

AWARD NUMBER:

W81XWH-14-1-0579

TITLE: Targeting Epigenetic Mechanisms in Pain due to Trauma and Traumatic Brain Injury (TBI)

PRINCIPAL INVESTIGATOR: David J. Clark, MD

RECIPIENT: VA Palo Alto Health Care System/PAVIR
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14. ABSTRACT Persistent pain after TBI, trauma to the extremities and in the situation where both types of injury exist is highly problematic. For example, persistent pain after surgery and other forms of soft tissue injury occurs in up to 50% of patients, and as many as 85% of those with TBI experience ongoing pain. Battlefield trauma, motor vehicle accidents and sports-related injuries are particularly likely to involve TBI, peripheral trauma or both. Disability due to pain and other causes is very high amongst such patients. We have no effective approaches to reducing the likelihood of developing chronic pain after TBI or peripheral injuries, and the mechanisms supporting such pain are poorly understood. Recent advances have suggested, however, that epigenetic changes occurring in the dorsal horn of the spinal cord after either brain or peripheral trauma may support chronic pain. Our work to-date has established a rodent model of TBI in combination with injury to a limb as a model for addressing this clinical problem. We have established the severity and time course of pain-related changes after TBI and incision. Critically, we have demonstrated that histone deacetylase inhibitors greatly exacerbate the pain problems while agents that block histone acetylation reduce the pain-related changes. Additional evidence suggests that changes in the levels of genes in the spinal cord along with brain-level changes after TBI may be responsible. These observations suggest novel approaches to treatment.					
15. SUBJECT TERMS Traumatic Brain Injury, Chronic Pain, Epigenetic, Chemokine, Disability, Analgesia, Spinal Cord					
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TABLE OF CONTENTS

	<u>Page No.</u>
1. Introduction	4
2. Keywords	4
3. Accomplishments	5
4. Impact	12
5. Changes/Problems	13
6. Products	14
7. Participants & Other Collaborating Organizations	15
8. Special Reporting Requirements	16
9. Appendices	16

1. INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Persistent pain after TBI, trauma to the extremities and in the situation where both types of injury exist is highly problematic. For example, persistent pain after surgery and other forms of soft tissue injury occurs in up to 50% of patients, and as many as 85% of those with TBI experience ongoing pain. Battlefield trauma, motor vehicle accidents and sports-related injuries are particularly likely to involve TBI, peripheral trauma or both. Disability due to pain and other causes is very high amongst such patients. We have no effective approaches to reducing the likelihood of developing chronic pain after TBI or peripheral injuries, and the mechanisms supporting such pain are poorly understood. Recent advances have suggested, however, that epigenetic changes occurring in the dorsal horn of the spinal cord after either brain or peripheral trauma may support chronic pain. Specifically, the acetylation of histone proteins with spinal cord dorsal horn neurons leads to the sustained up-regulation of pain-related chemokine receptor CXCR2 thereby supporting chronic pain. The objective of this project is to define the role of agents targeting epigenetic mechanisms in reducing pain and disability after trauma, particularly in the setting of TBI. This objective is closely in alignment with the pain management focus area of the CRM RP Neurosensory Research Award program. Specifically, these studies involve, 1) applied research on alternative non-opioid analgesic drugs, 2) strategies for management of acute and chronic pain under the care of a clinician in non-deployed settings (specifically in patients with TBI), and 3) research studies to evaluate novel analgesics and mechanisms of pain in relevant animal models. At the completion of the proposed studies we will have addressed our project's main objective using multiple approaches. We will have a refined mechanistic understanding of how tissue trauma, TBI and the combination lead to the experience of chronic pain. We will also have preclinically evaluated the complementary approaches of using HAT or chemokine signaling inhibition to reduce chronic pain and disability after TBI and soft tissue trauma.

2. KEYWORDS: Provide a brief list of keywords (limit to 20 words).

Traumatic Brain Injury
Chronic Pain
Epigenetic
Chemokine
Disability
Analgesia
Spinal Cord

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?

This project has an approved statement of work and anticipated timeline. The work described in those documents has nearly been completed. The relevant tasks and steps are provided below.

Specific Aim 1:

To evaluate the hypothesis that histone acetyl transferase (HAT) inhibitors reduce pain and disability after surgical incision, TBI and the combination of the two injuries

Major Task 1 (Pre-experimental animal approval)

Months

ST1.1 Local IACUC Approval

4 to -6 (100% complete)

ST1.2 DoD ACURO Approval

4 to Start (100% complete)

Major Task 2: Establish the roles of HAT inhibitors on simple measures of nociception after incision and TBI

Months

ST2.1 Measure effects of HAT inhibitors on nociceptive sensitization after incision

0-4 (100% complete)

ST2.2 Measure effects of HAT inhibitors on nociceptive sensitization in TBI model

3-8 (100% complete)

ST2.3 Measure effects of HAT inhibitors on nociceptive sensitization after incision and TBI.

6-12 (100% complete)

ST2.4 Test effective doses of drugs in open field and rotarod paradigms.

10-12 (30% complete)

What was accomplished under these goals?

In accordance with the Statement of Work and scientific plan, efforts during the first year of support were focused on initiating the project and addressing the goals of the first aim:

Aim and Objective: To evaluate the hypothesis that histone acetyl transferase (HAT) inhibitors reduce pain and disability after surgical incision, TBI and the combination of the two injuries

Task or Activity – We finalized the design of all anticipated animal experimental protocols and received approval both from both our local IACUC committee and from ACURO. These approvals are current.

Task or Activity – We optimized the surgical procedures for both creating the lateral fluid percussion lesions and performing the hindpaw incision on these animals. We optimized use of our fluid percussion device, a challenging technical undertaking. The outcomes were that we can more rapidly generate the required animal subjects, and fewer animals need to be excluded from cohorts due to neurological injury.

Task or Activity – We established the time courses of effect of TBI using various pressures as well as incision and the combination of injuries. These represent some of our first experiments fully establishing the characteristics of the fluid percussion model of TBI employed in this project. This was necessary as we purchased as a part of the approved equipment list a specialized TBI-generating device. As was stated in the approved application, these experiments used 8 rats/group (10/group was proposed for some of the more complex behavioral experiments). The fluid percussion lesions were performed (or sham surgery), and some rats also received hindpaw incision (or sham surgery). All fluid percussion experiments used the approved fluid percussion technique outlined in the application and ACORP. Pain (nociceptive) sensitization was followed using the von Frey method. Those measures were continued until the resolution of sensitization. We used pressures considered by the field to cause mild injury (1.3ATM), and a pressure reported to cause moderate TBI (2.0ATM). The data and statistical analysis is included in the modified report. Figure 1 in the revised Annual Report displays the data involving TBI injuries of the various pressures.

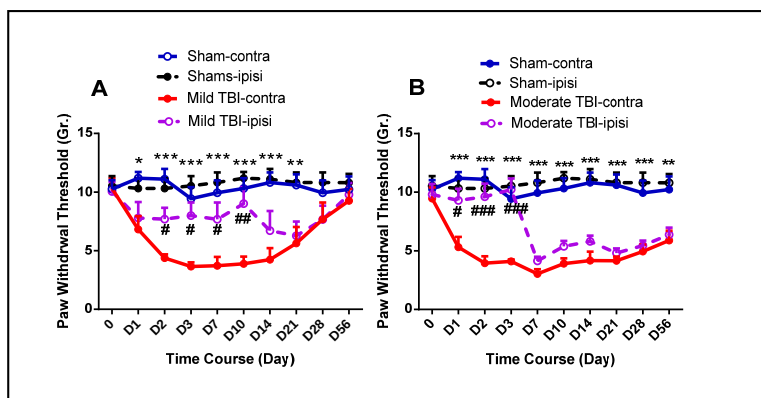


Figure 1. Figure 1: Pain (Mechanical Allodynia) Sensitization Resulting from Lateral Fluid Percussion. Animals were given mild (1.3 ATM) or moderate (2.0 ATM) lateral fluid percussion injuries on the right side of the brain under isoflurane anesthesia. Animals were followed for 8 weeks. Sham operated animals received anesthesia and preparation without fluid percussion. A: Mild TBI model; B: Moderate TBI model. Contra: contralateral side to TBI surgery site; Ipsi: Ipsilateral side to TBI surgery site. Pain sensitivity at the indicated time points was measured using the von Frey method. The data were expressed as mean $\pm$ SEM. N=8 rats/cohort; * p <0.05, ** p <0.01, *** p <0.001 sham contralateral versus TBI contralateral hindpaw thresholds; # p < 0.05, ## p <0.01, ### p <0.001 TBI- ipsilateral group versus TBI contralateral group.

We established that mild injury indeed causes contralateral sensitization that lasted weeks. Also displayed in Figure 1 in the revised Annual Report are data from limbs ipsi- and contralateral to the TBI injuries. We followed the sensitization of both limbs per the award's established plan. Consistent with our hypothesis, mild range TBI caused sensitization lasting 3-4 weeks. Eight rats were used per group, and statistics were performed using ANOVA with appropriate post-hoc testing as described in the figure. Mild injury rats did not show ipsilateral paw sensitization. Moderate intensity injury resulted in a delayed onset of ipsilateral sensitization. For these determinations, von-Frey testing was used.

Use of higher pressures caused bilateral sensitization in the rats. Moderate intensity injury resulted in a delayed onset of ipsilateral sensitization as measured using von Frey fibers (Figure 1). For these determinations, von-Frey testing was used. Moderate intensity lesions used 2.0 ATM pressure. These results improve our understanding of TBI as they suggest patients exposed to the lowest force injuries might be the ones most likely to demonstrate unilateral changes.

We also found that hindpaw incision alone causes sensitization lasting about one week, and that the combination of incision and TBI was feasible to study in our animals. A key part of the proposed studies is to examine the interactions of TBI and hindpaw incision as a model of co-existing peripheral injury. Therefore, we examined additional cohorts of 8 rats each following the effects of mild TBI, unilateral (contralateral from TBI) incision, and the combination of both lesions. Those results are presented in Figure 2. Mechanical von Frey testing was used to follow nociceptive sensitization. The results of these studies demonstrate that in fact we can study the individual injuries as well as the combination of injuries in rats. This represents an important accomplishment since much of the remaining investigation depends on our having access to both the individual and combination injury animals.

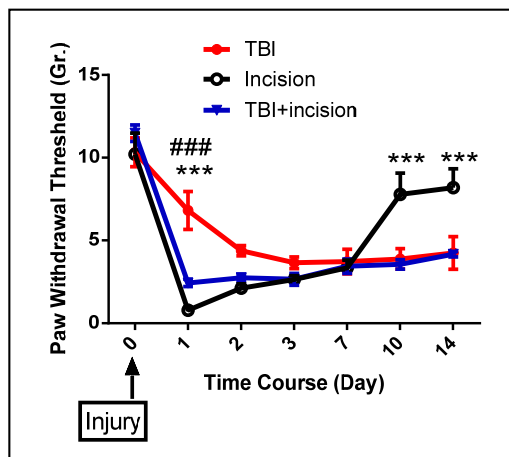


Figure 2. Pain (Mechanical Allodynia) Sensitization Resulting from TBI Combined with Peripheral Trauma (Hindpaw Incision). Animals received 1cm hindpaw incisions or sham incision contralateral to mild or sham TBI. Mechanical nociceptive thresholds at the indicated time points were assessed using the von Frey method. The values were displayed as mean $\pm$ SEM. N=8; ***p<0.001 comparison of paw incision with TBI alone group. ###p<0.001 comparison of TBI with TBI plus incision group.

Task or Activity – We established the ability of the HAT inhibitor curcumin to block a portion of the sensitization occurring after either TBI or hindpaw incision. Having established the basic model, we moved on in our studies to study the effects of the histone acetyltransferase inhibitor curcumin at the highest dose planned, 50mg/kg/day s.c. beginning just after TBI and lasting 7 days. In Figure 3, panels A and B we show that this dose of curcumin does in fact reduce mechanical sensitization after TBI or incision alone, at least with respect to mechanical sensitization.

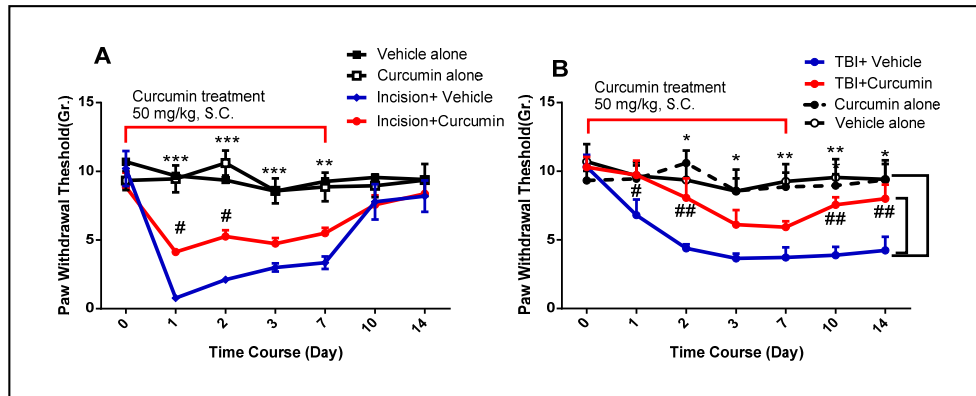


Figure 3. The Effects of Curcumin on TBI and Paw Incision Pain (Mechanical Allodynia). Curcumin (50 mg/kg) was injected subcutaneously daily from day 0 just after injury to day 7. Pain sensitivity was assessed with von Frey filaments before daily curcumin injection and continued to day 14. A: Effects of curcumin on incisional pain; B: Effects of curcumin on TBI pain. The values were displayed as mean $\pm$ SEM. N=8; * p <0.05, ** p <0.01, *** p <0.001 comparison of injury + vehicle with sham procedure + vehicle; # p < 0.05, ## p <0.01, ### p <0.001 comparison of injury plus vehicle to injury plus curcumin groups.

Our results showed that the selected dose of curcumin was not sufficient to prevent sensitization when both TBI and incision were experienced together. Using the highest planned dose of curcumin did not reduce nociceptive sensitization in the TBI/Incision combination cohort (Figure 4). This is discouraging for taking this approach to the treatment of pain after the combination of TBI and a peripheral injury (polytrauma), but it does suggest that there may be an interaction of incision and TBI to leave those affected with a more refractory pain state. This has potential clinical ramifications. We feel that these data make it important to consider examining the level of spinal cytokines in cohorts including the combination injury model later in the project. We also feel this may be due to our use of curcumin after injury which is consistent with how the drug would need to be used clinically. We are currently planning experiments with alternative HAT inhibitors as outlined in the application.

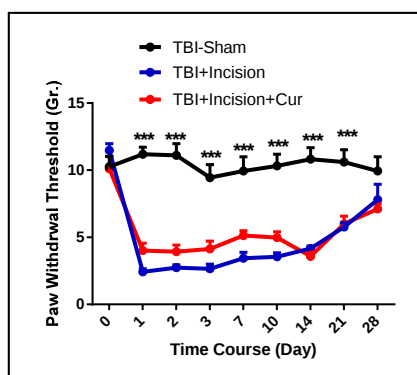
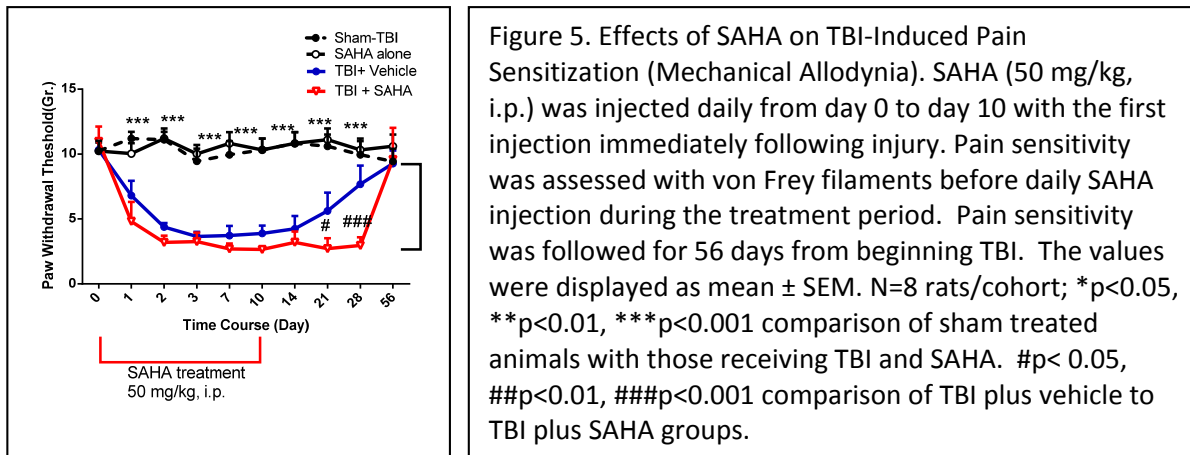
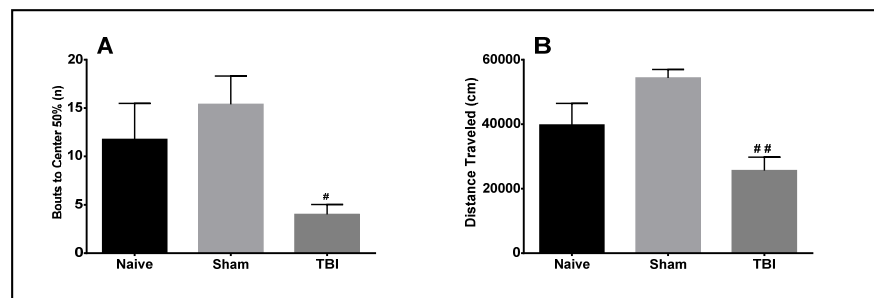


Figure 4. The Effects of Curcumin on the combination of TBI and Incision-Induced Pain Sensitization (Mechanical Allodynia). Curcumin (50 mg/kg) was injected subcutaneously daily from day 0 to day 7, and mechanical allodynia was followed as described in Figure 3. The values were displayed as mean $\pm$ SEM. N=8; * p <0.05, ** p <0.01, *** p <0.001 comparison of sham treated animals with those receiving TBI and hindpaw incisions.

We also have used the histone deacetylase inhibitor SAHA. This selective agent was predicted to have the effect of exacerbating nociceptive sensitization, and that in fact was seen in the TBI model. In Figure 5 of the modified report, we provide the results of the use of a selective histone deacetylase inhibitor (SAHA) on mechanical sensitization after TBI. Similar to the curcumin experiments, the drug administration began immediately following TBI and continued daily for 10 days as detailed in the figure legend. As can be seen in that figure, the early administration of the inhibitor caused a greater level of nociceptive sensitization in the rats weeks after the initial TBI. Group sizes and statistical approaches were similar in these experiments in comparison to those in which curcumin was used. This puts us in position to measure TBI and drug effects on spinal cord pain-related genes including CXCR2, the target for the pharmacological experiments planned for Aim 2.



Task or Activity – We have begun to establish the effects of TBI on open field activity. We have studied this index of anxiety in the control, sham and TBI animals. These experiments were done to determine whether TBI caused anxiety which is commonly associated with TBI in clinical populations as a part of the “polytrauma clinical triad” comprised of TBI, pain and PTSD (an anxiety disorder). We did in fact find that the TBI animals (8/group) were less mobile suggesting anxiety (**Figure 4**). Data presented in the same figure suggest that overall locomotor activity was reduced as well. The details of the analysis and statistical results are also found in that figure. In this segment of the work we used rats from the same cohorts as were used for some of the pain testing described in the previous figures. This represents a streamlining of the project’s procedures in that we may not need to generate individual cohorts for the anxiety testing procedures, and it suggests that for anxiety testing 8 rather than 10 rat might be sufficient. We have not to this point tested drug effects on anxiety or other behavioral measures.



Task or Activity – We have begun to study the expression of ACh3K9 in spinal tissue after TBI per the original SOW. Omission of mention of these data in the original report is regrettable, but our group was still optimizing experimental conditions and organizing the approach to the tissue processing and analysis. Statistical analysis of differences in staining between sham and TBI animals is ongoing. The typical appearance of spinal cord sections stained for ACh3K9 is provided below in Figure 5. Co-staining experiments are planned to determine whether ACh3K9 is localized to neurons, and whether ACh3K9 and CXCR2 are found in the same cells.

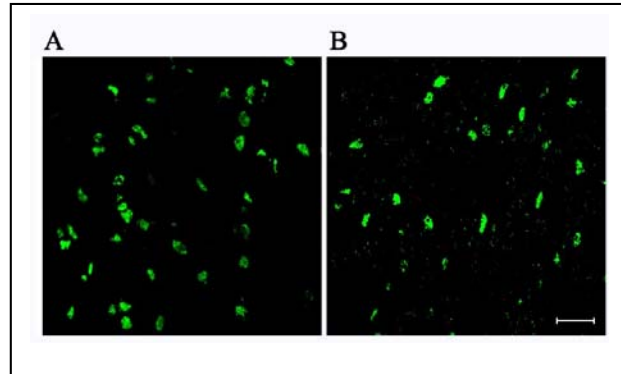


Figure 7: Expression of ACh3K9 and CXCR2 in spinal cord after TBI. (A) Immunostaining showed ACh3K9 in the dorsal horn of the spinal cord contralateral to TBI at 2 days after injuries. (B) Immunostaining showed that CXCR2 was also expressed in the contralateral spinal dorsal horn 2 days after TBI. Scale bar: 20 μ m.

One caveat of the work done in this area so-far is that a major amount of time is required to simply prepare the animals for experimentation. The actual TBI surgery is quite complex, and a major portion of the experimenter's time is being spent simply preparing the animals for testing. To address this problem we are separately looking at a mouse closed-head model that might be used to speed the pace of the experiments and to reduce the costs by reducing animal and husbandry charges.

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

Nothing to report that is not already listed in the following section.

How were the results disseminated to communities of interest?

The results of our studies were presented in poster format at a traumatic brain injury research conference held at the Palo Alto VA.

The results were presented to the Stanford Neuroscience Institute during their annual meeting.

The results of our studies were reported to the Stanford Neurosciences Institute.

The results of our studies were presented to the Stanford Department of Anesthesia, Pain and Perioperative Medicine both during the regular research meetings, and at the time of the annual Departmental meeting.

The results were presented at the joint Stanford/UCSF Anesthesiology Joint Research Meeting.

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

For the direct purpose of accomplishing the goals and objectives:

We will adhere to the written and approved research strategy. We will continue to use the TBI model as optimized in rats along with hindpaw incision. We intend to perform the same types of analysis and perform the same pharmacological studies as have been approved in the proposal.

We will look for opportunities to speed the work, and this may include using a mouse model of TBI along with the use of a model of more severe limb damage involving tibial fracture.

For the purpose of dissemination of information:

The topic of TBI-related pain is not one commonly discussed in the pain management community. Our plan is to work at the local and national levels to enhance awareness of this problem.

Locally we are one of the VA's 5 polytrauma sites. Each year our site holds a polytrauma conference, and we present results at that meeting. In addition, we present our results to that group at the time of the annual meeting as well. Finally, our own department has opportunities for the presentation of research findings during weekly conferences and at the time of the annual research celebration. The data from this project are presented at both of those.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

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

Our observations suggest that injury to the brain affects changes in the ways in which individuals experience pain. This had long been suspected as the prevalence of pain is very high in individuals with TBI. Furthermore, pain in patients with TBI even with well-healed additional injuries tends to be very long-lasting. Our data even at this early point suggest that epigenetic changes set in motion by the brain injury help to support this ongoing pain. This in turn opens the door for the development of treatment strategies directed against these epigenetic changes. Further investigation of the mechanisms involved could reveal mechanisms responsible for chronic pain of many types.

In the second year of the project we will move into the question of specific molecules that might be present at higher levels as the result of these epigenetic changes. Our first target will be CXCR2. This target is amenable to modulation using currently available agents. This raises the possibility of clinical translation of our results.

What was the impact on other disciplines?

When our project is even a little further along, our results are likely to impact the field of pain neuroscience. We feel that it is likely that we will be able to show that damage to specific pain pathways or, more likely, specific molecular processes like epigenetics mediate the link between TBI (or brain damage more broadly) and changes in sensory systems like pain.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change

Nothing to Report.

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

Nothing to report

Changes that had a significant impact on expenditures

We were slightly delayed in moving staff from other projects for this one at the beginning of the funding period. Those issues have been resolved, and we do not anticipate further problems.

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.

Not applicable

Significant changes in use of biohazards and/or select agents

Not applicable

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”

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

Report only the major publication(s) resulting from the work under this award.

Journal publications.

The results of our studies were presented in poster format at a traumatic brain injury research conference held at the Palo Alto VA.

The results were presented to the Stanford Neuroscience Institute during their annual meeting.

The results of our studies were reported to the Stanford Neurosciences Institute.

The results of our studies were presented to the Stanford Department of Anesthesia, Pain and Perioperative Medicine both during the regular research meetings, and at the time of the annual Departmental meeting.

The results were presented at the joint Stanford/UCSF Anesthesiology Joint Research Meeting.

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

Nothing to Report.

Other publications, conference papers, and presentations

Nothing to Report.

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

Nothing to Report.

- **Technologies or techniques**

Nothing to Report.

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

Nothing to Report.

- **Other Products**

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: David J. Clark
 Project Role: PI
 Annualized calendar months: 2
 Contribution to Project: This person is the project PI and administratively oversaw the completion of the regulatory requirements, the purchase of equipment, and the initiation of experimentation.

Name: David C Yeomans
 Project Role: Co-I
 Annualized calendar months: 1
 Contribution to Project: This person co-directs the experimentation. He reviews the progress of the experiments, provides scientific input and trouble-shoots scientific and technical issues.

Name: Deyong Liang
 Project Role: Investigator
 Annualized calendar months: 10
 Contribution to Project: This person conducts the majority of the experimentation. He has performed the TBI surgeries as well as the incisional model. He orders the animals and plans experiments. He processes and presents the data generated.

Name: Peyman Sahbaie
 Project Role: Research Associate
 Annualized calendar months: 6
 Contribution to Project: This person led the effort to acquire and set-up the TBI device. He is responsible for a portion of the animal testing, and will perform a portion of the surgeries.

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

The PI Dr. Clark has received a new award from the VA:

1I01RX001475-01A2 PI's Clark, Kingery 8/1/2015-7/31/2019 1.8 CM
VA-RR&D
Autoimmune Mechanisms Supporting Chronic Pain after Limb Injury

The major goals of this project are to: 1) delineate the contributions of neural control of autoimmunity to CRPS, and 2) to identify autoimmune mechanisms responsible for painful neuropathy in CRPS. No change in PI effort on the DoD award has resulted from the activation of this award.

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

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

QUAD CHARTS: See attached

- 9. APPENDICES:** Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.