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TITLE: Rescuing Our Warriors from Chronic Pain: A Battlefield-to-Nondeployment Means to Prevent Opioid-induced Amplification of Neuropathic Pain from Traumatic Injury

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14. ABSTRACT

Based on our preliminary data and a thorough review of the available scientific/clinical literature to date, *we hypothesize that:* (**a**) Trauma and opioids combine to amplify the intensity and duration of trauma-induced chronic pain. (**b**) This combined exposure to trauma plus opioids amplifies the creation and release of endogenous danger signals in spinal cord that create enduring release of TLR4 stimulatory substances as a consequence of cell stress/damage/death, leading to amplified trauma induced chronic pain.

Objective 1. Define the response to opioids commonly used for acute pain management, when these are administered early after trauma, prior to development of neuropathic pain

Objective 2. Define the response to opioids & non-opioids commonly used for neuropathic pain management, when these treatments are administered later after trauma, after development of neuropathic pain

Objective 3. Define whether the deleterious effects on neuropathic pain observed in Aims 1 & 2 can be prevented by targeting TLR4 and P2X7

Objective 4. Define whether the deleterious effects of analgesics, and positive effects of co-administered TLR4/P2X7 antagonists, extend beyond neuropathic pain to other indices of disability

15. SUBJECT TERMS chronic pain, opioid analgesics, non-opioid analgesics, toll-like receptor 4, return to duty					
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1. Introduction

In the current project we address the concern that opioids administered for the treatment of neuropathic pain after injury can result in a prolonged recovery period and increased intensity of pain. Our data from the previous year have shown that a five-day course of morphine can amplify neuropathic pain whether given in the acute period (one hour) after trauma, at the development of chronic pain (day 10 post-trauma), or late in the time course when chronic pain is well established (one month post-trauma). In addition to the amplification of pain, our data have shown that treatment with morphine has deleterious effects on other indices of recovery such as voluntary exercise and pilot data suggest long duration effects of morphine in increasing anxiety as well. We did not find a lasting effect of morphine on measures of gait, stamina and agility, suggesting that the decrease in voluntary exercise is due to loss of motivation or pain amplification rather than physical disability. We have also demonstrated that co-administration of the TLR4 antagonist (+)-Naloxone, and the P2X7 antagonist A438079 can improve the neuroinflammatory consequences of morphine as measured by voluntary exercise. Our pilot data from the previous year also suggest that the amplification of chronic pain observed in morphine treated rats also extends to the other opioids, Fentanyl and Oxycodone. The full extent of the effects of these two opioids on the development of chronic pain will continue to be explored in year two of this project.

2. Keywords

Neuropathic pain, opioid analgesics, non-opioid analgesics, return to duty, morphine, oxycodone, fentanyl, toll-like receptor 4, P2X7

3. Accomplishments:

What were the major goals of the project?

1. Define whether deleterious effects of opioids extend beyond neuropathic pain to other indices of disability. Determine impact on return to duty, normal gait, agility, and stamina by investigating effects of Morphine administered at day 10 post trauma on rotorod and horizontal ladder tasks in rats. Completed group one December 2016, completed group two March 2017

2. Determine the impact on return to duty, normal gait, agility, and stamina by investigating effects of Morphine administered at day 10 post trauma on voluntary wheel running in rats. Rats were habituated to running wheels with free access for 7 days, and baseline running data were collected. Rats then received unilateral 4 suture chronic constriction injury (CCI) of the sciatic nerve at mid-thigh level, or sham surgeries of the sciatic nerve. At day 10 post CCI, rats received a 5-day course of morphine (5-mg/kg b.i.d.) or saline. Running data were continuously collected by computer 24 hours per day, 7 days a week until

group differences resolved. Completed Pilot study January 2017, completed first experiment March 2017

3. Explore the use of the outbred Sprague Dawley strain to replace the genetically homogeneous Fischer 344 strain by adjusting the partial sciatic injury surgical model (chronic constriction injury; CCI) to further decrease allodynia by decreasing suture size of chromic gut, so that a more robust difference between groups is revealed much closer to the end of opioid dosing. Surgical models tested were: one suture 5-0, one suture 6-0, one, two, and three sutures 7-0 chromic gut. Completed March 2017

4. Test the effect of morphine begun at one hour post trauma (instead of the standard start of 10 days after trauma). Rats received CCI surgeries or sham surgeries of the sciatic nerve. At one hour post CCI, rats began a 5-day course of morphine (5-mg/kg b.i.d.) or saline. Assessment of mechanical allodynia by Von Frey testing occurred weekly. Completed September 2017

5. Test the effect of morphine begun at one month post trauma (instead of the standard start of 10 days after trauma). Rats received CCI surgeries or sham surgeries of the sciatic nerve. At one month post CCI, rats began a 5-day course of morphine (5-mg/kg b.i.d.) or saline. Assessment of mechanical allodynia by Von Frey testing occurred weekly following CCI, and continued weekly after the conclusion of morphine dosing. Completed August 2017

6. Test if antagonists administered with morphine prevent opioid induced suppression of voluntary wheel running. Rats were habituated to running wheels with free access for 7 days, and baseline running data were collected. Rats then received 4 suture CCI surgeries or sham surgeries of the sciatic nerve. At day 10 post CCI rats received a 5-day course of morphine (5mg/kg b.i.d.) or saline, along with the TLR4 antagonist (+)-Naloxone (20-mg/kg s.c.), the P2X7 antagonist A438079 (1-mg/kg s.c.), or equivolume s.c. saline vehicle. Running data were collected by computer 24 hours per day, 7 days a week. 50% completion

7. Test the opioids Fentanyl and Oxycodone given at day 10 post trauma. In this pilot, all rats received CCI surgeries of the sciatic nerve with one 6-0 suture. At day 10 post CCI rats began a 5-day course of Fentanyl (0.5mg/kg), Oxycodone (2.5mg/kg), or saline control. Assessment of mechanical allodynia by Von Frey testing occurred at day one post opioid completion and weekly thereafter. First set of rats completed September 2017

8. Create a subcontract for work to be done at MD Anderson by co-PI Dr. Peter Grace, now an Assistant Professor, Department of Critical Care & Respiratory Care Research. Completed May 2017. ACURO at MD Anderson for this project approved. Techniques required for running studies for this project have all been established and perfected at Grace's research lab.

9. What was accomplished under these goals?

1. **Define whether deleterious effects of opioids extend beyond neuropathic pain to other indices of disability. Determine impact on return to duty, normal gait, agility, and stamina by investigating effects of Morphine administered at day 10 post trauma on rotorod and horizontal ladder tasks in rats. Task 13, Objective 4B.**

We investigated the effect of Morphine (5-mg/kg b.i.d.) for 5 days starting at 10 days post trauma on behavioral tests of gait, agility and stamina. Rats were trained on rotorod and horizontal ladder, baseline data were collected, and 4 suture Chronic Constriction Injury (CCI) or sham surgeries of the sciatic nerve were then performed. Behavioral data were collected again at day 9 post CCI, before opioid administration. At day 10 post-surgery, rats began a 5-day regimen of morphine or saline. Behavioral data were collected again at day 1 post morphine completion, and weekly thereafter for 5 weeks. We found a significant surgical effect of CCI on these tasks at day 9 post CCI but did not find a significant effect of morphine on these tasks of agility at any of the time points tested, n=6 per group. Figures 1 and 2 graph the results of this experiment. Assessments of pain thresholds were also performed at the same time points by Von Frey testing to confirm allodynia.

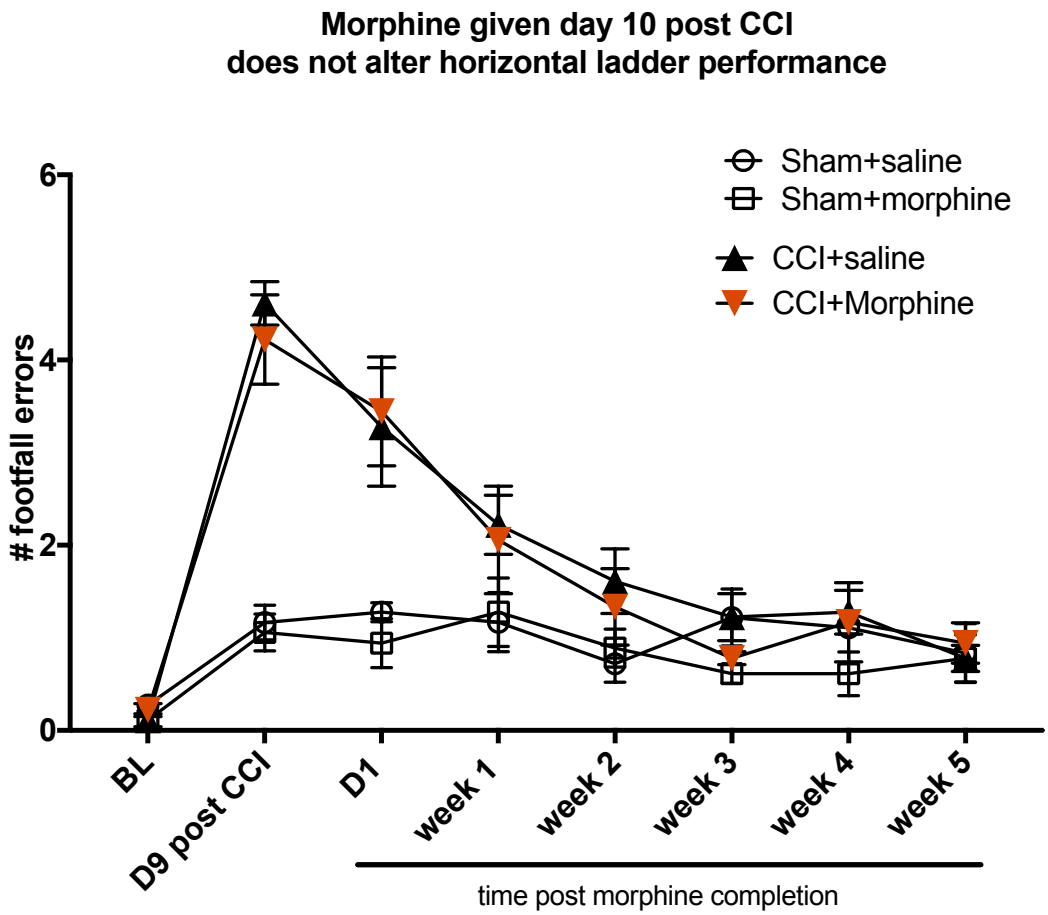

Figure 1. Chronic Constriction Injury (CCI) or sham surgeries were performed. Morphine (5-mg/kg b.i.d.) or saline were administered starting at day 10 post-surgery for 5 days. Behavior testing was conducted at day 1 post opioid completion, and weekly thereafter. Results presented are number of footfall errors when crossing horizontal ladder. ($p>.05$, Two Way ANOVA, n=6/group)

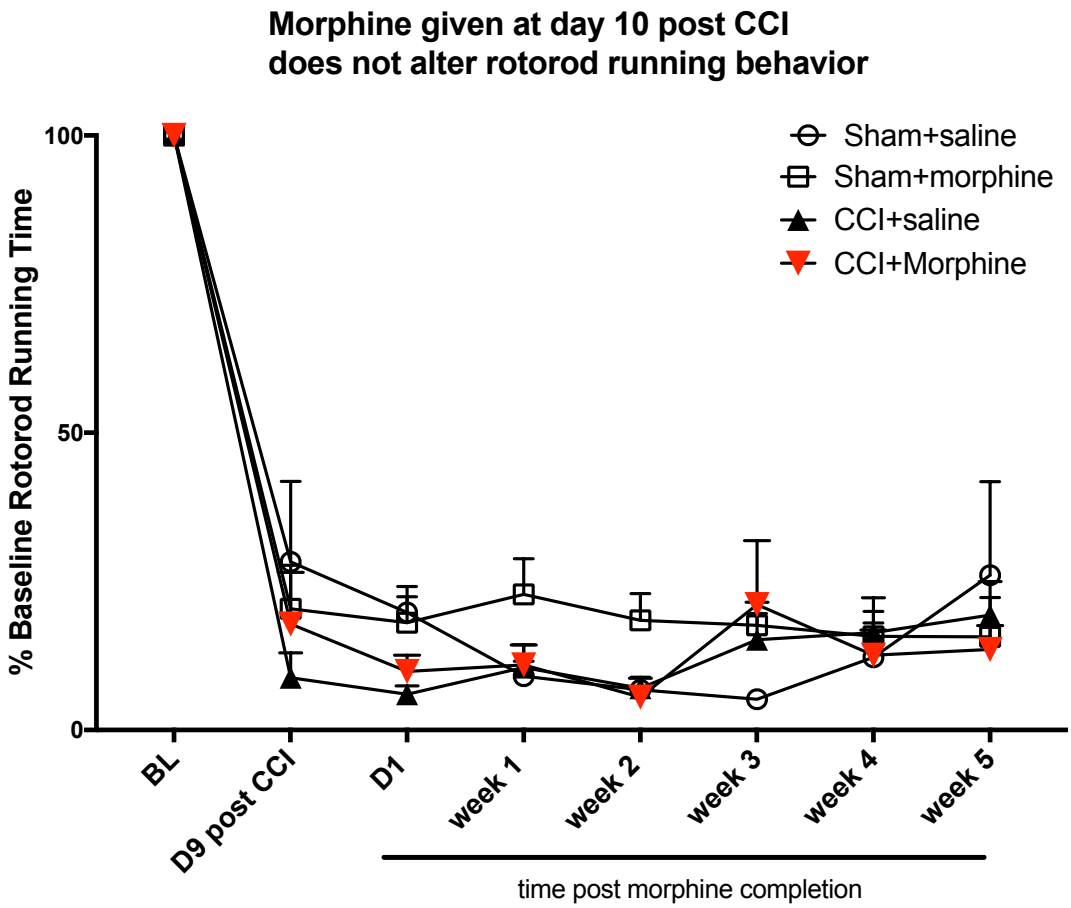

Figure 2. Chronic Constriction Injury (CCI) or sham surgeries were performed. Morphine (5mg/kg b.i.d.) or saline were administered at day 10 post-surgery for 5 days. Behavior testing was conducted at day 1 post opioid completion, and weekly thereafter. Results are reported as percentage of baseline performance time in seconds of rotorod running. ($p>.05$, Two way ANOVA, n=6/group)

This experiment was repeated with Sprague Dawley rats to rule out rat strain for the lack of effect of repeated morphine post-CCI that occurred in Fischer 344 rats, as no group of Fischer 344 rats (including shams saline rats) performed well on these tests, so there was a concern that the Fischer 344 results may not be trustable given the low performance and high variability. Although the Sprague Dawley strain performed remarkably better than the Fischer 344 rats on these behaviors, we still did not find a significant effect of CCI or morphine on these tasks of agility at any of the time points tested, n=6 per group. We conclude that there is no motor deficit resulting from morphine treatment on these tasks of gait, agility, and stamina. Figures 3 and 4 graph the results of this experiment. These data are important in putting the correct perspective on the suppression of voluntary wheel running (below) as factors such as intrinsic motivation and pain responsivity may be posited to underlie voluntary wheel running suppression, as forced stamina, agility and gait indices are not impaired.

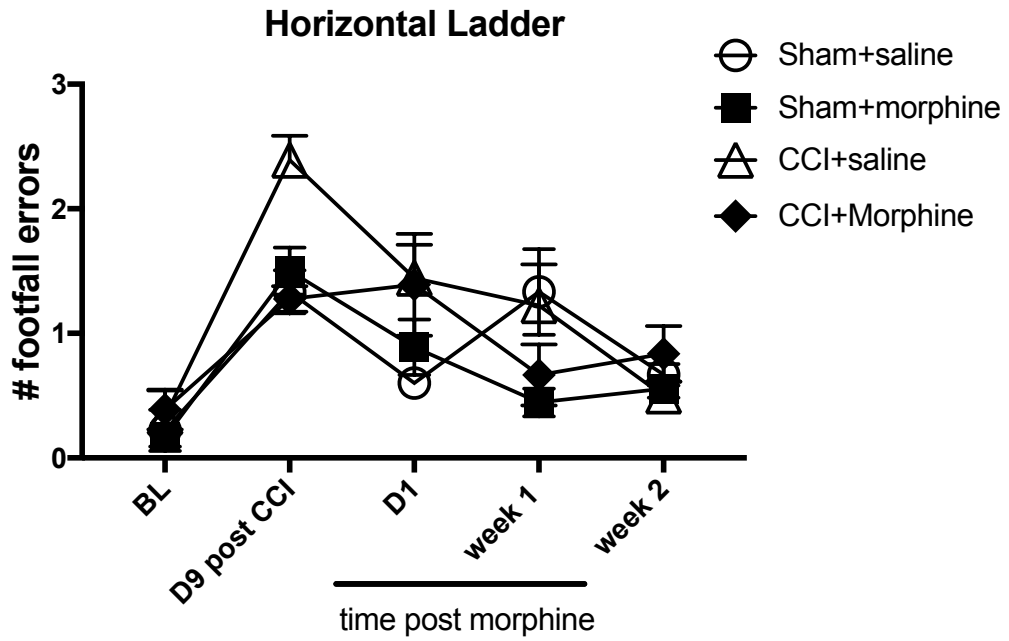

Figure 3. Chronic Constriction Injury (CCI) or sham surgeries were performed. Morphine (5mg/kg b.i.d.) or saline were administered at day 10 post-surgery for 5 days. Behavior testing was conducted at day 1 post opioid completion, and weekly thereafter. Results presented are number of footfall errors when crossing horizontal ladder. No significant effects of surgery or morphine treatment were observed in this task. ($p>.05$, Two Way ANOVA, n=6/group)

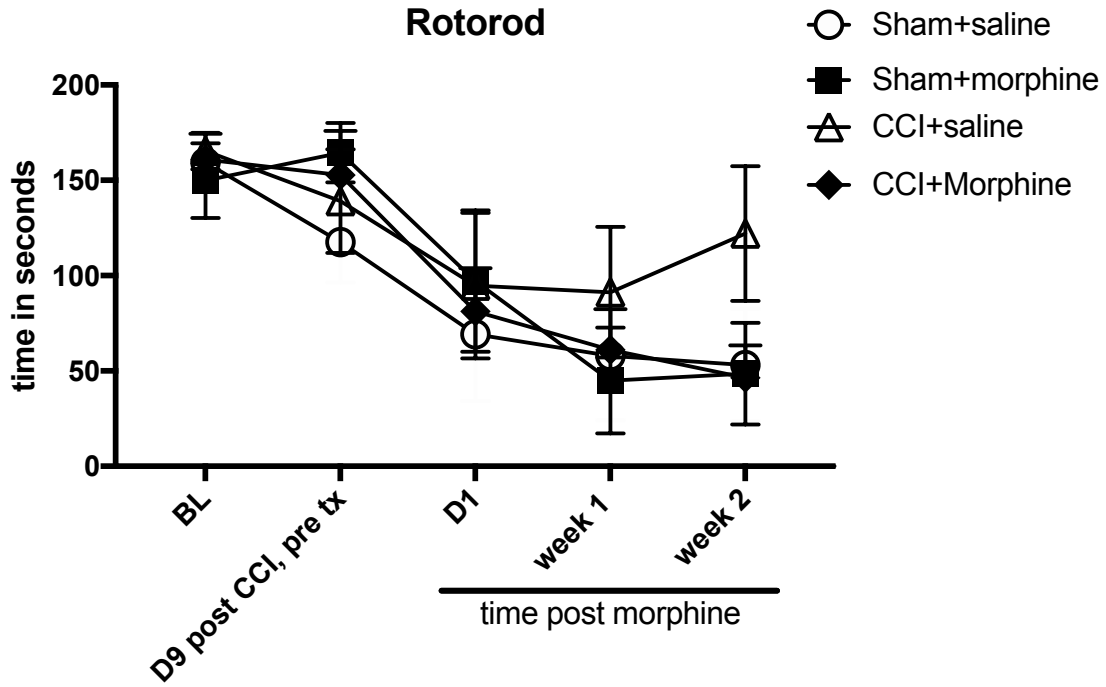

Figure 4. Chronic Constriction Injury (CCI) or sham surgeries were performed. Morphine (5mg/kg b.i.d.) or saline were administered at day 10 post-surgery for 5 days. Behavior testing was conducted at day 1 post opioid completion, and weekly thereafter. Results are reported as time in seconds of rotorod running. No significant effects of surgery or morphine treatment were observed in this task. ($p>.05$, Two way ANOVA, n=6/group)

2. **Define whether deleterious effects of opioids extend beyond neuropathic pain to other indices of disability. Determine the impact on return to duty, normal gait, agility, and stamina by investigating effects of Morphine administered at day 10 post trauma on voluntary wheel running. Task 13, Objective 4B.**

A separate group of rats were used to test the effect of morphine given at day 10 post trauma on voluntary wheel running. This pilot study looked at running behavior specifically during the first hour of lights on, with wheels only unlocked during this time-restricted running group. This selection of time for running is derived from (Whitehead, et. al, 2017, Chronic Sciatic Neuropathy in Rat Reduces Voluntary Wheel-Running Activity With Concurrent Chronic Mechanical Allodynia, Anesthesia & Analgesia: 124:346-355). A comparison group with access to running wheels 24 hours per day was included. All rats were habituated to wheels with free access for 3 days, then wheels were locked in the restricted running group, with access only during the first hour of lights on for 4 days. Rats then received 4 suture CCI surgeries of the sciatic nerve. Wheels were unlocked in the restricted group for the first hour of lights on (9-10am) at day 3 and 9 post CCI for data collection. Free running rats were allowed continual access to wheels 24 hours per day. At day 10 post CCI rats received a 5-day course of morphine (5-mg/kg b.i.d.) or saline. Wheels were unlocked at day 1 post morphine completion, and for 3 days per week thereafter during the first hour of lights on. Running data were collected by computer 24 hours per day for the entire study. The study was concluded at 4 weeks post opioid completion when differences between groups had resolved. We found a decrease in voluntary running behavior during opioid administration and for approximately 2 weeks after morphine cessation in rats with 24-hour access to running wheels. This effect was variable, and did not reach statistical significance in this pilot. These data are represented in Figure 5. No significant difference between groups was observed in restricted running groups (Figure 6).

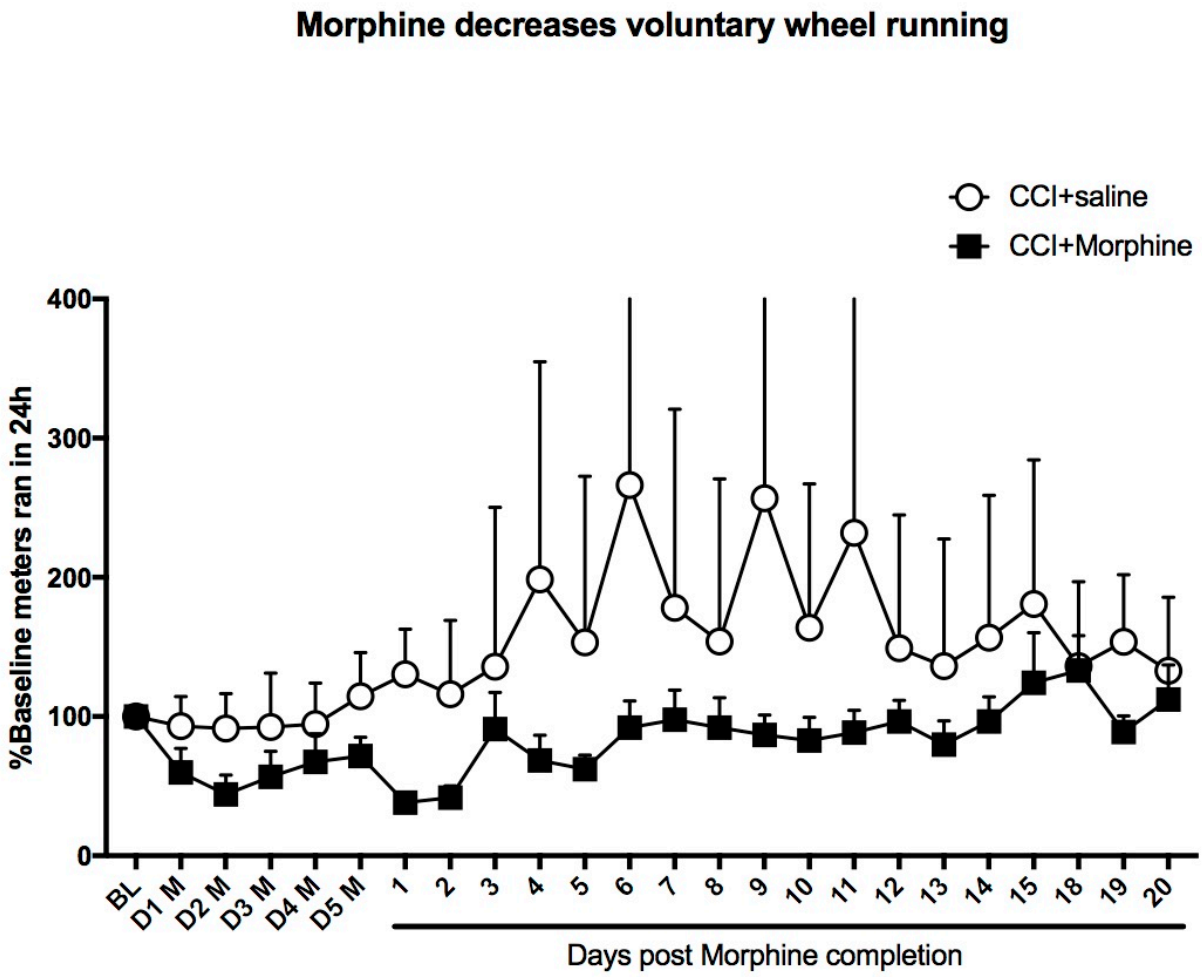

Figure 5. Chronic Constriction Injury (CCI) surgeries were performed. Morphine (5-mg/kg b.i.d.) or saline was administered at day 10 post-surgery for 5 days. Morphine decreased running behavior in this pilot n=4, in rats with free access to wheels 24 hours per day. Results are presented as percentage of baseline meters ran in 24 hours. ($p>.05$, Two way ANOVA, n=4/group pilot data)

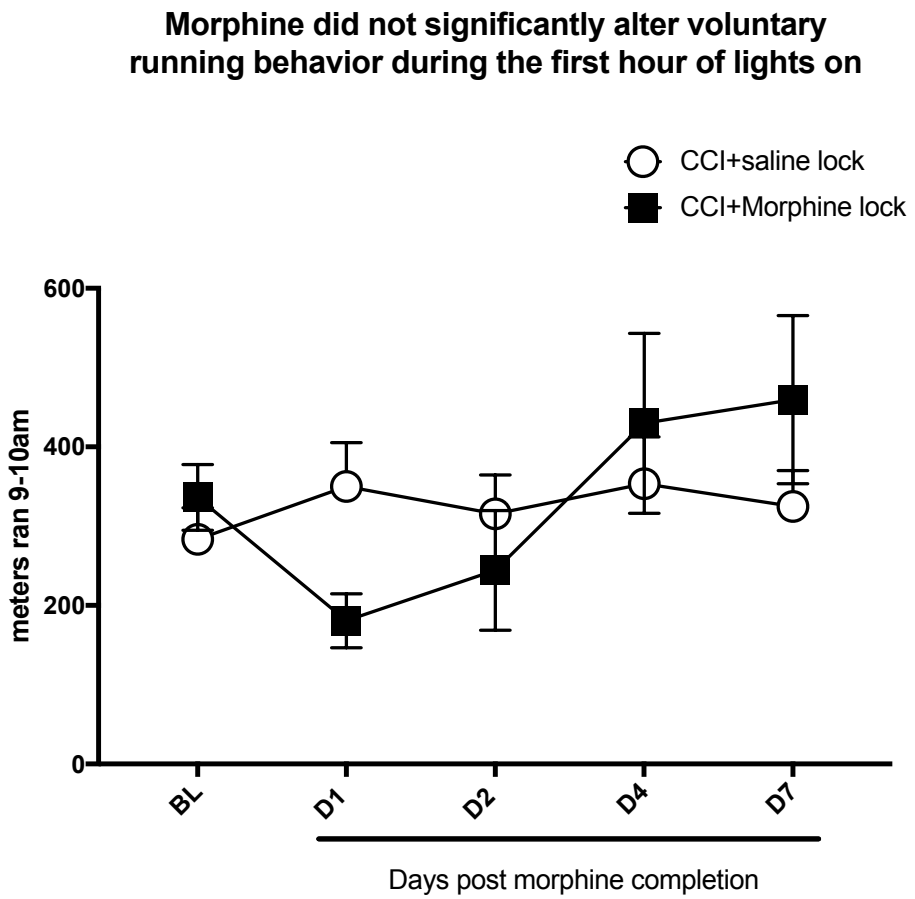

Figure 6. No significant effects of morphine were observed on voluntary wheel running behavior in rats given access to wheels only during the first hour of lights on. Data are presented as total meters ran from 9-10am. ($p>.05$, Two way ANOVA, n=4/group pilot data)

Following the pilot above, the effect of morphine given at day 10 post trauma on voluntary wheel running was tested in a complete study (n=8/group) of Fischer 344 rats. All rats were habituated to wheels with free access for 7 days. Rats then received 4 suture CCI surgeries of the sciatic nerve. At day 10 post CCI rats received a 5-day course of morphine (5-mg/kg b.i.d.) or saline. Running data were collected continuously by computer 24 hours per day for the entire study. Activity data was analyzed for total 24 hour running, active phase running (20:00-06:00), and inactive phase running (7:00-19:00). The study was concluded at 35 days post opioid completion when differences between groups had resolved. We found a significant decrease in voluntary running behavior during opioid administration and for approximately 4 weeks after morphine. These data are represented in Figure 7: meters ran in 24 hours as percent of baseline running, Figure 8: meters ran during active phase, Figure 9: meters ran during inactive phase, Figure 10: area under curve total meters ran in 24 hours, Figure 11: area under curve active phase running, Figure 12 area under curve inactive phase running.

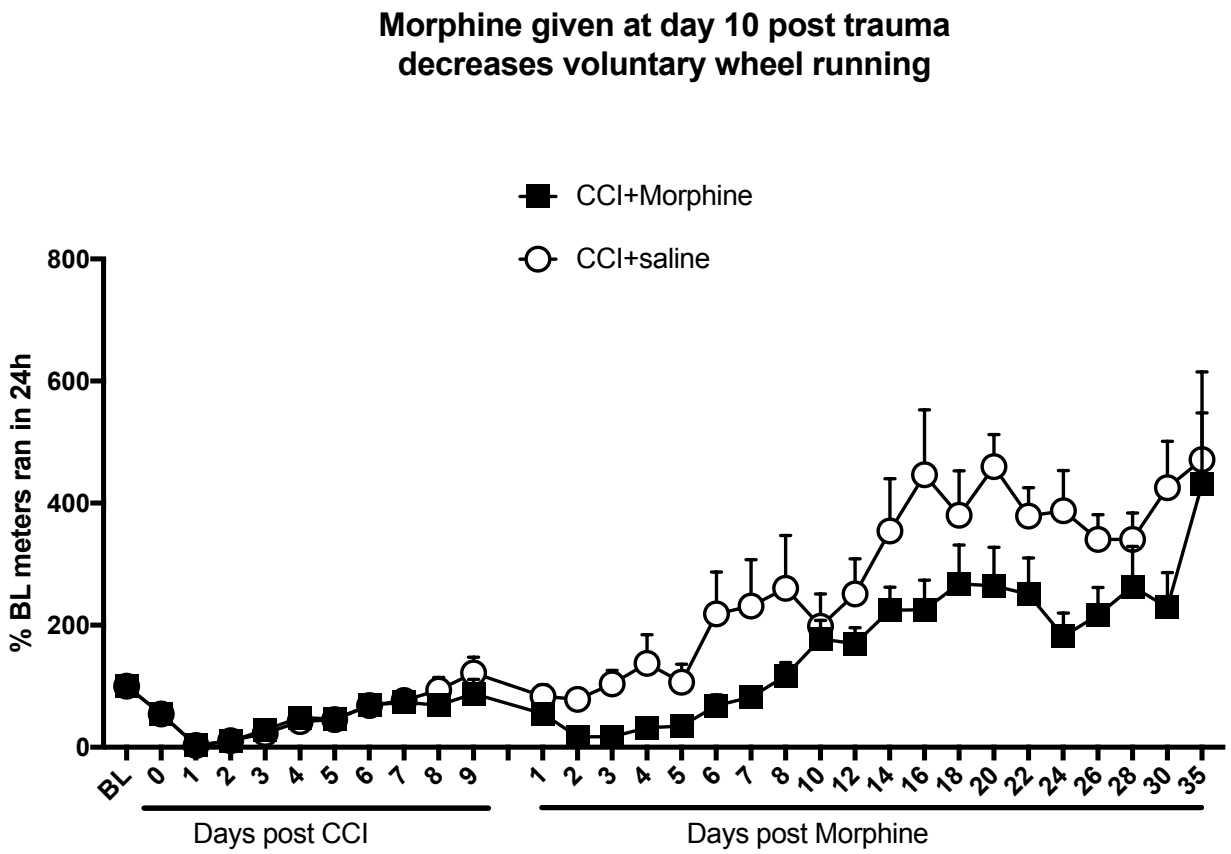

Figure 7. Chronic Constriction Injury (CCI) surgeries were performed. Morphine (5-mg/kg b.i.d.) or saline was administered at day 10 post-surgery for 5 days. Morphine significantly decreased running behavior in this study for approximately 4 weeks after morphine conclusion. Results are presented as percentage of baseline, meters ran in 24 hours.

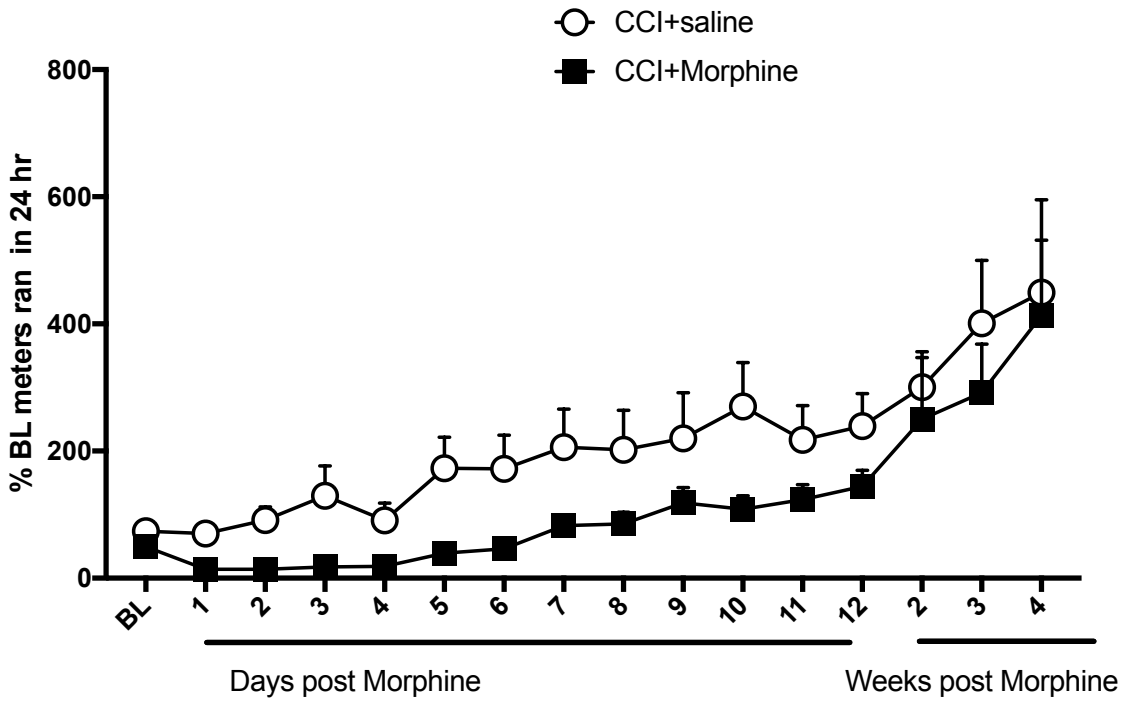

Figure 8. Chronic Constriction Injury (CCI) surgeries were performed. Morphine (5-mg/kg b.i.d.) or saline was administered at day 10 post-surgery for 5 days. Morphine significantly decreased running behavior during the active phase (20:00-06:00). Results are presented as percentage of baseline meters ran in 24 hours.

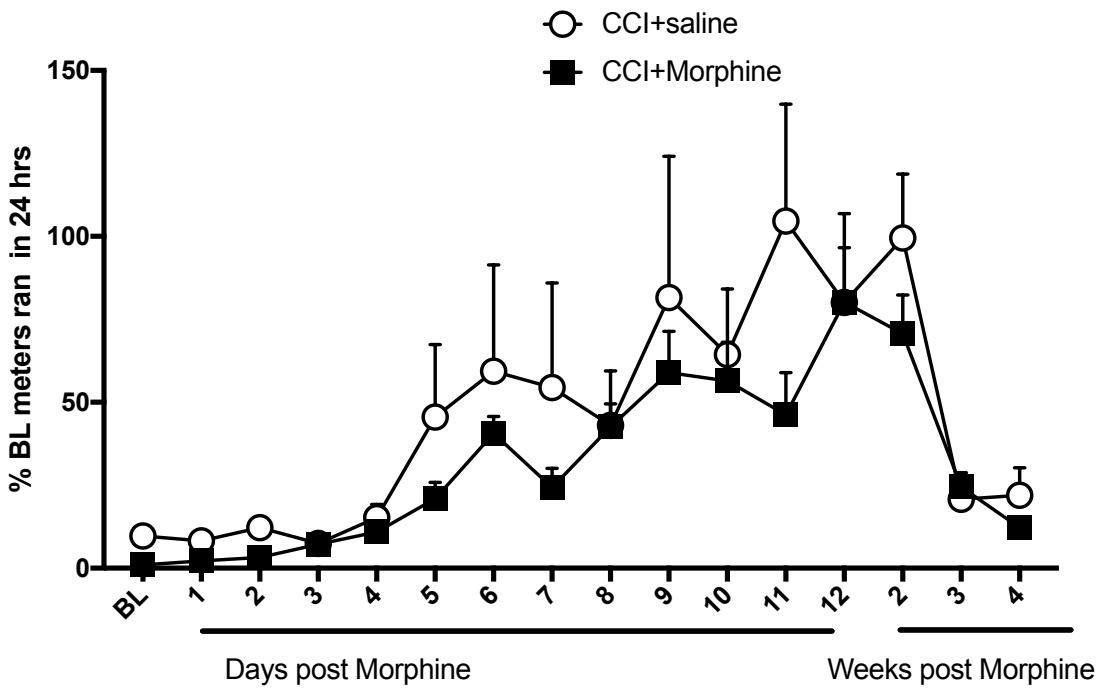

Figure 9. Chronic Constriction Injury (CCI) surgeries were performed. Morphine (5mg/kg b.i.d.) or saline was administered at day 10 post-surgery for 5 days. Morphine significantly decreased inactive phase (7:00-19:00) running behavior. Results are presented as percentage of baseline meters ran in 24 hours.

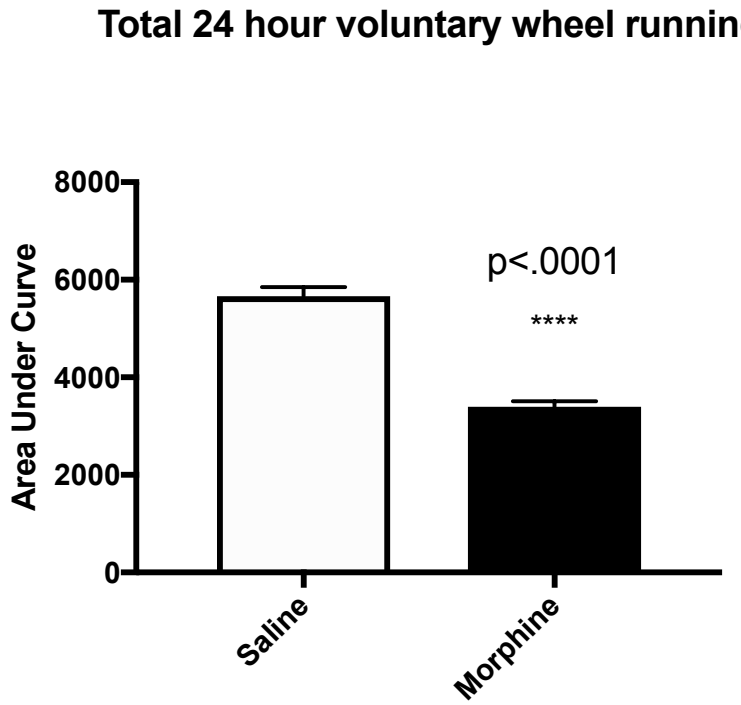

Figure 10. Chronic Constriction Injury (CCI) surgeries were performed. Morphine (5mg/kg b.i.d.) or saline was administered at day 10 post-surgery for 5 days. Morphine significantly

decreased total 24 hour running behavior. Results are presented as area under the curve. (p<.0001, unpaired t test, n=8/group)

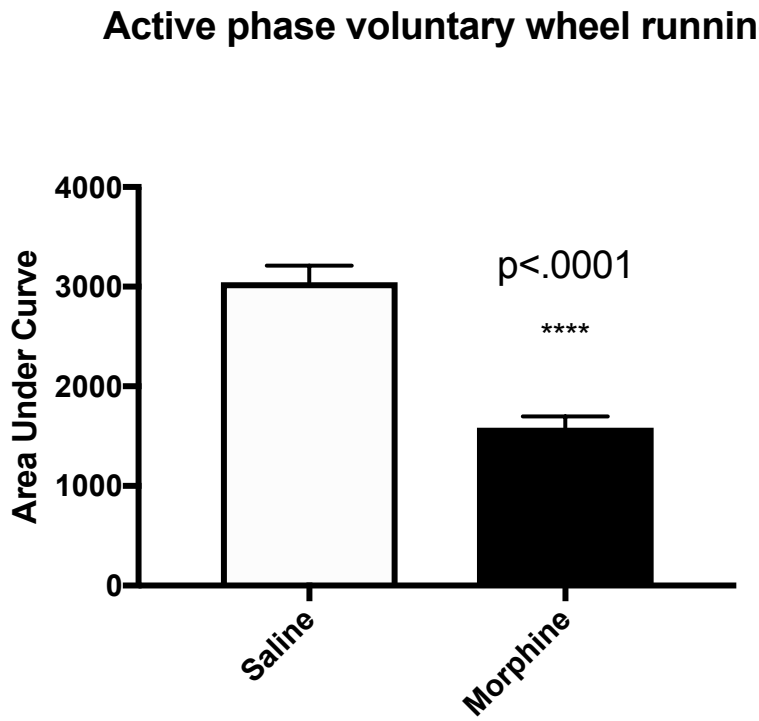

Figure 11. Chronic Constriction Injury (CCI) surgeries were performed. Morphine (5mg/kg b.i.d.) or saline was administered at day 10 post-surgery for 5 days. Morphine significantly decreased active phase (20:00-06:00) running behavior. Results are presented as area under the curve. (p<.0001, unpaired t test, n=8/group)

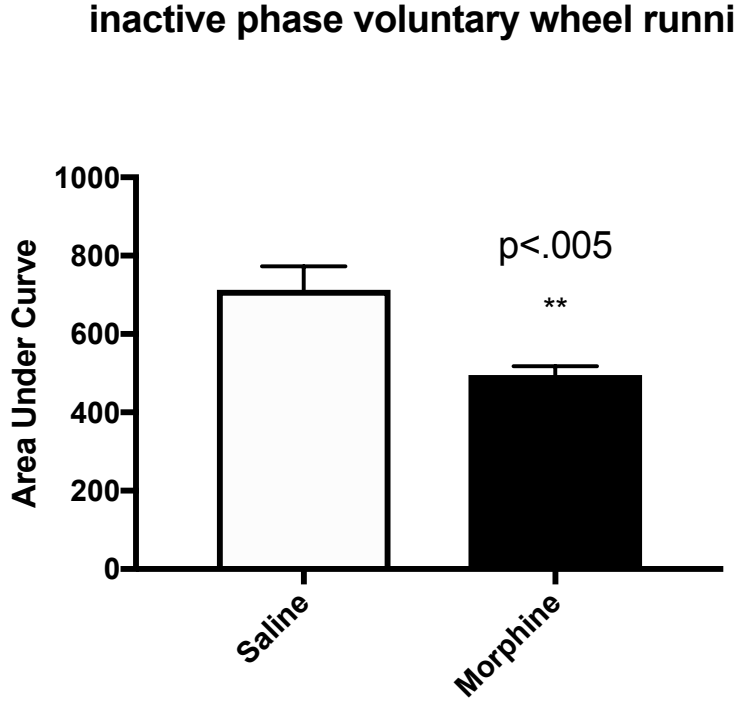

Figure 12. Chronic Constriction Injury (CCI) surgeries were performed. Morphine (5mg/kg b.i.d.) or saline was administered at day 10 post-surgery for 5 days. Morphine significantly

decreased inactive phase (7:00-19:00) running behavior. Results are presented as area under the curve. ($p<.005$, unpaired t test, n=8/group)

3. **Explore the use of the outbred Sprague Dawley strain to replace the genetically homogeneous Fischer 344 strain by adjusting the partial sciatic injury surgical model (chronic constriction injury; CCI) to further decrease allodynia by decreasing suture size of chromic gut, so that a more robust difference between groups is revealed much closer to the end of opioid dosing.**

The issue addressed was a behavioral test basement effect, which squashed the responses across groups to the very bottom of our testing range. The goal was to increase our ability to robustly detect and quantify differences between groups by *decreasing* the intensity of the neuropathic pain by *decreasing* the number and/or thickness of the suture material used to create the chronic constriction injury model under study. In the preliminary pilot experiment (n=4/group) to determine suture size effects, rats were given a one suture CCI of the sciatic nerve using either 5-0, 6-0 or 7-0 chromic gut, instead of the normal 4-0 chromic gut. All rats received a 5-day course of morphine (5mg/kg b.i.d) at day 10 post CCI. Weekly Von Frey assessments of mechanical allodynia were conducted to observe the level of allodynia created and the time frame of resolution in this new model. No significant differences were observed between 5-0 and 6-0 sutures in the first four weeks of testing, so 5-0 suture was dropped from the follow up experiments. These data are presented in Figure 13.

Based on these data we proceeded to a second pilot study to test one 6-0 suture compared to two and three 7-0 sutures (n=4/group). All of these rats received a 5-day course of morphine (5-mg/kg b.i.d.) or saline at day 10 post CCI. These data are presented in Figures 14 and 15. Given the longer separation of groups in the 6-0 suture study, compared to the quick resolution of the 7-0 suture groups, we chose the 6-0 suture model for future experiments.

We proceeded to use 6-0 model in Sprague Dawleys for future opioid studies based on these successful pilots, as effects from this outbred genetically heterogeneous strain would provide better generalization to humans. The Fischer 344 rat are also proving to be quite high strung, stressful animals which makes reliable testing of this strain problematic under our current laboratory conditions. As previously noted, our prior data using Spragues (Grace, et. al, 2016, PNAS) revealed a shorter period before groups separate post opioid completion, and a shorter overall effect compared to Fischers, which would expedite experiments and save costs on animal housing.

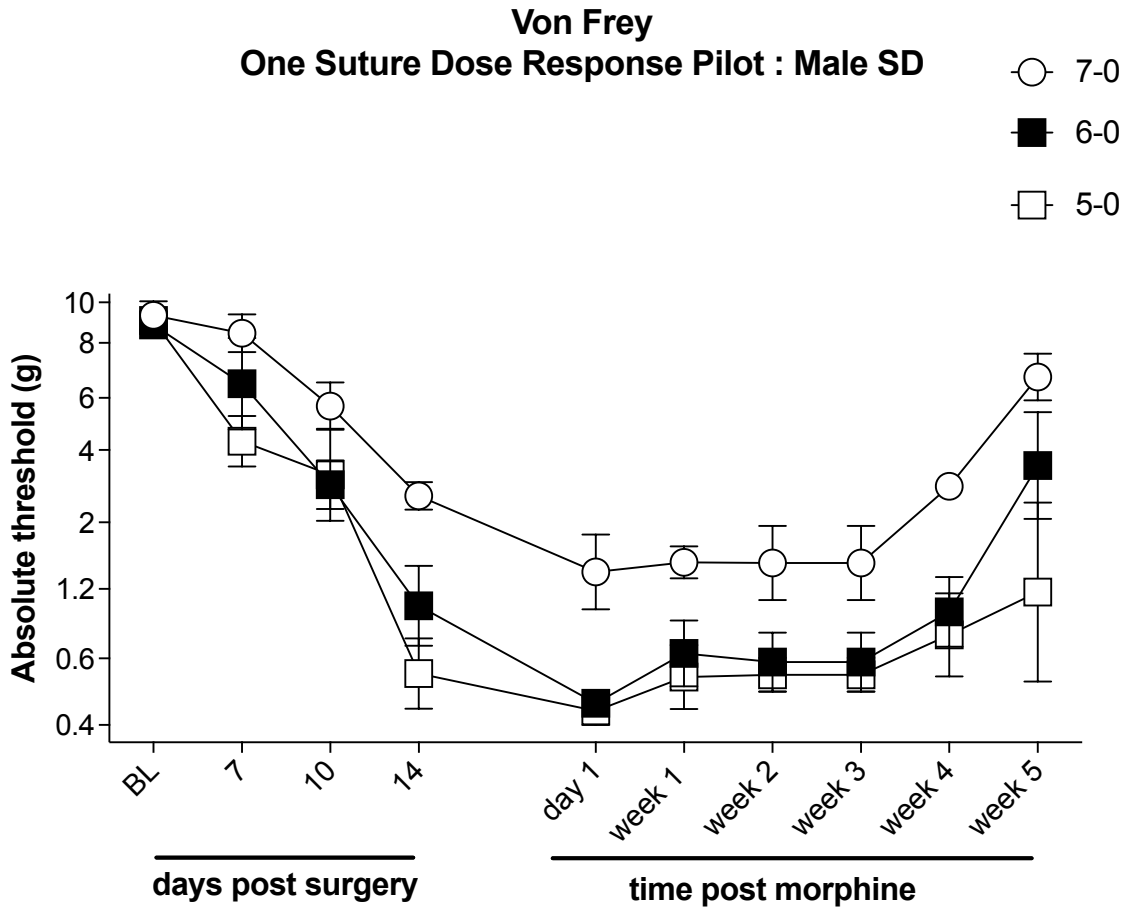

Figure 13. Chronic Constriction Injury (CCI) surgeries were performed with one 5-0, 6-0, or 7-0 suture. Morphine (5mg/kg b.i.d.) was administered at day 10 post-surgery for 5 days. Behavior testing was conducted at day 1 post opioid completion, and weekly thereafter.

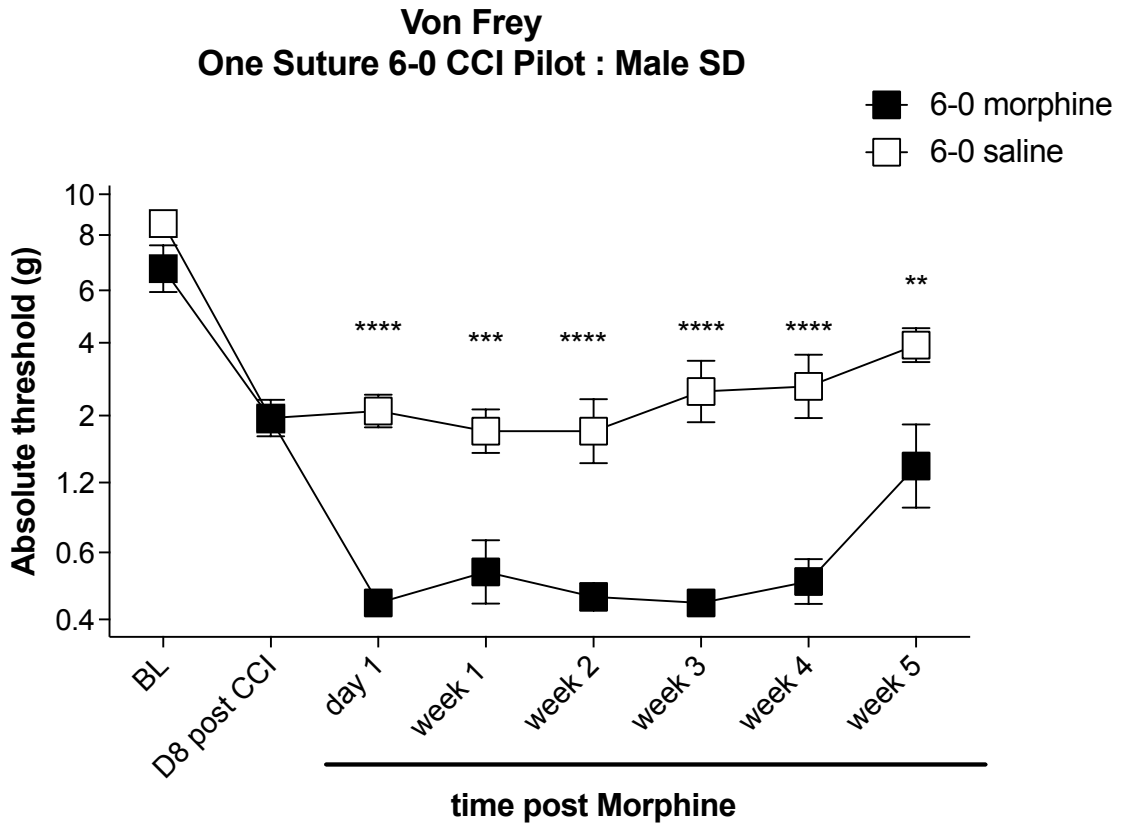

Figure 14. Chronic Constriction Injury (CCI) surgeries were performed with one 6-0 suture. Morphine (5mg/kg b.i.d.) or saline were administered at day 10 post-surgery for 5 days. Behavior testing was conducted at day 1 post opioid completion, and weekly thereafter. ($p<.0001$ Day 1 and Week 2-4, $p<.001$ Week 1, $p=.005$ Week 5, Two Way ANOVA, n=4/group)

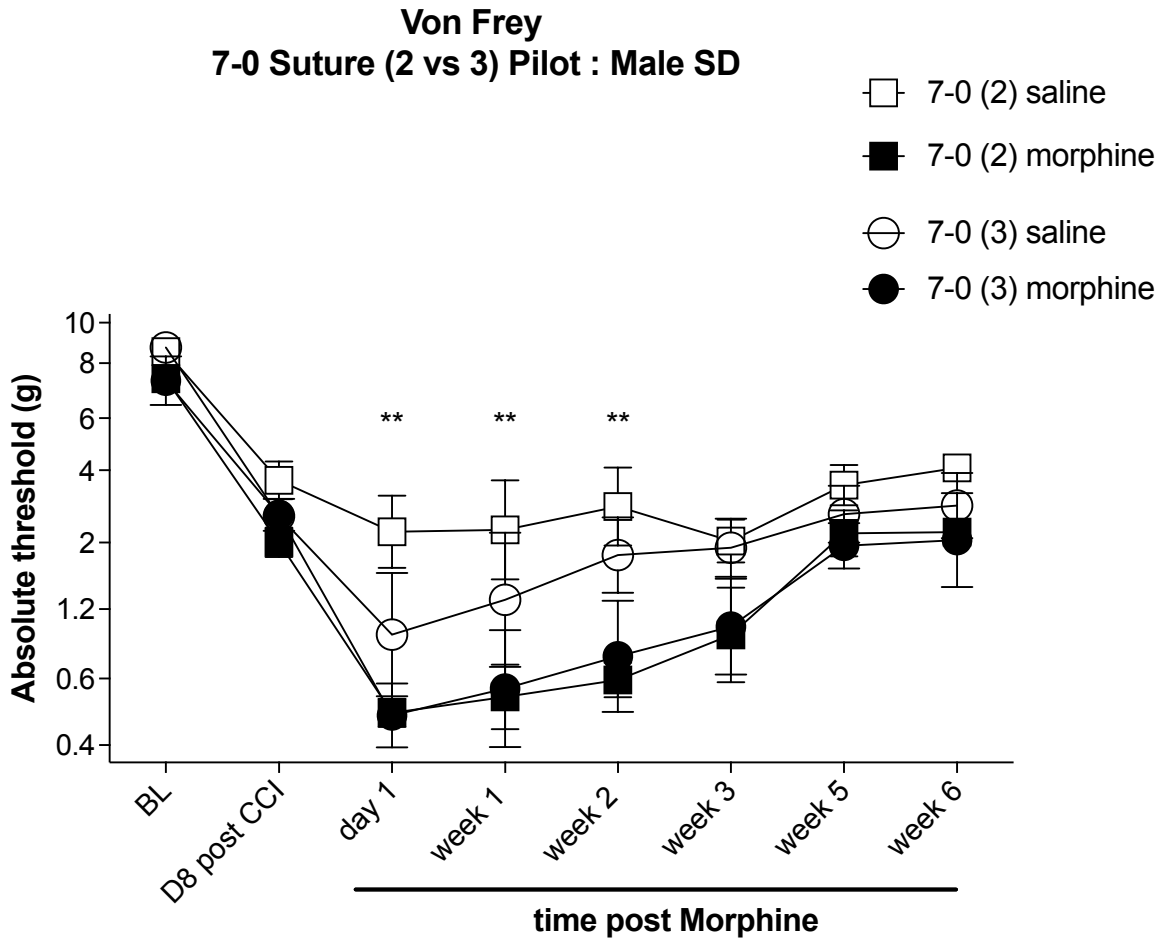

Figure 15. Chronic Constriction Injury (CCI) surgeries were performed with two or three 7-0 sutures. Morphine (5mg/kg b.i.d.) or saline were administered at day 10 post-surgery for 5 days. Behavior testing was conducted at day 1 post opioid completion, and weekly thereafter. ($p<.005$ 7-0(2) saline vs morphine Day1, Week 1, and Week 2, Two Way ANOVA, n=4/group,)

4. **Test the impact on recovery of 5 days of morphine begun at one hour post trauma instead of the standard start of 10 days after trauma. Task 2, Aim 1A.**

Rats received CCI surgeries or sham surgeries of the sciatic nerve. At one hour post CCI rats began a 5-day course of morphine (5-mg/kg b.i.d.) or saline. Assessment of mechanical allodynia by Von Frey testing occurred weekly until group differences resolved. Rats given morphine responded with lower pain thresholds (i.e. higher pain responsivity) than rats given saline, but group differences were not as robust as we see when treatment begins after the development of allodynia, such as treatment beginning at day 10 post trauma, or one month post trauma (presented below). Results of this experiment are presented in Figure 16. Area Under Curve analysis is presented in Figure 17. These data suggest that a short course of morphine shortly after trauma can worsen pain outcome, prolonging recovery.

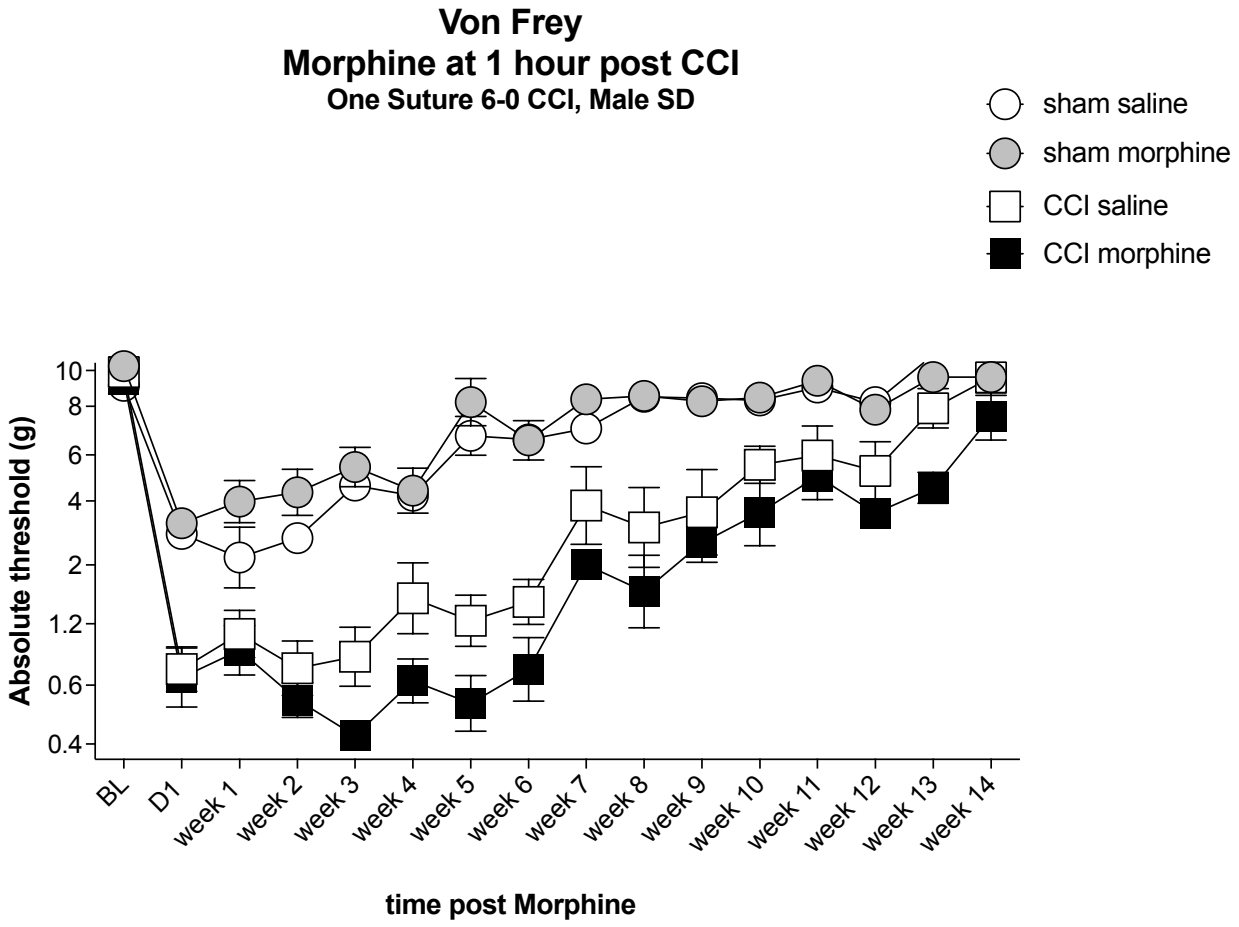

Figure 16. Chronic Constriction Injury (CCI) or sham surgeries were performed with one 6-0 suture. Morphine (5-mg/kg b.i.d.) or saline were administered at one hour post-surgery for 5 days. Behavior testing was conducted at day 1 post opioid completion, and weekly thereafter.

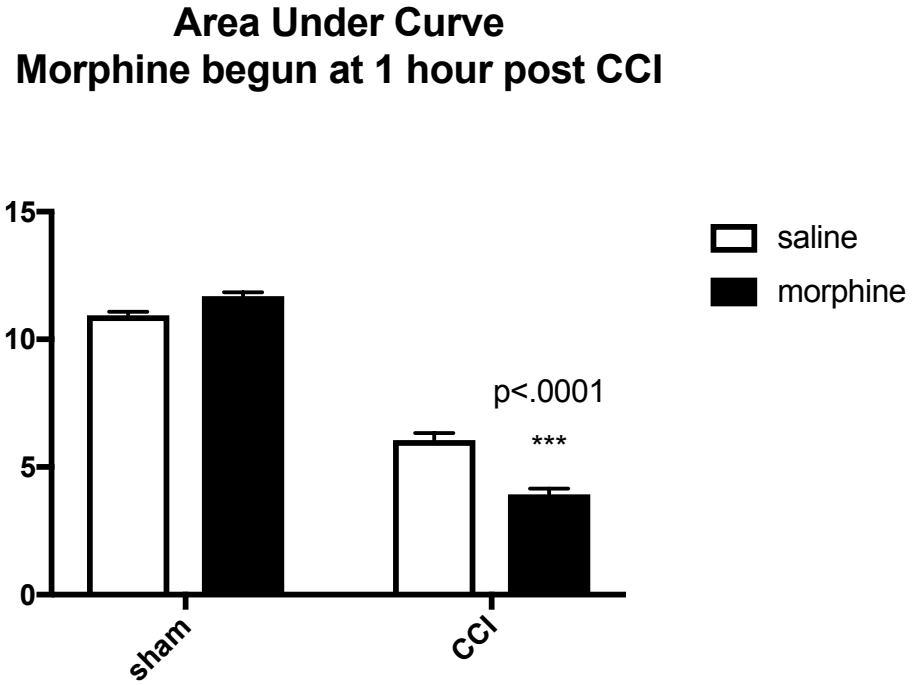

Figure 17. Chronic Constriction Injury (CCI) or sham surgeries were performed with one 6-0 suture. Morphine (5-mg/kg b.i.d.) or saline were administered at one hour post-surgery for 5 days. Behavior testing was conducted at day 1 post opioid completion, and weekly thereafter. ($p<.001$, CCI+Morphine vs CCI+Saline unpaired t test of Area Under Curve, n=6/group.)

5. **Test the impact on recovery of 5 days of morphine begun at one month post trauma instead of the standard start of 10 days after trauma. Task 7 Aim 2C**

Rats received CCI surgeries or sham surgeries of the sciatic nerve. At one month post CCI, rats began a 5-day course of morphine (5-mg/kg b.i.d.) or saline. Assessment of mechanical allodynia by Von Frey testing occurred weekly following CCI, and continued weekly after the conclusion of morphine dosing until group differences resolved. Rats given morphine beginning at one month post trauma responded with significantly lower pain thresholds (i.e. higher pain responsivity) than CCI rats given 5 days of saline post-surgery. Results of this experiment are presented in Figure 18. Area Under Curve analysis is presented in Figure 19. These data suggest that a short course of morphine even a month after injury worsens pain outcomes.

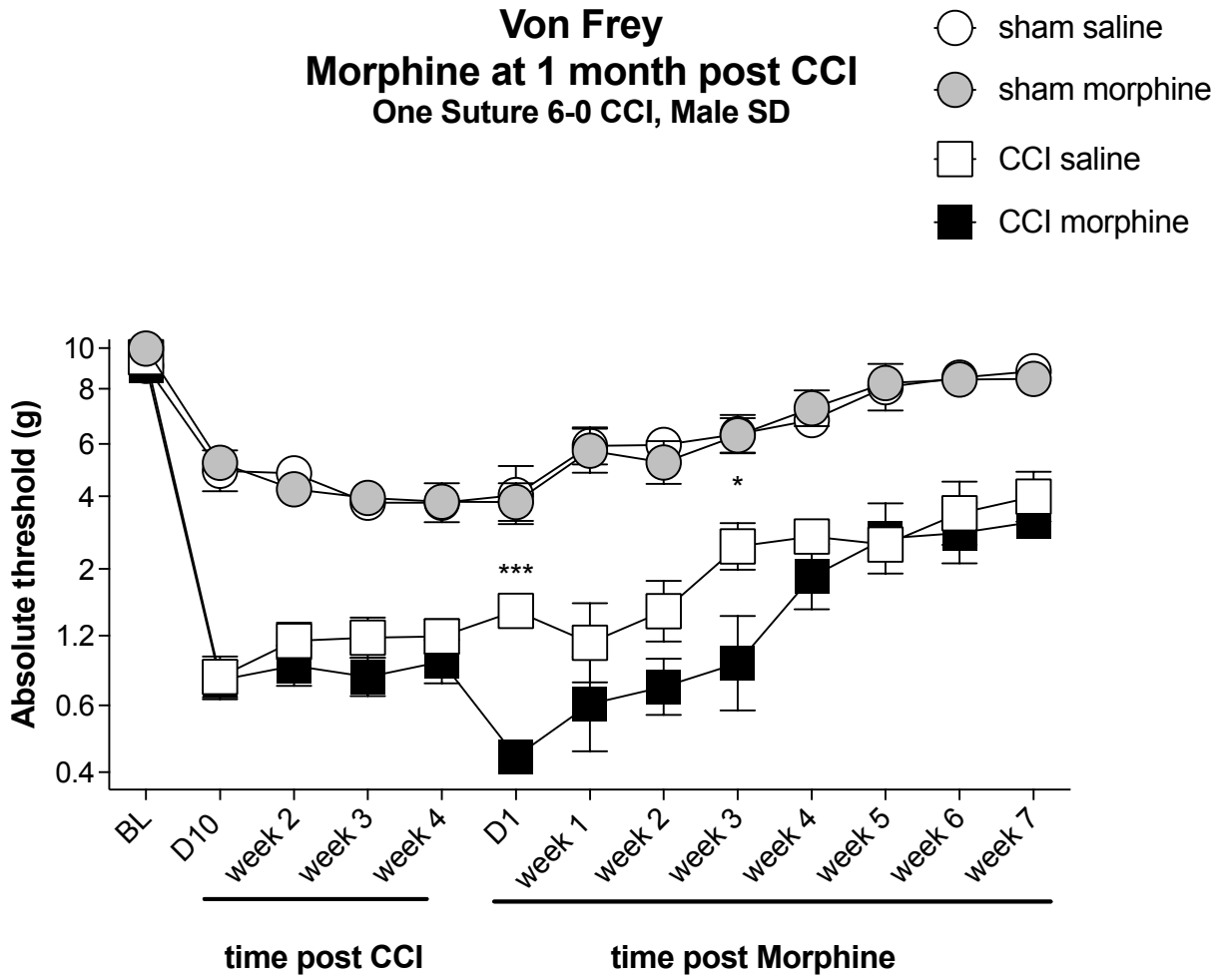

Figure 18. Chronic Constriction Injury (CCI) surgeries were performed with one 6-0 suture. Morphine (5-mg/kg b.i.d.) or saline were administered at one month post-surgery for 5 days. Behavior testing was conducted weekly after CCI, at day 1 post opioid completion, and weekly thereafter. (CCI+Morphine vs CCI+Saline $p<0.0005$ day 1, $p<0.05$ week 3, Two Way ANOVA, n=6/group)

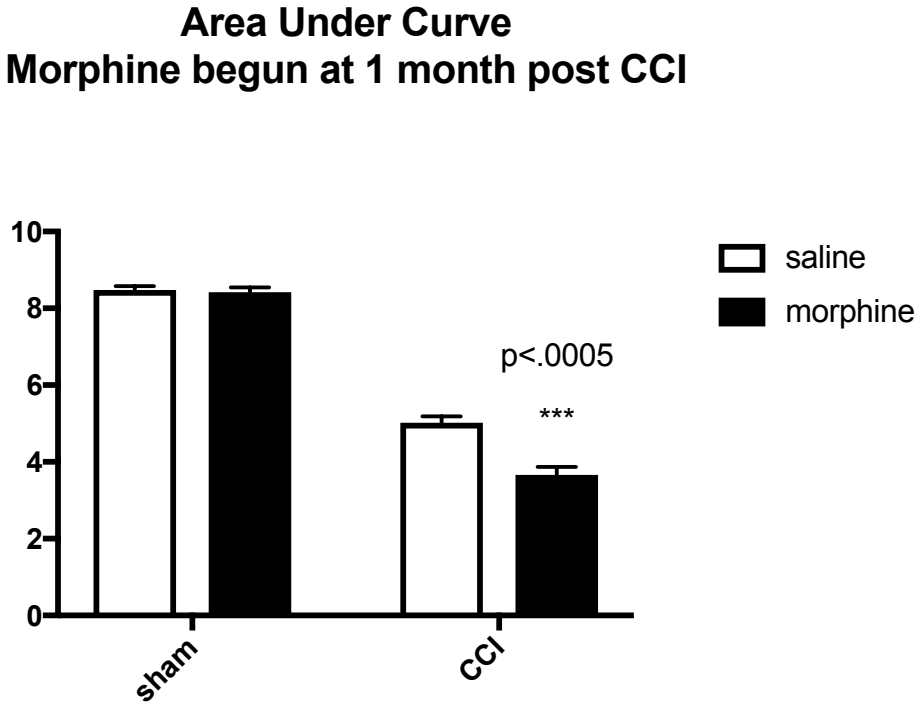

Figure 19. Chronic Constriction Injury (CCI) or sham surgeries were performed with one 6-0 suture. Morphine (5-mg/kg b.i.d.) or saline were administered at one hour post-surgery for 5 days. Behavior testing was conducted at day 1 post opioid completion, and weekly thereafter. ($p<.0005$, CCI+Morphine vs CCI+Saline, unpaired t test of Area Under Curve, n=6/group.)

6. **Test if antagonists administered with morphine prevent opioid induced suppression of voluntary wheel running. Task 14, Aim 4C**

Based on results from the voluntary wheel running pilot experiments, we proceeded to the full morphine antagonist study. The first group of this experiment began May 2017. The design for this study for each antagonist is 2 (CCI vs. sham) x 2 (morphine vs. saline) x 2 (antagonist vs. vehicle) x 7 rats/group. With 32 available running wheels that can be monitored by computer, this study will take place in three sequential cohorts of rats. The full study will take approximately 9 months to complete, depending on when group differences

resolve. Data will be reported each quarter as it progresses, but final results will remain inconclusive until all 3 cohorts of rats have been completed. All rats were habituated to wheels with free access for 7 days, and baseline running data was collected. Rats then received 4 suture CCI surgeries or sham surgeries of the sciatic nerve. At day 10 post CCI rats received a 5-day course of morphine (5-mg/kg b.i.d.) or saline, along with the TLR4 antagonist (+)Naloxone (20-mg/kg), the P2X7 antagonist A438079 (1-mg/kg), or saline vehicle. Body weights were recorded weekly. Running data was collected by computer 24 hours per day for the entire study, until differences between groups resolved. Parameters analyzed are total distance traveled, and maximum running speed.
Rats who received CCI surgery of the sciatic nerve ran significantly less, and at a lower speed than rats who received sham surgery from day 1 to day 9 post-surgery. No running differences were observed at baseline, prior to surgery. These results are presented in Figure 20 (distance ran in 24 hours) and Figure 21 (maximum speed). These data combined with the agility and gait testing data above indicate that chronic pain results in a decrease in voluntary exercise due to indices of motivation rather than physical impairment.

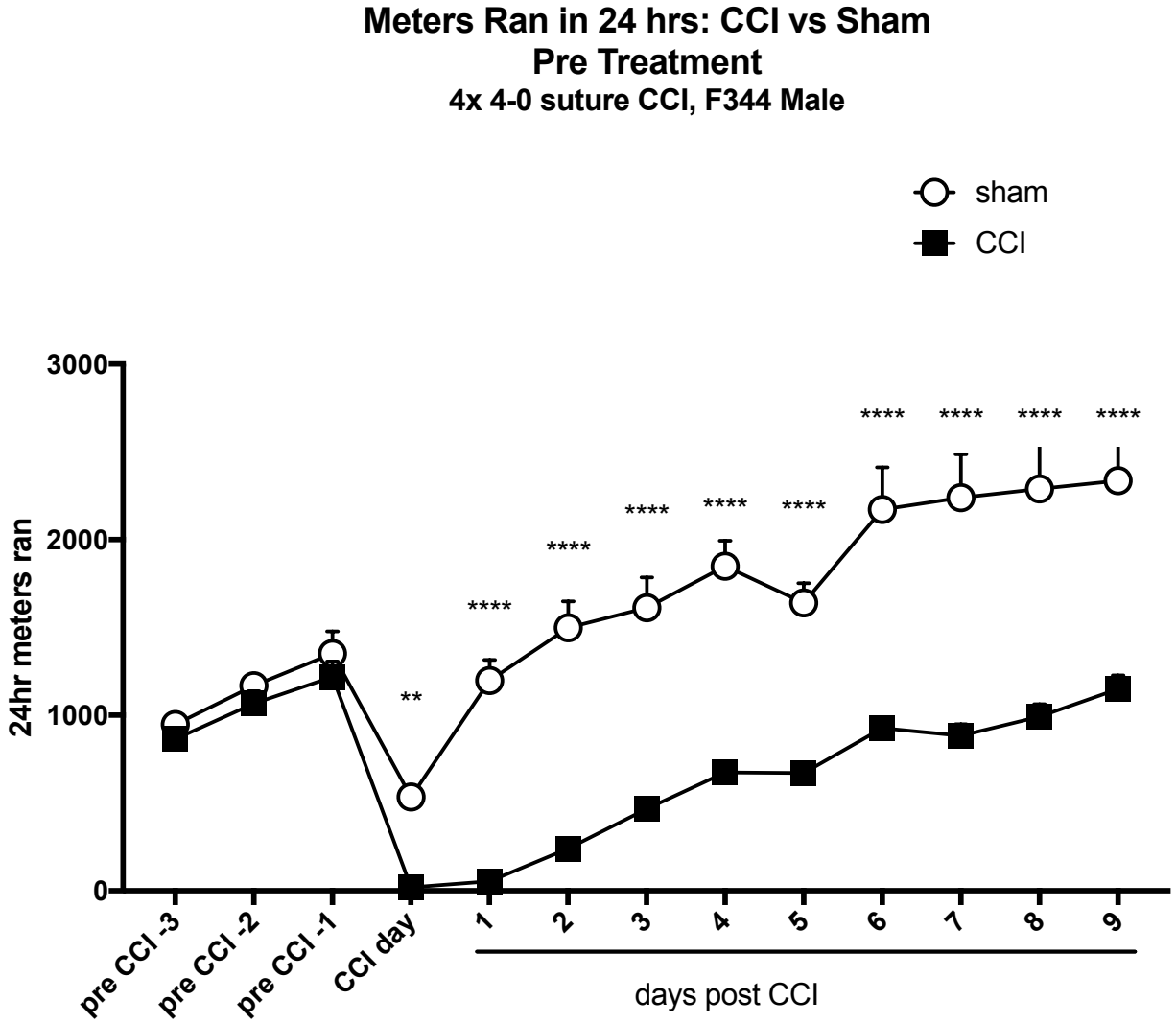

Figure 20. Running data was collected by computer 24 hours per day prior to and after surgery. Constriction Injury (CCI) or sham surgeries were performed with four 4-0 sutures. CCI

significantly reduced distance traveled. ($p<0.0001$ day 1-9 post-surgery, Two Way ANOVA, n=32/group)

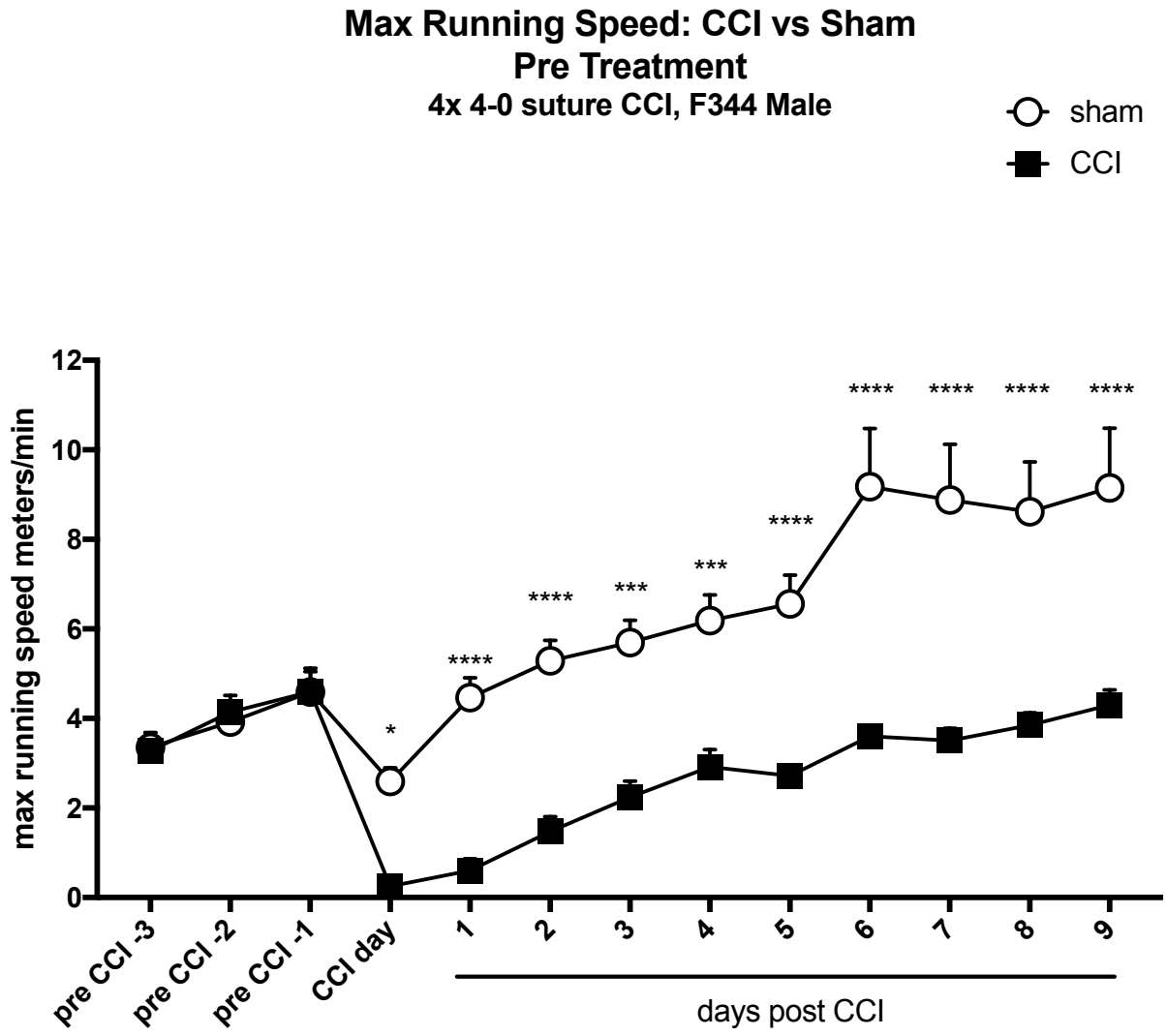

Figure 21. Running data was collected by computer 24 hours per day prior to and after surgery. Constriction Injury (CCI) or sham surgeries were performed with four 4-0 sutures. CCI significantly reduced running speed. ($p<.05$ surgery day, $p<.0005$ day 3 and 4 post-surgery, $p<0.0001$ day 1, 2, and 5-9 post-surgery, Two Way ANOVA, n=32/group)

At day 10 post-surgery a 5 day course of morphine (5-mg/kg b.i.d.) or saline, along with the TLR4 antagonist (+)-Naloxone (20-mg/kg), the P2X7 antagonist A438079 (1-mg/kg), or saline vehicle. Running data during treatment comparing only rats who received morphine vs saline without antagonists are presented in Figure 22 as percent of baseline meters ran in 24 hrs. Morphine decreased voluntary exercise during the 5 days of administration in rats that received both sham and CCI surgeries. Baseline is considered day 9 post-surgery, pre-treatment. Data on all groups during treatment are presented in Figure 23 (distance) and Figure 24 (speed).

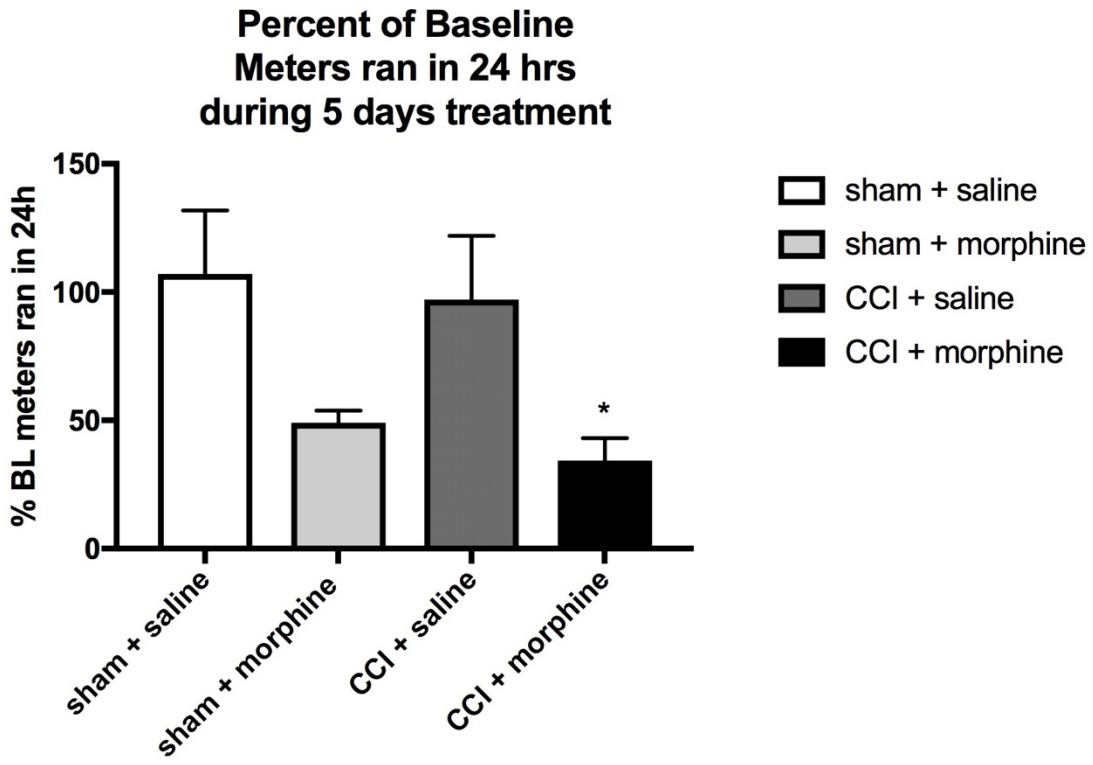

Figure 22. At day 10 post-surgery a 5 day course of morphine (5-mg/kg b.i.d.) or saline was given. Running data were collected by computer 24 hours per day. Morphine reduced running behavior during 5 days of treatment in sham and CCI rats. (p=.03 CCI+Morphine vs CCI+Saline, p=.051 Sham+Morphine vs Sham+Saline, unpaired t test, n=5-6/group)

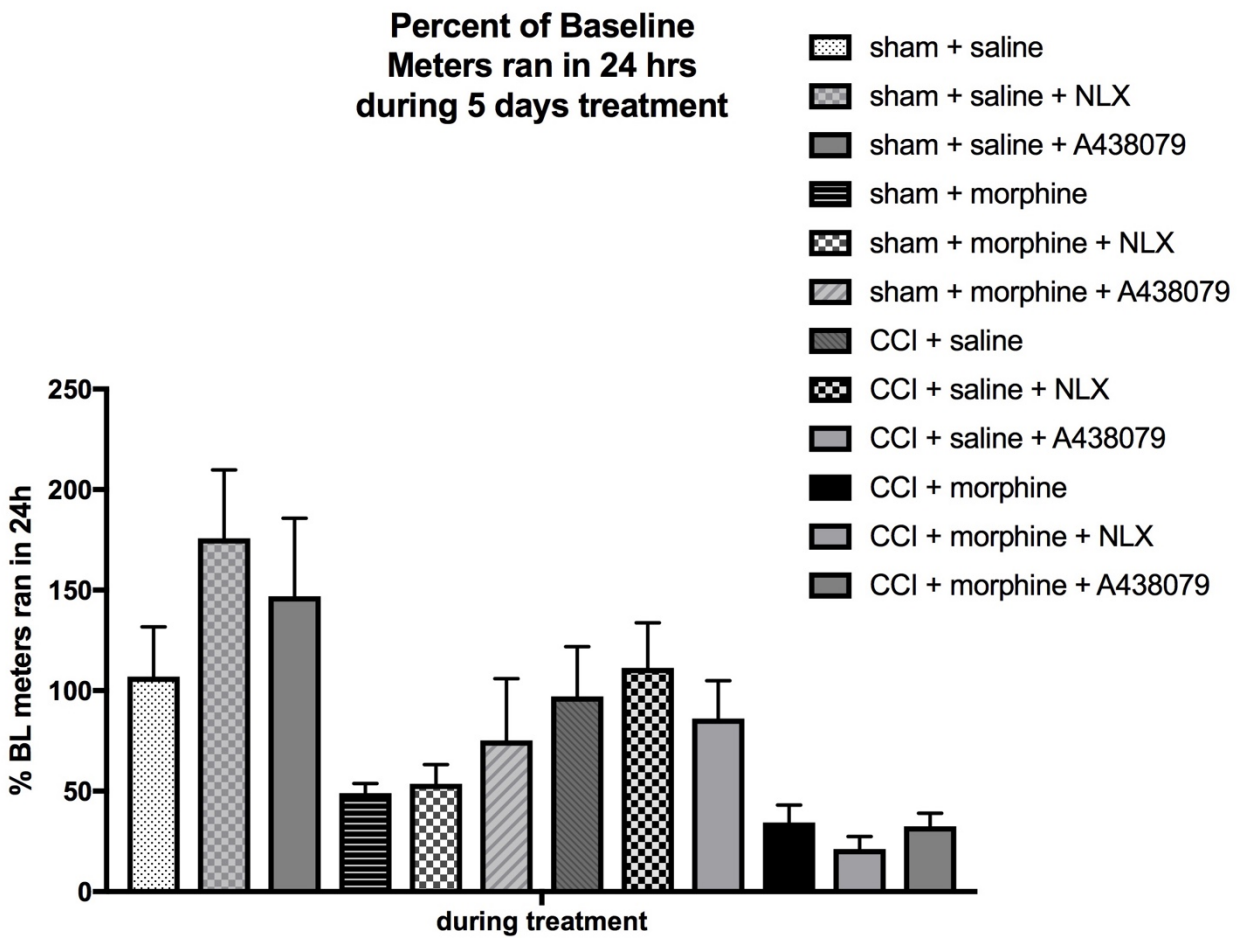

Figure 23. At day 10 post-surgery a 5 day course of morphine (5-mg/kg b.i.d.) or saline was given, along with the TLR4 antagonist (+)-Naloxone (20-mg/kg), the P2X7 antagonist A438079 (1-mg/kg), or saline vehicle. Running data of meters ran were collected by computer 24 hours per day. Data represent n=5-6 per group from the first two cohorts and statistical significance is not expected until groups are filled in with following replications.

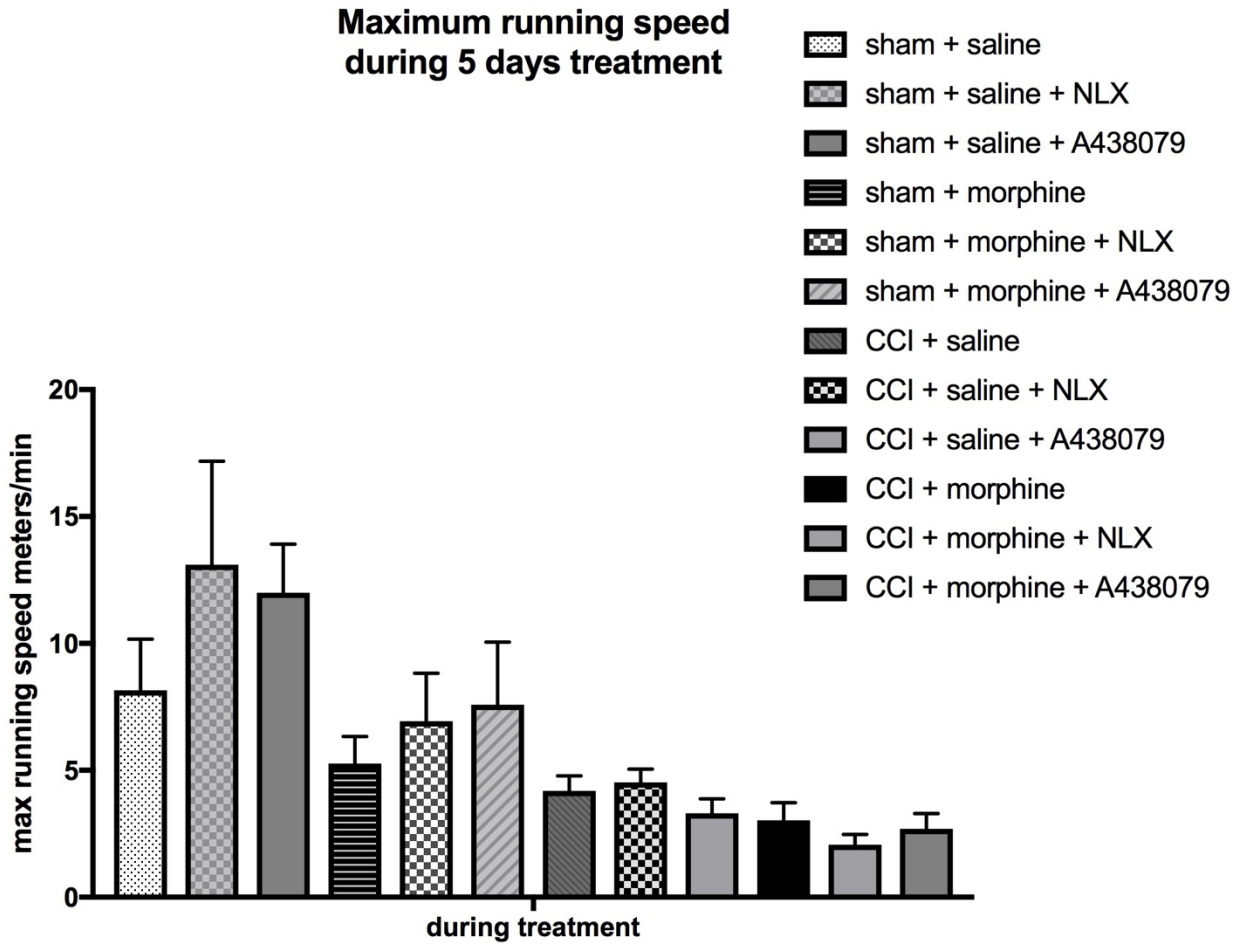

Figure 24. At day 10 post-surgery a 5 day course of morphine (5-mg/kg b.i.d.) or saline was given, along with the TLR4 antagonist (+)-Naloxone (20-mg/kg), the P2X7 antagonist A438079 (1-mg/kg), or saline vehicle. Running data of meters ran were collected by computer 24 hours per day. Data represent n=5-6 per group from the first two cohorts and statistical significance is not expected until groups are filled in with following replications.

Body weight data was collected weekly for the duration of the study. Rats show loss of body weight following CCI surgery, but return to baseline and gain weight by 2 weeks post-surgery. Morphine treatment prevented the return to baseline of body weight following treatment. Body weight data are presented in Figure 25 as change from baseline in grams.

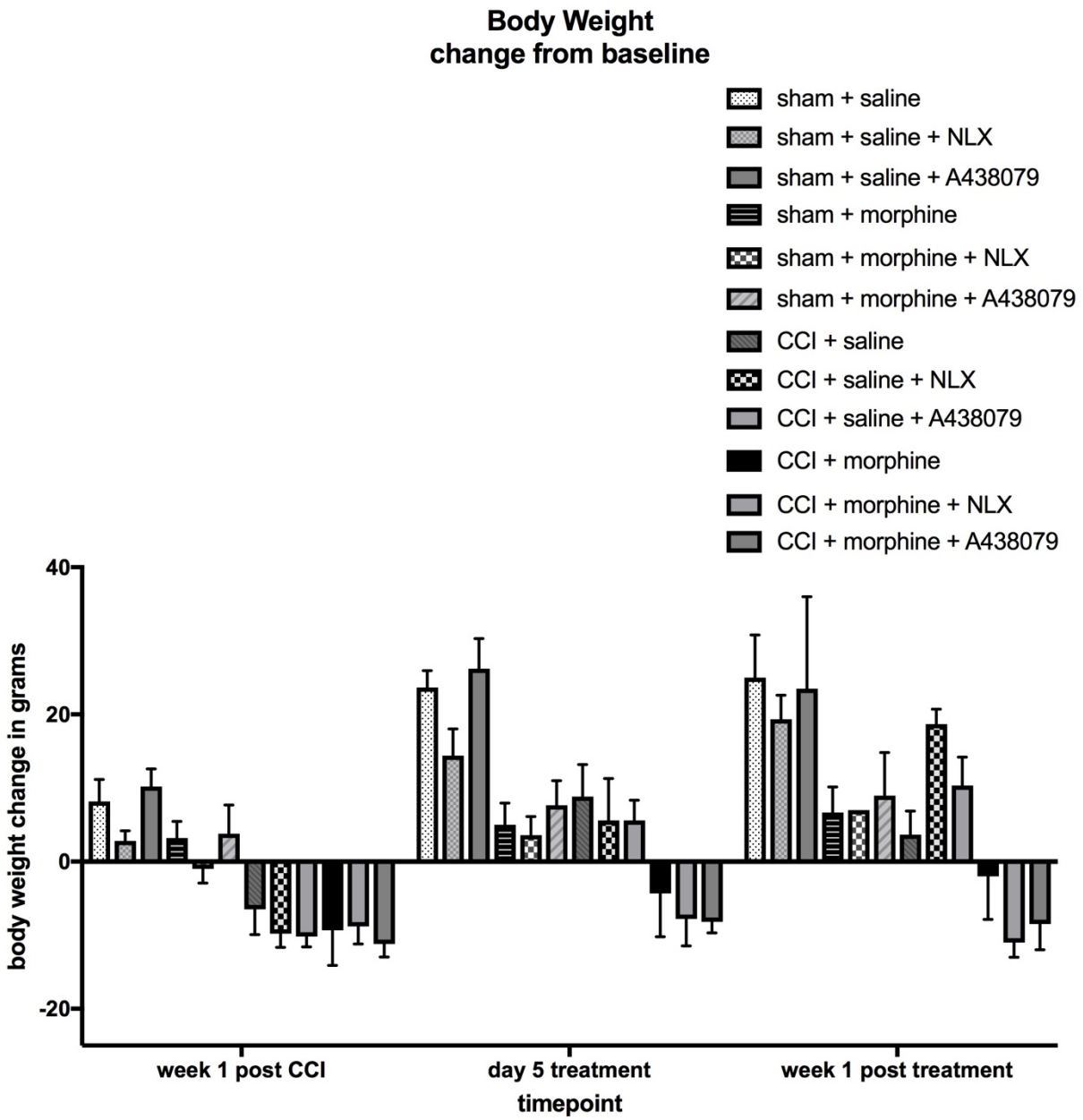

Figure 25. Body weights were collected at one-week post-surgery, and at the final day of dosing. A reduction in body weight was seen with CCI surgery compared to sham surgery at one-week post-surgery, and in groups that received CCI and morphine combined following treatment. Data represents n=5-6 per group from the current cohorts and statistical significance is not expected until groups are filled in with the following experiments.

Improvements in the suppression of voluntary exercise following treatment with morphine were observed by the co-administration of the TLR4 antagonist (+)-Naloxone, and the P2X7 antagonist A438079. Figure 26 presents meters ran in 24 hours as percent of baseline each week following treatment in rats that received CCI+morphine. Area Under Curve analysis is presented in Figure 27. No differences were observed by

the co-administration of either antagonist in rats that received CCI+saline. These data are presented in Figure 28.

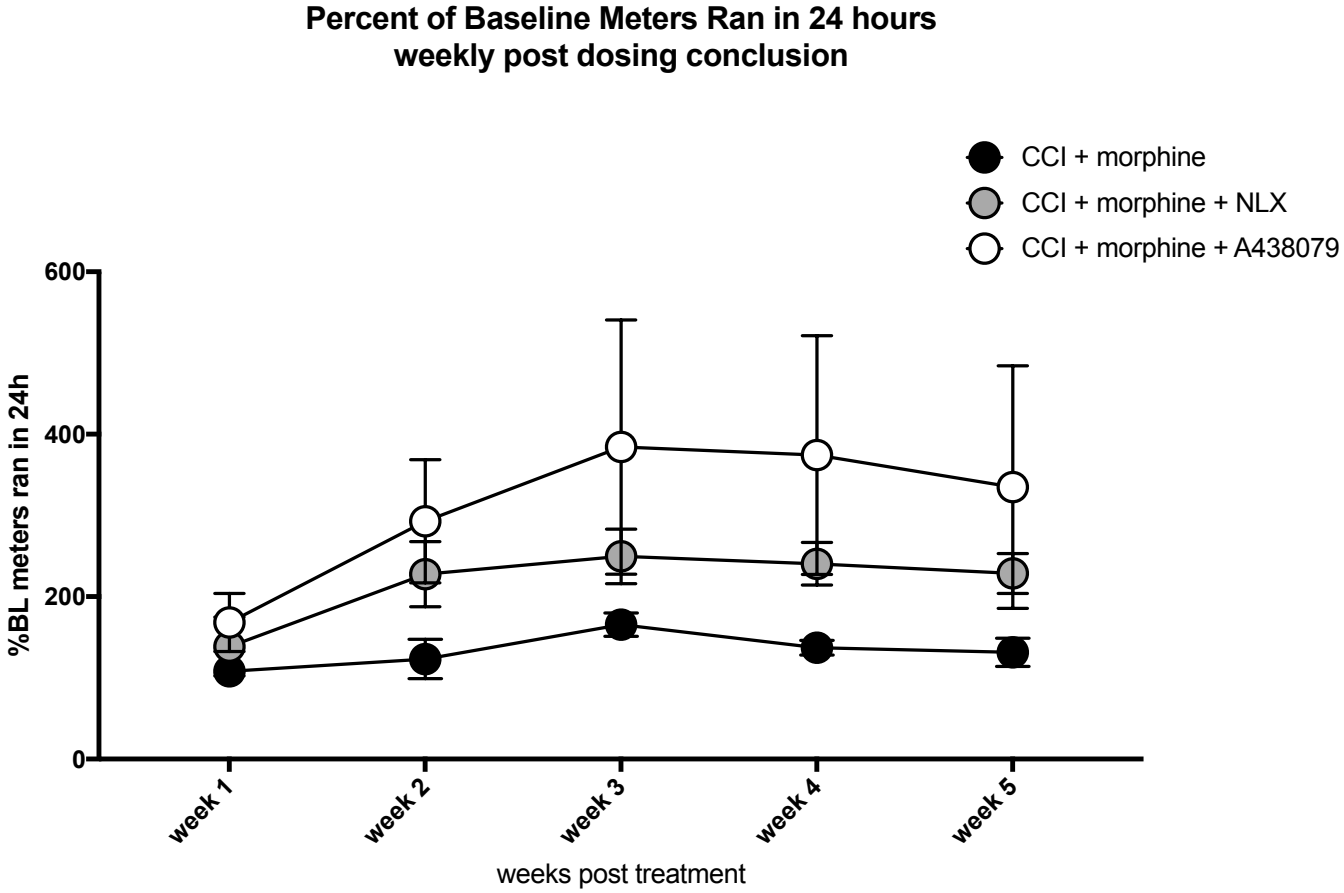

Figure 26. At day 10 post-surgery a 5 day course of morphine (5-mg/kg b.i.d.) or saline was given, along with the TLR4 antagonist (+)-Naloxone (20-mg/kg), the P2X7 antagonist A438079 (1-mg/kg), or saline vehicle. Running data of meters ran were collected by computer 24 hours per day. Treatment with the TLR4 antagonist (+)-Naloxone, and the P2X7 antagonist A438079 increased running distance in rats that received morphine following surgery.

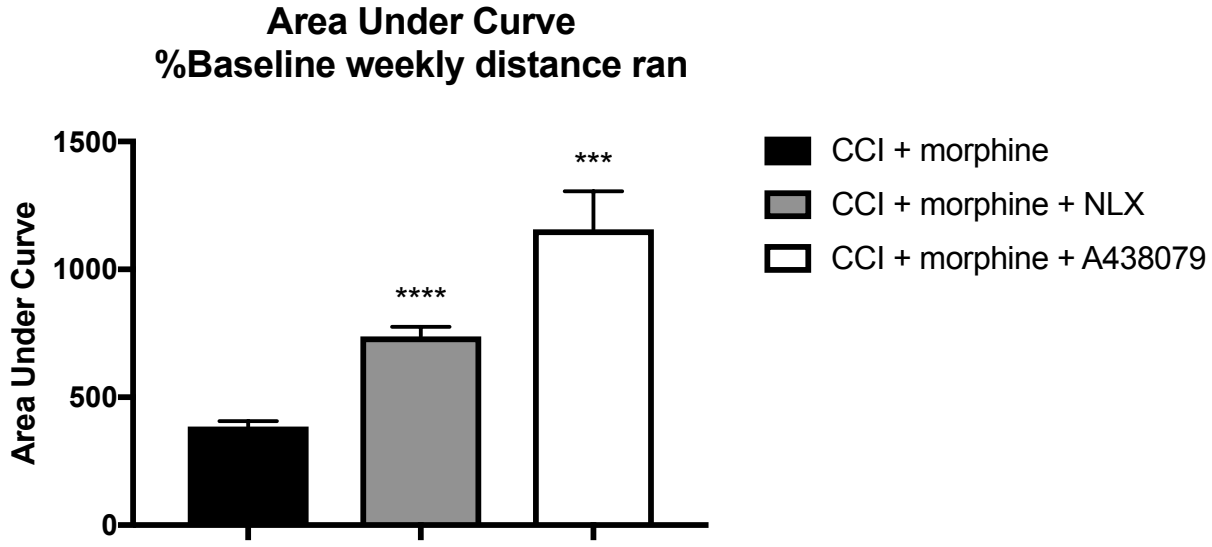

Figure 27. CCI+Morphine vs CCI+Morphine+NLX p<.0001 unpaired t test of Area Under Curve, n=5-6/group, CCI+Morphine vs CCI+Morphine+A438079 p<.0005 unpaired t test of Area Under Curve, n=5-6/group

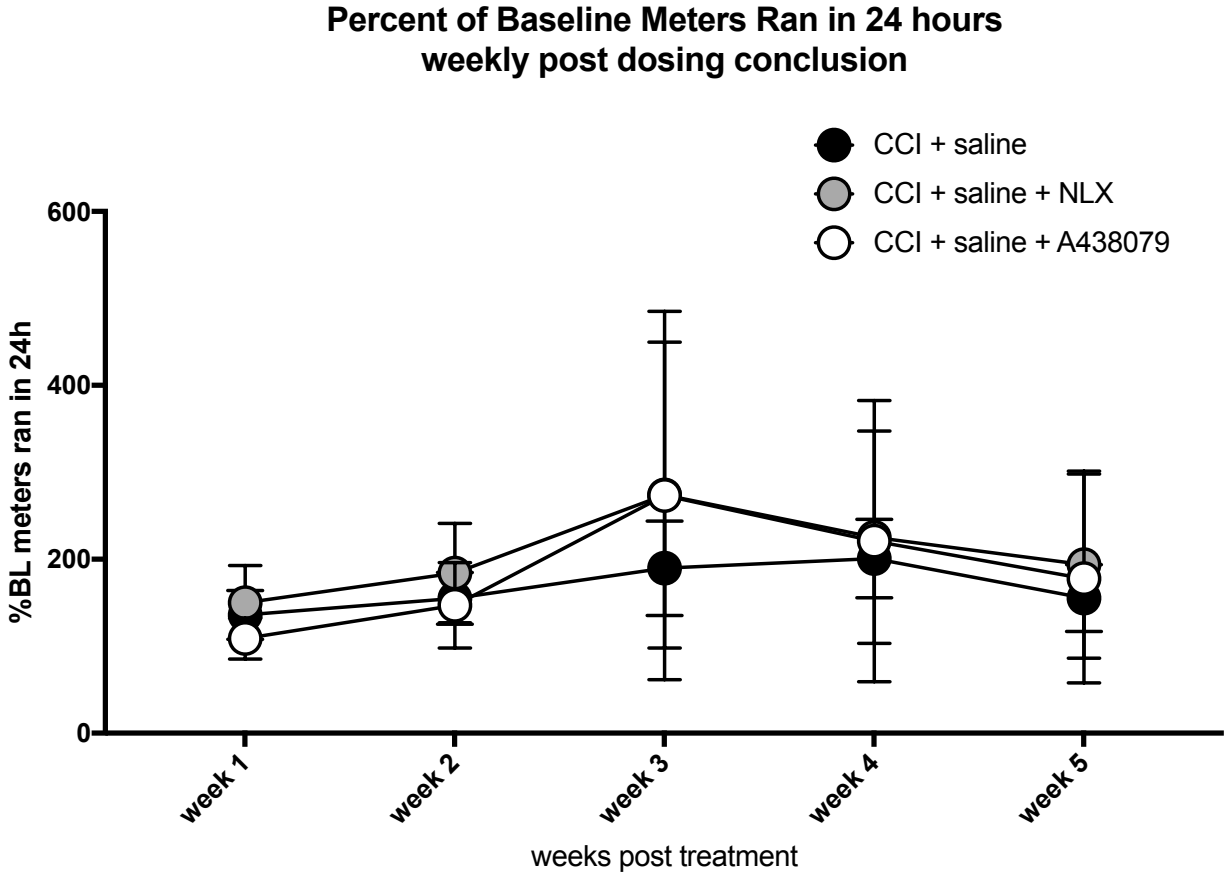

Figure 28. At day 10 post-surgery a 5 day course of morphine (5-mg/kg b.i.d.) or saline was given, along with the TLR4 antagonist (+)-Naloxone (20-mg/kg), the P2X7 antagonist A438079 (1-mg/kg), or saline vehicle. Running data of meters ran were collected by computer 24 hours per day. No significant differences were observed by the administration of either antagonist in rats that received saline following surgery.

Our lab has shown that the deleterious effects of opioids are not due to activation of the classical opioid receptor, but from their action on glia cells by binding to the TLR4 receptor (Watkins, Hutchinson, Rice, Maier, Trends Pharmacol Sci., 2009). Opioid induced glial activation at this site generates pro-IL1-B which amplifies neuropathic pain (Grace et al., Proc Soc Neurosci.,2013). P2X7 signaling plays a role in the induction and maintenance of neuropathic pain by activation of the TLR4 generated intra-cellular release of IL1-β and the resulting downstream neuroinflammatory cascade (He Y et al, Journal of Immunology, 2013, Weisman et al, Molecular neurobiology, 2012). Our data suggest that the long term deleterious effects of morphine can be improved by co-

administration of the TLR4 antagonist (+)-Naloxone, and the P2X7 antagonist A438079, while still allowing the analgesic effect of morphine by its neuronal opioid receptor activation.

7. **Test the opioids Fentanyl and Oxycodone given at day 10 post trauma. Task 5, Aim 2A.**

In this pilot, all rats received CCI surgeries of the sciatic nerve with one 6-0 suture. At day 10 post CCI rats began a 5-day course of Fentanyl (0.1-mg/kg/hr, subcutaneous osmotic minipump, Azlet model 2001), Oxycodone (2-mg/kg, b.i.d.), or saline control. Assessment of mechanical allodynia by Von Frey testing occurred at day one post opioid completion and weekly thereafter. Significant amplification of pain was observed in rats treated with both Fentanyl and Oxycodone compared to saline treated rats. Results are presented in Figure 29. Area Under Curve is presented in Figure 30. Given these preliminary results we will proceed to explore the full effect of these two opioids on the development of chronic pain following trauma with 8 rats per group in the following quarter after adjusting the surgery toward increased duration of allodynia.

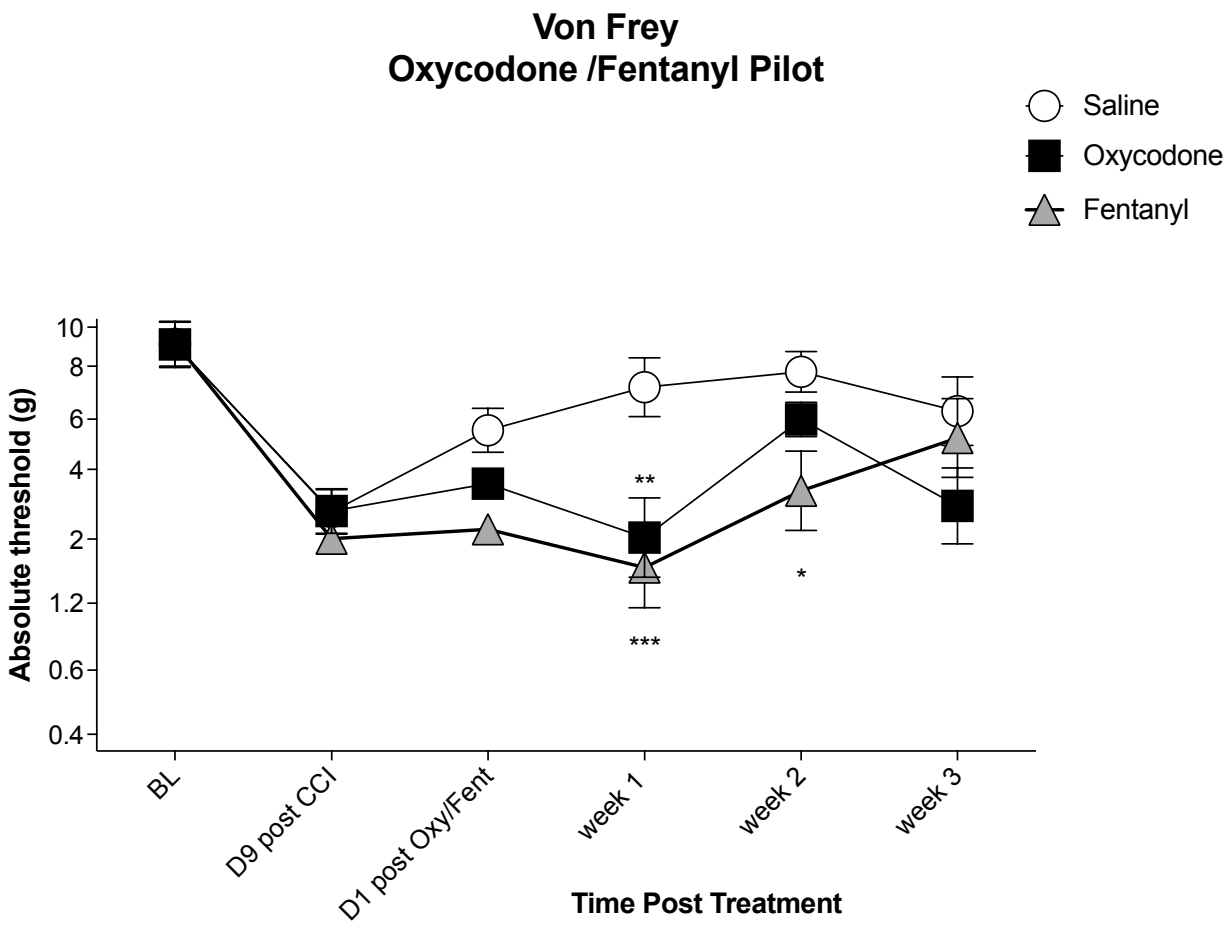

Figure 29. Chronic Constriction Injury (CCI) or sham surgeries were performed with one 6-0 suture. Fentanyl (0.1mg/kg/hr), Oxycodone (2mg/kg) or saline were administered at day 10 post-surgery for 5 days. Behavior testing was conducted at day 1 post opioid completion, and weekly thereafter. (week 1 post treatment $p<.0001$ saline vs Fentanyl, $p=.005$ saline vs Oxycodone, week 2 post treatment $p<.05$ saline vs Fentanyl, two way ANOVA, n=5-6/group.)

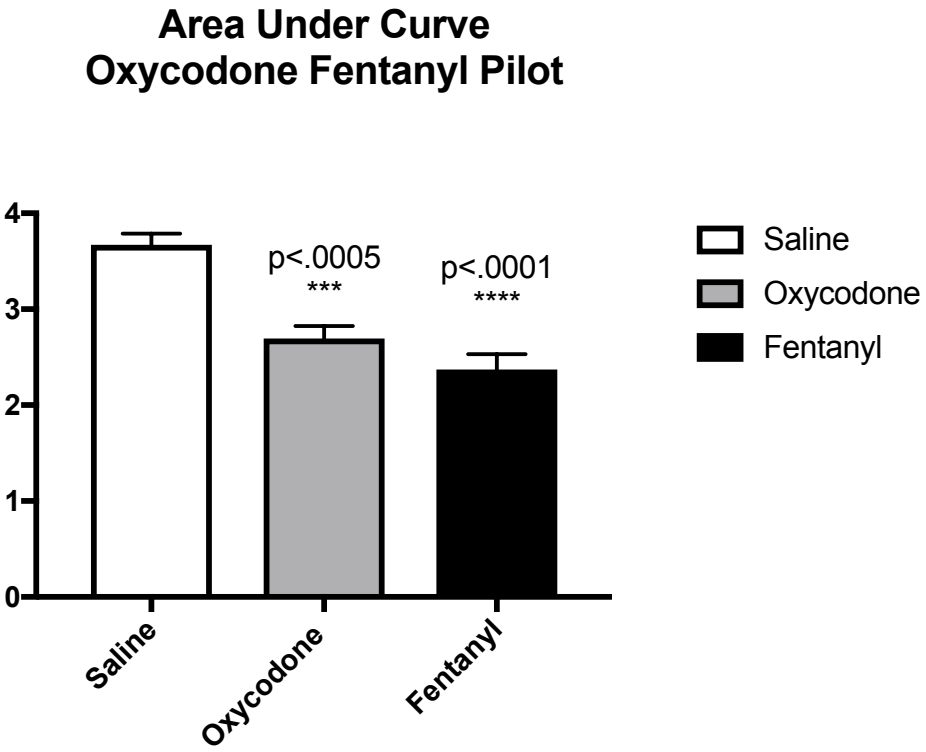

Figure 30. Chronic Constriction Injury (CCI) or sham surgeries were performed with one 6-0 suture. Fentanyl (0.1mg/kg/hr), Oxycodone (2mg/kg) or saline were administered at day 10 post-surgery for 5 days. Behavior testing was conducted at day 1 post opioid completion, and weekly thereafter. (p<.0005, CCI+ Oxycodone vs CCI+Saline, p<.0001 CCI+ Fentanyl vs CCI+Saline, unpaired t test of Area Under Curve, n=5-6/group.)

8. Establish Morphine-CCI model at MD Anderson Cancer Center.

New personnel (Fabisiak and Lacagnina) were trained to perform and test the morphine-CCI pain model. This was successfully achieved in Grace's new research lab.

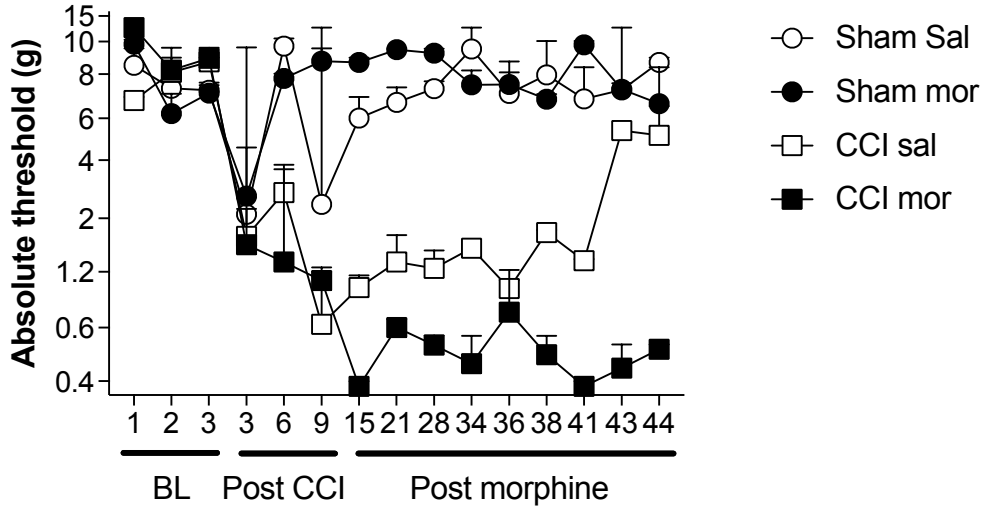

Figure 31. At day 10 post-surgery a 5 day course of morphine (5-mg/kg b.i.d.) or saline was given.

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

This project provided opportunities for the contracted professional research associates to advance professional skills such as Von Frey testing for mechanical allodynia, a skill that takes time and repetition to master. Research associates also advanced skills in data collection and analysis for the new Lafayette Instruments system of running wheels used to analyze morphine and antagonist effects on voluntary running behavior.

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?

1. **Task 14, Aim 4C: Test if antagonists administered with morphine prevent opioid induced suppression of voluntary wheel running.**

   The antagonist wheel running study will continue during the next quarter as planned, requiring 3 cohorts of 32 rats to fill the groups. When differences between groups resolve in the second group, the third group of 32 rats will begin immediately. The design will be the same as the first two cohorts, 2 (CCI vs. sham) x 2 (morphine vs. saline) x 2 (antagonist vs. vehicle) x 2-3 rats/group. Antagonists tested will again be the TLR4 antagonist (+)Naloxone (20-mg/kg), and the P2X7 antagonist A438079 (1-mg/kg).

2. **Task 5, Aim 2A: Test the opioids Fentanyl and Oxycodone with dosing beginning at day 10 post trauma.**

   Given the successful results on our pilot study, we plan to test the opioids Fentanyl and Oxycodone given at day 10 post trauma in the next quarter. In this pilot, all rats will receive CCI surgeries of the sciatic nerve with one 6-0 suture. At day 10 post CCI rats will begin a 5-day course of Fentanyl (0.1-mg/kg/hr),

Oxycodone (2-mg/kg), or saline control. Sham surgery groups will be excluded from this experiment as the recovery time course from sham surgery has been documented in previous experiments, and the purpose of this experiment is to investigate the effect of these opioids on neuropathic pain. This allows for a more efficient completion of the experiment with lower animal and drug costs. Assessment of mechanical allodynia by Von Frey testing will occur at day one post opioid completion and weekly thereafter until the resolution of pain.

3. **Test the non-opioids gabapentin and amitriptyline with dosing beginning at day 10 post trauma.**

We plan to test the non-opioids gabapentin and amitriptyline given at day 10 post trauma in the next quarter. In this study, all rats will receive CCI surgeries of the sciatic nerve. At day 10 post CCI rats will begin a 5-day course of gabapentin, amitriptyline, morphine (positive control) or saline (negative control). Sham surgery groups will be excluded from this experiment as the recovery time course from sham surgery has been documented in previous experiments, and the purpose of this experiment is to investigate the effect of these non-opioids on neuropathic pain. This allows for a more efficient completion of the experiment with lower animal and drug costs. Assessment of mechanical allodynia by Von Frey testing will occur at day one post opioid completion and weekly thereafter until the resolution of pain.

IMPACT:

What was the impact on other disciplines?

Nothing to report.

- **What was the impact on technology transfer?**

Nothing to report.

- **What was the impact on society beyond science and technology?**

The results of this project could have implications for the treatment of chronic pain in veterans, military personnel, and non-military general population. Peripheral nerve trauma can result in neuropathic pain that is debilitating and difficult to treat. Virtually all trauma patients receive opioids as the first therapeutic action after injury. Our data suggest that treatment with opioids prolongs the duration and intensity of neuropathic pain whether opioid administration is immediately after trauma, at the time of chronic pain development, or up to one month after injury. Our data suggest that this deleterious effect of opioids can be improved by the co-administration of the antagonists TLR4 antagonist (+)-Naloxone, and the P2X7 antagonist A438079 along with opioids. Targeting TLR4 and P2X7 with these antagonists blocks the neuroinflammatory cascade that occurs due to opioid induced glial activation at these sites, while still allowing the analgesic effects of opioids at the neuronal opioid receptor sites.

CHANGES/PROBLEMS:

Changes in approach and reasons for change

A minor decrease in proposed dosing for the opioids Fentanyl and Oxycodone was needed following dose response pilot studies. Dosing was optimized to produce analgesia while minimizing side effects such as respiratory depression, limb chewing, and overdose observed at originally proposed doses. Analgesia was confirmed with Von Frey and Hargreaves testing. Optimal doses were determined to be 2 mg/kg subcutaneous injection for Oxycodone, and 0.01 mg/kg/hr (0.24 mg/kg/day) via subcutaneous pump for Fentanyl. Both are given for 5 days as originally proposed. The subcutaneous pump allows for a slower release of the opioid which prevents side effects or over dose as well as being optimal for the shorter half-life of this opioid. These changes represent a slight *decrease* in dosing and drugs used.

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

The CU Boulder plans to move several rodent research labs, including ours, to a new facility in early 2018. Although this move will result in improvements in animal housing, behavior rooms, and surgical space, the move will require some down time between experiments as equipment is moved, new rat colonies are set up, and behavioral testing rooms optimized. We do not anticipate extreme delays in productivity because projects conducted at MD Anderson will still be operational, as well as the Morphine + Antagonist voluntary wheel running project, which is housed in a space at CU Boulder that will not be affected by the move initially and can continue to run until completion.

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**

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.

PRODUCTS:

Nothing to Report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

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

Name: Linda R. Watkins, Ph.D.
Project Role: Principal Investigator
Researcher Identifier (e.g. ORCID ID): none
Nearest person month worked: 10% effort for 12 months (1 month summer salary utilized)
Contribution to Project: Principal Investigator

Name: Peter M. Grace, Ph.D.
Project Role: Co- Principal Investigator
Researcher Identifier (e.g. ORCID ID): orcid.org/0000-0002-8999-1220
Nearest person month worked: 25% effort for 4 months (1 calendar month)
Contribution to Project: Co-Principal Investigator

Name: Suzanne M. Fulgham, M.S.
Project Role: Professional Research Assistant
Researcher Identifier (e.g. ORCID ID): none
Nearest person month worked: 6
Contribution to Project: Suzanne conducted experiments and analyzed data

Name: Timothy J Fabisiak, B.A.
Project Role: Professional Research Assistant
Researcher Identifier (e.g. ORCID ID): none
Nearest person month worked: 3
Contribution to Project: Timothy conducted experiments

Name: Michael Lacagnina
Project Role: Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID): none
Nearest person month worked: 50% time for 4 months = 2 calendar months
Contribution to the Project: Michael conducted experiments

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.

1. **SPECIAL REPORTING REQUIREMENTS**

- **COLLABORATIVE AWARDS:**

Nothing to report.

- **QUAD CHARTS:**

Nothing to Report.

2. **APPENDICES:**

Nothing to Report.