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# Oxycodone, fentanyl, and morphine amplify established neuropathic pain in male rats

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## Abstract

Opioids are widely prescribed for chronic pain, including neuropathic pain, despite growing evidence of long-term harm. Previous preclinical studies have documented exacerbation of nociceptive hypersensitivity, including that induced by peripheral nerve injury, by morphine. The present series of behavioral studies sought to replicate and extend our prior research, which demonstrated a multimonth exacerbation of nociceptive hypersensitivity by a 5-day course of morphine initiated 10 days after nerve injury. The current studies demonstrate that enduring exacerbation of nociceptive hypersensitivity is not restricted to morphine, but rather is also created by the clinically relevant opioids fentanyl and oxycodone when these are likewise administered for 5 days beginning 10 days after nerve injury. Furthermore, enduring exacerbation of nociceptive hypersensitivity is also observed when the same dosing regimen for either morphine, fentanyl, or oxycodone begins 1 month after nerve injury. Finally, a striking result from these studies is that no such exacerbation of nociceptive hypersensitivity occurs when either morphine, fentanyl, or oxycodone dosing begins at the time of nerve injury. These results extend our previous findings that morphine exacerbates nociceptive hypersensitivity to the clinically relevant opioids fentanyl and oxycodone when administered after the development of nociceptive hypersensitivity, while also providing possible clinically relevant insight into when these opioids can be safely administered and not exacerbate neuropathic pain.

### Keywords

rats; oxycodone; fentanyl; morphine; neuropathic pain; chronic constriction injury; behavior; allodynia

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Conflict of interest statement

The authors have no conflicts of interest to declare.

## 1. Introduction

Approximately one-third of Americans suffer from chronic pain, and this proportion is increasing.[41,43] Chronic pain, including neuropathic pain from peripheral nerve injury, is frequently treated with opioids.[3,12,41] Despite widespread opioid prescriptions, there is no evidence of the efficacy of long-term opioid use for management of chronic pain.[6] Furthermore, there is growing evidence of harm.[2,23,46,50]

In addition to clinical studies, preclinical studies demonstrate that opioids may cause long-term harm in models of chronic pain. We and others have shown that opioids such as morphine can paradoxically exacerbate nociceptive hypersensitivity for weeks to months after treatment ends in a range of experimental pain models. These models include inflammatory and postoperative pain,[11,14,25,35,36,52] and peripheral and centrally induced neuropathic pain.[10,18–20,24,25,36] We have also shown that exacerbation of allodynia by morphine is dependent on inflammatory signaling in the spinal cord because inhibition of microglia or proinflammatory cytokines during opioid administration can prevent exacerbation of such allodynia.[10,18–20] These pronociceptive spinal inflammatory signaling pathways have a well-documented role in the development and maintenance of chronic pain.[15,30,34,45]

Notably, the pattern-recognition receptor toll-like receptor 4 (TLR4) orchestrates the exacerbation of allodynia by morphine. This conclusion is based on the finding that morphine non-stereoselectively activates TLR4 and induces proinflammatory cytokines.[17,28,29,51] We showed that blockade of TLR4 during morphine treatment prevented exacerbation of nociceptive hypersensitivity without interfering with analgesia.[10,19,20] Importantly, the deleterious effects of morphine seemed to be independent of actions on mu-opioid receptors[20] because opioid-induced exacerbation of allodynia still occurs: (1) despite knockdown of spinal mu-opioid receptors, and (2) on administration of (+)-morphine, which activates TLR4 but not mu-opioid receptors because the latter are highly stereoselective for (−)-opioid isomers.[8]

The present series of behavioral studies focus on 3 interrelated objectives. The first objective arises from our prior demonstration that morphine enhanced allodynia in Sprague-Dawley rats after sciatic chronic constriction injury (CCI).[20] However, this single 4–0 diameter chromic gut suture model was constrained by a floor effect for measuring allodynia using the von Frey test. To overcome this constraint, the first objective of this study was to develop a model of morphine enhancement of CCI allodynia with greater dynamic range. The second objective arises as our previous studies of opioid exacerbation of allodynia focused exclusively on administration of morphine. It is important to define whether exacerbation of allodynia is restricted to morphine. To address this, the second objective was to extend our initial studies[20] so to now examine the effect of oxycodone and fentanyl, opioids that are widely used to treat chronic pain and that activate TLR4.[27,29,51] The third objective also arises from our previous studies of morphine exacerbation of nerve injury–induced allodynia because the original design was a short course (5 days) of opioid beginning on robust expression of allodynia, that is, initiating dosing 10 days after CCI. Thus, this last objective

explores the important issue of whether opioid-induced exacerbation of allodynia also occurs when dosing occurs at other times relative to nerve injury.

## 2. Materials and methods

### 2.1. Animals

Pathogen-free adult male Sprague-Dawley rats (Envigo, Indianapolis, IN), 10 weeks old on arrival, were used in all experiments. Rats were pair-housed in temperature-controlled (23 ± 3°C) and light-controlled (12-hour light–dark cycle; lights on at 07:00 hours) rooms with standard rodent chow and water available ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Colorado Boulder. This study adhered to the ARRIVE guidelines.

### 2.2. Chronic constriction injury surgery

Neuropathic pain was induced by a modification of the CCI model.[1] Briefly, surgery was performed under isoflurane anesthesia at the midthigh level of the left hind leg. The shaved skin was treated with Nolvasan, and the surgery was performed aseptically. The sciatic nerve was gently isolated, and a single sterile chromic gut suture of varying diameter (5–0 and 6–0 cuticular WebGut; Medtronic, Minneapolis, MN; 7–0 chromic gut; Ethicon, Somerville, NJ) was loosely tied around the nerve. Experiment 1 tested a single 5–0, 6–0, or 7–0 chromic gut suture, based on the observations that “dose”-dependent allodynia can be achieved by varying suture diameter[37] or suture number.[16] All following experiments used 1 × 6–0 sutures. Rats were monitored postoperatively until fully ambulatory before being returned to their home cage.

### 2.3. Drug administration

Morphine, fentanyl, and oxycodone were gifted from the National Institute on Drug Abuse Drug Supply Program Division of Therapeutics and Medical Consequences, Research Triangle Institute, NC. Morphine was administered subcutaneously (s.c.) at 5 mg/kg, twice daily for 5 days.[20] Oxycodone was administered s.c. at 2 mg/kg, twice daily for 5 days.[9,26] Fentanyl was continuously infused at 0.01 mg/kg/h using a s.c. osmotic minipump (Alzet, Cupertino, CA, model 2001) for 5 days.[38] Osmotic minipumps were implanted subcutaneously under brief isoflurane anesthesia and became active at the time of implantation. Because the half-life of fentanyl is shorter than morphine or oxycodone, fentanyl was administered through continuous infusion to avoid increased injections or more frequent withdrawals. Pilot studies were conducted to optimize dosage of oxycodone and fentanyl to confirm analgesia without observed side effects or overdose (data not shown). Drugs were prepared and are reported as free base concentrations. Equivolume saline vehicle was used as the control. Drug administration began 1 hour, 10 days, or 4 weeks after CCI surgery.

### 2.4. Mechanical allodynia

Rats received at least three 60-minute habituations to the test environment before behavioral testing. The von Frey test[5] was performed as previously described in detail[4,40] within the sciatic innervation region of the hind paws. Assessments were made before CCI surgery

(baseline), before opioid administration, at day 1 after opioid completion, and then at weekly intervals. Three separate experimenters, blinded to group assignments, performed von Frey testing: experimenter #1 performed testing for the data presented in Figures 1, 2, and 3; experimenter #2, the data in Figure 4, and experimenter #3, the data in Figures 5 and 6. A logarithmic series of 10 calibrated Semmes–Weinstein monofilaments (von Frey hairs; Stoelting, Wood Dale, IL) were applied randomly to the left vs right hind paws to define the threshold stimulus intensity required to elicit a paw withdrawal response. Log stiffness of the hairs ranged from manufacturer-designated 3.61 (0.40 g) to 5.18 (15.14 g) filaments. The behavioral responses were used to calculate the absolute threshold (the 50% probability of response) by fitting a Gaussian integral psychometric function using a maximum-likelihood fitting method[21,49] as described previously.[39,40] This fitting method allowed parametric analyses that otherwise would not be statistically appropriate.[21,49]

### 2.5. Statistics

Mechanical allodynia was analyzed as the interpolated 50% thresholds (absolute threshold). A Spearman correlation was used to determine the correlation between suture size and withdrawal thresholds, and a one-way analysis of variance (ANOVA) was used to determine differences between groups. One-way ANOVA was used to determine baseline differences between groups. One-way repeated-measures ANOVA was used to determine the effect of morphine on mechanical allodynia, compared with pretreatment thresholds, in suture dose–response tests. Differences in mechanical allodynia between treatment groups after opioid administration were determined using two-way repeated-measures ANOVA followed by Sidak post hoc test of opioids vs saline control. Unpaired t-tests were used to analyze area under curve for opioids vs saline control treatments.

## 3. Results

### 3.1. Behavioral characterization of morphine-potentiated allodynia in a mild CCI model

**3.1.1. Experiment 1: comparison of chronic constriction injury allodynia induced by 3 suture sizes—**The classic CCI model involves loose ligation of the sciatic nerve with four 4–0 chromic gut ligatures.[1] The maximal allodynia produced by this model confounds assessment of possible amplified mechanical allodynia by morphine; the drug may decrease the absolute thresholds below the limit of detection by von Frey filaments. To overcome this issue, we altered the CCI model to induce submaximal allodynia and therefore increase the dynamic range of allodynia. Previous studies have shown that allodynia induced by CCI can be attenuated if the suture size of the 4 ligatures is reduced,[37] or if the number of 4–0 chromic gut sutures is decreased.[16] Here, we combined the 2 approaches by applying only single ligatures of decreasing diameter (5–0, 6–0, or 7–0) to the sciatic nerve (n = 4/group). When tested at 14 days after CCI surgery, withdrawal threshold significantly correlated with suture size, with 7–0 suture producing the mildest allodynia (Fig. 1, Spearman $R = 0.791$, $P = 0.0022$; main effect of suture size F (2.00, 9.00) = 8.04, $P = 0.0099$). Post hoc tests did not reveal significant differences between groups.

**3.1.2. Experiment 2: comparison of chronic constriction injury + morphine allodynia induced by 3 suture sizes—**To test whether expression of mechanical

allodynia after morphine in the mild CCI model fell within the measurable range so to avoid floor effects, morphine (5 mg/kg, twice per day) was administered for 5 days, beginning 14 days after surgery (Fig. 2). There was a main effect of treatment for the 7–0 ($F(2.03, 6.08) = 10.7$, $P = 0.0102$) and 6–0 ($F(2.05, 6.16) = 9.79$, $P = 0.012$), but not for the 5–0 suture ($F(1.20, 3.60) = 1.36$, $P = 0.33$).

#### 3.1.3. Experiment 3: comparison of allodynia induced in 6–0 suture chronic constriction injury rats administered a 5-day regimen of morphine vs saline—

This study was conducted to assess whether morphine administered for 5 days beginning at day 10 after surgery could significantly increase magnitude and/or duration of allodynia, compared with saline in a mild CCI model. The 6–0 suture was chosen for the following experiments because it avoided behavioral floor effects before and after morphine treatment in the previous experiment (yet still had a suitable dynamic range for future assessment of reversal of allodynia, unlike 7–0 suture). Morphine significantly amplified mechanical allodynia in this model for at least 5 weeks after morphine completion (Figs. 3A and B; main effect of morphine $F(1.00, 6.00) = 32.7$, $P = 0.0012$, main effect of time $F(5.00, 30.00) = 13.9$, $P < 0.0001$, interaction $F(5.00, 30.00) = 2.63$, $P = 0.0435$). Thus, this optimized CCI procedure using 6–0 suture, which avoids behavioral floor effects, replicates and importantly improves detection sensitivity over our prior observation of morphine amplification of CCI allodynia in Sprague-Dawley rats.[20] With this improved procedure, the remaining studies examine generalization across opioids and time.

### 3.2. Fentanyl and oxycodone amplify chronic constriction injury allodynia when administered for 5 days beginning 10 days after mild chronic constriction injury

To define whether commonly used opioids other than morphine also exacerbate nerve injury–induced allodynia, the effects of fentanyl and oxycodone were tested. These opioids were administered for 5 days beginning day 10 after injury in our mild model of CCI (a single 6–0 ligature). Due to the short half-life, fentanyl and the vehicle control were continuously infused through a subcutaneous osmotic minipump. Both opioids amplified mechanical allodynia for approximately 9 weeks after treatment concluded, compared with saline treatment (Figs. 4A and B; main effect of opioids $F(2.00,19.00) = 13.8$, $P = 0.0002$, main effect of time $F(6.04,114.8) = 59.0$, $P < 0.0001$, interaction $F(22.00,209.0) = 2.16$, $P = 0.0028$). Hence, amplification of allodynia by opioids is not restricted to morphine ([20] and experiment 3 above), importantly demonstrating broad generality of this phenomenon to other clinically relevant opioid medications.

### 3.3. Opioids still amplify allodynia when dosing begins 1 month after chronic constriction injury, but not when dosing begins at the time of surgery

#### 3.3.1. Experiment 1: morphine, fentanyl, and oxycodone amplify allodynia when administered for 5 days beginning 28 days after mild chronic constriction injury—

Our previous publications[10,17–19] and the experiments above focused on assessing the impact of opioid administration at a time when allodynia is recently fully expressed (ie, day 10 after CCI). To test whether these opioids also amplify allodynia at a more extended timepoint, we administered a 5-day course of each opioid (same doses as above) beginning 28 days after mild CCI. Because no differences were found with saline

vehicle delivered by injection vs osmotic minipump in the previous experiment, all saline vehicles were delivered by minipump in this experiment to control for the more invasive drug delivery method. Morphine, fentanyl, and oxycodone each amplified allodynia for approximately 5 weeks after treatment concluded (Figs. 5A and B; main effect of opioids F (3.00, 21.0) = 12.7, $P < 0.0001$, main effect of time F (6.00, 126.0) = 96.7, $P < 0.0001$, interaction F (18.0, 126.0) = 4.13, $P < 0.0001$).

#### 3.3.2. Experiment 2: morphine, fentanyl, and oxycodone do not amplify allodynia when administration begins 1 hour after chronic constriction injury

—To determine whether opioids would also amplify CCI allodynia when delivery begins before the onset of nerve injury-induced allodynia, morphine, oxycodone, or fentanyl was administered for 5 days beginning 1 hour after CCI surgery. Under these conditions, we did not observe an increase in allodynia after treatment conclusion, compared with saline treatment (Figs. 6A and B, main effect of opioids F (3.00, 26.0) = 0.431, $P = 0.733$, main effect of time F (3.00, 78.0) = 26.6, $P < 0.0001$, interaction F (9.00, 78.00) = 1.07, $P = 0.395$).

## 5. Discussion

We have previously shown that a short course of morphine, beginning 10 days after CCI, can exacerbate nociceptive hypersensitivity induced by nerve injury for weeks to months after treatment concludes.[20] Here, we extend these observations to demonstrate that oxycodone and fentanyl—both widely prescribed opioids for pain treatment—induce similar effects in our rat model of neuropathic pain. We also demonstrate that exacerbation of allodynia occurs if morphine, oxycodone, or fentanyl is administered at an extended timepoint (ie, 28 days after CCI), in addition to early after allodynia develops (ie, 10 days after CCI). Importantly, none of these opioids exacerbated allodynia when administration began an hour after nerve injury.

Our working model for opioid exacerbation of nociceptive hypersensitivity is a “two-hit” hypothesis, that peripheral nerve injury (hit 1) confers a heightened neuroinflammatory response to subsequent opioids (hit 2).[7,13,20] This is based on literature of glial priming, wherein a first glial activating event (hit 1) primes glia to overrespond to a subsequent challenge (hit 2).[42] We argue that spinal TLR4 gates the immune signaling in our model because it is activated by damage-associated molecular patterns released by injured afferents as well as by opioids.[15,17,19,34] The exacerbation of nociceptive hypersensitivity by oxycodone and fentanyl is consistent with this working model because both of these opioids also activate TLR4 as does morphine.[27,29,51] Proinflammatory cytokines are produced as a consequence of TLR4 activation, which subsequently promote central sensitization through neuromodulation and dysfunctional synaptic plasticity. For example, tumor necrosis factor and interleukin-1β (IL-1β) enhance excitatory neurotransmission by mechanisms including neurotransmitter exocytosis and increased synaptic strength.[31,33,44,53] Glutamate homeostasis is also impaired by these mediators, which induce downregulation and posttranslational modifications of glutamate transporters and glutamine synthetase.[31,33,44,53] Our previous work indicates that these pronociceptive mechanisms are potentiated when opioids are administered after injury.[10,14,19,20]

The timing of opioid administration, relative to peripheral nerve injury, determines whether subsequent allodynia is exacerbated. Here, we found that 5 days of opioids only exacerbated allodynia when allodynia was already established at days 10 or 28 after CCI. We have previously found that a 5-day morphine treatment that ends 2 days before CCI surgery can also exaggerate ensuing allodynia.[36] In this study, we unexpectedly found that when morphine, oxycodone, and fentanyl administration was initiated one hour after CCI, subsequent allodynia was not adversely affected. This observation also raises other intriguing questions to be addressed in future studies. For example, whether immediate exposure to opioids after injury protects against the deleterious effects on subsequent reexposure. Also, importantly, we note that this temporally dependent result with the CCI neuropathic pain model contrasts with that from a laparotomy postoperative pain model. In this model, when morphine was administered for 7 days beginning immediately after surgery, morphine more than doubled the duration of allodynia induced by laparotomy.[14] It is not yet clear if our divergent results are due to the slightly longer course of morphine treatment in the laparotomy study, or if they are related to inherent differences between these models.

The mechanisms underlying the temporal differences remain unclear: spinal TLR4 mRNA is upregulated within 4 hours of peripheral nerve and persists for more than 28 days.[48] It is possible that other signaling components are not upregulated at this early timepoint after injury. For example, the purinergic receptor P2X7R is not upregulated in the lumbar spinal cord until 3 days after peripheral nerve injury.[22,32] Importantly, we have shown that P2X7R is required together with TLR4 for opioid exacerbation of allodynia because such potentiation is dependent on inflammasomes.[20] This could be one explanation for the lack of effect of opioid administration early after injury. Additional studies will be required to explore the important mechanistic differences underlying the lack of opioid effects when administered at the time of peripheral nerve injury vs after the expression of neuropathic pain.

We did note some between-experimenter variability in von Frey testing; the pretreatment thresholds in Figure 4 were slightly lower than those in other figures (~0.6 vs ~1.4 g). Such variability has been documented before.[47] However, the lower thresholds were also accompanied by a longer recovery in this experiment (~10 vs ~5 weeks). These differences may be explained by the greater detection sensitivity of this experimenter.

Despite being classified as third-tier therapeutics for the treatment of peripheral neuropathic pain, opioids are among the most common treatments prescribed for such pain.[3,12,41] The preclinical evidence presented here indicates that commonly used opioids may have adverse long-term consequences for the treatment of established neuropathic pain. By contrast, our results suggest that opioids may be appropriate to manage trauma pain acutely after peripheral nerve injury. The extent to which our findings extend from mechanical allodynia to other indices of neuropathic pain (eg, thermal hyperalgesia and ongoing pain) will be investigated in future studies. Overall, these studies provide a framework for a better understanding of the timing of administration and specific opioid medications that could lead to significantly different clinical treatment outcomes. These observations warrant future clinical testing in patients with chronic pain.

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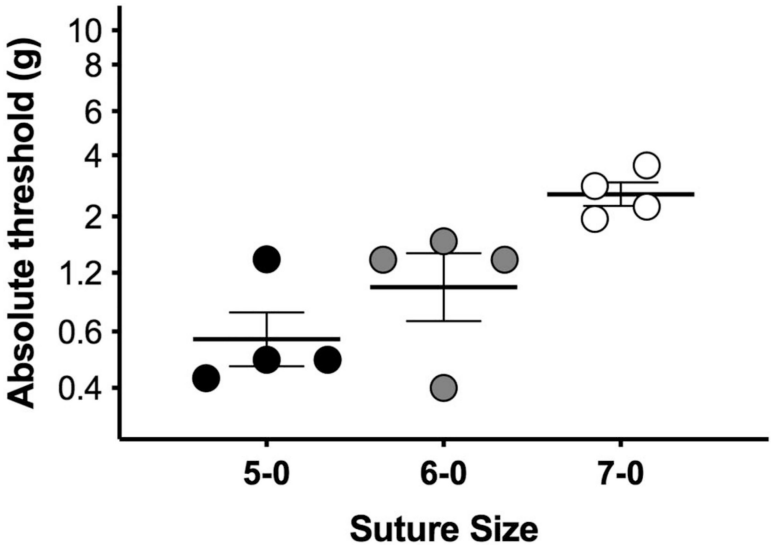


**Figure 1.**

"Dose"-dependent allodynia in response to sciatic CCI. Chronic constriction injury surgeries performed with one 5–0, 6–0, or 7–0 chromic gut sutures tied around the sciatic nerves of mail rats. Von Frey thresholds measured at day 14 after CCI size. n = 4/group. CCI, chronic constriction injury.

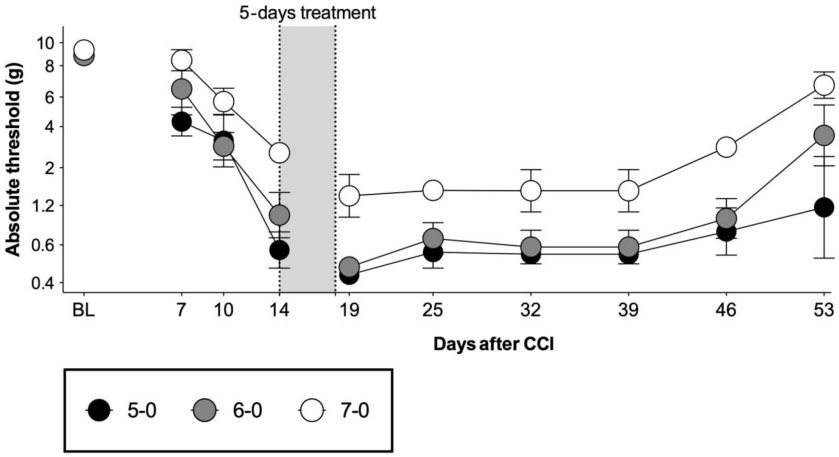


**Figure 2.**

Validation of the attenuated model of CCI for avoiding behavioral floor effects in the measurement of allodynia. Chronic constriction injury surgeries performed with one 5–0, 6–0, or 7–0 chromic gut suture. Morphine (5 mg/kg twice per day) was administered for 5 days, beginning at day 14 after surgery. Von Frey testing was conducted before and after CCI surgery, and after completion of opioid administration. BL: presurgery baseline. n = 4/group. CCI, chronic constriction injury.

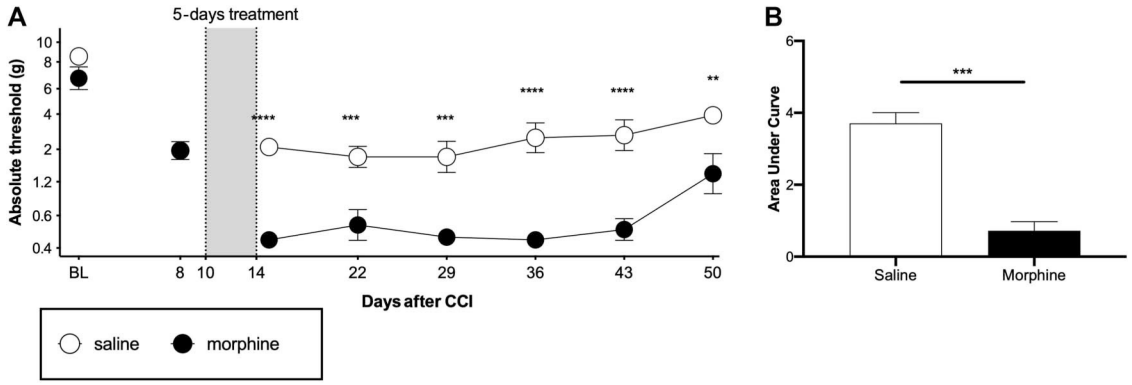


**Figure 3.**

Morphine exacerbates CCI allodynia when dosing begins 10 days after injury. Chronic constriction injury surgeries were performed with one 6–0 chromic gut suture. Morphine (5 mg/kg twice per day) or equivolume saline was administered for 5 days, beginning at day 10 after surgery. (A) Von Frey testing was conducted before and after CCI surgery, and after completion of opioid administration. (B) Area-under-the-curve analysis of von Frey threshold of all timepoints after morphine treatment. BL: baseline, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. n = 6/group. CCI, chronic constriction injury.

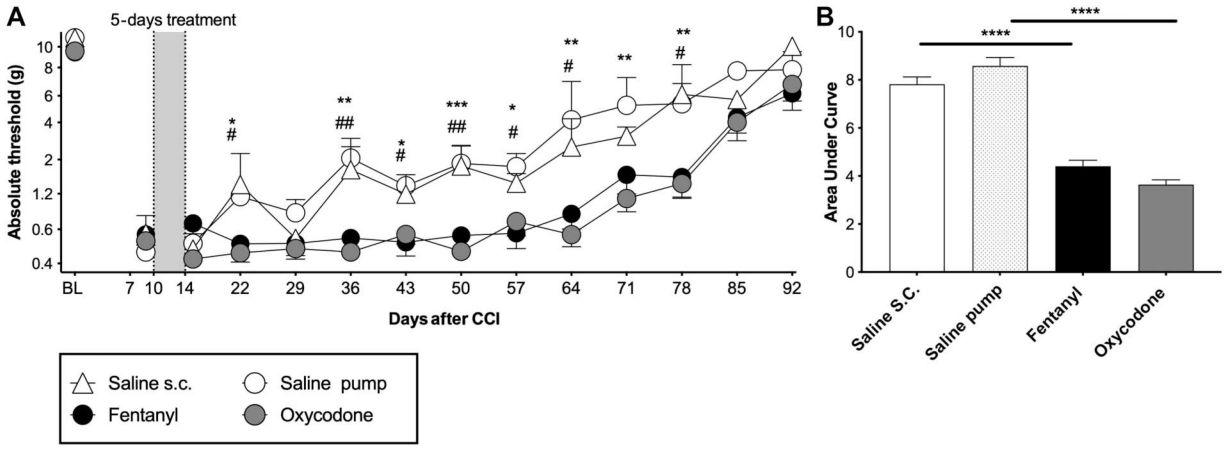


**Figure 4.**

Exacerbation of CCI allodynia by oxycodone and fentanyl when dosing begins 10 days after injury. Chronic constriction injury was performed with one 6–0 chromic gut suture. Fentanyl (0.01 mg/kg/h), oxycodone (2 mg/kg twice per day), or equivolume saline was administered for 5 days, beginning at day 10 after surgery. (A) Von Frey testing was conducted before and after CCI surgery, and after completion of opioid administration. (B) Area-under-curve analysis of von Frey threshold of all timepoints after opioid treatment. *Oxycodone vs all saline, #fentanyl vs all saline. BL, baseline, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. n = 6/group. CCI, chronic constriction injury.

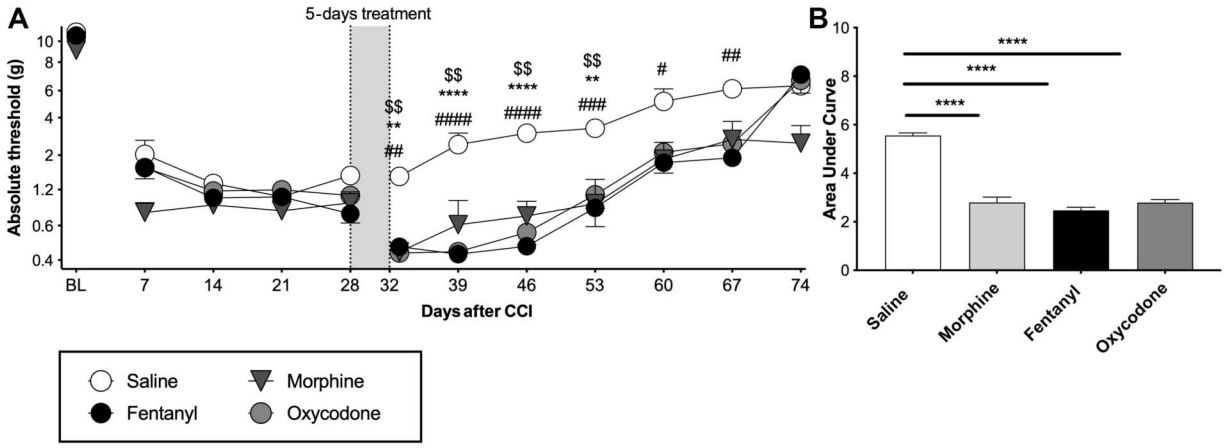


**Figure 5.**

Exacerbation of CCI allodynia by morphine, oxycodone, or fentanyl when dosing begins 1 month after injury. Chronic constriction injury was performed with one chromic gut 6–0 suture. Morphine (5 mg/kg twice per day), fentanyl (0.01 mg/kg/h), oxycodone (2 mg/kg twice per day), or equivolume saline was administered for 5 days, beginning at day 28 after surgery. (A) Von Frey testing was conducted before and after CCI surgery, and after completion of opioid administration. (B) Area-under-curve analysis of von Frey threshold of all timepoints after opioid treatment. *Oxycodone vs all saline, #fentanyl vs all saline #, $morphine vs all saline, BL: baseline, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. n = 6/group. CCI, chronic constriction injury.

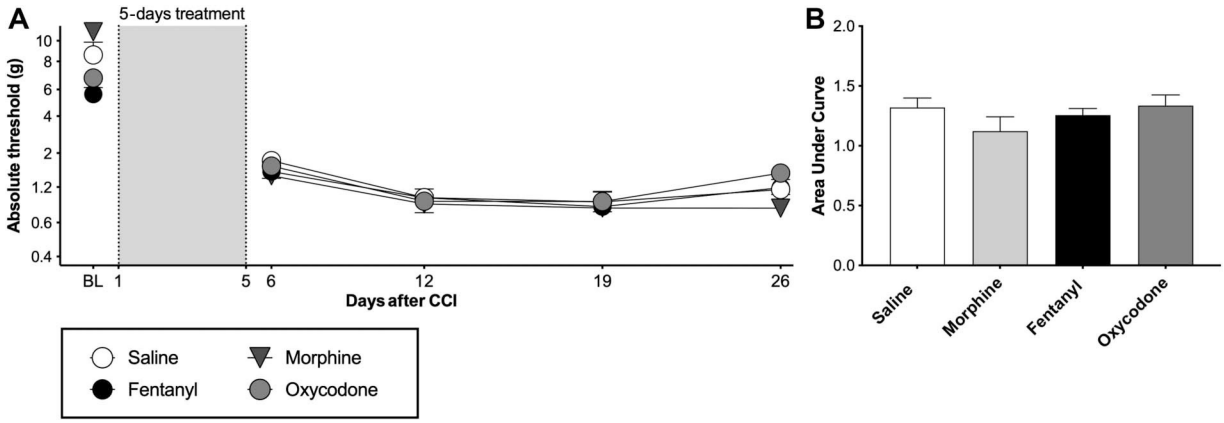


**Figure 6.**

No exacerbation of CCI allodynia by morphine, oxycodone, or fentanyl when dosing begins 1 hour after injury. Chronic constriction injury was performed with one chromic gut 6–0 suture. Morphine (5 mg/kg twice per day), (fentanyl (0.01 mg/kg/h), oxycodone (2 mg/kg twice per day), or equivolume saline was administered for 5 days, beginning at one-hour after surgery. (A) Von Frey testing was conducted before and after CCI surgery, and after completion of opioid administration. (B) Area-under-curve analysis of von Frey threshold of all timepoints after opioid treatment. BL, baseline. n = 6/group. CCI, chronic constriction injury.