Sex differences in pain: Spinal cord injury in female and male mice elicits neuropathic pain symptoms

Sydney E. Lee*[1,2], Emily K. Greenough*[1,2], Paul Oancea*[1,2], Ashley R. Scheinfeld[1,2], Apsaline M. Douglas[1,2], Andrew D. Gaudet[1,2]

[1] Department of Psychology, College of Liberal Arts
[2] Department of Neurology, Dell Medical School
University of Texas at Austin

* Authors contributed equally.

Address:
108 E. Dean Keeton Street, Stop A8000
Seay Building, Room 4.208
Austin, TX 78712

Conflict of interest: The authors declare no competing financial interests.

Acknowledgements:
We thank the Animal Resources Center (ARC) husbandry staff at the Health Discovery Building for excellent animal care. Partial support was provided by University of Texas at Austin start-up funds (ADG), the Wings for Life Foundation (ADG) and Mission Connect, a program of the TIRR Foundation (ADG).

Abstract

Spinal cord injury (SCI) in humans frequently causes intractable chronic pain. Females are susceptible to worsened pain compared to males, and females may show higher pain prevalence after SCI in humans. Despite this difference in clinical prevalence of SCI pain, few preclinical studies have systematically studied in rodents sex differences in SCI-elicited pain symptoms. Here, we leverage data from a large cohort of mice to test whether contusion SCI consistently causes pain symptoms in mice, and to establish whether female (vs. male) mice display heightened hypersensitivity after SCI. Mechanical and heat sensory thresholds were assessed using the von Frey test and Hargreaves test, respectively. In an initial experiment, female mice receiving moderate 60 kDyn SCI or moderate-to-severe 75 kDyn SCI both exhibited mechanical and heat pain symptoms compared to sham controls. 75 kDyn SCI caused excess motor deficits that confounded defining pain sensitivity at acute times, so the moderate SCI force was used for subsequent experiments. Next, adult female and male C57BL6/J mice received sham surgery or T9 contusion SCI. Comparing female to male mice after SCI, we reveal that mice of both sexes displayed mechanical and heat hypersensitivity compared to sham controls, from acute-to-chronic post-injury times. Females had amplified SCI-elicited hypersensitivity compared to males. Our data suggest that thoracic contusion SCI elicits consistent and persistent pain symptoms, which are more intense in female vs. male mice. These results have important implications for uncovering sex-specific mechanisms and therapeutic targets to ameliorate neuropathic pain after SCI.

Introduction

Neuropathic pain arises as a secondary complication in 65-80% of patients with spinal cord injury (SCI) (Siddall et al., 1999; Burke et al., 2017). Neuropathic pain is pain caused by damage to the nervous system and presents as a range of pain responses, including allodynia (pain resulting from non-painful stimuli), hyperalgesia, and spontaneous maladaptive pain (Shiao and Lee-Kubli, 2018). Unfortunately, clinical management of neuropathic pain is difficult, and the underlying mechanisms remain poorly understood (Anderson, 2004; Collinger et al., 2013). Accordingly, rodent models of SCI pain have been developed to discover mechanisms and potential therapeutic targets. For instance, midline thoracic contusion SCI in rats or mice causes below-level pain in the hindpaws (Crown et al., 2008; Detloff et al., 2008; Gaudet et al., 2017; Gaudet et al., 2021). Unilateral contusion SCI in the cervical spinal cord of rats or mice also drives neuropathic pain symptoms – both at and below the level of injury (Detloff et al., 2013; Putatunda et al., 2014; Dietz et al., 2022). Thus, effective SCI models that cause chronic pain have been established. Despite this, most SCI studies have not considered sex as a biological variable, so it remains unclear the extent that SCI pain symptoms are experienced by females vs. males.

Women are ~25% more susceptible to chronic pain than men (Munce and Stewart, 2007), and females have heightened sensitivities to pain in both humans and rodents (Mogil, 2012; Bartley and Fillingim, 2013). Indeed, accumulating evidence suggests that females with SCI are more likely to experience chronic pain – including increased prevalence of self-reported pain (Norrbrink Budh et al., 2003; Andresen et al., 2016) and pain that impairs ability to work (Cardenas et al., 2004). Further, neuropathic pain behavioral outcomes can have sex-specific underlying physiologic mechanisms (Sorge et al., 2015; Rosen et al., 2017), underscoring the importance of studying pain caused by SCI in both sexes. A few studies have assessed sex differences in preclinical models of SCI-elicited pain; however, research using both sexes has

shown conflicting results, with some reporting significant sex differences in pain after SCI (Dominguez et al., 2012; Gaudet et al., 2017), and some showing minimal to no apparent sex differences (Walker et al., 2019; McFarlane et al., 2020). Given the widespread use of rodents to unravel neurobiological mechanisms of pain, there is a need to better understand the extent that clinically relevant SCI elicits pain in mice of both sexes.

Here, we aim to establish whether thoracic contusion SCI causes acute-to-chronic neuropathic pain symptoms. In addition, we test the hypothesis that female mice will exhibit exacerbated SCI-elicited neuropathic pain symptoms compared to males. Mice were tested for mechanical allodynia and heat hyperalgesia prior to surgery, and weekly after sham or SCI surgery to 28 days post-operative (dpo). We collate a uniquely large dataset that includes mice of both sexes that receive sham or SCI surgery, which illuminates the extent and duration of pain symptoms at acute-to-chronic times after SCI. We reveal that SCI elicits neuropathic pain symptoms in mice of both sexes, and that females are predisposed to worsened mechanical and heat pain symptoms after SCI.

Materials and Methods

Surgery and Animal Care: All housing, surgery, and postoperative care adhered to guidelines set by The University of Texas at Austin Institutional Animal Care and Use Committee. Adult female and male C57BL/6J mice (8-12 weeks old; Jackson Laboratory, Stock 000664) were housed in pairs. Food and filtered tap water were provided *ad libitum,* and all animals were maintained on a 12:12 light/dark cycle.

All animals received buprenorphine hydrochloride (0.075 mg/kg; MWI Animal Health, Cat. 060969) analgesic immediately prior to surgery (to reduce acute post-surgery discomfort). Additional daily post-surgery analgesic doses were withheld to limit potential confounding effects on sensitivity-related behavioral tests. A T9 laminectomy was performed on both sham and SCI mice. Next, mice in the SCI group were subjected to contusion SCI using the Infinite Horizon impactor (Precision Systems and Instrumentation) (severity and forces defined below) (Gaudet et al., 2016; Gaudet et al., 2021). All surgeries occurred between Zeitgeber time (ZT) 2-10 (lights on at ZT0 and off at ZT12). Incisions were closed using sutures and wound clips. Post-operative care included daily subcutaneous injections of Ringer's solution (2, 2, 1, 1, 1 mL on the first 5 days post-operative (dpo), respectively; for both sham and SCI mice) to prevent dehydration, and manual voiding of bladders twice daily (shams handled similarly).

Experiments and mouse numbers: Experiment 1 sought to define an appropriate T9 SCI contusion force (midline injury; 0 s dwell time) for studying neuropathic pain symptoms. Female mice received sham surgery (n=10), 60 kDyn SCI (n=9; force: 62.3 ± 0.5 kDyn; displacement: 597 ± 35 μm), or 75 kDyn SCI (n=10; force: 76.7 ± 0.8 kDyn; displacement: 729 ± 36 μm).

Experiment 2 was designed to assess sex differences in neuropathic pain symptoms in female and male mice following moderate T9 SCI (midline injury; 0 s dwell). This experiment combined several independent smaller experiments all on a wildtype C57BL6/J strain. All experiments forming this study used a balanced combination of female/male mice with sham and SCI surgery. Mice received sham surgery (female-sham: n=44; male sham: n=48) or 60 kDyn T9 contusion SCI (female-SCI: n=32, force: 62.8 ± 0.4 kDyn; displacement: 500 ± 10 μm) (male-SCI: n=34, force: 63.0 ± 0.6 kDyn; displacement: 488 ± 9 μm). Mice were prospectively excluded from analysis if the Infinite Horizon force/displacement curves suggested a slip or bone hit; if SCI displacement was 100 μm higher or lower than the mean displacement; if mice died during surgery or prior to the experimental endpoint due to ill health; or if the BMS score was unusually high at 1 dpo (BMS of 3 or greater). 9 female-SCI mice were excluded (6 due to high displacement, 1 due to low displacement, 2 died during surgery), and 11 male-SCI mice were excluded (2 high displacement, 1 low displacement, 3 improper displacement curve, 1 died during surgery, 4 died after surgery). All animals received post-operative care as described above.

Locomotor Testing: Hindlimb motor function was measured using the Basso Mouse Scale (BMS) scores and subscores for locomotor recovery prior to surgery and at 1, 3/4, 7, 10, 14, 21, and 28 dpo. Hindlimb recovery was scored on the BMS scale of 0 (complete paralysis) to 9 (normal motor function) by two observers who were blind to treatment conditions.

Neuropathic pain testing: Neuropathic pain testing was performed as previously described (Gaudet et al., 2017; Gaudet et al., 2021). Mice were acclimated to the von Frey and Hargreaves apparatus for two 40-60 min sessions, followed by two pre-surgery tests (average of the two pre-surgery scores are presented here). After surgery, sensory testing occurred

weekly. The values for the left and right hindlimb were averaged to calculate a single score for each animal at each timepoint. Testing occurred within 2 hours between ZT2-6. To control for potential confounds of having both sexes tested simultaneously, female and male mice were tested in separate sessions. Both sham and SCI mice from a single sex were tested in the same session. Researchers randomized the order of mice in testing chambers to intersperse and remain blind to the treatment group.

Von Frey test for mechanical allodynia: Mechanical sensory thresholds were assessed using the simplified up-down (SUDO) method (Bonin et al., 2014) of von Frey testing to minimize stress and time outside of the homecage. Mice were placed in plastic compartments suspended above a wire mesh (Ugo Basile grid platform, Stoelting 57816) and allowed to acclimate for 40-60 min prior to testing. During testing, von Frey filaments (Touch Test Sensory Evaluator, Stoelting 58011) were pressed against the center of the plantar surface of the hindpaw until the filament buckled and held for a maximum of 3 s. A positive response was recorded if the mouse withdrew their hindpaw or flinched in response to the pressure of the filament. In order to minimize animal stress and ensure accurate scoring, von Frey testing occurred the day prior to Hargreaves testing.

Hargreaves test for heat hyperalgesia: Heat sensory thresholds (thermal hyperalgesia) were assessed using the Hargreaves test (Ugo Basile Thermal Plantar test, Stoelting 55370). Mice were placed in a plastic box on top of a glass surface. An infrared heat source (intensity of 25) was applied to the center of the mouse's hindpaw, and the latency to respond (lifting their paw or flinching) was automatically recorded. Maximum response latency was 30 s; the heat source automatically shut off at this time. Testing on each mouse's right and left hindpaw was alternated (three tests per time point - total of 6 tests), and each animal was allowed 5 min recovery between each test to minimize sensitivity.

Statistics: Results were analyzed using two-way or three-way repeated measures ANOVA followed by Bonferroni *post hoc* tests where appropriate. Specific tests used are described in the text for each dataset. Data were graphed using GraphPad Prism. All statistical analysis was performed using SigmaPlot 14.0, and results with $p < 0.05$ were considered significant. All data are plotted as mean ± SEM.

Results

Moderate or moderate-to-severe T9 contusion SCI in female mice elicits below-level neuropathic pain symptoms

To establish an effective model for studying SCI-elicited neuropathic pain, mice received sham surgery (T9 laminectomy), moderate 60 kDyn T9 contusion SCI, or moderate-to-severe 75 kDyn SCI. For locomotor recovery, SCI at different forces caused graded deficits and recovery of locomotor function (BMS) (**Fig. 1a**) (surgery x dpo effect: $F_{10,125}$=50.88, $p < 0.001$; sham vs. 60 kDyn, $p < 0.001$ from 1-28 dpo; sham vs. 75 kDyn, $p < 0.001$ from 1-28 dpo). 60 kDyn mice showed higher BMS scores vs. 75 kDyn mice from 7 to 28 dpo ($p < 0.001$ at each timepoint); at 28 dpo, average 60 kDyn mouse BMS scores represent frequent steps and mostly/all coordinated stepping, whereas average 75 kDyn mouse scores represent occasional plantar stepping (28 dpo BMS scores: 60 kDyn, 6.0 ± 0.3; 75-kDyn, 3.8 ± 0.3).

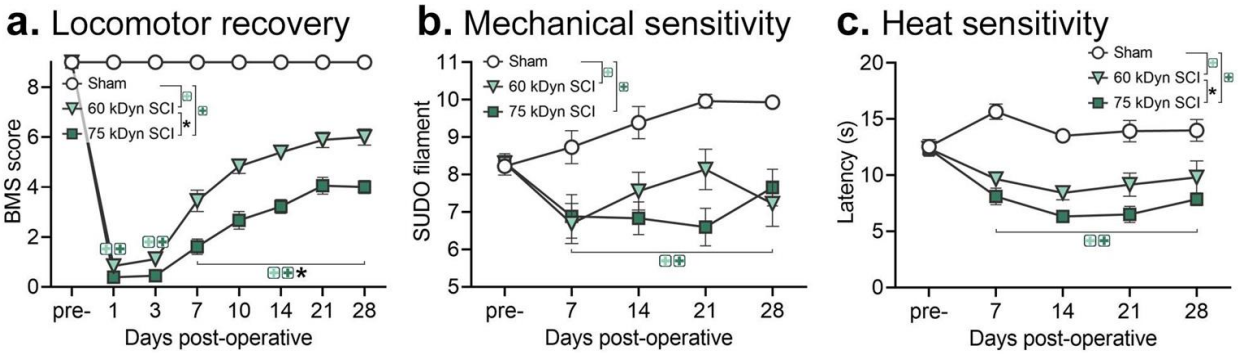

Figure 1. Moderate and moderate-to-severe T9 contusion SCI in female mice cause locomotor deficits and below-level neuropathic pain symptoms. **a.** BMS scores showing SCI-elicited hindlimb locomotor deficits after SCI. As expected, 75 kDyn SCI caused worse deficits than 60 kDyn SCI (from 7-28 dpo). **b.** SCI at both severities evoked mechanical hypersensitivity (von Frey test) from 7-28 dpo. **c.** SCI at both severities caused thermal hyperalgesia (Hargreaves test) from 7-28 dpo. **+** indicates p<0.05 between sham and SCI mice (color matched to force); * indicates p<0.05 between 60 kDyn and 75 kDyn SCI groups; ANOVA with Holm-Sidak *post-hoc* test.

SCI caused significant and persistent neuropathic pain symptoms. Mechanical allodynia was assessed using the von Frey test. Mice in both 60 and 75 kDyn groups exhibited mechanical hypersensitivity at every timepoint between 7 and 28 dpo (vs. sham mice; main effect of

surgery: $F_{2,78}$=15.54, $p < 0.001$; sham vs. 60 kDyn, $p < 0.05$ from 7-21 dpo and $p < 0.001$ at 28 dpo; sham vs. 75 kDyn, $p < 0.05$ at 7 dpo and $p < 0.001$ from 14-28 dpo) (**Fig. 1b**). There was no significant difference between 60 and 75 kDyn SCI mice in post-SCI mechanical thresholds ($p > 0.05$). Heat hyperalgesia was examined using the Hargreaves test. Both 60 and 75 kDyn SCI mice showed heat hypersensitivity at all times between 7 and 28 dpo (vs. sham mice; main effect of surgery: $F_{2,78}$=47.01, $p < 0.001$; sham vs. 60 kDyn, $p < 0.005$ from 7-28 dpo; sham vs. 75 kDyn, $p < 0.001$ from 1-28 dpo) (**Fig. 1c**). There was also a group effect of injury, with 75 kDyn mice showing overall increased sensitivity vs. 60 kDyn mice (Bonferroni *post-hoc*: $p < 0.05$). Although the 75 kDyn mice had increased heat hypersensitivity – which may suggest this would be a "better" pain model – these mice were also more prone to impaired plantar hindpaw placement at 7 and 14 dpo (e.g., see low BMS scores at these times). If the plantar paw surface was not exposed, testers had to stimulate the dorsal or side surfaces of the paw and nocifensive responses were not always as clear due to impaired sensorimotor function. Thus, given that 60 kDyn SCI caused comparable pain symptoms to 75 kDyn SCI – but with reduced confounding sensorimotor impairment – the moderate 60 kDyn SCI was chosen for further study of sex differences in SCI pain.

Moderate contusion SCI causes pain symptoms in mice of both sexes

Next, we used the 60 kDyn T9 contusion SCI validated above to define whether thoracic contusion SCI in mice consistently elicits pain symptoms. In addition, we aimed to assess whether SCI has differential effects on mechanical and heat sensitivity in female vs. male mice. Therefore, a comprehensive analysis of 100+ female and male mice from various studies in the lab were compiled; we reasoned that our large dataset would provide a robust foundation for revealing potential sex differences in SCI-elicited pain symptoms.

First, we evaluated locomotor recovery in female and male mice after SCI (**Fig. 2a-c**). Mice with

sham surgery displayed typical coordinated hindlimb locomotion. Mice with SCI displayed little hindlimb function at 1 dpo (slight hindlimb movement), which recovered to mostly consistent stepping by 28 dpo (Female-SCI vs. female-sham mice – surgery x dpo interaction: $F_{7,394}$=262.96, $p < 0.001$; male-SCI vs. male-sham mice – surgery x dpo interaction: $F_{7,515}$=244.59, $p < 0.001$) (both sexes – sham vs. SCI, $p < 0.001$ from 7-28 dpo). Females (vs. males) with SCI displayed worsened locomotor function at 4 and 7 dpo (two-way repeated measures ANOVA, $p < 0.05$); however, females with SCI ultimately plateaued at a similar level to males (no significant sex differences between 10-28 dpo).

Next, SCI-driven mechanical hypersensitivity was studied using the von Frey test (**Fig. 2d-f**). All groups had similar baseline pre-surgery mechanical thresholds. Mice with sham surgery showed consistently high mechanical thresholds that did not change much over time post-surgery. In contrast, mice with SCI exhibited decreased mechanical thresholds, which represents increased mechanical hypersensitivity (three-way ANOVA: dpo x sex x surgery interaction: $F_{4,612}$=2.49, $p < 0.05$). Indeed, mechanical hypersensitivity was observed in SCI mice across all post-injury times for females (**Fig. 2e**) and from 7-21 dpo for males (**Fig. 2f**), compared to sham controls (Female-SCI vs. female-sham mice – surgery x dpo interaction: $F_{4,277}$=11.65, $p < 0.001$; male-SCI vs. male-sham mice – surgery x dpo interaction: $F_{4,303}$=2.66, $p < 0.05$) (sham vs. SCI, $p < 0.001$ from 7-28 dpo (females) and from 7-21 dpo (males)).

To establish whether SCI in mice causes heat hypersensitivity, mice completed the Hargreaves test (**Fig. 3g-i**). SCI elicited significant heat hypersensitivity throughout the post-operative period; heat hypersensitivity was amplified in females vs. males (three-way ANOVA: dpo x surgery interaction, $p < 0.0001$; sex x surgery interaction, $p < 0.05$). Females with SCI had reduced latency to withdrawal due to heat at all post-surgery times compared to female-sham mice. Males also had SCI-elicited pain; however, the magnitude of SCI-elicited heat

hypersensitivity was reduced compared to females, and SCI-elicited heat pain symptoms presented on a subset of post-surgery days (7, 21, and 28 dpo – not 14 dpo) (Female-SCI vs. female-sham mice – surgery x dpo interaction: $F_{4,287}$=11.61, p < 0.001; male-SCI vs. male-sham

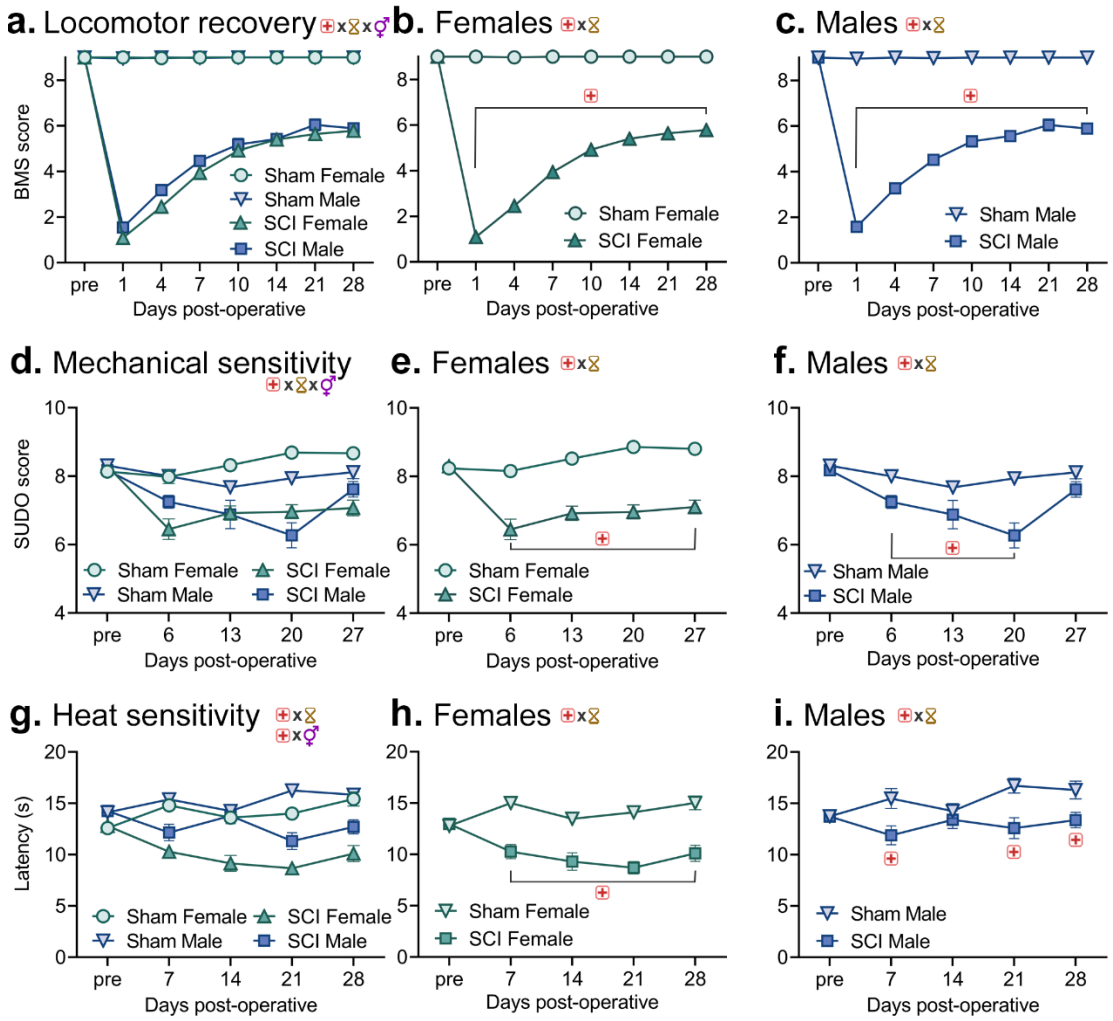

Figure 2. T9 thoracic contusion SCI elicits mechanical and heat hypersensitivity in mice of both sexes, with amplified pain symptoms in females compared to males. **(a-c)** Locomotor recovery after SCI or sham surgery in both sexes (a), females only (b), and males only (c). BMS scores reveal similar recovery curves after SCI between sexes, although females showed slightly delayed recovery at acute times. **(d-f)** Using the von Frey test, female and male mice exhibit SCI-elicited mechanical hypersensitivity compared to sham control mice. **(g-i)** The Hargreaves test reveals that female and male mice display heat hypersensitivity after SCI (vs. sham controls). + p<0.05 between sham and SCI mice; hourglass symbol indicates p<0.05 across timepoints; gender symbol indicates significant effect of sex; "x" symbol indicates significant interaction between respective groups. Symbols at the top of each panel indicate main effects. ANOVA (a,d,g: three-way; all other panels: two-way repeated measures) with Bonferroni post-hoc test.

mice – surgery x dpo interaction: $F_{4,253}$=5.19, $p < 0.001$) (sham vs. SCI, $p < 0.001$ from 7-28 dpo (females) and at 7, 21, and 28 dpo (males)). Therefore, our data suggest that moderate thoracic SCI in mice elicits significant below-level mechanical and heat hypersensitivity.

Spinal cord injury causes dynamic shifts in neuropathic pain symptoms

The size of our dataset enabled comparing variability between groups and sensitivity thresholds over time pre- and post-SCI (**Fig. 3**). For both mechanical and heat sensitivity, it is notable that pre-surgery thresholds were relatively consistent across mice and had low variability. In contrast, SCI induced higher variability, particularly during the acute-to-subacute times post-injury. For mechanical sensitivity, females with SCI exhibited higher sensitivity (vs. pre-SCI)

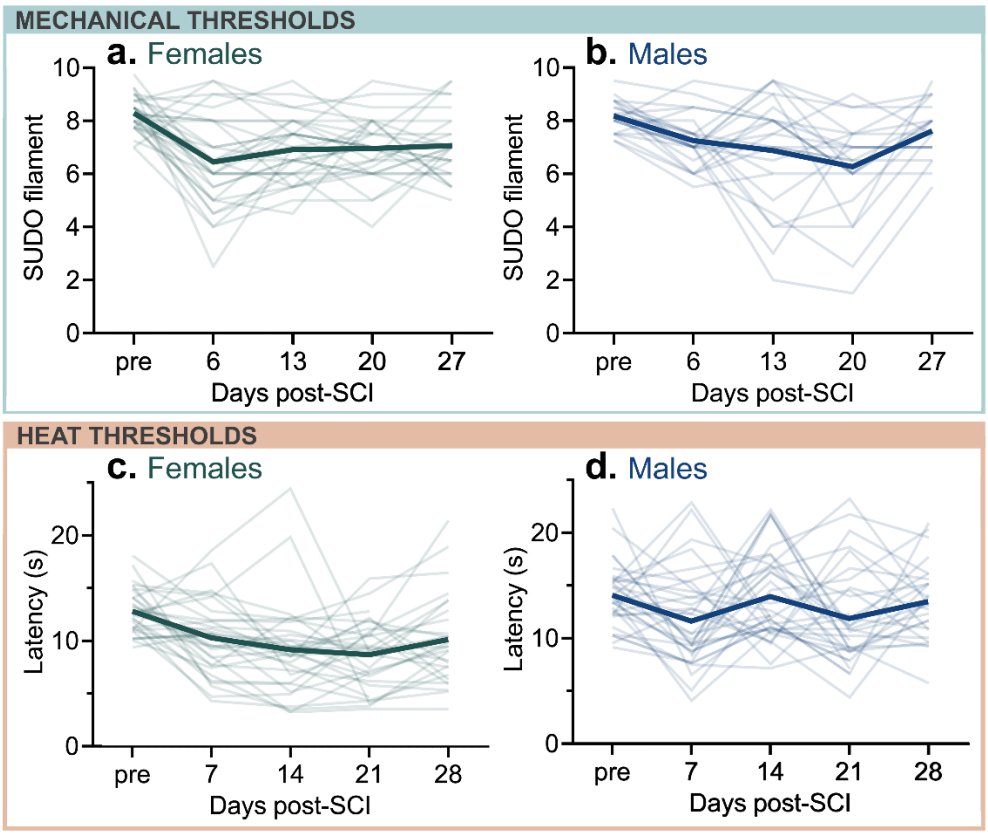

Figure 3. Mechanical and heat sensitivities compared to pre-surgery baseline highlight that SCI induces hypersensitivity in mice of both sexes. For each graph, the light trend lines represent one individual, and the dark trendline is the mean of all individuals. **(a,b)** SCI-elicited mechanical hypersensitivity in female (a) and male (b) mice. **(c,d)** SCI-elicited heat hypersensitivity in female (c) and male (d) mice.

and a small subset did not show hypersensitivity or were less responsive (**Fig. 3a**). Males after exhibited increased variability in mechanical sensitivity compared to females, with some SCI males developing intense hypersensitivity at 14-21 dpo (**Fig. 3b**). For heat stimulation, females with SCI again showed hypersensitivity (vs. pre-surgery) with relatively low variability (**Fig. 3c**). Some SCI females exhibited hyposensation that mostly resolved within 21 dpo. Males with SCI showed heat hypersensitivity, but the males showed increased post-SCI variability (**Fig. 3d**). This paradoxical combination of hypersensitivity vs. loss of sensation due to partial denervation may be driving the variability in the data after SCI.

SCI-driven mechanical pain symptoms are exacerbated in females compared to males

Next, we sought to directly compare pain symptoms in female vs. male mice. To enable comparing females vs. males, "difference scores" were calculated: sensitivity threshold values from SCI mice were subtracted from sham mouse values for each sex (i.e., sham scores minus SCI scores for both mechanical and heat thresholds) (**Fig. 4a**). Accordingly, SCI-elicited hypersensitivity is expressed as an increase on the graph. SCI caused mechanical hypersensitivity in both females and males compared to shams of the same sex. Females exhibited amplified SCI-elicited pain symptoms compared to males (main effect of sex ($F_{1,320}$=9.43, $p < 0.005$) and dpo ($F_{4,320}$=12.11, $p < 0.0001$); sex x dpo interaction; $p = 0.07$).

Comparing trends in individual mechanical sensitivity threshold values underscores the fact that SCI drives hypersensitivity (**Fig. 4b-e**). SCI elicited increased mechanical sensitivity at all timepoints. Females exhibited increased mechanical sensitivity at 6 dpo (two-way ANOVA: main effect of sex, $p < 0.005$; main effect of surgery, $p < 0.001$). At 27 dpo, females, but not males, displayed SCI-elicited mechanical hypersensitivity compared to sham controls (Two-way ANOVA: sex x surgery interaction: $F_{1,134}$=6.12, $p < 0.05$; female-sham vs. female-SCI, $p < 0.001$). Further, female-SCI mice had lower thresholds than male-SCI mice ($p < 0.005$).

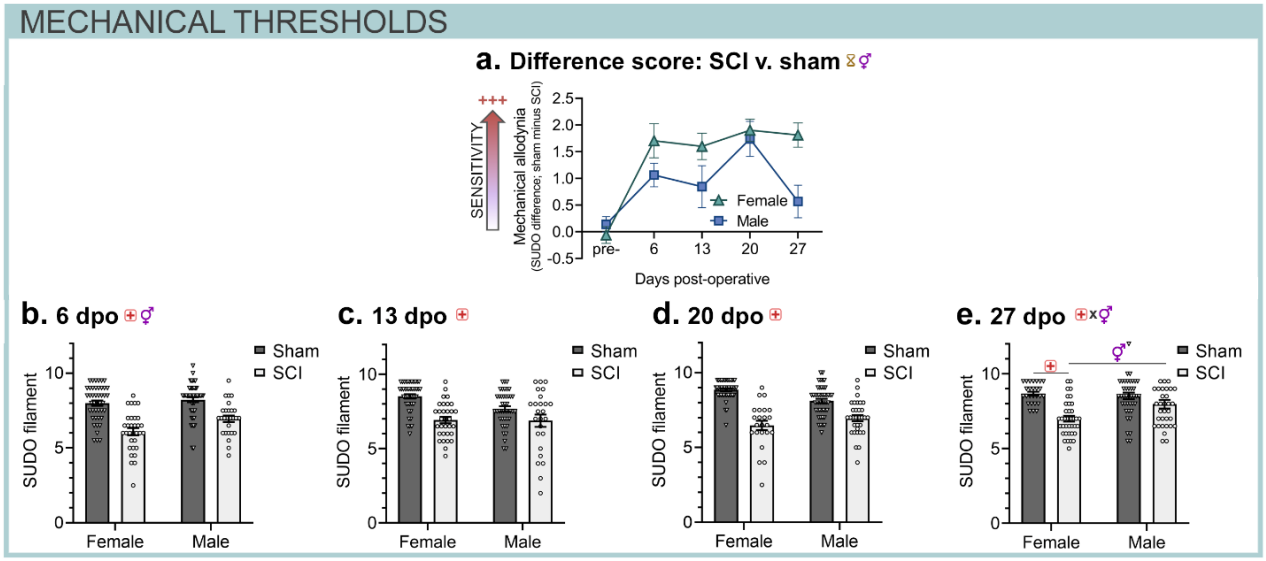

Figure 4. Difference scores and acute-to-chronic thresholds highlight the extent of SCI-elicited mechanical pain symptoms in females and males. **(a)** Difference scores in mechanical sensitivity: subtracting "sham minus SCI" SUDO von Frey scores for each sex shows that both female and male mice exhibit mechanical pain symptoms after SCI (increased pain = upwards on the y-axis). **(b-e)** SCI-driven mechanical allodynia in female and male mice. Individual von Frey scores at 6 dpo (b), 13 dpo (c), 20 dpo (d), and 27 dpo (e). Females exhibit increased SCI-elicited mechanical hypersensitivity compared to males. Hourglass symbol indicates p<0.05 across timepoints; gender symbol indicates significant effect of sex. Symbols at the top of each panel indicate main effects. Two-way repeated measures ANOVA with Bonferroni post-hoc test.

SCI-elicited heat hypersensitivity is worsened in females vs. males

SCI also induced heat hypersensitivity (**Fig. 5**). Differences in Hargreaves heat response latencies reveal that SCI drives heat pain symptoms in both female and male mice (compared to sham controls) (**Fig. 5a**) (main effect of sex ($F_{1,320}$=6.19, p < 0.05) and dpo ($F_{4,320}$=12.37, p < 0.0001); sex x dpo interaction; p = 0.14). However, there are some notable sex differences in pain behavior. Female mice with SCI exhibited early, consistent, and persistent heat hyperalgesia. Females had increased heat sensitivity compared to males. Male mice with SCI also show heat pain symptoms; however, they are not as consistent, with reduced hypersensitivity particularly at 14 dpo (**Fig. 5c**). At 14 dpo, females, but not males, exhibited

SCI-elicited heat hypersensitivity (Two-way ANOVA: sex x surgery interaction: $F_{1,142}$=6.16, p < 0.05; female-sham vs. female-SCI, p < 0.001). In addition, SCI-females had exacerbated hypersensitivity compared to SCI-males (p < 0.001). Overall, our data show that moderate contusion SCI causes below-level pain in female and male mice, and that females have amplified SCI-driven pain symptoms.

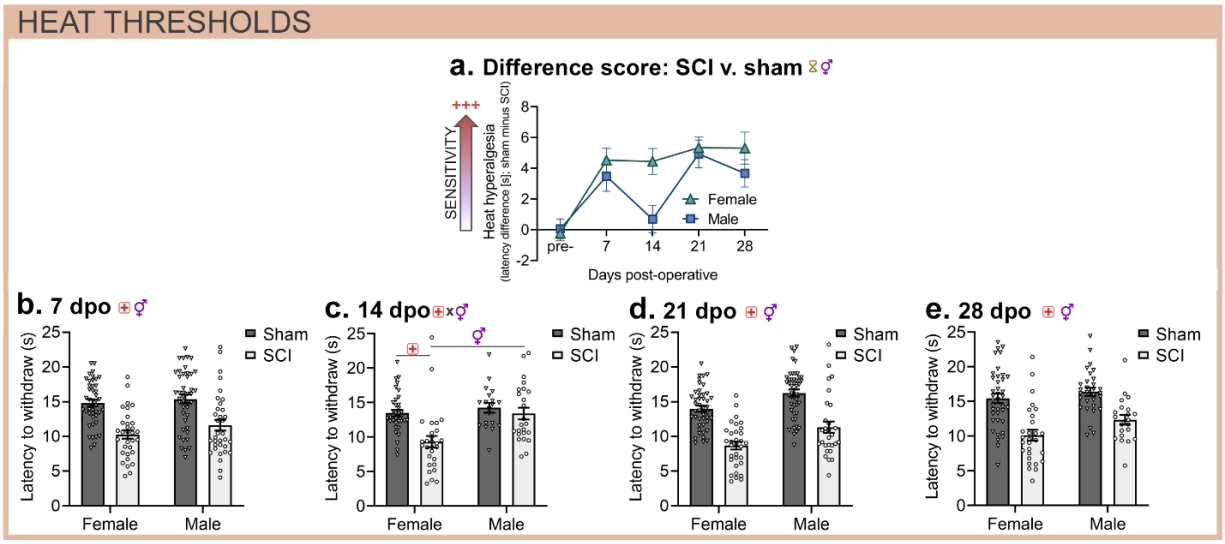

Figure 5. Sex similarities and differences in SCI-evoked heat hypersensitivity in mice: mice of both sexes display heat pain symptoms, and female (vs. male) mice exhibit exacerbated pain symptoms after SCI. **(a)** Difference scores in heat sensitivity: subtracting "sham minus SCI" withdrawal latencies for each sex reveals that both female and male mice exhibit heat pain after SCI (increased pain = upwards on the y-axis). **(b-e)** Individual Hargreaves withdrawal latencies at 7 dpo (b), 14 dpo (c), 21 dpo (d), and 28 dpo (e). Females (vs. males) generally display increased heat sensitivity, and show exacerbated SCI-elicited heat pain symptoms at 14 dpo. Hourglass symbol indicates p<0.05 across timepoints; gender symbol indicates significant effect of sex; + p<0.05 between sham and SCI mice; "x" symbol indicates significant interaction between respective groups. Symbols at the top of each panel indicate main effects. Two-way ANOVA with Bonferroni post-hoc test.

Discussion

Here, we leveraged our uniquely large dataset to illuminate whether contusion SCI in mice causes neuropathic pain symptoms, and whether SCI-elicited pain symptoms present differently in female vs. male mice. Our initial study revealed that two injury forces – a moderate 60 kDyn SCI and a moderate-to-severe 75 kDyn SCI – both caused mechanical and heat hypersensitivity in female mice. The moderate SCI caused fewer paradoxical motor deficits, so was used for subsequent studies. Moderate SCI in female and male mice caused mechanical allodynia and heat hyperalgesia. SCI increased hypersensitivity and also variability in pain thresholds within individuals. The extent and duration of SCI-mediated neuropathic pain was worsened in female compared to male mice. Overall, our data suggest that SCI in mice causes significant and long-lasting neuropathic pain symptoms; and that female mice are more susceptible to SCI-driven mechanical and heat hypersensitivity.

The purpose of our first experiment was to establish a model of SCI-elicited neuropathic pain. We found that mice with either 60 or 75 kDyn SCI had mechanical and heat pain symptoms, and the 75 kDyn SCI mice had modestly worsened pain symptoms compared to 60 kDyn SCI mice. This is an important validation study, because it shows that either force can be used experimentally to assess neuropathic pain and its modulation through genetic or therapeutic strategies. We did note that some 75 kDyn SCI mice had excess loss of sensorimotor function at 7 dpo, which masked or confounded establishing sensory thresholds at that timepoint. Further, some 75 kDyn SCI mice at 7 dpo did not always plantar place their hindpaw, so we modified our strategy and noted if we had to heat the dorsal or lateral aspects of the hindpaw (depending on what part of the paw was presented on the glass). Overall, these results corroborate with and extend previous results: we and others showed that thoracic contusion SCI at 60 kDyn (Wu et al., 2016) or 75 kDyn (Gensel et al., 2019; McFarlane et al., 2020; Gaudet et al., 2021) cause pain symptoms in mice, yet no known studies have systematically compared

pain that occurs with disparate SCI forces in the same experiment in mice (for force-pain relationships in rats, see Gaudet et al., 2017). Based on paradoxical sensorimotor impairments in 75 kDyn SCI mice at 7 dpo – an important time for recovery trajectory and epicenter inflammatory dynamics – we used 60 kDyn SCI for subsequent studies herein.

We found that moderate T9 contusion SCI caused mechanical and heat hypersensitivity in mice of both sexes from acute-to-chronic times post-injury. Similarly, others have explored neuropathic pain after SCI in rodents. Detloff et al. (2008) revealed that T8 SCI in rats elicited below-level mechanical allodynia similar to at-level allodynia caused peripheral nerve injury. Further, SCI-induced inflammatory activation in the lumbar spinal cord correlated with neuropathic pain symptoms. Carlton et al. (2009) showed that T10 SCI in rats caused above-level mechanical and heat hypersensitivity, which co-occurred with heightened glial reactivity in the thoracic and cervical spinal cord. In accordance, SCI causes neuropathic pain symptoms in mice, which can be dampened using anti-inflammatory modulators (thoracic SCI: Wu et al., 2016; McFarlane et al., 2020; Gaudet et al., 2021); cervical SCI: Brown et al., 2021; Dietz et al., 2022). Others have divided subjects into cohorts that exhibit pain or do not exhibit pain, then analyzed these cohorts separately (Nesic et al., 2005; Boroujerdi et al., 2011; Chhaya et al., 2019; Dietz et al., 2022). We and others find that this practice is unnecessary using the thoracic contusion injury model in mice; the SCI forces used herein cause significant and reproducible pain symptoms that can be modulated by predictable manipulations (e.g., anti-inflammatory strategies; Gensel et al., 2019; Gaudet et al., 2021).

We revealed that T9 contusion SCI in mice of both sexes caused pain symptoms, which were magnified in females. Indeed, females had worsened SCI-driven mechanical and heat hypersensitivity compared to males. Further, males with SCI (compared to male-sham mice) showed increased hypersensitivity at most timepoints, but not all timepoints after SCI. Others

have shown mixed results related to sex differences after SCI. SCI using different models in rats can have divergent sex-specific outcomes (Dominguez et al., 2012; Gaudet et al., 2017). In mice, McFarlane et al. (2020) assessed mechanical allodynia and heat hyperalgesia once every two weeks post-surgery – they found that T9 75 kDyn SCI caused pain symptoms in mice of both sexes to 6 weeks post-SCI, but there were no significant differences between sexes. Interestingly, females showed slightly (but not significantly) increased mechanical hypersensitivity in both von Frey and Hargreaves tests, and 6-9 mice were used per group (McFarlane et al., 2020). This differed from our study in that we used large cohorts of mice, a moderate 60 kDyn injury force, and tested thresholds weekly after SCI. There could also be slight differences across labs due to surgeon technique, the behavioral tester's experience and technique, and even the gender of the behaviorist (Sorge et al., 2014). Overall, our large cohort, more moderate SCI model, and weekly sensitivity testing likely enabled us to illuminate that female mice exhibit exacerbated SCI-elicited pain symptoms.

Even similar pain outcomes between sexes can have differential underlying cellular and physiologic mechanisms. Peripheral nerve injury in mice causes mechanical allodynia in mice of both sexes. Some studies have revealed sexually divergent cellular drivers of injury-induced pain, suggesting that pain in males is mediated by microglia, whereas pain in females is caused mainly by adaptive immune cells (Sorge et al., 2015; Fernandez-Zafra et al., 2019; Tawfik et al., 2020). Other studies suggest that microglia are required for neuropathic pain induction in both sexes (Peng et al., 2016), or facilitate pain but on sex-specific post-injury timelines (delayed microglial response in females) (Huck et al., 2022). Neuropathic pain can also be elicited by altered excitability of spinal cord circuits via altered dorsal horn neuron excitability or inhibition; dampened descending inhibition; detrimental shifts in excitation-inhibition balance; and altered expression of neuromodulators and immunomodulators (Hulsebosch et al., 2009; Brown et al., 2022). Although sex-specific mechanisms causing SCI pain remain understudied, sex

differences in hormones and inflammatory responses after SCI could underpin distinct mechanisms (Stewart et al., 2021; Stewart et al., 2022). Overall, this highlights the importance of incorporating subjects of both sexes throughout all studies (Shansky, 2019; Stewart et al., 2020; Shansky and Murphy, 2021): if no significant sex differences exist then results from both sexes can be combined for analysis, whereas if sex differences become apparent they can be studied further. Therefore, future studies should consistently include both sexes and will further crystallize our understanding of sex-specific mechanisms that underlie neuropathic pain, which could have crucial implications for development of targeted analgesic therapies.

Our study provides an extensive exploration of how SCI in mice of both sexes affects evoked pain symptoms. Although these acute-to-chronic measures of pain-like behavior aid in identifying relevant mechanisms underlying complex chronic pain conditions, tests that rely on reflexive response do not fully capture the affective components of the pain experience (Kramer et al., 2017). These straightforward tests have limits in their utility to identify mechanisms underlying the human neuropathic pain experience. Recently, researchers have been working to develop relevant pain-like behavioral assays that incorporate affective components of the pain experience. Despite their efforts, developing appropriate rodent models of affective pain-like behavior has proven difficult (Burma et al., 2017).

Two promising strategies include incorporating conflict tests, which pair a pain-inducing stimulus against a conflicting stimulus, and studying signs of spontaneous pain behavior (see reviews: Burma et al., 2017; Kramer et al., 2017). Conflict tests can be used to evaluate differing motivational states through the introduction of approach-avoidance situations. The mechanical-conflict avoidance paradigm is a recently developed conflict test used to assess pain behavior in rats (Chhaya et al., 2019; Gaffney et al., 2022). The apparatus consists of a long rectangular runway connecting a light chamber to a dark chamber. This connecting runway is equipped with

small mechanical spikes that produce mechanical sensitivity when crossed. Conditioned place preference has also been used to reveal pain behavior in rats (Yang et al., 2014). Regarding rodent spontaneous pain, one recently developed assay is the mouse grimace scale. The mouse grimace scale was developed to assess mouse pain behavior through differing mouse facial expressions (Langford et al., 2010; Mogil et al., 2020), including after SCI (Wu et al., 2016; Schneider et al., 2017; Heinsinger et al., 2020). Pain-like behavior is assessed using machine learning to identify characteristic orbital tightening, cheek bulge, nose bulge, and whisker position; however, its effectiveness may be limited by animals' tendency to hide behaviors that signal pain (Burma et al., 2017). Another challenge with assessing SCI-driven pain in particular is that the injury itself simultaneously affects sensory function (paradoxical loss of sensation and/or pain) and motor function, which can confound interpreting results of promising pain-related tests that assume similar motor function across mice/groups. Overall, conflict tests and spontaneous pain measures could complement reflexive pain assays in future studies to better understand the complex mechanisms underlying pain behavior in chronic pain conditions.

Conclusions and future directions

We employed a commonly used, clinically relevant contusion SCI model to establish whether SCI elicits neuropathic pain symptoms in female and/or male mice. We found that both moderate and moderate-to-severe SCI in mice caused neuropathic pain symptoms. Further, our data suggest that midline thoracic SCI in mice causes persistent neuropathic pain symptoms in mice of both sexes, and that females are predisposed to worsened sensitivity to mechanical and heat pain. Given the high prevalence of pain after SCI in humans, there is a need to develop and validate rodent models of SCI-induced neuropathic pain. Our study provides a crucial foundation for preclinical research related to sex and SCI; future studies should explore whether SCI-elicited pain in females vs. males is due to similar or distinct cellular mechanisms. Future

preclinical SCI studies could combine complementary nociceptive tests, validated for SCI here, with newer but more complex strategies that incorporate the affective aspect of the pain experience. Development of clinically relevant models and tests of SCI-elicited pain is critical to identify new therapeutic targets for quenching chronic pain.

References

Anderson KD (2004) Targeting recovery: priorities of the spinal cord-injured population. *Journal of Neurotrauma* 21:1371-1383.

Andresen SR, Biering-Sørensen F, Hagen EM, Nielsen JF, Bach FW, Finnerup NB (2016) Pain, spasticity and quality of life in individuals with traumatic spinal cord injury in Denmark. *Spinal Cord* 54:973-979.

Bartley EJ, Fillingim RB (2013) Sex differences in pain: a brief review of clinical and experimental findings. *British Journal of Anaesthesia* 111:52-58.

Bonin RP, Bories C, De Koninck Y (2014) A simplified up-down method (SUDO) for measuring mechanical nociception in rodents using von Frey filaments. *Molecular Pain* 10:26.

Boroujerdi A, Zeng J, Sharp K, Kim D, Steward O, Luo DZ (2011) Calcium channel alpha-2-delta-1 protein upregulation in dorsal spinal cord mediates spinal cord injury-induced neuropathic pain states. *Pain* 152:649-655.

Brown EV, Malik AF, Moese ER, McElroy AF, Lepore AC (2022) Differential Activation of Pain Circuitry Neuron Populations in a Mouse Model of Spinal Cord Injury-Induced Neuropathic Pain. *The Journal of Neuroscience* 42:3271-3289.

Brown EV, Falnikar A, Heinsinger N, Cheng L, Andrews CE, DeMarco M, Lepore AC (2021) Cervical spinal cord injury-induced neuropathic pain in male mice is associated with a persistent pro-inflammatory macrophage/microglial response in the superficial dorsal horn. *Experimental Neurology* 343:113757.

Burke D, Fullen BM, Stokes D, Lennon O (2017) Neuropathic pain prevalence following spinal cord injury: A systematic review and meta-analysis. *European Journal of Pain* 21:29-44.

Burma NE, Leduc-Pessah H, Fan CY, Trang T (2017) Animal models of chronic pain: Advances and challenges for clinical translation. *Journal of Neuroscience Research* 95:1242-1256.

Cardenas DD, Bryce TN, Shem K, Richards JS, Elhefni H (2004) Gender and minority differences in the pain experience of people with spinal cord injury. *Archives of Physical Medicine and Rehabilitation* 85:1774-1781.

Carlton SM, Du J, Tan HY, Nesic O, Hargett GL, Bopp AC, Yamani A, Lin Q, Willis WD, Hulsebosch CE (2009) Peripheral and central sensitization in remote spinal cord regions contribute to central neuropathic pain after spinal cord injury. *Pain* 147:265-276.

Chhaya SJ, Quiros-Molina D, Tamashiro-Orrego AD, Houlé JD, Detloff MR (2019) Exercise-Induced Changes to the Macrophage Response in the Dorsal Root Ganglia Prevent Neuropathic Pain after Spinal Cord Injury. *Journal of Neurotrauma* 36:877-890.

Collinger JL, Boninger ML, Bruns TM, Curley K, Wang W, Weber DJ (2013) Functional priorities, assistive technology, and brain-computer interfaces after spinal cord injury. *Journal of Rehabilitation Research and Development* 50:145-160.

Crown ED, Gwak YS, Ye Z, Johnson KM, Hulsebosch CE (2008) Activation of p38 MAP kinase is involved in central neuropathic pain following spinal cord injury. *Experimental Neurology* 213:257-267.

Detloff MR, Wade RE, Jr., Houle JD (2013) Chronic at- and below-level pain after moderate unilateral cervical spinal cord contusion in rats. *Journal of Neurotrauma* 30:884-890.

Detloff MR, Fisher LC, McGaughy V, Longbrake EE, Popovich PG, Basso DM (2008) Remote activation of microglia and pro-inflammatory cytokines predict the onset and severity of below-level neuropathic pain after spinal cord injury in rats. *Experimental Neurology* 212:337-347.

Dietz V, Knox K, Moore S, Roberts N, Corona KK, Dulin JN (2022) Dorsal horn neuronal sparing predicts the development of at-level mechanical allodynia following cervical spinal cord injury in mice. *Experimental Neurology* 352:114048.

Dominguez CA, Strom M, Gao T, Zhang L, Olsson T, Wiesenfeld-Hallin Z, Xu XJ, Piehl F (2012) Genetic and sex influence on neuropathic pain-like behaviour after spinal cord injury in the rat. *European Journal of Pain* 16:1368-1377.

Fernandez-Zafra T, Gao T, Jurczak A, Sandor K, Hore Z, Agalave NM, Su J, Estelius J, Lampa J, Hokfelt T, Wiesenfeld-Hallin Z, Xu X, Denk F, Svensson CI (2019) Exploring the transcriptome of resident spinal microglia after collagen antibody-induced arthritis. i160:224-236.

Gaffney CM, Muwanga G, Shen H, Tawfik VL, Shepherd AJ (2022) Mechanical Conflict-Avoidance Assay to Measure Pain Behavior in Mice. *Journal of Visualized Experiments : JoVE.*

Gaudet AD, Fonken LK, Ayala MT, Maier SF, Watkins LR (2021) Aging and miR-155 in mice influence survival and neuropathic pain after spinal cord injury. *Brain, Behavior, and Immunity* 97:6.

Gaudet AD, Ayala MT, Schleicher WE, Smith EJ, Bateman EM, Maier SF, Watkins LR (2017) Exploring acute-to-chronic neuropathic pain in rats after contusion spinal cord injury. *Experimental Neurology* 295:46-54.

Gaudet AD, Mandrekar-Colucci S, Hall JC, Sweet DR, Schmitt PJ, Xu X, Guan Z, Mo X, Guerau-de-Arellano M, Popovich PG (2016) miR-155 Deletion in Mice Overcomes Neuron-Intrinsic and Neuron-Extrinsic Barriers to Spinal Cord Repair. *The Journal of Neuroscience* 36:8516-8532.

Gensel JC, Donahue RR, Bailey WM, Taylor BK (2019) Sexual Dimorphism of Pain Control: Analgesic Effects of Pioglitazone and Azithromycin in Chronic Spinal Cord Injury. *Journal of Neurotrauma* 36:2372-2376.

Heinsinger NM, Spagnuolo G, Allahyari RV, Galer S, Fox T, Jaffe DA, Thomas SJ, Iacovitti L, Lepore AC (2020) Facial grimace testing as an assay of neuropathic pain-related behavior in a mouse model of cervical spinal cord injury. *Experimental Neurology* 334:113468.

Huck NA, Donovan LJ, Shen H, Jordan CE, Muwanga GPB, Bridges CM, Forman TE, Cordonnier SA, Haight ES, Dale-Huang F, Takemura Y, Tawfik VL (2022) Sex-distinct microglial activation and myeloid cell infiltration in the spinal cord after painful peripheral injury. *Neurobiology of Pain* 12.

Hulsebosch CE, Hains BC, Crown ED, Carlton SM (2009) Mechanisms of chronic central neuropathic pain after spinal cord injury. *Brain Research Reviews* 60:202-213.

Kramer JL, Minhas NK, Jutzeler CR, Erskine EL, Liu LJ, Ramer MS (2017) Neuropathic pain following traumatic spinal cord injury: Models, measurement, and mechanisms. *Journal of Neuroscience Research* 95:1295-1306.

Langford DJ, Bailey AL, Chanda ML, Clarke SE, Drummond TE, Echols S, Glick S, Ingrao J, Klassen-Ross T, Lacroix-Fralish ML, Matsumiya L, Sorge RE, Sotocinal SG, Tabaka JM, Wong D, van den Maagdenberg AM, Ferrari MD, Craig KD, Mogil JS (2010) Coding of facial expressions of pain in the laboratory mouse. *Nature Methods* 7:447-449.

McFarlane K, Otto TE, Bailey WM, Veldhorst AK, Donahue RR, Taylor BK, Gensel JC (2020) Effect of Sex on Motor Function, Lesion Size, and Neuropathic Pain after Contusion Spinal Cord Injury in Mice. *Journal of Neurotrauma* 37:1983-1990.

Mogil JS (2012) Sex differences in pain and pain inhibition: multiple explanations of a controversial phenomenon. *Nature Reviews Neuroscience* 13:859-866.

Mogil JS, Pang DSJ, Silva Dutra GG, Chambers CT (2020) The development and use of facial grimace scales for pain measurement in animals. *Neuroscience and Biobehavioral reviews* 116:480-493.

Munce SE, Stewart DE (2007) Gender differences in depression and chronic pain conditions in a national epidemiologic survey. *Psychosomatics* 48:394-399.

Nesic O, Lee J, Johnson KM, Ye Z, Xu GY, Unabia GC, Wood TG, McAdoo DJ, Westlund KN, Hulsebosch CE, Regino Perez-Polo J (2005) Transcriptional profiling of spinal cord injury-induced central neuropathic pain. *Journal of Neurochemistry* 95:998-1014.

Norrbrink Budh C, Lund I, Ertzgaard P, Holtz A, Hultling C, Levi R, Werhagen L, Lundeberg T (2003) Pain in a Swedish spinal cord injury population. *Clinical Rehabilitation* 17:685-690.

Peng J, Gu N, Zhou L, U BE, Murugan M, Gan WB, Wu LJ (2016) Microglia and monocytes synergistically promote the transition from acute to chronic pain after nerve injury. *Nature Communications* 7:12029.

Putatunda R, Hala TJ, Chin J, Lepore AC (2014) Chronic at-level thermal hyperalgesia following rat cervical contusion spinal cord injury is accompanied by neuronal and astrocyte activation and loss of the astrocyte glutamate transporter, GLT1, in superficial dorsal horn. *Brain Research* 1581:64-79.

Rosen S, Ham B, Mogil JS (2017) Sex differences in neuroimmunity and pain. *Journal of Neuroscience Research* 95:500-508.

Schneider LE, Henley KY, Turner OA, Pat B, Niedzielko TL, Floyd CL (2017) Application of the Rat Grimace Scale as a Marker of Supraspinal Pain Sensation after Cervical Spinal Cord Injury. *Journal of Neurotrauma* 34:2982-2993.

Shansky RM (2019) Are hormones a "female problem" for animal research? *Science* 364:825-826.

Shansky RM, Murphy AZ (2021) Considering sex as a biological variable will require a global shift in science culture. *Nature Neuroscience* 24:457-464.

Shiao R, Lee-Kubli CA (2018) Neuropathic Pain After Spinal Cord Injury: Challenges and Research Perspectives. *Neurotherapeutics* 15:635-653.

Siddall PJ, Taylor DA, McClelland JM, Rutkowski SB, Cousins MJ (1999) Pain report and the relationship of pain to physical factors in the first 6 months following spinal cord injury. *Pain* 81:187-197.

Sorge RE et al. (2014) Olfactory exposure to males, including men, causes stress and related analgesia in rodents. *Nature Methods* 11:629-632.

Sorge RE et al. (2015) Different immune cells mediate mechanical pain hypersensitivity in male and female mice. *Nature Neuroscience* 18:1081-1083.

Stewart AN, Glaser EP, Bailey WM, Gensel JC (2022) Immunoglobulin G Is Increased in the Injured Spinal Cord in a Sex- and Age-Dependent Manner. *Journal of Neurotrauma* 39:1090-1098.

Stewart AN, MacLean SM, Stromberg AJ, Whelan JP, Bailey WM, Gensel JC, Wilson ME (2020) Considerations for Studying Sex as a Biological Variable in Spinal Cord Injury. *Frontiers in Neurology* 11:802.

Stewart AN, Lowe JL, Glaser EP, Mott CA, Shahidehpour RK, McFarlane KE, Bailey WM, Zhang B, Gensel JC (2021) Acute inflammatory profiles differ with sex and age after spinal cord injury. *Journal of Neuroinflammation* 18:113.

Tawfik VL, Huck NA, Baca QJ, Ganio EA, Haight ES, Culos A, Ghaemi S, Phongpreecha T, Angst MS, Clark JD, Aghaeepour N, Gaudilliere B (2020) Systematic Immunophenotyping Reveals Sex-Specific Responses After Painful Injury in Mice. *Frontiers in Immunology* 11:1652.

Walker CL, Fry CME, Wang J, Du X, Zuzzio K, Liu NK, Walker MJ, Xu XM (2019) Functional and Histological Gender Comparison of Age-Matched Rats after Moderate Thoracic Contusive Spinal Cord Injury. *Journal of Neurotrauma* 36:1974-1984.

Wu J, Zhao Z, Zhu X, Renn CL, Dorsey SG, Faden AI (2016) Cell cycle inhibition limits development and maintenance of neuropathic pain following spinal cord injury. *Pain* 157:488-503.

Yang Q, Wu Z, Hadden JK, Odem MA, Zuo Y, Crook RJ, Frost JA, Walters ET (2014) Persistent pain after spinal cord injury is maintained by primary afferent activity. *The Journal of Neuroscience* 34:10765-10769.