AWARD NUMBER: W81XWH-18-1-0807

TITLE: Nocicepitve (Pain) Input After Spinal Cord Injury (SCI) Enhances Secodary Injury: Identifying Treatments that can be Translated to Clinical Practice

PRINCIPAL INVESTIGATOR: James W. Grau
Psychological and Brain Sciences
Texas A&M University

CONTRACTING ORGANIZATION: Texas A&M University
400 Harvey Mitchell Pkw, STE 300
College Station, TX 77845-4375

REPORT DATE: October 2019

TYPE OF REPORT: Annual Report

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2019	Annual Report	30SEP2018 - 29SEP2019

4. TITLE AND SUBTITLE

Nociceptive (Pain) Input After Spinal Cord Injury (SCI) Enhances Secondary Injury: Identifying Treatments that Can Be Translated to Clinical Practice

5a. CONTRACT NUMBER
W81XWH1810807

5b. GRANT NUMBER
GRANT12524207

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
James W. Grau

E-Mail:

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Texas A&M University
400 Harvey Mitchell Parkway Suite 300
College Station, TX 77845-4375

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, MD 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Using an animal model, we have shown that nociceptive (pain) input after spinal cord injury (SCI) amplifies secondary injury and undermines long-term recovery. These observations are important because SCI is often accompanied by additional tissue damage (polytrauma) that provides a source of nociceptive input. The consequences of pain (nociceptive) input on recovery after a lower thoracic contusion injury have been studied using an animal (rat) model. Prior work has shown that engaging nociceptive fibers 24 hr after injury increases tissue loss, undermines long-term recovery, and fosters the development of pain and spasticity. Recent studies have related these effects to pain induced hemorrhage at the site of injury and shown that this can be blocked by means of a local (lidocaine) or general (pentobarbital) anesthesia. Experiments will determine when nociceptive input affects tissue loss, the time period over which hemorrhage occurs, whether these effects are observed in both male and female animals, and whether chemically engaging pain (C) fibers with the irritant capsaicin has the same effect. Having demonstrated that capsaicin induces hemorrhage and impairs long-term recovery, we are assessing whether this effect can be prevented by means of a pharmacological transection or general anesthesia. To further evaluate the clinical relevance of these findings, we will test whether they are effective when initiated soon after injury or the initiation of painful stimulation.

15. SUBJECT TERMS

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	12	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

1. INTRODUCTION

Using an animal model, we have shown that nociceptive (pain) input after spinal cord injury (SCI) amplifies secondary injury and undermines long-term recovery. These observations are important because SCI is often accompanied by additional tissue damage (polytrauma) that provides a source of nociceptive input. The consequences of pain (nociceptive) input on recovery after a lower thoracic contusion injury have been studied using an animal (rat) model. Prior work has shown that engaging nociceptive fibers 24 hr after injury increases tissue loss, undermines long-term recovery, and fosters the development of pain and spasticity. Recent studies suggest that nociceptive stimulation increases tissue loss because it induces a breakdown in the blood spinal cord barrier (BSCB) that allows blood to infiltrate spinal cord tissue. Because red blood cells are neurotoxic, this hemorrhage augments cell death (secondary injury). Importantly, we have shown that pretreatment with a local anesthetic (lidocaine), applied at the site of injury, blocks nociception-induced hemorrhage and the impairment in long-term recovery. Further work revealed that cutting communication with the brain, by means of a rostral transection, also blocks pain-induced hemorrhage. This suggests that the adverse effects of nociceptive input depend, in part, on surviving fibers and the brain—that brain-dependent processes can fuel tissue loss at the site of injury. Supporting this, preliminary data were presented to show that a pharmacological transection (induced by slowly infusing lidocaine rostral to injury) also has a protective effect. So too does placing the animals in a state analogous to a medically-induced coma, by administering the general anesthetic pentobarbital. The work outlined within the present proposal is designed to verify these observations and evaluate their generality. Experiments will determine when nociceptive input affects tissue loss, the time period over which hemorrhage occurs, whether these effects are observed in both male and female animals, and whether chemically engaging pain (C) fibers with the irritant capsaicin has the same effect. Having demonstrated that capsaicin induces hemorrhage and impairs long-term recovery, we are assessing whether this effect can be prevented by means of a pharmacological transection or general anesthesia. To further evaluate the clinical relevance of these findings, we will test whether they are effective when initiated soon after injury or the initiation of painful stimulation.

2. KEYWORDS: spinal cord injury, contusion, recovery, polytrauma, pain, anesthesia, lidocaine, pentobarbital, anesthesia, coma

3. ACCOMPLISHMENTS

Major Goals

Task	Months	Status
IACUC/ACURO approval	2	Aug. 2018
Aim 1		
Major1: Detail the emergence of hemorrhage over time	3-6	75%
Major2: Detail when nociceptive stimulation affects tissue loss	7-10	50%
Major3: Does the effect of tonic pain (capsaicin) depend on brain systems	11-13	75%
Sub1: Is hemorrhage blocked by spinal transection		July 2019
Sub2: Does afferent input initiate the breakdown of the BSCB		50%
Major4: Are similar effects observed in male and female animals	14-16	July 2019
Aim 2		
Major1: Is pentobarbital effective if given after stimulation?	17-19	Sept. 2019
Major2: Does anesthesia block the effect of capsaicin treatment?	20-22	75%
Major3: Does anesthesia after surgery attenuate damage?	23-26	
Aim 3		
Major1: Does epidural lidocaine block the effect of capsaicin?	27-29	
Major2: Does rostral lidocaine block the effect of capsaicin?	30-33	
Major3: Does epidural lidocaine after surgery reduce tissue loss?	34-36	

Summary of What was Accomplished (Year 1)

Subtask 1: IACUC/ACURO approval

We had indicated that this would be accomplished within the first two months of the project. We completed the task in August of 2018, before the project was funded.

Aim 1: When does nociceptive input have an adverse effect?

Major Task 1: Emergence of hemorrhage over time

Prior work has shown that application of a noxious stimulus (intermittent electrical stimulation or the irritant capsaicin) a day after rats have received a contusion injury induces hemorrhage. Preliminary data, available at the time of grant submission, indicated that treatment with the irritant capsaicin produced hemorrhage when tissue was collected 3 hrs after treatment. The present experiment was designed to assess whether the capsaicin-induced hemorrhage is accompanied by an increase in blood pressure and the duration of these effects. Here, and in subsequent experiments, rats received a moderate contusion injury at T10. Hemorrhage was assessed using standard methods, which include the assessment of absorbance at the wavelength associated with hemoglobin (420 nm) and the Drabkin assay. In some cases, the data are reinforced with Western blotting.

Elucidation of a time-course requires a positive control, in this case a demonstration of the effect at 3 hrs after treatment. We coupled this to the assessment of hemorrhage at 24 hrs. Whether subsequent time points were needed was to be determined by the data from the 24 hr condition. Having found a positive effect, we assessed hemorrhage at a later time point, keeping the interval between conditions equivalent (with an 8 fold change (3, 8, 192 hrs)].

Below we present the key findings for the 3 and 24 hr conditions in detail. The 192 hr data were still being collected at the time this report was written, and so they are presented in a briefer format. For completeness, we present graphs depicting the full pattern of the results. To keep the length of this text manageable, only select statistics are presented. Our results indicate that the effects of noxious stimulation are evident 24 hours later, but not at a later time point.

3 Hours After Capsaicin Treatment (details):

Locomotor scores were significantly lower after capsaicin injection. Locomotor scores did not differ between groups prior to treatment, F (1, 14) = 0.071, p = 0.794. Locomotor scores were obtained immediately, one, two, and three hours after injection. To examine whether capsaicin affected locomotor recovery over time, a repeated measures ANCOVA was used with injection as the between subjects variable, SCI locomotor scores as the covariate, and time as the repeated measure. Overall analysis revealed a main effect of injection, F (1, 13) = 9.428, p = 0.009 (Figure 3A). No other effects were statistically significant, all Fs < 1.973, p > 0.134. *Post hoc* analysis showed a reduction in locomotor scores in subjects receiving a capsaicin injection compared to vehicle controls.

Capsaicin injection had no effect on hypertension after SCI. Blood pressure measurements did not differ between groups prior to treatment, all Fs < 1.020, p > 0.394. Blood pressure measurements were obtained immediately, one, two, and three hours after injection to examine the effect of capsaicin on hypertension. A repeated measures ANCOVA with injection as the between subjects variable, SCI systolic blood pressure as a covariate, and time as a repeated measure found no significant effects, all Fs < 0.617, p > 0.608 (Figure 3B). Analysis of diastolic blood pressure and MAP showed similar null effects, all Fs < 1.020, p > 0.394 (data not shown).

Capsaicin treatment increased indices of hemorrhage at the SCI site within three hours after capsaicin injection. An ANOVA examining the magnitude of the peak in absorbance at 420 nm with injection as the between subject variable found a significant main effect of injection, F (1, 14) = 13.186, p = 0.003 (Figure 3E). Subsequent analysis found that subjects treated with capsaicin showed an increase in hemorrhage. This effect

was confirmed through Drabkin's analysis, F (1, 14) = 12.651, p = 0.0032 (Figure 3F). Additionally, the level of hemoglobin within the spinal cord tissue was analyzed using quantitative Western blotting. An ANOVA with injection as the between subjects variable found a significant difference between groups, F (1, 14) = 5.070, p = 0.041 (Figure 3G).

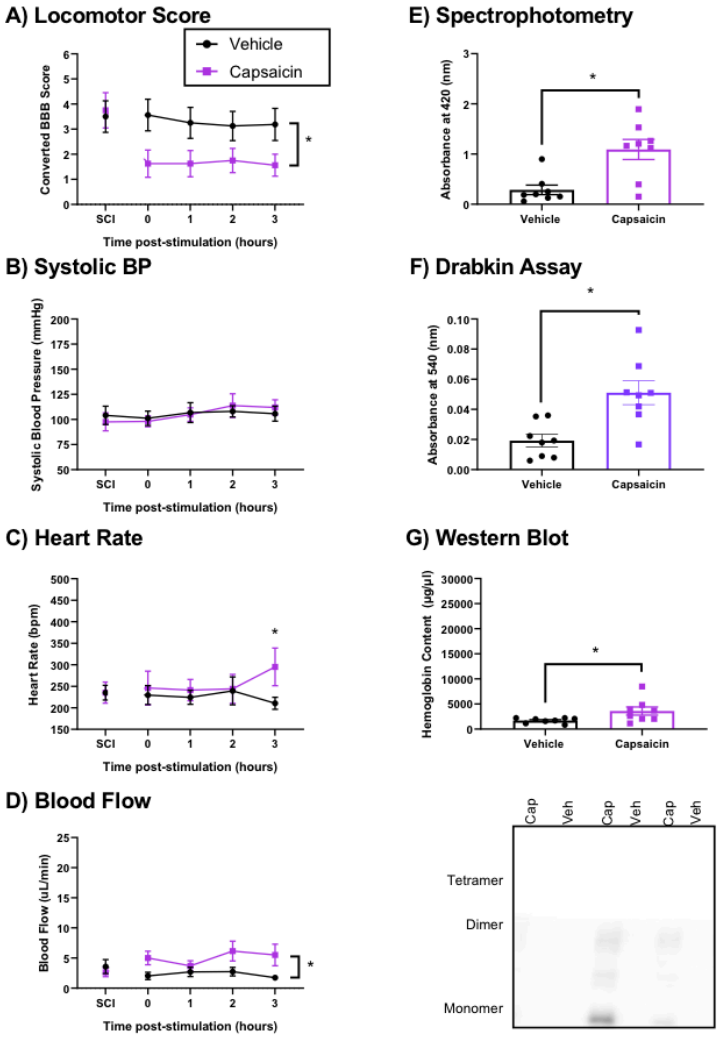


Figure 3. Locomotor performance over the three hours after capsaicin injection. BBB locomotor scores taken during the three hours post stimulation period decreased in subjects receiving capsaicin (A). Capsaicin treatment did not increase systolic blood pressure (B), but increased heart rate and blood flow (C and D). The absorbance at 420 nm taken at three hours was increased after capsaicin treatment (E). The amount of hemoglobin-α did not differ between groups (F). Representative western blot showing the three main bands associated with hemoglobin (G). Error bars represent SEM (n = 8).

24 Hours After Capsaicin Treatment (details):

Locomotor performance was reduced in capsaicin treated subjects. Locomotor scores did not differ between groups prior to treatment, F (1, 14) = 0.069, p = 0.796. Locomotor scores were obtained three, six, 12, and 24 hours after injection to examine whether a capsaicin injection would affect locomotor recovery. A repeated measures ANCOVA was used with injection as the between subjects variable, SCI locomotor score as the covariate, and time as the repeated measure. Overall analysis revealed a main effect of injection, F (1, 13) = 12.839, p = 0.003. No other effects were statistically significant, all Fs < 0.327, p > 0.602. *Post hoc* analysis revealed a significant decrease in locomotor performance in animals injected with capsaicin (Figure 4A).

A capsaicin injection had no effect on hypertension in the first 24 hours after injection. Blood pressure measurements did not differ between groups prior to treatment, all Fs < 3.254, p > 0.093. Blood pressure measurements were obtained at three, six, 12, and 24 hours after injection to examine the effect of injection on hypertension. A repeated measures ANCOVA with injection as the between subjects variable, SCI systolic blood pressure as the covariate, and time as a repeated measure found no significant changes in systolic blood pressure, F < 4.259, p > 0.060. Although the main effect of injection was not significant, there is a notable

increase in blood pressure for subjects given a capsaicin injection compared to vehicle controls, F (1, 13) = 4.259, p = 0.060 (Figure 4B). Neither diastolic blood pressure nor MAP showed any significant effects, F < 2.914, p > 0.112.

A capsaicin had no effect on heart rate in the first 24 hours after injection. Heart rate did not differ between groups prior to treatment, F (1, 14) = 2.919, p = 0.110. Heart rates were obtained at three, six, 12, and 24 hours after injection. A repeated measures ANCOVA with injection as the between subjects variable, SCI heart rate as the covariate, and time as a repeated measure, found no significant changes, all Fs < 1.200, p > 0.323 (Figure 4C). Preplanned analysis of the three-hour time point using an ANCOVA revealed no significant increase in heart rate, F (1,14) = 1.121, p = 0.309.

A capsaicin had no effect on blood flow in the first 24 hours after injection. Blood flow did not differ between groups prior to treatment, F (1, 14) = 1.121, p = 0.308. Blood flow measurements were obtained at three, six, 12, and 24 hours after injection. A repeated measures ANCOVA with injection as the between subjects variable, SCI blood flow as a covariate, and time as a repeated measure found no significant changes, all Fs < 2.698, p > 0.124 (Figure 4D). Preplanned analysis of the three-hour time point using an ANCOVA revealed no significant increase in blood flow, F (1, 14) = 3.353, p = 0.09.

Capsaicin increased the amount of hemorrhage in the spinal cord 24 hours after injection. The magnitude of the peak in absorbance at 420 nm was analyzed using an ANOVA with injection as the between subjects variable. A significant main effect of injection was observed, F (1, 14) = 11.515, p = 0.004. Subsequent analysis found that subjects treated with capsaicin showed an increase absorbance at 420 nm (Figure 4E). Drabkin's reagent was used to confirm the increased concentration of hemoglobin in animals treated with capsaicin, F (1, 14) = 87.341, p = 0.0001 (Figure 4F). A quantitative Western blotting also found significantly different levels of hemoglobin-α within the spinal cord tissue, F (1, 14) = 4.809, p = 0.046 (Figure 4G).

We found that capsaicin treated animals exhibited greater absorbance at 420 nm, which is indicative of hemorrhage. An ANOVA yielded a significant main effect of injection was observed, F (1, 14) = 11.515, p = 0.004. Subsequent analysis found that subjects treated with capsaicin showed an increase absorbance at 420 nm (Figure 4E). Drabkin's reagent was used to confirm the increased concentration of hemoglobin in animals treated with capsaicin, F (1, 14) = 87.341, p = 0.0001 (Figure 4F). A quantitative Western blotting also found significantly different levels of hemoglobin-α within the spinal cord tissue, F (1, 14) = 4.809, p = 0.046.

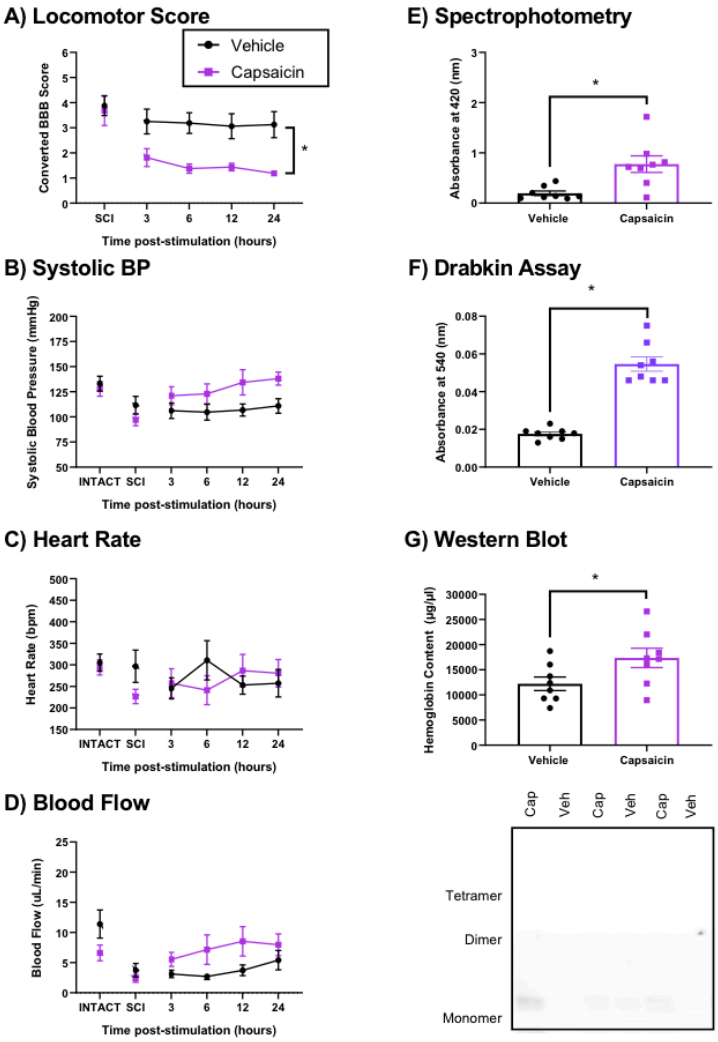
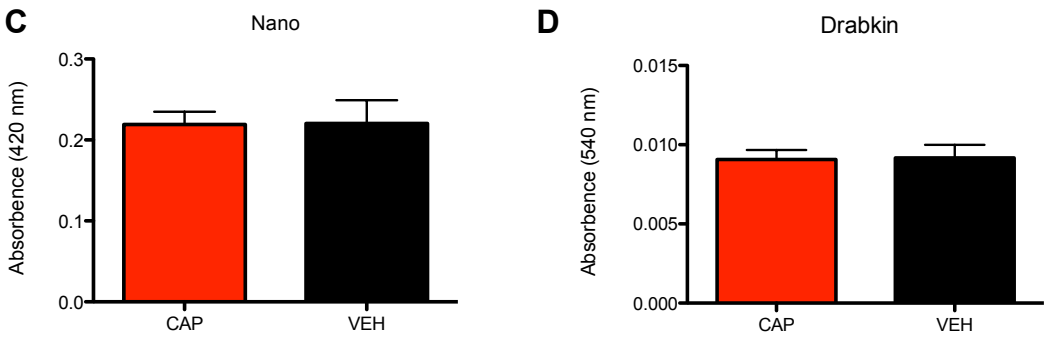


Figure 4. Hemorrhage 24 hours after capsaicin injection.
BBB locomotor scores were reduced in subjects treated with capsaicin (A). Capsaicin treatment did not increase blood pressure measurements (B-D). The absorbance at 420 nm taken at 24 hours was increased after capsaicin treatment (E). The amount of hemoglobin-α did not differ between groups (F). Representative western blot showing the three main bands associated with hemoglobin (G). Error bars represent SEM (n = 8).

192 Hours After Capsaicin Treatment:

Because biological processes typically decay in an exponential manner, we kept the ratio of time intervals constant across conditions (multiples of 8). Thus, our last time point examined hemorrhage at 192 hrs. At this time point, levels of hemorrhage in the spinal cord were not increased by capsaicin injection. Absorbance at 420 nm was assessed, and no significant differences between groups were found by an ANOVA, F (1,14) = 0.002, $p > 0.05$ (Figure 5C). Further evaluation with Drabkin's assay yielded similar results after an ANOVA was conducted, F (1,14) = 0.020, $p > 0.05$ (Figure 5D). Capsaicin also did not have a significant effect on blood pressure, F (1,14) = 0.116, $p = 0.739$.



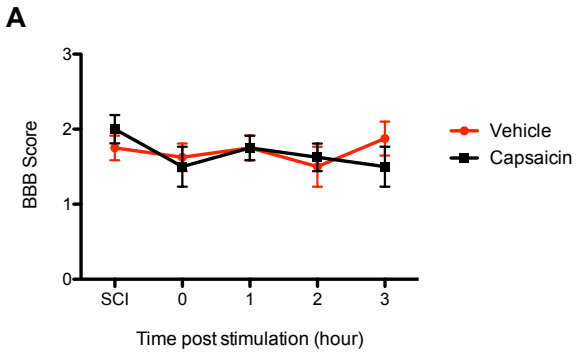
Major Task 2: Detail when nociceptive stimulation affects tissue loss

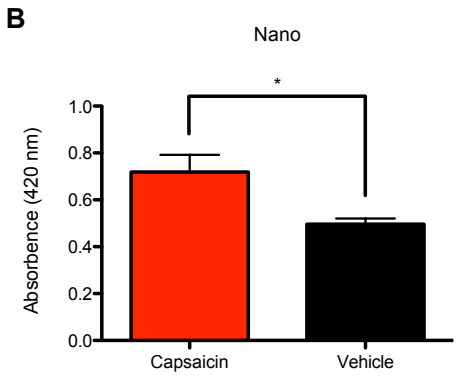
At the time of grant submission, we had collected preliminary data indicating that noxious stimulation (electrical stimulation or capsaicin) induces hemorrhage when applied 24 hrs after injury. The experiment outlined under this task was designed to detail when pain input has an adverse effect. Again, the exact time intervals selected were adjusted on basis of the initial results. Our results indicate that a robust effect is observed when pain input occurs from 6-24 hrs after injury. Data for the 96 hr condition are currently being collected.

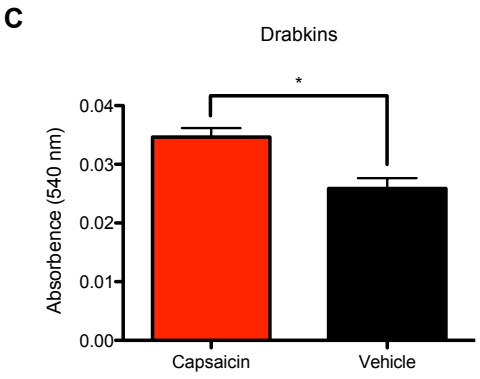
Capsaicin Treatment 6 Hours After Injury:

When injected 6 hours after injury, capsaicin did not significantly impact locomotor function. Prior to treatment, locomotor scores did not differ between groups, F (1,14) = 0.081, p < 0.7803. An ANCOVA found no significant differences between groups after injection, F (1,13) = 0.905, p > 0.05 (Figure 6A).

Hemorrhage at the injury site was significantly increased in animals administered capsaicin. An ANOVA found significantly greater absorbance in samples from animals treated with capsaicin compared to vehicle controls, F (1,14) = 7.372, p = 0.0168. Analysis with Drabkin's assay confirmed this effect, F (1,14) = 12.401, p = 0.0034.



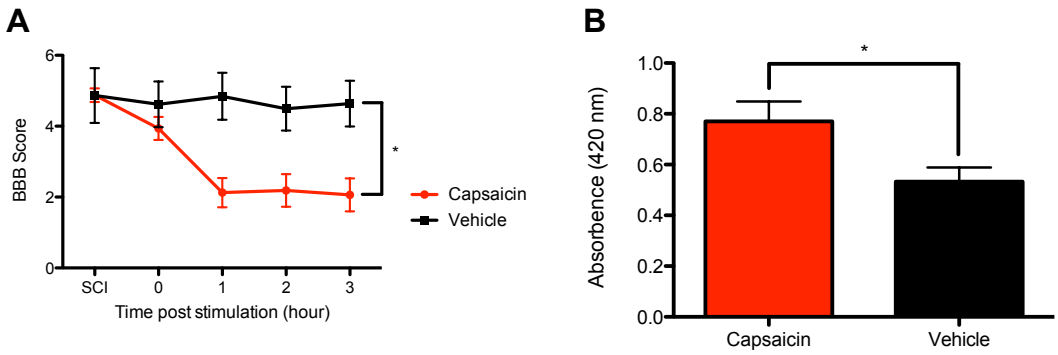




Capsaicin Treatment 24 Hours After Injury:

In animals injected with capsaicin, locomotor function was significantly impaired relative to vehicle controls. Baseline locomotor scores did not differ between groups prior to treatment, F (1,13) = 2.974, p = 0.108. After treatment, an ANCOVA, using baseline scores as a covariate, found that animals treated with capsaicin had significantly lower BBB scores, F (1, 12) = 34.807, p = 0.0001 (Figure 7A).

Hemorrhage at the injury site was increased in animals treated with capsaicin. After spectrophotometric analysis of protein samples from the injury site, an ANOVA found a significant difference between groups, F (1,13) = 5.716, p < 0.05 (Figure 7B).



Major Task 3: Does the effect of tonic pain (capsaicin) depend on brain systems

Prior work has shown that disrupting communication with the brain by means of a spinal transection blocks hemorrhage in animals exposed to noxious electrical stimulation. The present experiment was designed to test whether a similar effect is observed when animals are treated with capsaicin. Here, and in subsequent experiments, hemorrhage was assessed 3 hrs after noxious stimulation. We found that this effect was completely blocked by a complete transection at T2.

Detailed description:

Prior to transection surgery, systolic blood pressure ranged from 99.27 ± 7.09 to 101.88 ± 10.31 (mean ± SE) across groups. These differences were not statistically significant (all Fs < 1.0, p > .05). Contused animals that were treated with capsaicin showed no change in blood pressure and this was true independent of whether they had also received a spinal cord transection (Fig. 3). An ANCOVA confirmed that neither capsaicin nor spinal transection had a significant effect (all Fs < 1.0, p > .05).

Contused rats that had not undergone a spinal cord transection (Sham) exhibited greater absorbance at the wavelength associated with hemoglobin and this effect was blocked by spinal cord transection (Fig. 4A). An ANOVA analysis confirmed that the main effects of capsaicin ($F_{(1, 28)}$ = 5.40, p < .05) and transection ($F_{(1, 28)}$ = 8.34, p < .01) treatment, as well as their interaction ($F_{(1, 28)}$ = 5.65, p < .05), were statistically significant. *Post hoc* comparisons confirmed that the sham group that received shock differed from the other three groups (p > .05).

A similar pattern of results was obtained on with the Drabkin assay. Again, capsaicin increased hemoglobin concentration at the site of injury in contused rats that were not transected (Sham). This effect was blocked by a spinal cord transection (Fig. 4B). An ANOVA confirmed that the main effects of capsaicin ($F_{(1, 28)}$ = 4.73, p < .05) and transection ($F_{(1, 28)}$ = 9.42, p < .01) treatment, as well as their

interaction ($F_{(1, 28)} = 5.75$, $p < .05$), were statistically significant. *Post hoc* comparisons confirmed that the sham operated group that was treated with capsaicin differed from the other three ($p < .05$).

Western blotting confirmed that contused rats that received capsaicin had higher concentrations of hemoglobin at the site of injury relative to both the unshocked controls and transected rats that received shock (Fig. 4C). Because there was greater variability in behavioral performance after injury in this experiment, we analyzed the data using an analysis of covariance with baseline BBB score entered as a covariate. The main effects of transection and capsaicin treatment, and their interaction, were statistically significant (all *F*s > 4.33, p > .05). *Post hoc* comparisons showed that the non-transected (Sham) group that received capsaicin differed from the other three ($p < .05$). No other group comparison was significant ($p > .05$).

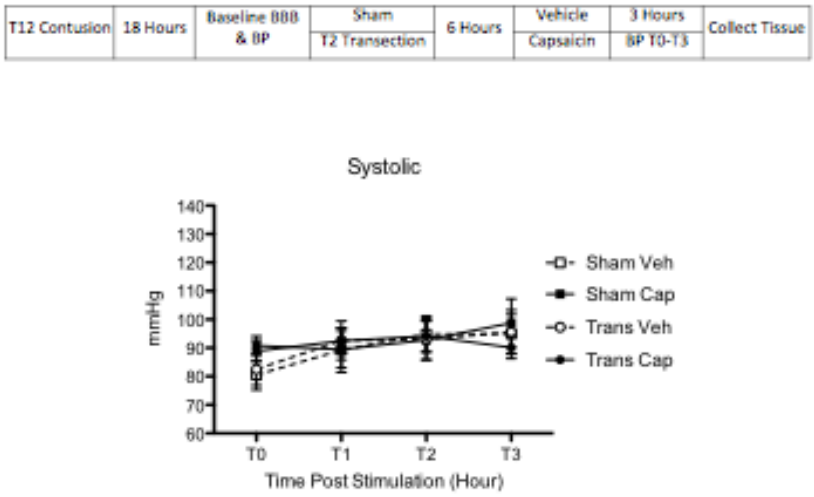


Fig. 3.. Application of capsaicin (Cap) to one hind paw did not induce a change in blood pressure (n = 8).

Figure 4

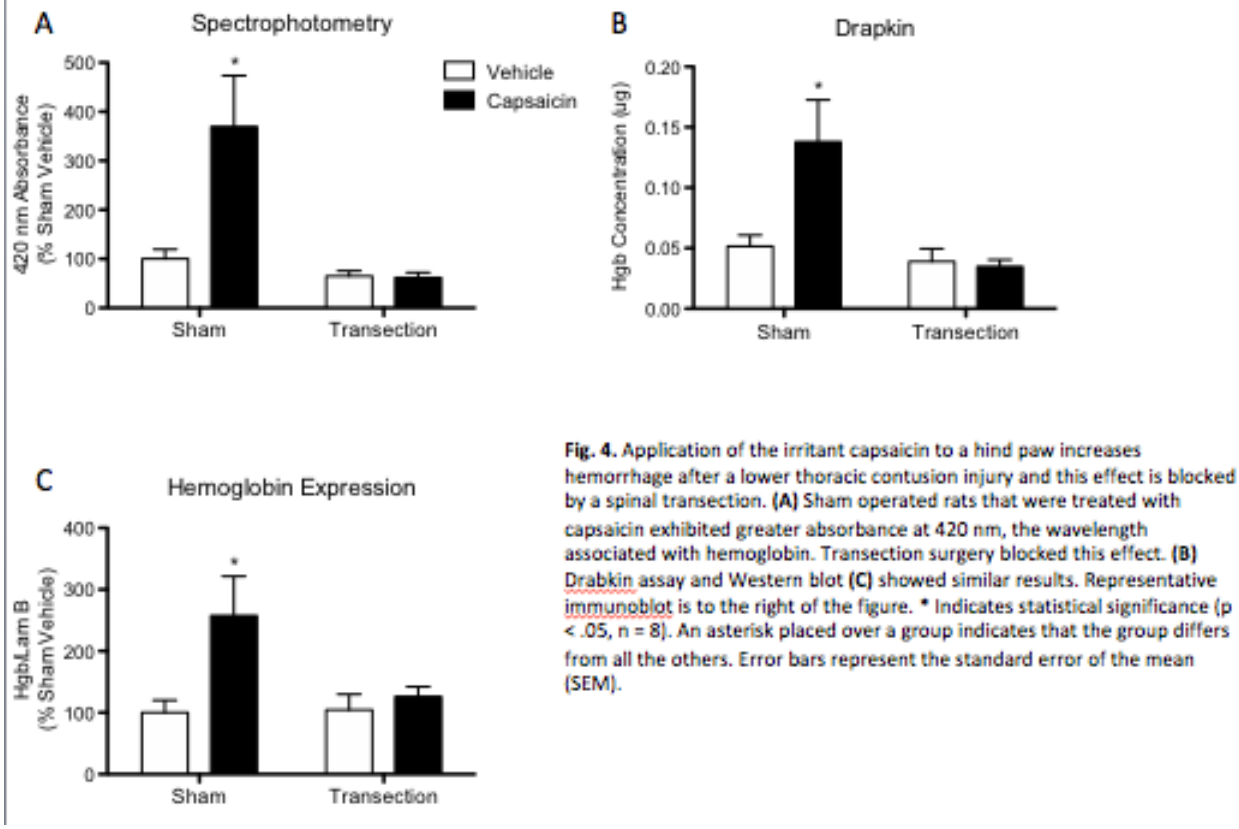


Fig. 4. Application of the irritant capsaicin to a hind paw increases hemorrhage after a lower thoracic contusion injury and this effect is blocked by a spinal transection. **(A)** Sham operated rats that were treated with capsaicin exhibited greater absorbance at 420 nm, the wavelength associated with hemoglobin. Transection surgery blocked this effect. **(B)** Drabkin assay and Western blot **(C)** showed similar results. Representative immunoblot is to the right of the figure. * Indicates statistical significance (p < .05, n = 8). An asterisk placed over a group indicates that the group differs from all the others. Error bars represent the standard error of the mean (SEM).

Additional analyses are being performed to determine whether: 1) capsaicin treatment increases the expression of SUR1-TRPM4, which is indicative of progressive hemorrhagic necrosis; and 2) whether this effect is blocked by spinal transction.

Major Task 4: Are similar effects observed in male and female rats.

At the time of grant submission, all of our data had been collected using male rats. The present experiment was designed to determine whether treatment with capsaicin induces hemorrhage induces this effect in female animals.

Detailed description:

Experiment 1 observed the effect of intradermal capsaicin injection in female rats after SCI. Prior to treatment, baseline BBB scores did not differ between groups [F (1,12) < 1.215, p > 0.05], ranging from 3.79 (± 0.52) to 3.36 (± 0.28). Following treatment, locomotor function was impaired in animals that received capsaicin injection, F (1,11) = 23.532, p = 0.0005. This effect varied in a time-dependent manner, emerging after the first hour following injection and persisting until the animals were sacrificed, F (3,33) = 11.289, p = 0.0001 (Fig. 1A).

Nociceptive stimulation has also been shown to elevate blood pressure in male animals following spinal cord injury. Before injection of capsaicin in Experiment 1, MAP values did not differ between groups, F (1,12) < 1.0, p > 0.05. Following injection, an ANCOVA, using baseline MAP as a covariate, found no significant differences between groups [F (1,11) < 1.0, p > 0.05], though at two hours after injection, animals that received capsaicin showed significantly higher MAP than their controls, F (3,33) = 3.136, p < 0.05 (Fig. 1C).

Following the three hours of behavioral assessments in Experiments 1 and 2, animals were sacrificed and their spinal cord tissue collected for protein extraction and analysis. Past work has demonstrated increased amounts of hemorrhage at the injury site in animals treated with nociceptive stimuli. To determine the effects of capsaicin and shock on hemorrhage in female animals, the concentration of α-hemoglobin in samples was assessed with spectrophotometry and western blotting. Protein extracts from animals injected with capsaicin in Experiment 1 exhibited greater absorbance at 420 nm, the wavelength associated with hemoglobin, F (1,12) = 6.77, p < 0.05 (Fig. 2A). Immunoblotting was then used to confirm this effect. Levels of α-hemoglobin were significantly elevated in animals that received pain input, F (1,12) = 10.873, p < 0.01 (Fig. 2C).

Given that both forms of nociceptive input produced similar deficits acutely in female rats as shown in previous experiments in male rats, Experiments 3 and 4 examined the effects of capsaicin and shock on recovery over a 28-day period. It was hypothesized that the neuroprotective effect of estrogen might protect against the damage inflicted by secondary injury. As a result, any sex-dependent differences may only be apparent beyond the acute phase of injury, requiring observation of recovery over a longer period of time. Again, locomotor function was assessed using the BBB method. Animals were scored prior to noxious stimulation, each day for the week following injury, on day 10, then once a week thereafter until day 28. Prior to injection of capsaicin in Experiment 3, BBB scores did not differ between groups, F (1,16) < 1.0, p > 0.05, ranging from 2.222 (±0.278) to 2.444 (±0.242). An ANCOVA using baseline BBB scores as the covariate revealed that animals injected with capsaicin recovered less function over time, F (1,15) = 5.764, p <0.05 (Fig. 3A). No other within subject factors were significant, all Fs < 1.525, p > 0.05.

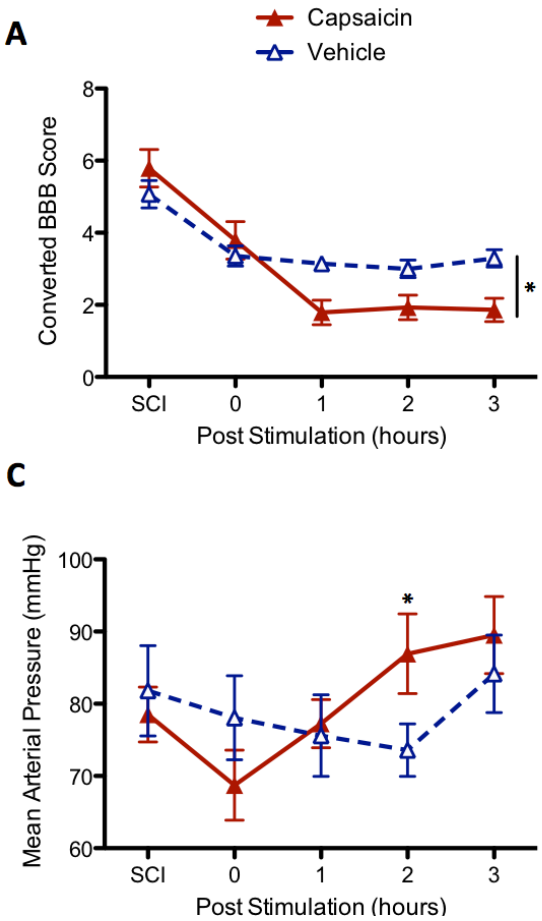


Figure 1. Acute effects of nociceptive input on locomotor function and blood pressure in female rats. (A) Animals that received intradermal injection of capsaicin to the hindpaw showed greater deficits in locomotor function after treatement. (C) MAP was only elevated for animals that received capsaicin at two hours after stimulation. * Indicates statistical significance ($p < 0.05$). Error bars represent standard error of the mean (Capsaicin n = 7, Vehicle n = 7, Shock n = 8, Unshock n = 8).

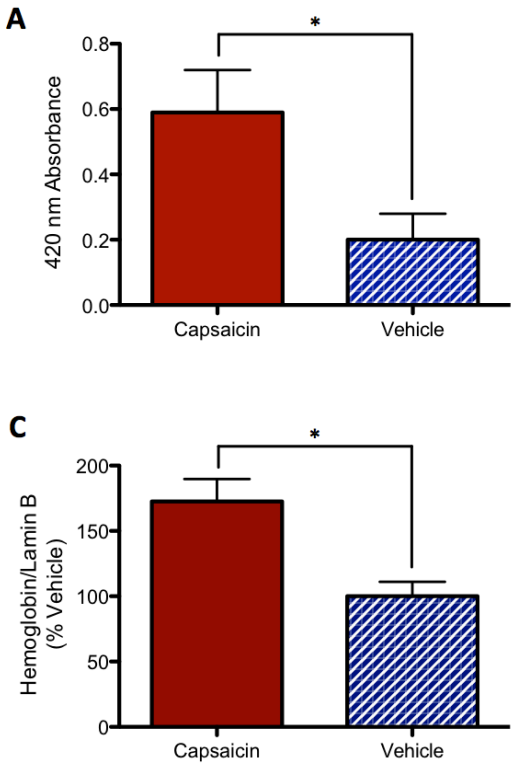


Figure 2. Acute effects of nociceptive input on hemorrhage after SCI in female rats. (A) Spectrophotometric analysis found greater absorbance of protein samples at 420 nm for animals that receive intradermal capsaicin injection. (C) Western blot analysis found greater hemoglobin content in capsaicin-treated animals. * Indicates statistical significance ($p < 0.05$). Error bars represent standard error of the mean (Capsaicin n = 7, Vehicle n = 7, Shock n = 8, Unshock n = 8).

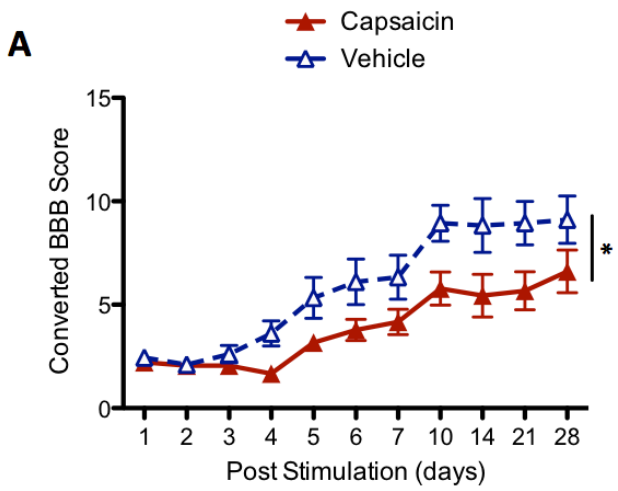


Figure 3. Effects of nociceptive stimulation on long-term recovery of locomotor function and weight. (A) Capsaicin-treated animals recovered less function over 28 days than their vehicle-treated controls. Error bars represent standard error of the mean (Capsaicin n = 9, Vehicle n = 9).

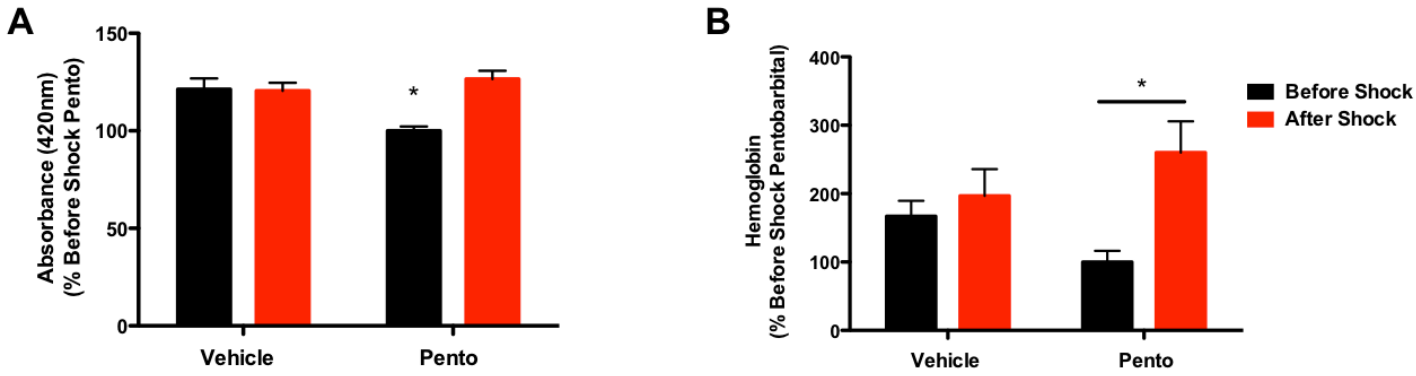
Specific Aim 2: Promoting recovery using general anesthesia

Major Task 1: Is anesthesia only effective if given prior to noxious stimulation

At the time of grant submission, we had shown that nociception induced hemorrhage and impaired long-term recovery can be blocked by pretreatment with the anesthetic pentobarbital. The current experiment examined whether inducing a state of anesthesia after noxious stimulation attenuates its adverse effect. Because the onset and offset of electrical stimulation is readily controlled, it was used here rather than capsaicin. We found that pentobarbital given before, but not after, nociceptive stimulation attenuates hemorrhage.

Detailed description:

Rats that received pentobarbital prior to shock treatment exhibited lower levels of hemoglobin at the site of injury. This protective effect was not observed when animals were anesthetized after shock treatment. An analysis of the absorbance at 420 nm confirmed that the effect of pentobarbital treatment depended upon when it was admininstered, $F(3,28) = 4.913$, $p < 0.05$. Likewise, an analysis of the western blotting data confirmed that anesthesia given before, but not after, reduced hemoglobin content, $F(3,28) = 4.047$, $p < 0.05$.



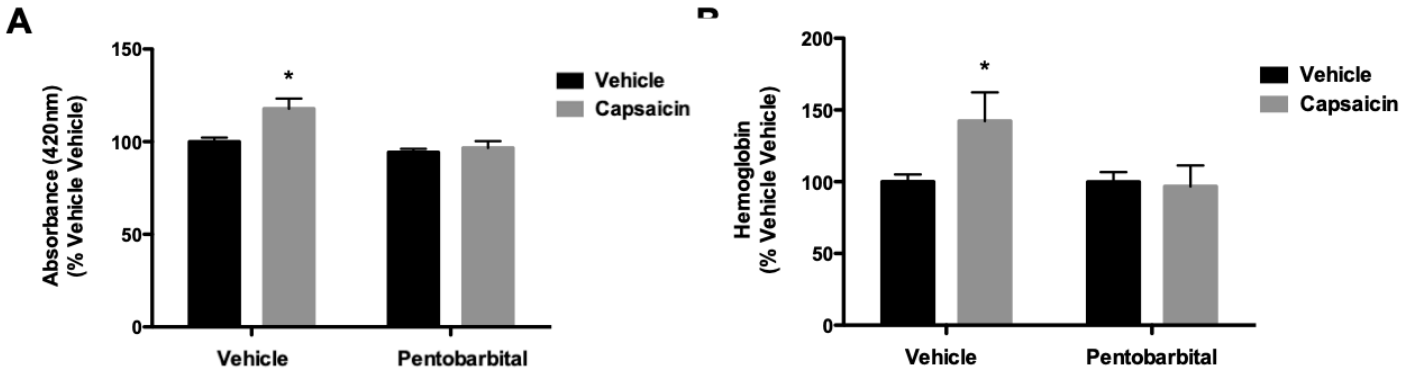
Hemorrhage was assessed via absorbance (A) and western blotting (B) in contused rats that had received noxious electrical stimulation. This effect was attenuated by pentobarbital given before, but not after shock.

Major Task 2; Does anesthesia block the effect of capsaicin treatment?

We have shown that anesthesia blocks the effect of noxious electrical stimulation. The present experiment was designed to test whether this treatment also attenuates capsaicin-induced hemorrhage. We found that it did.

Detailed description:

Protein extracted from injured spinal cord tissue of unanesthetized (vehicle treated) rats given hindpaw capsaicin exhibited significantly greater hemoglobin content. This effect was blocked by pretreatment with pentobarbital. A analysis of absorbance at 420 nm confirmed that the effect of capsaicin depended upon pentobarbital treatment, F (3,32) = 9.374, $p < 0.05$. Immunoblotting targeting hemoglobin yielded the same effect, F (3,32) = 3.431, $p < 0.05$.



Hemorrhage was assessed via absorbance (A) and western blotting (B) in contused rats 3 hrs after capsaicin was applied. Capsaicin induced hemorrhage and this effect was blocked by pentobarbital anesthesia.

Overview of the results in year 1:

We have worked to integrate the findings described above into four papers. Each of these papers has required additional experiments, which I have been able to fund from other sources (e.g., my professorship). One has been submitted for publication and three are close to submission. The papers show: (1) a rostral transection eliminates the nociception-induced rise in blood pressure and hemorrhage (Fauss et al.); (2) nociceptive stimulation induces hemorrhage in female rats (Baine et al.); (3) lidocaine applied rostral to injury has a protective effect (Davis et al.); and (4) pentobarbital anesthesia has a protective effect (Davis et al.). We should have all four papers submitted by the end of the second year with at least two accepted for publication.

Our overall progress on the project has been excellent and we are ahead of schedule on many components. Regarding Major Tasks 1 and 2 of Aim 1, which are detailing the emergence and time course of our effects, we had originally predicted that these components would be done within the first year. I have indicated Major Task 1 is not yet complete because we want to make sure that we have accurately identified the decay of the effect between 24 and 192 hrs. Likewise, data collected under Major Task 2 have shown that noxious stimulation has a robust effect from 6 to 24 hrs after injury. Data currently being collected suggest a surprising outcome—stimulation at earlier time points may have less effect. Because this is very important to clinical treatment, we want to complete this time point before we consider the experiment complete. Other components of the grant were not scheduled till the second

year. As shown in the table provided above, we are in the process of completing experiments that were originally targeted for months 18-22. Given this, we believe that our progress has been quite good.

While we are not in a position to translate these findings to humans, the work has begun to generate considerable interest, fueled in part by our frequent presentation of the material at national conferences. Indeed, the data are central to two keynote addresses at the spinal cord centers in Louisville and Vancouver. I also presented the data for Grand Rounds at Houston Methodist, a leading center in spinal cord injury. Other invited talks include ASIA and KSCHIRT.

Training Opportunities

Though not explicitly designed to offer training opportunities, the project has yielded value in this domain. First, one graduate student (Jacob Davis, 9 mos.) has been supported by the project and is completing his Ph.D. based on material within this proposal. In addition, a new graduate trainee (Travis Johnston, 2 mos.) has contributed to the project. In both cases, the work has provided a learning opportunity, introducing the students to a host of behavioral, surgical, and cellular techniques. It has also provided a forum for discussing research impacting hemorrhage and recovery after spinal cord injury.

In addition, the proposal has bolstered undergraduate training in two ways. First, a number of students have elected to stay on after they graduate, prior to applying to medical school. These students work as technicians and have been instrumental to moving the project forward. Like the graduate students, they have gained expertise in a host of new domains, including surgery, behavioral testing, statistical analyses, experimental design, and cellular assays. They have also had an opportunity to prepare papers for publication. This year, one trainee (Melissa Henwood, 7.5 mos.) began M.D./Ph.D. at the University of Texas Medical Branch. A second trainee (Paris Bean, 2 mos.) has just been accepted to medical school. Two others (Rachel Baine, 1 mo.; Megan Tarbet, 2 mos.) will be applying to medical/graduate school this coming year. Beyond this, the work helps to support independent study projects by undergraduates. Each semester, approximately 4-5 students gain individualized training through this mechanism.

Dissemination of Results

As noted above, we have completed data collection for 3 new papers that should be submitted by the end of the year. An additional paper is nearly completed and should be submitted early in 2020.

An article that reviews this work has been submitted to *Experimental Neurology* ("Learning to promote recovery after spinal cord injury"). The project also helped to support a review article ("Achieving adaptive plasticity in the spinal cord") that is in press, to be published in the *Oxford Research Encyclopedia of Neuroscience.*

Finally, the work has been presented at two national conferences: the National Neurotrauma Society in Pittsburgh PA (July, 2019) and the Society for Neuroscience in Chicago IL (October, 2019):

Baine, R.E., Strain, M.M., Henwood, M.K., Johnston, D.T., Davis, J.A., Grau, J.W. (2019). Pain input after spinal cord injury increases tissue loss and impairs long-term recovery in female rats. *Journal of Neurotrauma. 36,* A-72*.*

Fauss, G.N.K., Strain, M.M., Huang, Y.J., Reynolds, J.A., Davis, J.A., Henwood, M.K., West, C.R., & Grau, J.W. (2019). Role of blood pressure in pain-induced hemorrhage after spinal cord injury: Brain systems may drive an increase in blood pressure, but this effect was not sufficient to induce hemorrhage. Chicago, IL: Society for Neuroscience, 2019.

Davis, J.A., Henwoow, M.K., Cox, C., Baine, R., & Grau, J.W. (2019). Pain-induced hemorrhage after spinal cord injury: Pentobabital anesthesia and local application of lidocaine prevent hemorrhage when give before, but not after, noxious stimulation. Chicago, IL: Society for Neuroscience, 2019.

Baine, R., Strain, M.M., Reynolds, J.A., Henwood, M.K., Lout, E., Haribhakti, P., West, C.R., & Grau, J.W. (2019). Role of blood pressure in pain-induced hemorrhage after spinal cord injury: Effect of shock and capsaicin treatment on blood pressure, behavioral performance, and hemorrhage over time. Chicago, IL: Society for Neuroscience, 2019.

Plans for the Next Reporting Period

Our overall progress has been excellent. What has deviated slightly from our original plan is the order in which the experiments have been completed. We are now well into parts of Aim 2, which we had not planned to start until month 17 of the project. At the same time, some components of Aim 1 (Major Tasks 1-2) have taken longer to complete. Nonetheless, progress on these tasks is moving along in a predictable manner and we do not anticipate any problems with completing Aim 1 by month 18 of the project. Meeting this goal, together with the progress made on Aim 2, would put us well ahead of schedule.

After the above is complete, we will begin to assess whether lidocaine affects capsaicin induced hemorrhage (Aim 3). In addition, we proposed to test whether pentobarbital or lidocaine affects recovery if given soon after injury (Major Task 3, Aims 2 and 3).

4. IMPACT

Impact on Principal Discipline

Our results are transforming how researchers and clinicians think about tissue loss after spinal cord injury in three ways. First, our work shows that the development is not a passive process, dictated solely by the magnitude of the initial physical injury. Instead, it is an active process that unfolds over the course of hours to days after injury, a process that is modulated by pain input.

Another way that our work is impacting the discipline is by showing that brain-dependent processes contribute to tissue loss after spinal cord injury. To our knowledge, this is the first evidence that the brain can have such an adverse effect. Indeed, prior work (including our own) suggested that brain systems act to quell neural excitation within the spinal cord, which should have a protective effect. Our work overturns this view and leads the field to think about tissue loss after spinal cord injury as a systemic process that is modulated by other on-going physiological processes.

Finally, our work is impacting the field by demonstrating two new procedures that could be translated to practice, to reduce tissue loss after injury. At present, the only method available to reduce neural excitation at the site of injury is hypothermia. This procedure is difficult to implement, especially in a battlefield situation, and hard to maintain over days-weeks after injury. Anesthesia, especially through the local application of lidocaine, offers an attractive alternative that could be readily administered by technicians soon after injury.

Impact on Other Disciplines

By moving the focus of research beyond the site of injury, to consider brain mechanisms and systemic processes, our work touches on a wide range of fields. What brain systems are critical? Are the effects of pain input mediated by lower-level processes within the brainstem or midbrain, or do higher-level

structures (e.g., amygdala, cortical areas) contribute? Are these effects related to stress and activation of the pituitary adrenal axis?

Likewise, our work is relevant to work exploring the medical benefits of placing an individual in a coma-like state. Is complete anesthesia required or would a sedative be sufficient? Future studies will be exploring these issues.

Technology Transfer

The project does not involve components that would require technology transfer. The procedures used are regularly employed in a medical setting, albeit for different reasons. Implementation of the procedures would involve an "off-label" use and clinical verification of treatment effectiveness.

5. CHANGES/PROBLEMS

Changes in Approach

There have been no significant changes in our overall approach. As the research has progressed, it has become evident that the Drabkin assay provides an efficient means of quantifying hemorrhage extent and, for this reason, we have been using it more. For assays examining the expression of pro-inflammatory cytokines, we have increasingly focused upon interleukin 1ß and 18 because they are robustly expressed, provide an internal control (active vs. inactive form), and impact a number of important down-stream pathways. As the project continues, we will continue to refine our procedures.

Problems or Delays

As noted above, detailing the duration of hemorrhage and window of vulnerability has proven a bit more challenging than we anticipated (Aim 1, Major Tasks 1 and 2). In both cases, the short-term effects are clear: nociceptive stimulation induces hemorrhage when applied 6-24 hrs after injury; and hemorrhage is evident 3-24 hrs after the application of nociceptive stimulation. What has been somewhat less clear is the rate at which these effects dissipate, an inherent problem whenever one attempts to derive whether a treatment has "no effect". As might be expected, later time points have yielded intermediate results. To clearly define the time courses, we are taking a conservative approach and replicating key observations.

Changes that Impact Expenditures

As would be expected, replicating components of Aim 1 (Major Tasks 1 and 2) has inflated our animal budget. However, we anticipated some problems when we developed the proposal and, to date, it appears that this will provide sufficient cushion to complete the project.

Significant Changes in Vertebrate Animals

None

6. PRODUCTS

Journal Publications

Submitted

Grau, J. W., Baine, R. E., Bean, P. A., Davis, J. A., Fauss, G. N. K., Henwood, M. K., Hudson, K. E., Johnston, D. T., Tarbet, M. M., & Strain, M. M. (submitted). Learning to promote recovery after spinal cord injury. *Experimental Neurology.*

In Preparation

Davis, J. A., Bopp, A. C., Henwood, M. K., Baine, R. E., Cox, C. C., & Grau, J. W. (in preparation). Pharmacological transection of brain-spinal cord communication blocks pain-induced hemorrhage and locomotor deficits after spinal cord injury.

Fauss, G. N. K., Strain, M. M., Huang, Y.-J., Reynolds, J. A., Davis, J. A., Henwood, M. K., West, C. R., & Grau, J. W. (in preparation). Spared fibers, but not hypertension, mediate development of pain-induced hemorrhage after spinal cord injury.

Baine, R. E., Strain, M. M., Henwood, M. K., Davis, J. A., Johnston, D. T., Reynolds, J. A., & Grau, J. W. (in preparation). Nociceptive input in females: Estrogen does not protect against the harmful effects of pain after spinal cord injury.

Davis, J. A., Bopp, A. C., Tarbet, M. M., Strain, M. M., & Grau, J. W. (in preparation). Pain-induced hemorrhage after spinal cord injury is blocked by pentobarbital anesthesia.

Other Publications

Grau, J. W. (forthcoming) Achieving adaptive plasticity in the spinal cord. *Oxford Research Encyclopedia of Neuroscience,* ed. Murray Sherman. New York and Oxford: Oxford University Press. doi:10.1093/acrefore/9780190264086.013.00243

Baine, R.E., Strain, M.M., Henwood, M.K., Johnston, D.T., Davis, J.A., Grau, J.W. (2019). Pain input after spinal cord injury increases tissue loss and impairs long-term recovery in female rats. *Journal of Neurotrauma. 36,* A-72*.*

Websites

Fauss, G.N.K., Strain, M.M., Huang, Y.J., Reynolds, J.A., Davis, J.A., Henwood, M.K., West, C.R., & Grau, J.W. (2019). Role of blood pressure in pain-induced hemorrhage after spinal cord injury: Brain systems may drive an increase in blood pressure, but this effect was not sufficient to induce hemorrhage. Chicago, IL: Society for Neuroscience, 2019.

https://www.abstractsonline.com/pp8/#!/7883/presentation/52484

Davis, J.A., Henwood, M.K., Cox, C., Baine, R., & Grau, J.W. (2019). Pain-induced hemorrhage after spinal cord injury: Pentobabital anesthesia and local application of lidocaine prevent hemorrhage when give before, but not after, noxious stimulation. Chicago, IL: Society for Neuroscience, 2019.

https://www.abstractsonline.com/pp8/#!/7883/presentation/52482

Baine, R. E., Strain, M.M., Reynolds, J.A., Henwood, M.K., Lout, E., Haribhakti, P., West, C.R., & Grau, J.W. (2019). Role of blood pressure in pain-induced hemorrhage after spinal cord injury:

Effect of shock and capsaicin treatment on blood pressure, behavioral performance, and hemorrhage over time. Chicago, IL: Society for Neuroscience, 2019.

https://www.abstractsonline.com/pp8/#!/7883/presentation/52483

Technologies

None

Inventions

None

Other Products

None

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Individuals Who have Worked on the Project

Name:	James Grau
Project Role:	PI
Research Identifier (ORCID ID):	orcid.org/0000-0002-6191-0433
Person Month Worked:	1.1 cal. mos.
Contribution to Project:	Coordinates project, assists in designs/analyses, paper preparation
Funding Support:	National Institutes of Health

Name:	Jacob Davis
Project Role: PI	Graduate Student
Research Identifier (ORCID ID):	orcid.org/0000-0002-9293-9853
Person Month Worked:	10 cal. mos.
Contribution to Project:	Executes experiments, behavioral testing, hemorrhage assays
Funding Support:	National Institutes of Health

Name:	Travis Johnston
Project Role: PI	Graduate Student
Research Identifier (ORCID ID):	orcid.org/0000-0003-3071-7996
Person Month Worked:	6 cal. mos.
Contribution to Project:	Executes experiments, behavioral testing, hemorrhage assays
Funding Support:	National Institutes of Health

Name:	Rachel Baine
Project Role: PI	Graduate Research Assistant
Research Identifier (ORCID ID):	orcid.org/0000-0002-8576-6368
Person Month Worked:	1 cal. mo.
Contribution to Project:	Executes experiments, behavioral testing, hemorrhage assays
Funding Support:	National Institutes of Health

Name:	Melissa Henwood
Project Role: PI	Technician
Research Identifier (ORCID ID):	orcid.org/0000-0003-2358-972X
Person Month Worked:	6 cal. mos.
Contribution to Project:	Executes experiments, behavioral testing, hemorrhage assays
Funding Support:	National Institutes of Health

Name:	Paris Bean
Project Role: PI	Technician
Research Identifier (ORCID ID):	orcid.org/0000-0002-1268-3588
Person Month Worked:	6 cal. mos.
Contribution to Project:	Executes experiments, behavioral testing, hemorrhage assays
Funding Support:	National Institutes of Health

Name:	Megan Tarbet
Project Role: PI	Technician
Research Identifier (ORCID ID):	orcid.org/0000-0001-7802-9071
Person Month Worked:	6 cal. mos.
Contribution to Project:	Executes experiments, behavioral testing, hemorrhage assays
Funding Support:	National Institutes of Health

Has there been a Change in Other Support Since the Last Reporting Period?

No

What Other Organizations were Involved as Partners

None

8. SPECIAL REPORTING REQUIREMENTS

Collaborative Awards: None

Quad Chart: Attached

9. APPENDICES

Because the grant has only been active for one year, a copy-edited version of the one accepted paper is not yet available. A second paper has been submitted for publication and three others are within a month or two of submission.

Nociceptive (pain) input after spinal cord injury (SCI) enhances secondary injury: Identifying treatments that can be translated to clinical practice

Award Number: GRANT12524207 Log Number: SC170241

PI: James W. Grau **Org:** Texas A&M University **Award Amount:** $498,619 (direct costs)

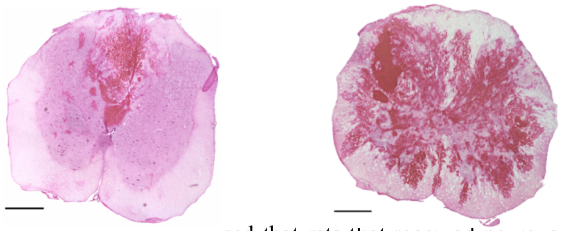
Study/Product Aim(s)

- **Aim 1: When does nociceptive input have an adverse effect?**
- **Aim 2: Promoting recovery using general anesthesia.**
- **Aim 3: Promoting recovery using local anesthesia.**

Approach

Pain input after spinal cord injury drives tissue loss by increasing the area of secondary injury/hemorrhage. Three aims were proposed that will define when pain input affects injury processes and how to block this effect. It is proposed that general anesthesia, or blocking spinal activity with lidocaine, will reduce pain-induced tissue loss and thereby promote recovery. These treatments could be rapidly translated to practice.

Contusion Alone Contusion+Pain Input



Typical sections from contused that rats that received noxious stimulation after injury (right) or nothing (left). Pain input dramatically increases the area of hemorrhage (dark red). (4x; bar = 500µm).

Accomplishments: Prior work had shown that noxious electrical stimulation induces hemorrhage at the site of injury and undermines long-term recovery. This effect is eliminated by a rostral transection. We have shown that an irritant that engages pain (C) fibers has a similar effect and that comparable results are observed in both male and female rats (Aim 1, Major Tasks 3 and 4). We also showed that administration of anesthesia after painful stimulation does not attenuate hemorrhage (Aim 2, Major Task 1). On-gong work is detailing the time-course of hemorrhage and when nociceptive stimulation affects tissue loss (Aim 1, Major Tasks 1 and 2).

Timeline and Cost

Activities	Year 1	Year 2	Year 3
Major Aim 1: Determine when does nociceptive input has an adverse effect?			
Major Aim 2: Determine when general anesthesia has a protective effect.			
Major Aim 3: Determine when the local application of lidocaine has a protective effect.			
Estimated Budget ($K):	$162,423	$166,165	$170,031

Updated: October 31, 2019

Goals/Milestones

Mos. 1-2: Complete animal protocols
■ IACUC approval; ■ ACURO approval
Mos. 3-16: Aim 1—When does nociceptive input affects recovery?
☐ Task 1 (75%); ☐ Task 2 (50%); ☐Task 3 (75%); ☐■ Task 4
Mos. 17-26: Aim 2 – Evaluate effectiveness of general anesthesia.
■ Task 1; ☐ Task 2 (75%); ☐ Task 3;
Year 3/Aim 3 – Evaluate effectiveness of local lidocaine.
☐ Task 1; ☐ Task 2; ☐ Task 3

Concerns

- To date, no problems have been encountered.

Budget Expenditure to Date

Expenditures: $161,237 (direct; 32.3% of the net budget)