

## Research Paper

## Exploring acute-to-chronic neuropathic pain in rats after contusion spinal cord injury☆



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## ARTICLE INFO

## Article history:

Received 13 March 2017  
Received in revised form 25 April 2017  
Accepted 25 May 2017  
Available online 25 May 2017

## Keywords:

Central nervous system  
Neuropathic pain  
Nervous system trauma  
Spinal cord injury  
Sex differences

## ABSTRACT

Spinal cord injury (SCI) causes chronic pain in 65% of individuals. Unfortunately, current pain management is inadequate for many SCI patients. Rodent models could help identify how SCI pain develops, explore new treatment strategies, and reveal whether acute post-SCI morphine worsens chronic pain. However, few studies explore or compare SCI-elicited neuropathic pain in rats. Here, we sought to determine how different clinically relevant contusion SCIs in male and female rats affect neuropathic pain, and whether acute morphine worsens later chronic SCI pain. First, female rats received sham surgery, or 150 kDyn or 200 kDyn midline T9 contusion SCI. These rats displayed modest mechanical allodynia and long-lasting thermal hyperalgesia. Next, a 150 kDyn (1 s dwell) midline contusion SCI was performed in male and female rats. Interestingly, males, but not females showed SCI-elicited mechanical allodynia; rats of both sexes had thermal hyperalgesia. In this model, acute morphine treatment had no significant effect on chronic neuropathic pain symptoms. Unilateral SCIs can also elicit neuropathic pain that could be exacerbated by morphine, so male rats received unilateral T13 contusion SCI (100 kDyn). These rats exhibited significant, transient mechanical allodynia, but not thermal hyperalgesia. Acute morphine did not exacerbate chronic pain. Our data show that specific rat contusion SCI models cause neuropathic pain. Further, chronic neuropathic pain elicited by these contusion SCIs was not amplified by our course of early post-trauma morphine. Using clinically relevant rat models of SCI could help identify novel pain management strategies.

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## 1. Introduction

65–80% of individuals with spinal cord injury (SCI) experience chronic neuropathic pain (Siddall et al., 1999). Unfortunately, SCI-elicited pain is difficult to manage, and pain relief remains an important priority for individuals with SCI (Anderson, 2004; Collinger et al., 2013; Lo et al., 2016). Using rodent SCI models to study neuropathic pain mechanisms could help identify new pain modulatory targets. Several rat models of SCI-exacerbated evoked pain have been studied. Detloff et al. (2008) found that midline thoracic contusion SCI (moderate force) caused persistent mechanical allodynia in the hindpaw that correlated

with increased inflammation in the lumbar spinal cord. Crown et al. (2008) showed that a T10 contusion SCI (150 kDyn force, 1 s dwell) caused mechanical allodynia by 35 days post-injury (dpi). Finally, unilateral cervical contusion SCI in rats caused persistent neuropathic pain the forepaw and hindpaw (Detloff et al., 2013; Putatunda et al., 2014). It is clear that midline and unilateral contusion SCI in rats causes some pain; however, the onset, duration, and relative magnitude of pain in SCI rat models has not been compiled in a single study.

Individuals with SCI are often treated with the opioid morphine to reduce post-SCI pain. Of individuals with post-SCI pain, 52–60% of patients received post-injury morphine (Cardenas and Jensen, 2006; Warms et al., 2002). According to patients, opioids are among the most helpful pain relievers (Cardenas and Jensen, 2006; Warms et al., 2002). Although morphine has beneficial acute analgesic effects that last hours (Cardenas and Jensen, 2006), opioid treatment also has adverse effects that include nausea, drowsiness, and constipation (Baastrup and Finnerup, 2008). In addition, studies from our group (Ellis et al., 2016; Grace et al., 2016) and others (Fletcher and Martinez, 2014; Laboureyras et al., 2014) suggest that opioids can have later paradoxical pain-enhancing effects. SCI rats treated with

*Abbreviations:* BBB, Basso-Beattie-Bresnahan (locomotor recovery scale); CNS, central nervous system; dpi, days post-injury; s.c., subcutaneous; SCI, spinal cord injury; SUDO, simplified up-down; T-, thoracic segment.

☆ **Summary:** Spinal cord injury pain models were compared. In female and male rats, midline spinal contusion caused persistent pain. Acute morphine did not worsen chronic pain.

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high-dose morphine show larger SCI lesions and impaired recovery of locomotor function (Hook et al., 2007; Hook et al., 2009; Hook et al., 2017). Using T13 dorsal root avulsion, our group found that subcutaneous morphine injections in rats for 7 days exacerbated later mechanical allodynia (Ellis et al., 2016). Extending these findings, here we test whether morphine also worsens later chronic pain in a rat model of clinically relevant contusion SCI.

Past studies assessed neuropathic pain in specific SCI models or timepoints. However, a systematic timecourse and comparison showing how different rat SCI models affect neuropathic pain has not been done. Here, we sought to establish an effective, clinically relevant rat model of SCI-elicited chronic neuropathic pain. Several thoracic contusion SCI models were tested, including moderate-to-severe midline contusion SCIs and a unilateral contusion SCI. A moderate force (150 kDyn) SCI with 1 s dwell was particularly effective at eliciting pain symptoms in male and female rats. Because morphine treatment in other nervous system trauma models can exacerbate later neuropathic pain, we hypothesized that acute post-injury morphine would worsen later chronic pain symptoms in our SCI models. Acute morphine treatment had expected analgesic effects and caused tolerance; however, later morphine-exacerbated pain was not observed.

## 2. Material and methods

### 2.1. Surgery and animal care

All housing, surgery, and postoperative care were approved by the University of Colorado Boulder Institutional Animal Care and Use Committee. All animals were fed standard chow and filtered tap water ad libitum and maintained on a 12:12 light/dark cycle. For all experiments, sham/SCI surgeries were interspersed throughout the day (during the light cycle). Female and male Sprague-Dawley rats (females: 200–250 g, males: 320–380 g; 2–3 months old; Harlan Laboratories) were anesthetized with isoflurane inhalation anesthesia, and both sham and SCI rats were treated with prophylactic antibiotics (gentamicin sulfate (Butler Schein), 1.25 mg s.c. in 0.25 mL sterile water). A partial T9 or T13 laminectomy was performed prior to SCI. The periosteum, but not the dura, was removed for all surgeries (this is the end of the surgery for sham rats). SCI rats were subjected to a contusion SCI using the Infinite Horizon device (Precision Systems and Instrumentation). Each experiment had distinct design and rat numbers: Experiment 1: 150 or 200 kDyn force, 0 s dwell with 1.5 mm impactor tip at midline T9 [females only: sham  $n = 8$ , 150 kDyn  $n = 6$ , 200 kDyn  $n = 5$ ]; Experiment 2: 150 kDyn force, 1 s dwell with 1.5 mm impactor tip at midline T9 [females: sham-saline  $n = 6$ , sham-morphine  $n = 6$ , SCI-saline  $n = 5$ , SCI-morphine  $n = 4$ ; males: sham-saline  $n = 7$ , sham-morphine  $n = 6$ , SCI-saline  $n = 5$ , SCI-morphine  $n = 4$ ]; Experiment 3: 100 kDyn force, 1 s dwell with 1.0 mm-diameter impactor tip at left side T13 [females only: sham-saline  $n = 6$ ; SCI-saline  $n = 5$ ; SCI-morphine:  $n = 6$ ].

Post-operative animal care included daily administration of gentamicin (both sham and SCI rats; 1 mL/d for 5 d), subcutaneous injection of Ringer's solution (5, 5, 4, 3, 2 mL on each of the first 5 days post-injury (dpi), respectively; for both sham and SCI rats) to prevent dehydration, and manual voiding of bladders twice daily (until recovery of bladder function at 2–3 weeks) (Gaudet et al., 2015). Animals were monitored daily for infection or other signs of suboptimal recovery. Rats were housed in pairs. Rats in all treatment groups were numbered randomly to ensure researchers were blind to group.

### 2.2. Morphine treatment

Rats were treated with morphine (or control saline) as previously described (Loram et al., 2012). (-)-Morphine sulfate (gift from NIDA Drug Repository; Research Triangle, NC, USA) was dissolved in sterile saline. Rats received 5 mg/kg s.c. morphine (or saline)  $2 \times$  per day for 7 d (around the beginning and end of light phase), beginning on the

first day post-surgery. To test for analgesic efficacy of morphine in Experiment 2, rat heat thresholds were tested prior to and 60 min after saline/morphine injection (on final day of morphine course, in the morning). To test for morphine analgesic efficacy and tolerance in Experiment 3, rat heat thresholds were tested prior to and 30, 60, 90, and 120 min after saline/morphine injection (after first injection and on final day of morphine course, in the morning).

### 2.3. Locomotor testing

Locomotor recovery was assessed using the Basso-Beattie Bresnahan (BBB) locomotor rating scale (Basso et al., 1995). A BBB score of zero represents no hindlimb movement; the highest BBB score of 21 represents typical coordinated and stable rat walking. Rating was performed by two researchers who were blind to treatment group. BBB scores were recorded at 1, 4, 7, 10, and 14 days post-injury (dpi), then weekly thereafter.

### 2.4. Neuropathic pain testing

Neuropathic pain assessment was completed as previously described (Ellis et al., 2016; Grace et al., 2016; McGraw et al., 2005). Rats were pre-acclimated to the von Frey and Hargreaves beakers for three sessions, and then had two pre-surgery tests. For each individual testing session, rats acclimated to their beaker for 40–60 min. Post-injury sensory testing occurred at least weekly post-injury. Unless otherwise noted, values for left and right hindpaws were averaged for each animal at each timepoint. The order of the rats in the beakers was randomized to ensure that the tester was blind to treatment group.

To assess mechanical sensory thresholds, the simplified up-down (SUDO) method (Bonin et al., 2014) of von Frey testing was used. This was used to limit rat stress and time out of home cage. Rats were placed on an elevated wire mesh, under a clear plastic beaker. After 40–60 min, the von Frey filaments (a logarithmic series of 10 calibrated Semmes-Weinstein monofilaments; Stoelting, Wood Dale, IL) were pressed against the center of the plantar surface of the rat hindpaw until they buckled and were held for a maximum of 5 s. The log stiffness of the hairs ranged from 3.61 (0.40 g) to 5.18 (15.14 g) filaments; the SUDO method started with filament 10 (4.31, 2.0 g). If the force of the filament elicited a definite paw withdrawal or notable flinch, a positive response was recorded. Whenever possible, von Frey testing was completed the day prior to Hargreaves testing to ensure accurate threshold measurements for both tests and to minimize rat stress.

To assess heat sensory thresholds (thermal hyperalgesia), the Hargreaves test was performed. Rats were placed on a glass platform under plastic beakers with an open top. An infrared source (intensity of 35) was placed under the center of their hindpaw and activated, then stopped once the rat moved their paw or flinched (clear nocifensive response to heat stimulus). Latency to response was automatically recorded. Testing on left and right hindpaws was alternated (three tests per timepoint), and 5–10 min elapsed between each test to limit sensitization. For morphine analgesia/tolerance, only two tests per paw per timepoint were used to limit hypersensitivity. Maximum response latency was set at 25 s; rats that did not respond at all over an individual test scored 25 s. This particularly occurred in some rats after acute morphine treatment.

### 2.5. Statistics

Data were analyzed using Student's *t*-test or non-parametric Mann-Whitney *U* test; or a one- or two-way ANOVA (repeated-measures, as appropriate), followed by Holm-Sidak post hoc test. Data were analyzed using SigmaPlot 12.0 (SPSS), and were considered significant when  $p < 0.05$ . All data are plotted as mean  $\pm$  SEM.

### 3. Results

#### 3.1. Midline T9 spinal cord contusion (150 vs. 200 kDyn), with no dwell, in female rats elicits modest below-level pain symptoms

To determine how clinically relevant contusion SCI in a rodent model affects neuropathic pain symptoms, female rats were subjected to sham surgery, moderate 150 kDyn contusion SCI (no dwell time), or moderate-to-severe 200 kDyn contusion SCI (no dwell). Locomotor recovery (BBB scale for locomotor recovery) and pain symptoms (mechanical allodynia and thermal hyperalgesia) were studied over time.

Female rats showed expected differences in BBB locomotor score, with the females subjected to moderate 150 kDyn SCI showing improved hindlimb movement compared to females that received moderate-to-severe 200 kDyn SCI (overall group difference; also specific timepoints: 1 dpi, 7 dpi, 35 dpi, 42 dpi;  $p < 0.05$ ) (Fig. 1a). The average 150-kDyn BBB score at the final 42 dpi timepoint was 12, which corresponds to hindlimb plantar stepping with frequent coordination, whereas the average 200-kDyn BBB score at 42 dpi was approximately 11, corresponding to hindlimb plantar stepping with occasional coordination.

Rats with these SCIs showed modest post-injury neuropathic pain symptoms in the hindpaw. For mechanical allodynia (von Frey test), the more severe 200 kDyn group displayed significant hypersensitivity at 20 and 34 dpi (asterisks, compared to sham surgery rats at same timepoint;  $p < 0.05$ ) (Fig. 1b). The 150 kDyn rats did not show significant SCI-induced mechanical allodynia. The Hargreaves test was used to assess SCI-induced thermal hyperalgesia in the hindpaw. Both 150- and 200-kDyn SCI groups displayed heat hypersensitivity at several timepoints (200-kDyn at 14, 28, and 35 dpi, asterisks; and 150-kDyn at 28, 35, and 42 dpi, asterisks) (Fig. 1c). Thus, the moderate and moderate-to-severe SCIs elicited neuropathic pain symptoms; however, neuropathic pain was not consistent or particularly robust. Because neuropathic pain after 150 kDyn (0 s dwell) SCI was only modest, males were not tested in this model and we sought to explore SCI pain in another injury model.

#### 3.2. Midline T9 spinal cord contusion (150 kDyn), with 1 s dwell, in female and male rats causes neuropathic pain

There are reports that neuropathic pain can be elicited by adding a one-second “dwell time” to the impact (Carlton et al., 2009; Gwak et al., 2012). With dwell time, the contusion probe impacts the spinal cord to a known force; then, it remains in place compressing the spinal cord for 1 s prior to retracting. Thus, next we tested whether performing midline 150-kDyn contusion SCI at T9 with 1 s dwell elicited more robust pain symptoms. Both females and males were tested in this model. For locomotor recovery (BBB scale), females and males showed expected deficits and recovery patterns (Fig. 2a).

For neuropathic pain, rats displayed some neuropathic pain symptoms with the 1 s dwell time – particularly in male rats. For mechanical allodynia (Fig. 2b), female rats did not show significant neuropathic pain (compared to sham rats at same timepoint), whereas male rats had significant mechanical allodynia beginning at 14 dpi and persisting through 42 dpi ( $p < 0.05$ ). For thermal hyperalgesia (Fig. 2c), female rats had significant neuropathic pain (compared to sham rats at same timepoint) at 14, 35, and 42 dpi ( $p < 0.05$ ). Male rats showed significant thermal hyperalgesia at 14, 21, 28, 35, and 42 dpi ( $p < 0.05$ ). Thus, we found that this SCI model elicited significant thermal hyperalgesia in females and males, and mechanical allodynia specifically in males.

#### 3.3. After midline T9 spinal cord contusion (150 kDyn, 1 s dwell), acute morphine did not exacerbate neuropathic pain in either male or female rats

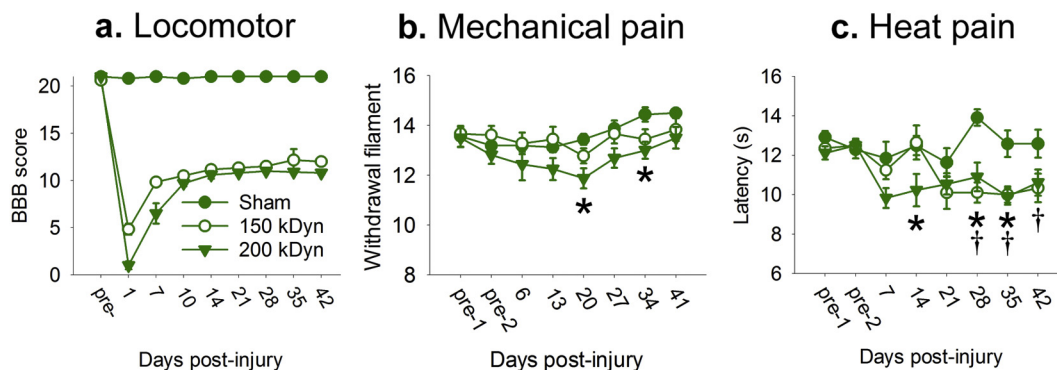
Based on previous work by our lab and others, we expected that an acute course of morphine would slow locomotor recovery and exacerbate SCI-induced neuropathic pain symptoms. To examine this, rats were subjected to sham or SCI surgery (as in Fig. 2 – same animals; including all groups [saline/morphine] here to enable effective explanation) then were injected with control saline solution or morphine (5 mg/kg, 2× per day for 7 d beginning at 1 dpi) (Fig. 3). Our lab previously showed that acute morphine treatment worsens later neuropathic pain in peripheral pain models (Grace et al., 2016; Hutchinson et al., 2008; Johnston et al., 2004; Loram et al., 2012) and in a dorsal root avulsion CNS injury model (Ellis et al., 2016). The morphine treatment had a negative effect on the rats' appearance and condition; SCI rats treated with morphine had more piloerection (raised fur) and more squinted eyes, suggesting their recovery of health took longer.

Acute morphine had little effect on later locomotor recovery and neuropathic pain symptoms. For locomotor recovery (Fig. 3a), SCI females on morphine did not show significant differences in BBB scores over the timecourse. Males with SCI on morphine performed significantly worse at 14 dpi.

Mechanical allodynia and thermal hyperalgesia were assessed (Fig. 3b, c). We predicted that the acute morphine course would worsen later (i.e., chronic) neuropathic pain. Here, there were no significant differences between saline- and morphine-treated rats, both in females and males.

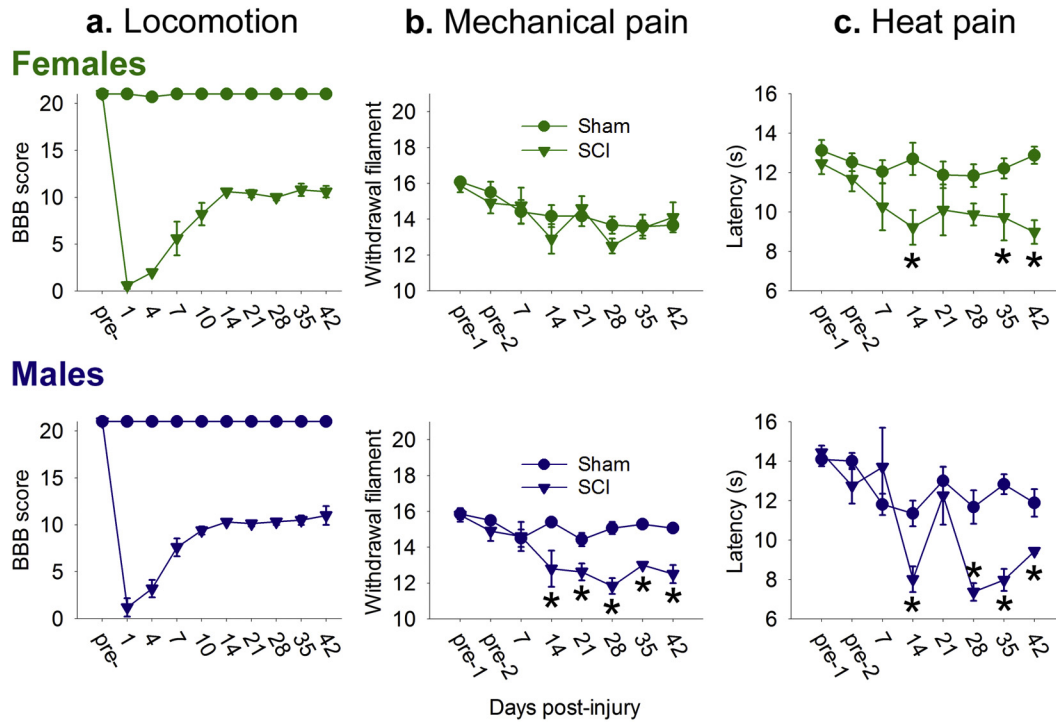
#### 3.4. Morphine had expected acute analgesic effects

Since morphine had no significant effect on later chronic neuropathic pain, we sought to confirm whether the morphine was effective. Rats were tested for mechanical and thermal thresholds prior to, and 40–60 min after treatment with morphine or saline at 7 dpi (since mechanical thresholds showed no significant morphine analgesia at this time, only heat sensitivity is presented for clarity). Note that the 7 dpi data presented in Fig. 3 are immediately prior to morning saline/morphine



**Fig. 1.** After moderate or moderate-severe midline contusion SCI (no dwell), female rats show expected post-SCI locomotor deficits (a) and modest neuropathic pain symptoms (b: mechanical allodynia; c: thermal hyperalgesia). \*Indicates sham vs. 200 kDyn,  $p < 0.05$ ; †Indicates sham vs. 150 kDyn,  $p < 0.05$ .

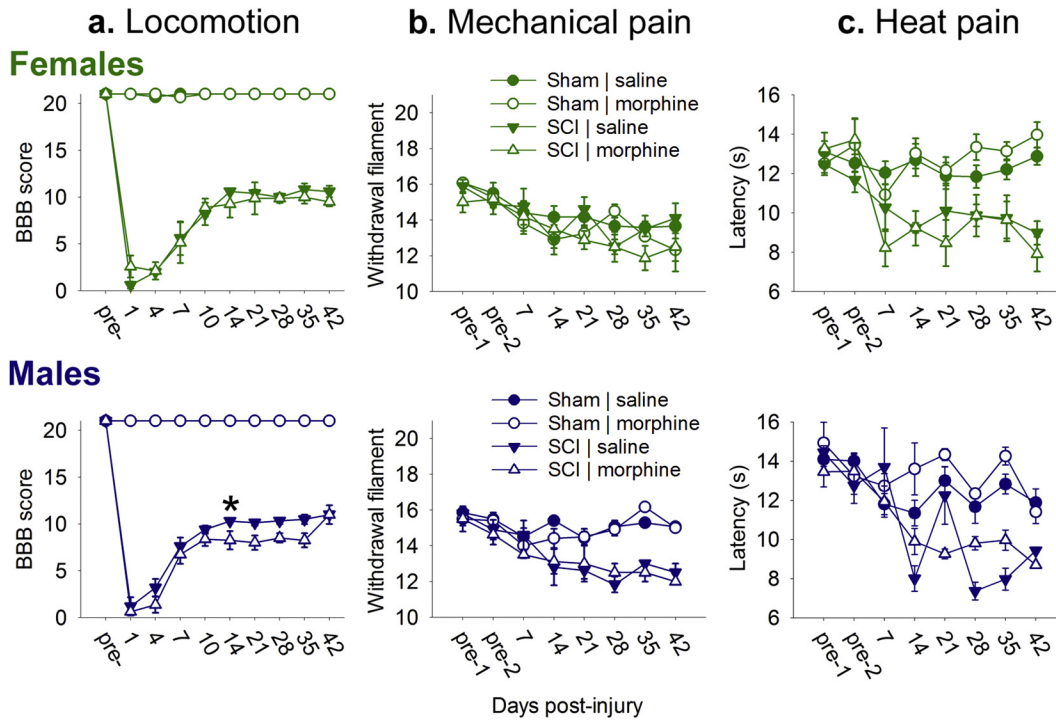




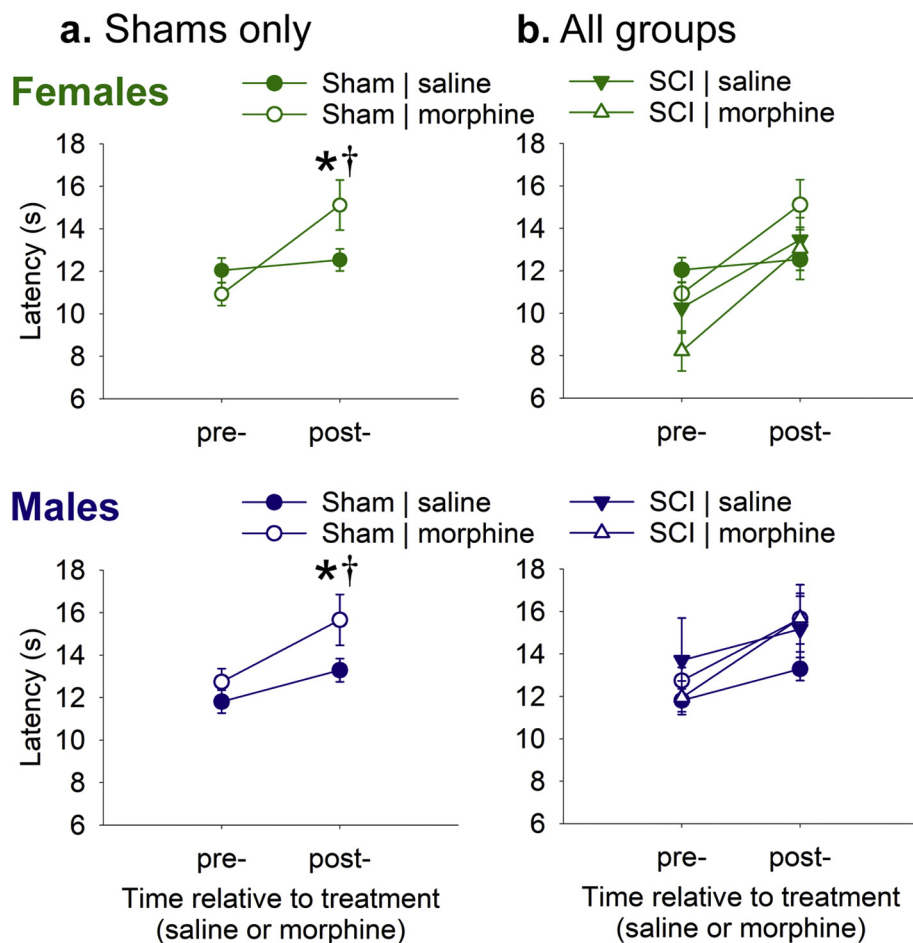
**Fig. 2.** Midline 150-kDyn SCI with 1 s dwell causes locomotor deficits and neuropathic pain in female (top panels) and male (bottom panels) rats. (a) Female and male rats with SCI show similar locomotor deficits and recovery after SCI. (b) Male, but not female SCI rats exhibit significant mechanical allodynia. (c) Both female and male SCI rats display significant thermal hyperalgesia at several post-surgery timepoints. \*Indicates  $p < 0.05$  vs. sham at same timepoint.

treatment; acute effects of the previous doses had worn off. There was a significant analgesic effect of morphine on thermal thresholds (Fig. 4). Indeed, both female and male sham rats treated with morphine showed significantly lengthened latencies to respond to thermal stimuli (compared both to pre-injection values and to saline-injected rats at the post-injection time) (Fig. 4a). SCI rats treated with morphine had delayed

responses compared to saline-treated rats, although this was not significant (Fig. 4b). There was no significant effect of morphine treatment on mechanical thresholds and on heat thresholds in SCI rats, and there are several possible explanations. First, they were tested at 40 min post-morphine; perhaps a timecourse could have identified analgesia. Second, the rats were only tested on the penultimate day of morphine delivery;



**Fig. 3.** Morphine did not exacerbate neuropathic pain after midline contusion SCI in female and male rats. (a) Acute treatment with morphine (5 mg/kg  $2 \times$  per d for 7 d post-surgery, beginning at 1 dpi) had no significant effect on post-SCI locomotor recovery in females, and a modest detrimental effect on BBB score in males. (b) SCI-elicited mechanical allodynia was not significantly worsened by morphine. (c) SCI-induced heat pain was not exacerbated by acute morphine treatment. \*Indicates SCI-saline vs. SCI-morphine,  $p < 0.05$ .



**Fig. 4.** Morphine had expected analgesic effects on heat thresholds in sham rats. To test morphine efficacy, female and male rat heat thresholds were tested on the 7th day of morphine treatment prior to injection and at ~60 min post-injection. (a) Acute post-morphine heat thresholds were significantly extended in female and male sham rats, highlighting its efficacy. (b) Acute post-saline/morphine heat thresholds in female and male rats with sham or SCI surgery. SCI rats did not show significant post-morphine analgesia. In future studies, testing morphine efficacy at earlier times and testing morphine tolerance were included. \*Indicates sham-morphine pre- vs. post-injection,  $p < 0.05$ ; †Indicates sham post-injection morphine vs. saline,  $p < 0.05$ .

morphine tolerance had likely already set in. Therefore, these possibilities were accounted for in the design of our next experiment (using the same morphine batch; see below), which confirms the acute analgesic and tolerance effects of morphine under these conditions. These results suggest that morphine was having its intended effects, and that morphine does not appear to worsen chronic SCI pain in this injury model.

### 3.5. Unilateral T13 spinal cord contusion (100 kDyn, 1 s dwell) causes chronic neuropathic pain in male rats that is not altered by an acute course of morphine

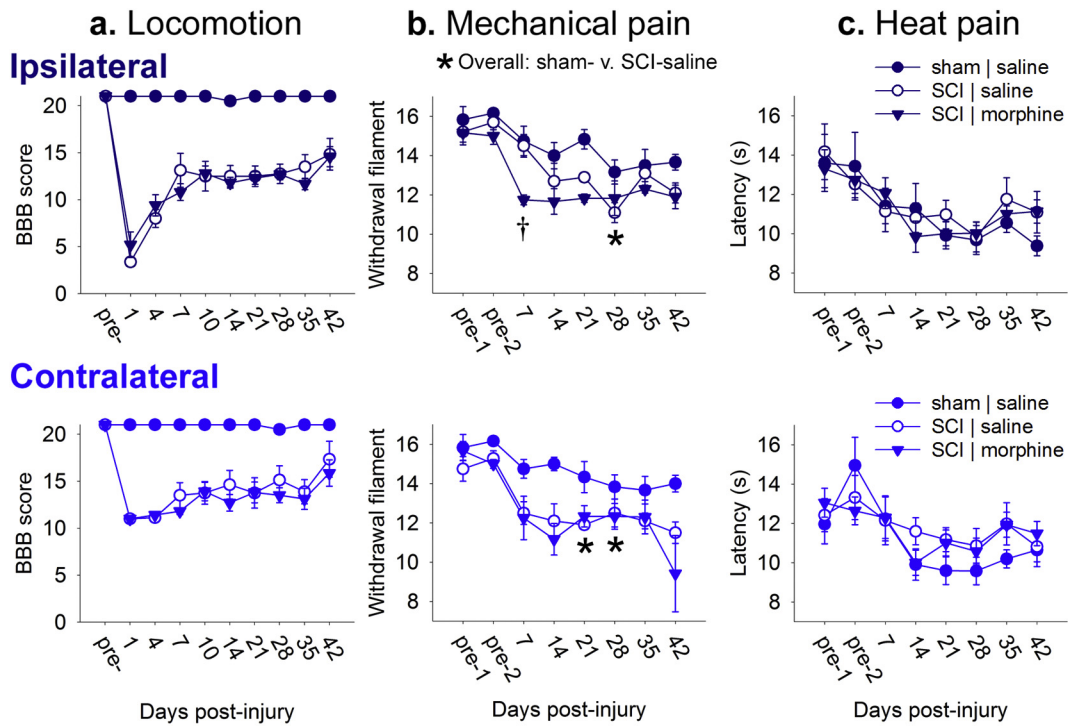
Morphine-exacerbated chronic neuropathic pain may be observed in other SCI models. Another contusion SCI model known to cause neuropathic pain is a unilateral spinal contusion (Putatunda et al., 2014; Watson et al., 2014). Here, we adapted the unilateral injury to the thoracic level, which would enable comparing results to our previous experiments and testing below-level pain in the male rat hindpaw. Thus, a 100-kDyn contusion SCI (with a 1.0-mm diameter impactor tip) was performed on the left side of the T13 spinal cord and compared to sham surgery (laminectomy) male rats. In addition, we tested the hypothesis that acute morphine would worsen locomotor recovery and chronic neuropathic pain. Male rats were used, since they showed more robust pain symptoms in the 150 kDyn (1 s dwell) model.

Rats with unilateral SCI exhibited hindpaw locomotor deficits that were more severe on the side ipsilateral to injury. The ipsilateral hindpaw of saline-treated SCI rats had an average BBB score of 2.8 at

1 dpi, and recovered to 13.25 by the final 42 dpi timepoint (Fig. 5a). The hindpaw contralateral to injury in saline-treated SCI rats had an average BBB score of 11 at 1 dpi, and recovered to 15.5 by the final 42 dpi timepoint. Morphine had no significant effect on recovery of function. Thus, our data show that rats have expected worsened below-level motor function on the side ipsilateral to injury, and that morphine did not modify locomotor recovery.

Unilateral SCI caused mechanical allodynia in both ipsilateral and contralateral hindpaws. On the ipsilateral hindpaw, saline-treated rats with SCI exhibited significant ipsilateral mechanical neuropathic pain (compared to sham rats; overall group difference,  $p < 0.05$ ). Saline-SCI rats also showed significant ipsilateral mechanical pain specifically at 28 dpi (vs. sham-saline rats;  $p < 0.05$ ). Unilateral SCI-induced pain appeared to be more robust on the contralateral side (vs. sham-saline rats;  $p < 0.05$ ). On the contralateral hindpaw, SCI-saline rats displayed significant mechanical allodynia at 21 and 28 dpi.

Morphine modestly exacerbated unilateral SCI-induced pain. On the side ipsilateral to SCI, morphine treatment worsened SCI mechanical pain at 7 dpi ( $p < 0.05$ ). Similarly, on the contralateral hindpaw, morphine worsened SCI-induced neuropathic pain (vs. SCI-saline; overall group effect and specifically at 7 dpi). Thermal hyperalgesia was also examined in these unilateral SCI rats. Both ipsilateral and contralateral to SCI, there was no significant SCI-elicited hypersensitivity observed. Although there was a slight pattern of apparent sham-saline rat thermal hypersensitivity, their thresholds were not significantly different from SCI-saline rats (SCI-saline vs. sham-saline rats;  $p > 0.05$ ). In addition,



**Fig. 5.** Unilateral SCI in male rats caused locomotor deficits and mechanical allodynia. (a) After unilateral SCI, locomotor deficits are worse on the side ipsilateral to injury (\* sham-saline vs. SCI-saline). Morphine did not affect recovery. (b) Unilateral SCI caused mechanical neuropathic pain on both ipsilateral and contralateral hindpaws (\*). Morphine († vs. SCI-saline) worsened post-SCI pain at 7 dpi. (c) Unilateral SCI did not elicit significant thermal hyperalgesia on either hindpaw; morphine did not alter responses in the Hargreaves test.

there was no significant effect of morphine on heat thresholds over the timecourse (vs. SCI-saline;  $p > 0.05$ ).

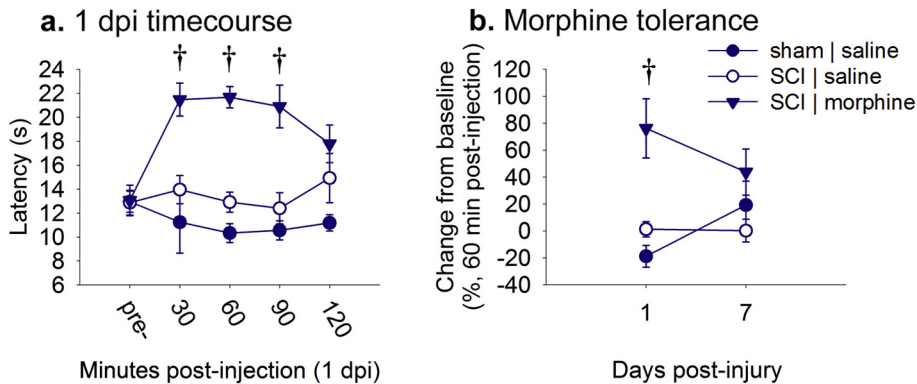
**3.6. In the unilateral SCI model, morphine had expected analgesic effects and tolerance properties**

To confirm that morphine was having its known effects, a more thorough examination of analgesic and tolerant properties was performed using the Hargreaves test (Fig. 6). Hindpaw responses were averaged. At 1 dpi – immediately after the first morphine injections, when it is expected to have its strongest analgesic potency – morphine increased heat thresholds (analgesia) as soon as 30 min post-injection and maintained strong analgesic effects through 90 min (Fig. 6a). At 120 min, the analgesic effects of the single morphine dose began to decay. To test for tolerance, thresholds were tested again at 7 dpi (after 6 d of twice-daily saline/morphine injections) (Fig. 6b). Sham-saline rats showed minor (non-significant) hypersensitivity over the brief timecourse; this could be due to repeated heat testing over a short period that more strongly

affected this group. Thresholds were tested prior to and 60 min post-injection – a time of maximal morphine efficacy. (Again, the pre-injection 7 dpi data are also presented as thresholds in Fig. 5) Comparing the percent change from baseline at 1 dpi and 7 dpi, morphine was the only treatment that had significant analgesic effects at 1 dpi ( $p < 0.05$ ). At 7 dpi, these analgesic effects were less robust (~50% less analgesia at 7 dpi compared to 1 dpi; percent change not significantly different from saline-treated group). Therefore, morphine had analgesic properties, and the 7 d course of morphine led to expected morphine tolerance in the unilateral SCI model.

**4. Discussion**

This study assessed neuropathic pain caused by various clinically relevant types of SCI in male and female rats, and established that acute morphine (5 mg/kg s.c., 2 × per day, from 1–8 dpi) did not exacerbate later SCI pain in these models. To start, typical moderate (150 kDyn force) and moderate-to-severe (200 kDyn) T9 contusion SCIs were



**Fig. 6.** After unilateral SCI, morphine had expected analgesic effects and tolerance properties. (a) Male rats treated with their first injection of morphine show lengthened latency to response to heat stimuli. (b) Rats treated with morphine for 6 d exhibit analgesic tolerance. Heat thresholds were tested prior to and 60 min post-injection; morphine had significant analgesic effects at 1, but not at 7 dpi. †Indicates SCI-saline vs. SCI-morphine,  $p < 0.05$ .

studied in female rats. These SCIs caused modest mechanical allodynia and thermal hyperalgesia, so another SCI model was assessed. Next, a T9 SCI contusion (150 kDyn, 1 s dwell) was tested in female and male rats. This caused thermal hyperalgesia in females and males, and mechanical allodynia in males. An acute course of morphine did not exacerbate later chronic pain in this model. A unilateral T13 SCI (100 kDyn, 1 s dwell) was tested next in male rats. This caused modest mechanical allodynia, but not thermal hyperalgesia, in both hindpaws. Again, acute morphine had little effect on later chronic pain elicited by unilateral contusion SCI. Morphine efficacy was confirmed by assessing acute analgesia, and tolerance to morphine over 7 d. Thus, we identified useful and clinically relevant rat SCI models for use in females and males. Our data suggest that acute morphine (using this dosing regimen) has limited effects on later SCI pain in these models.

The SCI models used in this study were chosen based on previous research. Most of these studies had a different scope, which included fewer behavioral timepoints, rats of only one sex, and/or only one SCI model. Here, we present a comparison of different SCI models in a single study. Previous studies showed that a T9 contusion SCI (no dwell) elicits long-lasting mechanical allodynia (Detloff et al., 2008); however, this was not observed to the same degree in our study. This could be due to using a different spinal cord impactor or using different methods for measuring mechanical thresholds. Similarly, in our study, the unilateral T13 SCI did not elicit robust, long-lasting pain. This model was adapted from a cervical unilateral SCI model, which consistently elicits neuropathic pain in the forepaws (representing at-level pain) and hindpaws (below-level pain) (Detloff et al., 2013; Falnikar et al., 2016; Putatunda et al., 2014; Watson et al., 2014). Thus, there could be level-dependent differences in SCI-elicited; that is, different post-SCI mechanisms depending on spinal segment. The thoracic contusion SCI model that most effectively elicited neuropathic pain in this study was the T9 contusion SCI with 1-second dwell. This model has been used previously to study below-level mechanical allodynia in male rats (Carlton et al., 2009; Crown et al., 2008; Gwak et al., 2012). Here, we confirm these previous findings, and extend them by examining both mechanical and heat nociceptive thresholds and by studying female rats.

Differential neuropathology in these SCI models likely underlies their disparate effects on chronic pain. After midline SCI, thermal hyperalgesia (in females and males) and mechanical allodynia (particularly in males) were observed. Past studies have linked SCI to remote neuroinflammation, which likely exacerbates pain. This has been observed with the T9 SCI (0 s dwell) (Detloff et al., 2008) and with the T9 SCI (1 s dwell) (Gwak et al., 2012). Thus, it is likely that thoracic SCI causes distant activation in the lumbar spinal cord of microglia and astrocytes, which produce cytokines and other pain modulators to heighten pain sensitivity (Grace et al., 2014). Using the unilateral T13 SCI, mechanical allodynia (but not thermal hyperalgesia) was elicited on both ipsilateral and contralateral hindpaws. This may have been slightly more pronounced on the contralateral hindpaw, which is the side that sends noxious thermal and mechanical hindpaw information through the ipsilateral spinothalamic tract (Palecek et al., 1992; Vandenberg et al., 2014). Thus, it is possible that damage to the ipsilateral spinothalamic tract renders these neurons sensitized to nociceptive input (concomitant with predicted unilateral SCI-elicited neuroinflammatory changes) (Wasner et al., 2008; Watson et al., 2014).

There were sex differences in SCI-elicited neuropathic pain. Male rats with T9 SCI (1 s dwell) displayed both mechanical allodynia and thermal hyperalgesia, whereas female rats with this T9 SCI exhibited thermal hyperalgesia, but not mechanical allodynia. There are known sex differences in neuropathic pain (Berkley, 1997); the etiology of sex differences in pain could be due to experiential and/or neurobiological differences (Mogil, 2012). In humans with SCI, females are more likely to report pain as a major concern (Lo et al., 2016). One study examined sex differences in male and female rats with photochemical

(laser) SCI at T13; females showed higher mechanical sensitivity than males (Dominguez et al., 2012). In contrast, another study showed that after severe T8 contusion SCI, more male rats (78%) than female rats (26%) exhibited at-level mechanical hypersensitivity (Hubscher et al., 2010). Thus, there is some variability in SCI pain observed between sexes, likely due to specific injury model and pain tests used. In rat models of peripheral nerve injury-elicited pain, female rats typically show worse and/or extended neuropathic pain symptoms (Coyle et al., 1995; LaCroix-Fralish et al., 2006; Tall et al., 2001). Given that SCI rats of both sexes in our study exhibited thermal hyperalgesia, but only males had mechanical allodynia, it seems that distinct modalities can be differentially affected in each sex. Neuropathic pain in male and female rodents can have divergent underlying cellular mechanisms (Sorge et al., 2015; Taves et al., 2016), which could explain why there is variability in pain intensity using different models. As in all biology fields, it is clear that more SCI pain studies should assess sex differences (Clayton and Collins, 2014; Kim et al., 2010).

One limitation of this study was that two of the three pain models tested were only examined in one sex; however, there was a logical progression underlying study design. Our first study (T9 midline 150 kDyn, 0 s dwell) was performed in female rats only. Since minimal neuropathic pain symptoms were observed in this model, another promising SCI pain model was tested (rather than using additional rats to test the first not-so-promising pain model on males). The second model (T9 midline 150 kDyn, 1 s dwell) caused SCI pain: both females and males showed significant SCI-elicited neuropathic pain (males showed heat and mechanical hypersensitivity; females showed heat hypersensitivity). It was expected that acute morphine would worsen later chronic pain; however, this was not observed. To establish whether acute morphine-elicited pain occurred in another SCI model, we adapted a promising cervical unilateral SCI to the T13 level (T13 unilateral 100 kDyn, 1 s dwell). Males were used, since they showed more robust pain symptoms in our second model. This SCI caused mechanical (but not heat) hypersensitivity on the contralateral and ipsilateral hindpaws; however, no acute morphine-elicited chronic pain exacerbation was observed after unilateral T13 SCI. Since male rats with T13 unilateral SCI did not exhibit acute morphine-exacerbated chronic pain, females were not tested in this model. In addition, some of the sham rats exhibited hypersensitivity over time. It is possible that the rats were more sensitive from being in the behavioral apparatus for several sessions, that the behavioral room temperature/pressure fluctuations influenced sensory thresholds (Sato et al., 1999; Sato et al., 2000), or that they were simply responding more quickly after having been tested for several weeks. Regardless, our data highlight the importance of including sham rat controls (instead of simply comparing to pre-injury baseline values).

Opioids can be used for management of post-SCI neuropathic pain, and there exists some evidence of opioid efficacy in post-SCI pain (Attal et al., 2002; Eide et al., 1995; Norrbrink and Lundeberg, 2009; Siddall, 2009; Siddall and Loeser, 2001; Teasell et al., 2010). However, opioids are not an ideal long-term solution to neuropathic pain (Siddall, 2009). First, opioids must be injected, limiting their usefulness. Second, opioids cause constipation and tolerance (Cahill et al., 2016; Camilleri et al., 2014; Kim et al., 2014). Finally, these molecules can facilitate opioid-induced hyperalgesia (Yi and Pryzbylowski, 2015) or later chronic pain (Johnston et al., 2004). Indeed, a recent study showed that intravenous morphine (escalating dose beginning at 1 dpi for 7 days) elicited modest tactile (but not thermal) hypersensitivity after contusion SCI (Hook et al., 2017), suggesting that post-SCI opioid-induced hyperalgesia can be modeled in rats.

In this study, we hypothesized that post-SCI morphine treatment would worsen later chronic pain. Although morphine analgesia and tolerance were observed, there was only sparse evidence of morphine-exacerbated later neuropathic pain. This was somewhat unexpected, given that our group previously established that morphine can worsen later pain symptoms for many weeks in models of dorsal root avulsion (Ellis et al., 2016) and peripheral nerve injury (Grace et al., 2016;



Hutchinson et al., 2008; Johnston et al., 2004; Loram et al., 2012). It is possible that different pathological mechanisms underlie pain in these models, and/or that different dosing strategies elicit distinct responses. Our dosing (5 mg/kg s.c., 2× per day from 1–8 dpi) was used to increase morphine levels across the day (rather than at a single timepoint; a single higher dose would only be effective for 3–4 h once per day and may not be as clinically relevant) and was based on our group's recent study (Grace et al., 2016). However, other studies used different regimens (e.g., 10 mg/kg s.c., 1× per day from 1–8 dpi (Ellis et al., 2016); 5 mg/kg s.c., 2× per day from 1–6 dpi (Loram et al., 2012); 15 µg intrathecal morphine (Hutchinson et al., 2008)). Morphine may also slightly worsen pain and locomotor outcomes after contusion SCI (Hook et al., 2007); however, this study used different contusion SCI models (moderate T13 contusion with MASCIS device) and dosing regimens (single intraperitoneal morphine dose at 10 or 20 mg/kg) than used in the current study. Another article showed that acute intrathecal morphine (0, 30, or 90 µg at 1 dpi) treatment after moderate T13 contusion SCI (MASCIS device) worsened locomotor outcomes, but not chronic pain symptoms (Hook et al., 2009) (similar pain-enhancing effects to what was observed here, with significant morphine-elicited pain at 7 d post-unilateral SCI, but not at other times). Thus, acute morphine does not always worsen later chronic pain symptoms. Similarly, opioids used to manage pain in humans may not always worsen later chronic pain (Eisenberg et al., 2015). Regardless, current SCI pain management strategies suggest that anticonvulsants (gabapentin, pregabalin) could provide more effective and long-lasting relief than opioids (Finnerup et al., 2015; Mehta et al., 2016a; Mehta et al., 2016b; Teasell et al., 2010).

We defined a clinically relevant thoracic contusion SCI model of neuropathic pain. Of course, given the variability in type, severity, and location of human SCIs, it is important to understand pain after different types of injuries. For instance, studying unilateral SCI in the cervical region has proven useful for understanding at-level pain, recovery of fine motor function, and axon plasticity. Thus, thoracic and cervical SCI studies could be complementary and informative. Pain researchers are increasingly recognizing the value of studying non-evoked pain measures (Burma et al., 2016; Kramer et al., 2016). Potential spontaneous affective measures of rodent pain include the mouse grimace scale (Langford et al., 2010), conditioned place preference (Little et al., 2015), burrowing/nesting frequency (Andrews et al., 2012; Jirkof et al., 2010), home cage monitoring for abnormal behaviors (Goulding et al., 2008; Houghton et al., 1997) and the sucrose preference test (Fonken et al., 2016; Fonken et al., 2012; Luedtke et al., 2014). Although there are obvious confounds for SCI rats with pain-related tasks involving mobility (including whether a treatment affects both pain symptoms and mobility in parallel), future studies could further examine how SCI affects non-evoked measures of neuropathic pain.

## 5. Conclusions

Rat models of SCI-elicited neuropathic pain remain understudied. Here, we explored three separate clinically relevant thoracic contusion SCI models, and tested the most effective pain model in both females and males. Our data suggest that sex differences in SCI pain exist; both females and males with SCI experienced persistent thermal hyperalgesia, whereas only males presented with mechanical allodynia. Our study also established whether acute morphine treatment worsened later SCI-elicited neuropathic pain. In the SCI models examined, we saw little evidence of morphine-exacerbated neuropathic pain. Together, our data identify an effective model for studying SCI-elicited pain, and highlight the importance of studying sex differences in SCI pain. Future studies could use these models to understand the mechanisms underlying post-SCI pain, and to identify new potential treatments to relieve pain after SCI.

## Acknowledgements

The authors would like to thank Dr. Laura Fonken for critically reviewing this manuscript, and the animal care staff at University of Colorado Boulder for their expert assistance. This work was supported by the Paralyzed Veterans of America (LRW: #3004) and by the United States Department of Defense (LRW: W81XWH-13-1-0277/SC120066). Additional support was provided by the Craig H. Neilsen Foundation (SFM: 382497) and the Wings for Life Foundation (LRW/ADG; Project number 139). The authors have no conflicts of interest related to this research.

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