indicate increased and decreased expression, respectively, in SNI compared to sham controls. Black indicates no change exceeding 2-fold. **B)** The 5 panels in (A) combined and sorted according to time and abundance. **C)** Log fold-change plots for the most abundant pair of patterns (up and down) for each day, broken down by brain region. Number of DEGs represented in each graph are indicated. Green arrows highlight the expression patterns for tissues having the greatest number of DEGs for each time point. Green box indicates day 21 thalamus dataset depicted in the gene network analysis in figure 2. Animals per group, $N=6$.

What opportunities for training and professional development has the project provided?

Dr. Natasha M. Sosanya, a postdoctoral fellow who worked on this project, received extensive training in developing and standardizing the SNI model of neuropathic pain and also in pain behavioral testing methods. Other specialized training included instruction in brain microdissection techniques at the USACEHR. She has also gained significant experience in presenting these results at internal meetings and teleconferences, and is planning to present the results at upcoming scientific conferences. Dr. Sosanya also worked on Project 2, which is related to this project.

Dr. Emily Workman is a postdoctoral fellow at the USAISR. She received similar training in neuropathic pain models, pain testing, and microdissection.

How were the results disseminated to communities of interest?

In addition to reporting the results internally at both USACEHR and USAISR, these results have been presented at multiple international scientific conferences, including the MHSRS (multiple years, includes posters and talks), Neurotrauma 2018, Experimental Biology 2018, Society for Neuroscience 2018, and others. There has been one publication (in press) and two others currently in preparation. See below.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report – final report

7. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

This work has made two primary impacts on the base knowledge and research in the pain field, in particular research on chronic neuropathic pain. Both of these impacts are based on our previous finding that administering an analgesic drug, for only a few minutes at the point of injury, in the rat spinal nerve ligation (SNL) model, could completely block the development of chronic neuropathic pain weeks later.

First, by determining the differences in gene expression in specific brain regions, between rats that have been surgically induced to experience chronic pain, and mock surgery controls, we have identified gene expression ‘hot spots’, which are times and places in the brain where there is a concentration of activity that is specific for chronic pain development. Among the more striking changes observed were a downregulation of gene expression that suggests a global suppression of neural signaling in the thalamus (desensitization), much of which does not commence until after 14 days post injury.

Second, we identified miRNAs (small noncoding RNA molecules - microRNAs) that were differentially expressed between animals with chronic pain and sham controls. These miRNAs are carried in membrane bound extracellular vesicles in the blood called exosomes. miRNAs regulate the expression of genes through interaction with protein coding mRNAs. We have identified the putative mRNA targets for many of the differentially expressed miRNAs, revealing genes implicated in mitochondrial dysfunction, inflammation, synaptic plasticity, and nervous system development and function. The miRNAs could serve both as biomarkers for chronic pain, as well as provide either targets for pain therapy, or potentially could be therapeutics themselves.

What was the impact on other disciplines?

The mRNA sequencing techniques used in this project are considered state-of-the-art and the detailed description of the computational methods used for the mRNAseq part of the study (the analysis ‘pipeline’) have been described in detail in a poster presented at the 2018 MHSRS meeting. These results are contained in a manuscript that is in preparation for publication in a bioinformatics journal, which will be read by researchers in a broad range of fields that rely on mRNA sequencing technology. It is expected that through this exposure, our work will impact other disciplines.

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

We anticipate that identification of candidate blood-based *biomarkers of chronic pain* will be a near immediate product of this study. Such biomarkers have the potential to not only gauge chronic pain in an objective manner, which is currently lacking in the clinic, but also provide a diagnostic marker for the effectiveness of pain therapies. This should greatly improve clinical pain management practice.

20. CHANGES/PROBLEMS:**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**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

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

Journal publications.

1. Sosanya NM, Kumar R, Clifford JL, Chavez R, Dimitrov G, Srinivasan S, Gautam A, Trevino AV, Williams M, Hammamieh R, Cheppudira BP, Crimmins SL, Christy RJ, Crimmins SL. Identifying Plasma Derived Extracellular Vesicle (EV) Contained Biomarkers in the Development of Chronic Neuropathic Pain, Journal of Pain, in press.
2. Kumar R, Campbell R, Gautam A, George Dimitrov G, Srinivasan S, Hamad D, Sosanya NM, Cheppudira BP, Christy RJ, Crimmins S, Clifford JL, Hammamieh R. Data Agnostic Knowledge Exploration and Integration for the Identification of Chronic Neuropathic Pain Gene Networks from the Transcriptome Profile of Brain Regions. In preparation.
3. Clifford JL, Kumar R, Srinivasan S, Dmitrov G, Gautam A, Yang R, Williams J, Workman E, Chavez R, Sosanya N, Cheppudira B, Christy R, and Hammamieh R. Global Transcriptomic Analysis of the Rat Brain During Chronic Neuropathic Pain Development. In preparation.

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

Nothing to report

Other publications, conference papers and presentations

1. Clifford JL, Kumar R, Srinivasan S, Dmitrov G, Gautam A, Walsh A, Workman E, Chavez R, Sosanya N, Christy R, and Hammamieh R. Gene Expression in the Rat Thalamus following Chronic Neuropathic Pain Development: mRNA sequencing analysis. Experimental Biology annual conference, 2018.
2. Clifford JL, Kumar R, Srinivasan S, Dmitrov G, Gautam A, Yang R, Williams J, Workman E, Chavez R, Sosanya N, Cheppudira B, Christy R, and Hammamieh R. Gene Expression in Multiple Regions of the Rat Brain During Chronic Neuropathic Pain Development: mRNA Sequencing Analysis. Neurotrauma 2018.
3. Sosanya NM, Kumar R, Clifford JL, Workman E, Srinivasan S, Dmitrov G, Gautam A, Walsh A, Chavez R, Alrufaiee O, Trevino AV, Cheppudira BP, Christy RJ, Crimmins S, Hammamieh R. Gene Expression in Key Pain Processing Brain Regions During the Development of Chronic Neuropathic Pain. Military Health System Research Symposium (MHSRS), 2018.*
4. Kumar R, Campbell R, Gautam A, George Dimitrov G, Srinivasan S, Hamad D, Sosanya NM, Cheppudira BP, Christy RJ, Crimmins S, Clifford JL, Hammamieh R. Data Agnostic Knowledge Exploration and Integration for the Identification of Chronic Neuropathic Pain Gene Networks from the Transcriptome Profile of Brain Regions. . Military Health System Research Symposium (MHSRS), 2018.*
5. Clifford JL, Kumar R, Srinivasan S, Dmitrov G, Gautam A, Yang R, Williams J, Workman E, Chavez R, Sosanya N, Cheppudira B, Christy R, and Hammamieh R. Global Transcriptomic Analysis of the Rat Brain During Chronic Neuropathic Pain Development. Society for Neuroscience Annual Meeting, 2018.*

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

The mRNA sequencing techniques used in this project are considered state-of-the-art and the detailed description of the computational methods used for the mRNAseq part of the study (the analysis ‘pipeline’) have been described in detail in a poster presented at the 2018 MHSRS meeting.

In this part of the study we applied a systematic, customized yet agnostic analytical approach to explore molecular mechanisms from the SNI neuropathic pain model, that included multiple dimensions such as time, treatment, and different brain regions. This analytical method can integrate the multiple datasets, such as differentially expressed genes, gene networks/pathways, proteins, miRNAs, gene-to-gene interactions and regulatory region-specific annotations.

These results are also contained in a manuscript that is in preparation for publication in a bioinformatics journal.

- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

Nothing to report

22. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

No change.

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

23. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Nothing to report.

QUAD CHARTS: Nothing to report.

24. APPENDICES: Nothing to report.