Title: Comprehensive Phenotyping of Cutaneous Afferents Reveals Rapid-Onset Alterations in Nociceptor Response Properties Following Spinal Cord Injury

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Abstract

Spinal cord injury (SCI) is a complex syndrome that has profound effects on patient well-being, including the development of medically-resistant chronic pain. The mechanisms underlying SCI pain have been the subject of thorough investigation but still remain poorly understood. While the majority of the research has focused on changes occurring within and surrounding the site of injury in the spinal cord, there is now a consensus that alterations within the peripheral nervous system, namely sensitization of nociceptors, contribute to the development and maintenance of chronic SCI pain. Here we demonstrate that thoracic spinal contusion injury results in the emergence of autotomy and spasticity, both indicators of spontaneous pain, in areas below the level of the injury within 24 hr of SCI. These behaviors were associated with hindpaw edema and elevated cutaneous calcitonin gene-related peptide (CGRP) concentration. Electrophysiological recordings using an *ex vivo* skin/nerve/DRG/spinal cord preparation demonstrated that SCI increased mechanical and thermal sensitivity, as well as the incidence of spontaneous activity (SA) and afterdischarge (AD), in below-level C-fiber nociceptors 24 hr following injury. Interestingly, the distribution of nociceptors that exhibit SA and AD are not identical, and the development of SA was observed more frequently in nociceptors with low thermal thresholds, while AD was found more frequently in nociceptors with high thermal thresholds. These results demonstrate that SCI causes rapid-onset of peripheral inflammation-like processes that sensitize nociceptors, which may contribute to the early emergence and persistence of chronic SCI pain.

Key words: Acute spinal cord injury; below level; pain; DRG; sensory neuron; inflammation

1. Introduction

Spinal cord injury (SCI) can have devastating consequences that are most commonly associated with loss of motor function. Individuals with SCI experience a number of complications including bowel and bladder dysfunction, metabolic syndrome, sleep disturbances, depression, and fatigue [1-4]. However, the overwhelming majority (up to 80%) of patients experience chronic pain, and half of these patients rate this pain as being severe and unrelenting [2, 5-9]. Evidence also suggests that ongoing pain may impair recovery for those undergoing rehabilitation [10-14]. Current pain management strategies rely heavily on opioids, despite research demonstrating that these drugs potentiate the development of SCI pain as well as undermine bladder and locomotor recovery [15-20]. There is a need to better understand the mechanisms underlying SCI pain so we can discover new treatment options and improve the quality of life of SCI patients.

Pain following SCI is identified regionally by the relationship of pain experience to the site of injury. At-level pain is reported in dermatomes and myotomes that are innervated by sensory neurons originating from the site of SCI. Below-level pain by comparison, originates from tissue below the level of injury [21, 22], and is unique because it occurs despite disrupted connectivity in the ascending neural pathways, and in the absence of normal somatosensation [23]. This type of pain occurs in the majority of SCI pain patients [24], and is characterized as a spontaneous, intense burning or stabbing sensation, and is refractory to medical intervention [24].

The mechanisms underlying SCI-induced pain, including below-level pain, are complex and poorly defined. The majority of research has focused on the role that central nervous system structures play in SCI pain generation, but there is now an established literature demonstrating that the peripheral nervous system (PNS), and specifically the noxious stimulus transducing nociceptive afferents, also contribute to the establishment and maintenance of SCI-induced pain [25-28]. Furthermore, while the majority of SCI pain patients don't report pain for weeks to months after injury, research suggests that nociceptor excitability may result within days following SCI [25-28]. This excitability occurs in the form of spontaneous activity (SA), which is thought to be the physiological correlate of spontaneous pain [26, 28-34]. However, there has been little research examining whether the emergence of SA is also associated with the development of modality-specific evoked sensitivity, the presence of nociceptor afterdischarge (AD), or whether these phenomena are restricted to specific functional afferent subtypes.

In the present study, we show that indicators of spontaneous below-level pain develop between 1-7 days following SCI, and that these behaviors are associated with increased hindpaw edema and increased levels of calcitonin gene related peptide (CGRP), suggesting that SCI increases peripheral inflammatory processes during the acute phase of injury. Using our *ex vivo* skin/nerve/DRG/spinal cord preparation, we demonstrate that SCI increases mechanical and thermal sensitivity, as well as the incidence of SA and AD in unmyelinated, but not myelinated, cutaneous afferents. Moreover, SA and AD are shown to be present in similar but not overlapping distributions of cells that can be predicted by thermal, but not mechanical, sensitivity. This work is an important step in establishing the mechanisms underlying SCI pain by

further supporting the role of the PNS in the development of pathological pain following SCI.

2. Methods

2.1. Animals

Experiments were conducted using 8-12 week-old (20-30 g) female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) that were group housed (4-5 mice/cage) and maintained in a temperature-controlled environment on a 12:12 light:dark cycle with free access to food and water. All studies were approved by the UConn Health and University of Kansas Medical Center Institutional Animal Care and Use Committee and treated in accordance with published NIH standards.

2.2. Spinal contusion injury

Mice were randomly assigned to SCI and naïve conditions and SCI mice were anesthetized with isoflurane (2%). A sham condition was not included because prior work has shown that laminectomy alone is sufficient to produce nociceptor sensitization and pain in mice [32, 35]. A rostro-caudal incision was made to expose the thoracic level vertebrae and surrounding musculature. Tissue was carefully removed from around the vertebral column, and a laminectomy was performed at the level of T8-T11 using fine surgical scissors and forceps (Fine Science Tools; Foster City, CA). The spinal column was then secured into position on the impactor using modified forceps (Fine Science Tools; Foster City, CA) clamped to vertebral tissue rostral and caudal of T8 and T11, respectively. Dorsal spinal contusions were produced at the T9 spinal level using the Infinite Horizons IH-0400 Spinal Cord Impactor (Precision Systems and Instrumentation; Lexington, KY) equipped with a standard mouse impact tip (1.3 mm diameter) at a severity of 65 kDynes and a 1 s dwell time. This level of injury is sufficient to produce hindlimb paralysis that results in a moderate level of recovery of locomotor function over time. The T9 spinal level was chosen to leave the lumbar spinal cord and dorsal root projections intact. Following the contusion, the wound was closed with 7-0 vicryl sutures, mice were injected with 2 mL of 0.9% saline and 5 mg/kg of gentamicin sulfate and were placed on warm heating pads until regaining consciousness. After displaying ambulatory behavior, mice were individually housed in cages with enrichment materials (plastic huts, burrowing material, etc.) and food, water, and supplemental gel diet (ClearH_2O, Westbrook, ME) placed in specially designed containers on the cage floor. Cages were placed on warm heating pads such that half of the cage remained off of the heating pad in quiet rooms away from foot traffic and were isolated from other animals for the 7-day duration of experiments. Mouse bladders were manually voided twice daily, supplemental injections of 0.9% saline and antibiotic were administered for 5 days as described in [36]. Bladder voiding was discontinued if reflexive bladder emptying recovered.

2.3. Spontaneous Pain Behaviors (autotomy and spasticity)

Autotomy and spasticity were recorded as present or absent for each subject at 24 hr intervals across the first week. Presence of autotomy was based on criteria previously described[37]. Briefly, loss of one or more toenails and/or any evidence of injuries to the proximal or distal half of any phalanx qualified as autotomy. Presence of spasticity was defined as spontaneous muscle twitch associated with the hindlimbs and/or tail. Mice were observed for 5 minutes/trial.

2.4. Ex vivo preparation and electrophysiological recordings

The *ex vivo* skin/nerve/DRG/spinal cord preparation (depicted in **Figure 1**) is similar to what has been described elsewhere [38-41]. Briefly, mice were anesthetized with a 90/10 mg/kg mixture of ketamine and xylazine (i.m.) and were transcardially perfused with ice cold oxygenated (95% O2–5% CO2) artificial cerebrospinal fluid (aCSF; in mM: 1.9 KCl, 1.2 KH2PO4, 1.3 MgSO4, 2.4 CaCl2, 26.0 NaHCO3, 10.0 D-glucose, and 127.0 NaCl). The spinal cord, saphenous nerve, and DRG were dissected in continuity and transferred to a recording chamber also containing oxygenated aCSF. The skin was then placed on an elevated metal platform with the epidermis exposed to air for mechanical and thermal stimulation. The dermis was continually perfused with bath aCSF that was maintained at 31°C. Electrophysiological recordings were performed by impaling individual sensory neuron somata with sharp quartz microelectrodes containing 1 M potassium acetate. Orthograde electrical search stimuli were administered through a suction electrode placed on the saphenous nerve to locate sensory neuron somata innervating the skin. Receptive fields (RF) were localized on the skin using mechanical (e.g., paintbrush) or thermal stimuli (~51 °C or ~0 °C 0.9% saline). Response characteristics of DRG neurons were determined by applying digitally controlled mechanical and thermal stimuli. The mechanical stimulator consisted of a tension/length controller (Aurora Scientific, Aurora, ON, Canada) attached to a 1-mm diameter plastic disk. Computer controlled 5-s square waves of 1, 5, 10, 25, 50, and 100 mN were applied to the cells RF. After mechanical stimulation, a controlled thermal stimulus was applied using a 3- 2 mm contact area Peltier element (Yale University Machine Shop, New Haven, CT, USA). The temperature stimulus consisted of a 12 s heat ramp from 31 to 52 °C, followed by a 5-s holding phase, after which the temperature was ramped back down to 31 °C over a 12 s period. A 30 s resting period was inserted between stimulus presentations. In some instances, fibers could not be characterized by computer-controlled mechanical and/or thermal stimulation but were able to be phenotyped using von Frey and/or saline stimuli, respectively. These cells were not included in threshold determination. All elicited responses were recorded digitally for offline analysis (Spike 2 software, Cambridge Electronic Design, Cambridge, UK).

Peripheral conduction velocity was then calculated from spike latency and the distance between stimulation and recording electrodes (measured directly along the saphenous nerve). A-fibers were identified as sensory neurons with conduction velocities > 1.2 m/s, while C-fibers were identified as sensory neurons with conduction velocities of <1.2 m/s (Lawson and Waddell, 1991; Lawson et al., 1993). C-fibers were classified as follows: (1) those that responded to mechanical and heat stimuli (CMH); (2) cells that responded to mechanical, heat and cold stimulation (CMHC); (3) those that

responded only to mechanical stimulation of the skin (CM); (4) those that responded to mechanical and cooling stimuli (but not heating) (CMC); (5) those that were cold and mechanically insensitive, but heat sensitive (CH); and (6) those that were heat and mechanically insensitive but responded to cooling of the skin (CC).

2.5. Tissue collection

Mice were anesthetized with a lethal exposure to isoflurane and intracardially perfused with ice cold 0.9% saline prior to the dissection and collection of hindpaw skin.

2.6. Enzyme-Linked Immunosorbent Assays (ELISA)

Hindpaw hairy skin was homogenized in ice-cold RIPA buffer/protease inhibitor cocktail and centrifuged for 20 min (4°C; 18,000 rcf). Each sample's total protein concentration was determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific; Waltham, MA). ELISAs for calcitonin gene-related peptide (CGRP; MyBioSource;), substance P (SP; Enzo; Farmingdale, NY), and nerve growth factor β (NGFβ; Boster Bio; Pleasanton, CA) were run according to manufacturer's instructions. All samples were run in duplicate and absorbance ratios were read at 450 nm. Protein concentration was determined by comparison to linearized protein concentration standards, and analyses were conducted using the mean concentration for the duplicate wells for each sample.

2.7. Statistical Analysis

Data were analyzed using Student's t-test, and one-way or mixed designs univariate analysis of variance (ANOVA). *Post hoc* testing was performed using Tukey honestly significant difference (HSD) t-tests where appropriate. Afferent population data were analyzed using Chi square (χ^2), and linear regression was used to determine relationships between nociceptor response thresholds and alterations in spontaneous firing, as well as SA and AD. Significance was determined using p values $\leq .05$.

3. Results

3.1. SCI increases the occurrence of spontaneous pain behaviors in injured mice

SCI is often accompanied by the development of spontaneous pain, indicated by the presence of autotomy and muscle spasticity [42-49]. Therefore, we assessed the presence of both behaviors in a cohort of 9 SCI mice for 7 days following SCI. We found that 5/9 SCI mice exhibited signs of autotomy during this time period, with wounding to the lower abdomen, hindpaws, and/or tail. We also observed hindlimb spasticity in 3/9 mice, all of which also exhibited autotomy (**Figure 2**), further suggesting an overlap in underlying mechanism for these two behavioral outcomes. Neither autotomy nor spasticity were observed in naïve mice (n=8).

Inflammation is a significant driver of both central and peripheral pain states [50-55]. In the periphery, inflammation is often observed in the form of edema. To determine

whether hindpaw autotomy was associated with inflammatory processes that developed during the acute phase of SCI, we measured paw thickness in a separate cohort of 10 naïve and 15 SCI mice during the first 7 days of recovery following SCI. We found that hindpaw diameter increased significantly following SCI relative to naïve mice. Because the day in which subjects' hindpaws reached their maximum thickness did not differ between individual mice, we analyzed average paw thickness and found significantly greater paw thicknesses in SCI (2.20 $\pm$ 0.09 mm) relative to naïve (1.85 $\pm$ 0.09 mm) mice, $F(1,24)=7.34$, $p<.05$ (**Figure 3A**).

Hindpaw edema results from increased release of proinflammatory molecules within the microenvironment of the skin, including CGRP, nerve growth factor (NGF), and substance P (SP) [56-60]. Concentration of each of these respective proteins was measured in hindpaw skin lysate from a cohort of 14 naïve and 12 SCI mice 24 hr following SCI. We chose this timepoint based on our earlier observations that autotomy, spasticity, and edema all develop within this short timeframe. Our analysis did not detect any change in NGF or SP concentrations following SCI. However, CGRP levels in hindpaw skin were significantly elevated following SCI, $F(1,25) =4.41$, $p<.05$ (**Figures 3B-D**), suggesting the presence of acute-onset CGRP-dependent inflammation within the skin.

3.3. Functionally identified afferent populations in naïve and SCI mice 24 hr following injury

Thus far our data suggest a role for peripheral processes in the emergence of spontaneous pain during the acute phase of injury and recovery. To further explore this possibility, we performed *ex vivo* intracellular sharp electrode electrophysiological recordings on 71 cutaneous primary afferents from 19 naïve mice and 81 primary afferents from 27 SCI mice. All recordings were performed 24 hr following T9 spinal contusion injury (65 kD, 1s dwell time). We first analyzed afferent response characteristics based, broadly, on responses to specific stimulus modalities. We found that 61.19% of naïve neurons responded to temperature, and of that population 58.21% responded to heat and 11.94% responded to cold (**Table 1**). We also found that 88.06% were mechanically sensitive, with 46.27% responding to both mechanical and heat stimulation, 11.94% responding to mechanical and cold stimulation, and 8.96% responding to mechanical, heat, and cold stimulation. Additional analyses demonstrated that 62.40% of SCI neurons were temperature sensitive, with 56.79% responding to heat and 18.52% responding to cold. Furthermore, 83.95% were sensitive to mechanical stimulation, with 44.44% being sensitive to mechanical and heat stimulation, 14.81% responding to mechanical and cold stimulation, and 11.11% were sensitive to mechanical, heat, and cold stimulation, $\chi^2=4.08$, $p<.05$ (**Table 1**). Statistical comparison of these distributions revealed a significantly greater proportion of SCI afferents that were sensitive to cold stimulation compared to the naïve. No other significant relationships were found.

We further characterized neurons by fiber type (A vs C) and specific stimulus-evoked response using the methods described above to distinguish fiber types (see **Table 2**). The afferent phenotype distribution for naïve neurons included 16.42% A-LTMR, 13.43% A-HTMR, 8.96% CM, 11.94% CH, 0% CC, 2.99% CMC, 37.31% CMH,

and 8.96% CMHC neurons. The distribution of SCI afferents included 12.35% A-LTMR, 16.05% A-HTMR, 7.41% CM, 12.35% CH, 3.70% CC, 3.70% CMC, 33.33% CMH, and 11.11% CMHC neurons. Analysis of the distribution of cells between each group revealed a greater percentage of CC neurons present in SCI mice, χ^2=9.00, p<.01. No other comparisons reached statistical significance (**Table 2**).

3.4. SCI increases mechanical sensitivity in unmyelinated C-fiber nociceptors 24 hr following injury

We assessed mechanical thresholds from 11 naïve and 10 SCI A-LTMR afferents 24 hr following SCI and found no difference in average mechanical thresholds between naïve (4.27 ± 0.47 mN) and SCI (3.67 ± 0.63 mN) neurons (**Figure 4A**). Similar results were also observed when comparing mechanical thresholds of 9 naïve (49.09 ± 17.34 mN) and 13 SCI (33.08 ± 6.58 mN) A-HTMR afferents (**Figure 4B**). Additional analysis of A-LTMR and A-HTMR firing rates in response to mechanical stimulation of the skin at 1, 5, 10, 25, 50, and 100 mN of force revealed no significant differences in firing rates in either population of neurons between naïve and SCI conditions (**Figures 4C, 4D**).

Analysis of average mechanical thresholds from mechanically-sensitive C-fibers revealed similar results. Specifically, we found that thresholds of 6 naïve (87.50 ± 11.41 mN) and 6 SCI (75.00 ± 27.54) CM, 2 naïve (62.50 ± 26.52 mN) and 3 SCI CMC (15.00 ± 7.07 mN), 25 naïve (37.60 ± 10.61 mN) and 27 SCI CMH (24.23 ± 5.04 mN), and 6 naïve (36.67 ± 12.78 mN) and 9 SCI CMHC (11.50 ± 5.29 mN) neurons were not significantly different between conditions (**Figures 5A, 5B**). Subsequent analysis of mechanically-evoked firing rates of CMH and CMHC nociceptors showed increased firing in response to 5 and 50 mN of force applied to the skin in SCI CMH neurons and increased responding to 1 and 5 mN of force in SCI CMHC neurons, all Fs>4.86, p<.05 (**Figures 5C, 5D**). Despite our ability to obtain mechanical response thresholds from CM and CMC neurons, analysis of firing rates was not performed because there was an insufficient number of characterized neuros to perform reliable statistical analysis. Nonetheless, our results suggest that SCI increases mechanical sensitivity in C-fiber nociceptors within 24 hr of injury.

3.5. SCI increases thermal sensitivity in C-fiber nociceptors 24 hr following injury

Temperature thresholds were also examined 24 hr following SCI in 42 naïve and 46 SCI heat sensitive neurons, 8 naïve and 15 SCI cold sensitive neurons, and 4 naïve and 9 SCI neurons that exhibited warming responses. Response to warming was defined as any firing present following an increasing temperature ramp from 0°C to 31°C. We found no differences in response thresholds within heat, cold, or warm temperature-responsive neuronal subtypes. Therefore, we combined afferent subtypes that responded to each stimulus modality and calculated and analyzed average heat, cold, and warm thresholds. Analysis of these data failed to detect any significant differences in temperature thresholds between naïve and SCI afferents (**Figure 6A-C**). However, we did find a significantly greater percentage of heat-sensitive SCI (21.74%) nociceptors that responded to warming stimuli than naive (10.00%) heat sensitive

nociceptors, χ^2=6.25, p<.05. We then analyzed responding to warming of the skin following cold stimulation in heat and cold sensitive neurons and found neither naïve nor SCI CH nociceptors responded to warming of the skin. Conversely, warming of the skin elicited responses from a significantly greater percentage of SCI CMH and CMHC (22.22% and 44.44%, respectively) nociceptors relative to naïve CMH and CMHC (8.00% and 33.33%, respectively) nociceptors, all χ^2>3.68, p<.05. Further supporting this result, analysis of thermally-evoked firing rates showed that CMH and CMHC nociceptors exhibited significantly increased firing rates in response to temperatures ranging from 31-38°C and 41-51°C. Additional analysis found that SCI resulted in a significant increase in firing rate in CH nociceptors following 33-35°C and 49-50°C relative to naïve CH nociceptors, all Fs>2.27, p<.01 (**Figure 7A-C**). Collectively, these results suggest that heat sensitivity is increased in thermally responsive nociceptors following SCI.

3.6. SCI increases spontaneous firing in C-fiber nociceptors 24 hr following injury

Previous research has shown that SCI increases the incidence of SA and AD in putative nociceptors within days of injury and that SA and AD can persist for several months [25, 26, 28, 61, 62]. It is not currently known whether SA and AD are characteristics of a certain functional population of afferents, whether SA and AD are interrelated, or whether an association exists between SCI- induced alterations in thermal and mechanical response thresholds and the incidence of SA or AD. To examine these issues, we examined the presence of SA and AD in all electrophysiologically characterized neurons and found a significantly greater percentage of SCI neurons (39.02%) exhibited SA relative to naïve neurons (1.35%), χ^2=684.5, p<.0001. When we analyzed the presence of SA in specific functional subtypes of afferents, we found that 0/11 naïve and 2/10 SCI LTMRs, 0/9 naïve and 0/13 SCI HTMRs, 1/6 naïve and 5/6 SCI CMs, 0/11 naïve and 6/10 SCI CHs, 0/2 naïve and 1/3 SCI CMCs, 0/25 naïve and 14/27 SCI CMHs, and 0/6 naïve and 4/9 SCI CMHCs exhibited SA. Analysis of these data revealed a significant increase in the occurrence of SA in SCI LTMR, CM, CH, CMH, and CMHC neurons relative to the same functional populations of naïve neurons, all χ^2>4.00, p<.05 (**Figure 8A**). Analysis of the prevalence of AD and obtained similar results. While AD was absent in all naïve neurons, we did observe AD in 41.46% of SCI neurons. Quantification of the presence of AD within in each functional subclass of SCI neurons showed that 1/10 LTMRs, 0/13 HTMRs, 4/6 CMs, 0/10 CHs, 1/3 CMCs, 23/27 CMHs, and 5/9 CMHCs exhibited AD (**Figure 8B**). Analysis revealed statistically significant increases in AD occurrence in SCI CM, CMH, and CMHC relative to naïve neurons in the same functional categories.

It is possible that the increase in SA and AD following SCI was due to alterations in the intrinsic electrophysiological properties of neurons, potentially resulting in a relative state of depolarization and a subsequent decrease in firing threshold. To examine this possibility, we compared resting membrane potentials (RMPs) in each neuronal subtype across injury conditions. We found no significant differences in RMPs in any subpopulation of afferents following SCI compared to naïve subjects (**Table 2**).

These above results demonstrate that SA and AD occur in similar functional distributions of nociceptive afferents in response to SCI. Pearson correlations were then

performed to determine whether there was an association between the presence of SA and AD in a given cell. Analysis failed to detect a significant relationship between SA and AD (r_P=0.048, p>.05). We then examined whether nociceptor phenotype, mechanical sensitivity, or heat sensitivity was significantly correlated with SA or AD using linear regression. We failed to resolve any significant association between specific nociceptor subtype or mechanical sensitivity with the incidence of SA or AD (all r_P <0.32, p<.05). However, our analysis did reveal a significant inverse relationship between heat sensitivity and the presence of SA (r_P = -0.290, p<.05) and significant positive relationship between heat sensitivity and the presence of AD (r_P = 0.300, p<.05). Collectively, these results suggest that SA and AD are unrelated to each other and that a population of low threshold heat-responsive nociceptors are more likely to exhibit SA, while a separate population of high threshold nociceptors are more likely to exhibit AD.

4. Discussion

4.1. SCI increases spontaneous pain behavior and neurogenic-like inflammation.

Chronic pain occurs at an alarmingly greater percentage in the spinally injured relative to the general chronic pain population [2, 5-9]. Upwards of 80% of chronic SCI patients report and receive medical treatment for their pain, ranking as one of the most significant secondary health concerns [1]. Still, pain is often poorly managed and represents an unmet need that negatively impacts quality of life. Furthermore, chronic SCI-pain is also associated with poor prognoses for recovery [10-14], and therefore requires greater understanding of the underlying molecular mechanisms in order to develop novel therapeutics. The mechanisms underlying SCI-induced pain begin to develop rapidly following injury, and our present results demonstrate that autotomy and spasticity below the level of the lesion occur between 1 and 7 days following injury, suggesting that injured animals experience ongoing pain early following SCI. We also found that spontaneous pain behavior was associated with edema and increased levels of cutaneous CGRP during the same period, suggesting that an early neurogenic inflammation-like process contributes to this pain.

4.2. SCI increases mechanical and temperature sensitivity in unmyelinated nociceptors

Despite resulting from damage to the central nervous system, there is now consensus that nociceptors innervating peripheral tissues play an important role in the generation and maintenance of SCI-induced chronic pain [25-28]. The precise manner in which nociceptors function in this capacity still has yet to be characterized, and given that SCI resulted in spontaneous pain behavior, increased cutaneous CGRP release, and hindpaw edema, we examined how spinal contusion injury altered nociceptor response properties 24 hr following SCI. We found that the majority of characterized neurons from both naïve and SCI mice were mechanically sensitive, with a smaller, but significant, proportion of afferents that were temperature sensitive. The distribution of temperature-responsive neurons was similar between injury conditions, with the exception of an increase in cold-sensitive SCI afferents. However, this finding should be

interpreted with some caution because we did not characterize any CC neurons from naïve mice, which is unusual.

The above SCI-induced alterations in general afferent response characteristics appear to emerge due to increased sensitivity within specific functional populations of neurons. Interestingly, we did not observe changes in mechanical response thresholds or firing rate of A-fiber afferents, but we did find specific modifications in action potential (AP) firing characteristics in unmyelinated C-fibers. Specifically, SCI did not affect average C-fiber mechanical response thresholds but did cause an increase in AP firing in response to mechanical force applied to the skin. In particular, CMH and CMHC nociceptors demonstrated a leftward shift in mechanical sensitivity, with CMH nociceptors exhibiting increased firing to 5 mN of force and CMHC nociceptors exhibiting increased firing in response to 1 and 5 mN of force applied to the skin. Importantly, these increases in nociceptor firing rates occurred at or near innocuous forces of stimulation, suggesting that normally innocuous or minimally noxious mechanical stimulation can produces heightened nociceptor responses and potentially increased pain (e.g., allodynia). SCI also increased firing to 50 mN of applied force in CMH nociceptors, suggesting the presence of increased sensitivity to noxious mechanical stimulation as well (e.g., hyperalgesia).

Application of hot, warm, and cold stimuli to the skin did not reveal any significant differences in average response thresholds. However, we did find an increase in the number of nociceptors that responded to warming of the skin, suggesting an overall increase in thermal sensitivity, particularly at lower temperatures. Supporting this conclusion, we found that all thermally sensitive nociceptor populations (e.g., CH, CMH, and CMHC) exhibited leftward shifts in their respective temperature response curves; SCI CH nociceptors began firing at 34°C, while naïve CHs began firing at 35°C; SCI CMH nociceptors began firing at 31°C, while naïve CMHs began firing at 34°C; and SCI CMHC nociceptors showed modest, but significant, increases in firing at 32°C, while naïve CMHCs began firing at 39°C. As temperature increased, we observed a complex relationship in stimulation-response curves, and it is difficult in many cases to interpret specific changes in nociceptor response characteristics following SCI. These complex relationships may arise from disruptions in the ability of injured nociceptors to maintain higher firing rates during the early stages of injury, when the nervous system is adapting to the injured environment.

Alterations in nociceptor responses to stimulation can result in certain functional populations of nociceptors, such as the mechanically-insensitive CH nociceptors gaining mechanical sensitivity, for example [63-65]. The consequence of these phenotypic switches is that there is a redistribution of functional populations in response to injury. While phenotypic switching has been reported in response to peripheral inflammation and neuropathic injury, we did not observe such population changes in the current experiments. This may be due to divergent mechanisms responsible for alterations in nociceptor response properties following peripheral and central injuries. Given that we are the first to perform this type of comprehensive phenotyping of nociceptor responses, further research is required to determine whether phenotypic switching is a functional consequence of SCI.

4.3. SCI increases spontaneous activity and afterdischarge in specific populations of C-fiber nociceptors

Prior work has shown that SA is increased in dissociated afferent cell bodies within 3 days of SCI, that it persists for at least 8 months, and that it contributes to the development of chronic SCI pain [25, 26, 28]. These findings were instrumental in increasing our understanding of the effects of SCI on primary afferent neuron function, as well as establishing a role for peripheral neurons in the development and maintenance chronic SCI pain. The results detailed here are in agreement with this previous work, but also build upon those findings by showing that SA and AD are present 24 hr following SCI, which is earlier than previously demonstrated. We also found that SA is not observed in all primary afferents, but is restricted to CM, CH, CMH, CMHC, and to a lesser extent, in A-LTMR neurons. We also showed that AD is observed in CM, CMH, and CMHC neurons, demonstrating that while SA and AD are found in similar functional classes of neurons, these distributions do not completely overlap. Supporting this we found a statistical association between thermal, but not mechanical, sensitivity and the presence of SA or AD. In particular we found that SA was correlated with lower heat thresholds, while AD was correlated with higher heat thresholds. This result has important functional implications, suggesting that nociceptors with higher heat continue to fire in an aberrant manner following the termination of high heat stimulation, which may lead to miscoding of sensory information, for example. Along with the results detailed above, these observations are significant because they allow us to begin characterizing which neuronal populations are impacted by SCI, as well as the specific functional properties (e.g., stimulus sensitivity, presence of SA and AD, etc.) change in response to SCI.

4.4. Potential mechanisms responsible for central recruitment of nociceptive afferents and subsequent sensitization

The development of SA has been shown to result from neurophysiological mechanisms including depolarizing RMPs, reductions in AP generation threshold, and the occurrence of depolarizing spontaneous fluctuations (DSFs) [32]. We did not observe any obvious DSFs, nor did we find any significant differences in RMPs. This may be due to the nature of our recordings. Alternatively, absence of RMP depolarization may have resulted from our ability to leave the peripheral sensory circuit intact, which leaves the central and peripheral targets of axon termination intact during our experiments. As such, it is possible that during the acute phases, nociceptor excitability is regulated by changes in the central nervous tissue near the site of afferent terminal projection, or at the site of peripheral tissue innervation. Supporting this, prior work has shown that SCI increases the early release of proinflammatory cytokines, such as IL-1β, that increase afferent sensitivity [66]. Here, we also show that SCI increases the concentration of CGRP in the skin, which not only sensitizes nociceptors, but also recruits inflammatory/immune cells that release other neuroactive molecules, such as prostaglandins, that can further increase nociceptor excitability [67-70]. Consequently, ongoing central and peripheral stimulation of nociceptors may not only increase firing

rates during the acute period of SCI but may also serve as a potential mechanism that may result in later nociceptor depolarization.

Ongoing central and peripheral inflammatory stimulation may also contribute to the onset of SA and AD, and whether these neurophysiological changes in afferent firing occur prior to, in conjunction with, or following increased evoked afferent responding is not currently known. However, it is possible that ongoing inflammatory central and peripheral stimulation may decrease stimulus response thresholds via spontaneous AP firing. And while not identical to naturalistic, physiological processes by which SA/AD or "ectopic" afferent activity occurs, similar results have been observed following experimental induction of wind-up, central sensitization, and long-term potentiation [71-84]. It is not our intent to determine the nature, role, or relationships between each of these phenomena, as this has been a long-standing debate covered in depth elsewhere. It is, however, possible that SA and AD cause reductions in nociceptor response thresholds, and that nociceptor sensitivity increases over time with continued aberrant firing. Furthermore, given that SA and AD occur more frequently in heat-sensitive nociceptors, SA and AD may account for patient-reported ongoing spontaneous burning sensation below the level of injury. Additionally, a significant proportion of cutaneous heat sensitive afferents also respond to mechanical stimulation [85-89], suggesting that SA and AD in these populations of nociceptors may result in the aberrant encoding of concomitant mechanical and burning pain in the absence or cessation of stimulation.

The molecular mechanisms underlying the emergence of SA and AD following SCI remain to be fully elucidated. However, the presence of SA occurs in cell bodies of a population of $Na_v1.8$ and TRPV1-expressing, isolectin B4 (IB4) binding, afferents, which also requires activation of adenylyl cyclase-dependent signaling cascades [25, 28, 90, 91]. Given that TRPV1 has been characterized as a heat-transducing channel, and that heat sensitivity is associated with the development of SA and AD, these results collectively suggest an important role for TRPV1 in the generation of SA and AD. However, much more work is required to fully understand how nociceptor sensitization occurs following SCI.

4.5. Functional and clinical importance of altered evoked and spontaneous nociceptor firing properties

Simultaneous central and peripheral activation of nociceptors could have additional functional consequences that are clinically relevant. Specifically, others have hypothesized that SCI creates a "reverberatory loop", where antidromic nociceptor APs generated in the injured spinal cord causes release of neuroactive molecules from peripheral terminals into their targets of innervation [92]. This peripheral neurogenic inflammation-like process allows for generation of orthodromic nociceptor APs that propagate into the spinal cord and foster the development and/or maintenance of an excitatory environment within the spinal cord. Consequently, breaking this self-perpetuating loop of nociceptive communication could serve as a valuable clinical tool for treating chronic SCI pain. This approach has at least two readily discernable advantages: first, pain may be reduced, or even eliminated, if nociceptor input is silenced. Second, the peripheral nervous system can be easily accessed relatively

compared to the central nervous system, potentially reducing the need for performing highly invasive procedures. Moreover, given that nociceptor excitability contributes to the development and maintenance of chronic SCI pain, and that this excitability occurs within hours of SCI, peripheral administration of a therapeutic that attenuates nociceptor excitability could occur soon following SCI, which may lead to greater efficacy in the management of chronic SCI pain. Supporting this, numerous studies have shown that prevention of chronic pain development is highly successful in preclinical models, while reversal of chronic pain is incredibly difficult. SCI is one of the unique injury scenarios where the time of injury is known, and intervention could occur relatively close to the time of injury.

Collectively, our data demonstrate that SCI creates an environment that increases evoked and spontaneous nociceptor activity. These changes in afferent response properties occur within the context of a proinflammatory environment (edema) and in conjunction with the presence of autotomy, which has previously been associated with spontaneous pain. Our findings are clinically significant because we observe a leftward shift in the temperature-dependent activation of heat-sensitive nociceptors, increasing nociceptor firing at temperatures that are near below body temperature. This would suggest that “normal” body temperature is an important determinant of nociceptor excitability and contributes to the difficulty in managing chronic SCI pain. Our results further suggest that difficulty in pain management could result from the constant barrage of endogenous and environmental stimuli that activate sensitized nociceptors. For example, contact with wheelchairs, bedsheets, clothing, necessary medical procedures (e.g., catheterization, IV placement, etc.), forced joint movement, development of pressure sores, spasticity, etc. all could contribute to evoked and spontaneous pain that may have detrimental effects for patient pain burden and recovery. Consequently, therapeutics designed to quiet nociceptor activity during the acute phase of injury may have broad reaching effects improve patient recovery prognosis and quality of life.

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Table 1

Stimulus Modality	Naïve % Responders	SCI % Responders
Temperature	61.19	62.40
Heat	58.21	56.79
Cold	11.94	**18.52***
Mechanical	88.06	83.95
Mechanical + Heat	46.27	44.44
Mechanical + Cold	11.94	14.81
Mecahnical + Heat + Cold	8.96	11.11

Table 1. Distribution (%) of naïve and SCI afferents that respond to temperature, mechanical, and the combination of temperature and mechanical, stimuli. The percentage of afferents within each category did not differ between conditions except for the percentage of cold responding afferents from SCI mice (bold). *=significantly different from naïve, $p<.05$.

Table 2

Phenotype	Fiber Type	Function	% Naïve (N=67)	%SCI (N=81)	Naïve RMP (mV)	SCI RMP (mV)
A-LTMR	A	Low threshold mechanoreceptor	16.42	12.35	-46.61 ± 3.63	-56.13 ± 4.20
A-HTMR	A	High threshold mechanoreceptor	13.43	16.05	-53.45 ± 4.28	-60.12 ± 3.69
CM	C	Mechanoreceptor	8.96	7.41	-42.13 ± 6.36	-50.30 ± 3.41
CC	C	Cold sensitive	0.00	**3.70***	--	-39.43 ± 5.43
CH	C	Heat sensitive	11.94	12.35	-39.65 ± 4.25	-44.69 ± 4.69
CMC	C	Mechanical + cold sensitive	2.99	3.70	-42.25 ± 0.67	-58.50 ± 5.93
CMH	C	Mechanical + heat sensitive	37.31	33.33	-46.13 ± 1.93	-44.23 ± 2.02
CMHC	C	Mechanical + heat + cold sensitive	8.96	11.11	-53.07 ± 3.56	-49.40 ± 2.47

Table 2. Functional population distributions of characterized primary afferent populations from naïve (A) and SCI (B) mice 24 hr following injury. Both groups of mice show similar distributions of A- and C-fibers as well as resting membrane potentials (RMP). However, analysis did reveal an increase in the percentage of CC neurons characterized from SCI mice, *=statistical significance relative to naïve mice, $p<.05$.

Figure 1

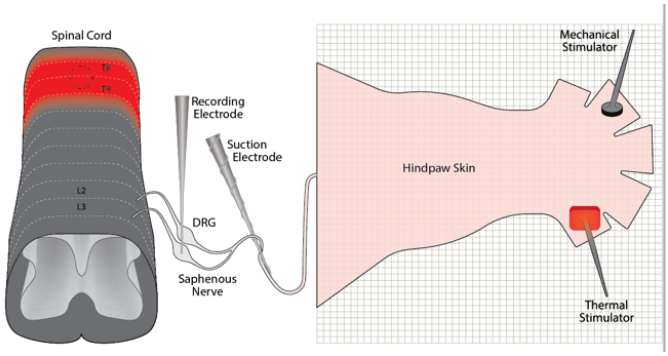

Figure 1. Schematic depicting *ex vivo* skin/nerve/DRG/spinal cord preparation. Spinal contusion injury was performed at T8-T9. Spinal cord tissue rostral to the lesion site and caudal of L4 was dissected, leaving L2 and L3 DRG and the saphenous nerve in continuity with the hairy skin of the hindpaw. Sharp electrode recordings were performed by impaling neuron cell bodies contained with L2 and L3 DRG while heat, cold, and mechanical stimuli were delivered within the cutaneous receptive field of the neuron.

Figure 2

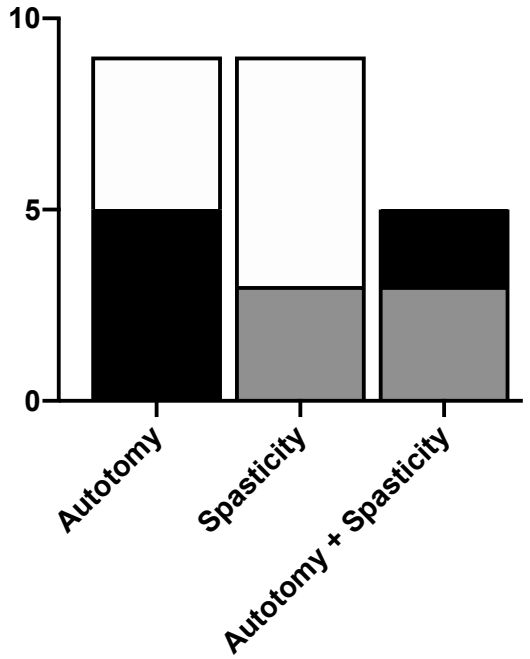

Figure 2. Quantification of autotomy and spasticity for 7 days following SCI. Autotomy and spasticity were characterized as being present or absent in each of 9 SCI mice. The graph depicts the total number of mice that exhibited autotomy (black) and spasticity (gray) as a proportion of the total number of mice (white). The final bar depicts the number of mice in the autotomy group (black) that also exhibited spasticity (gray).

Figure 3

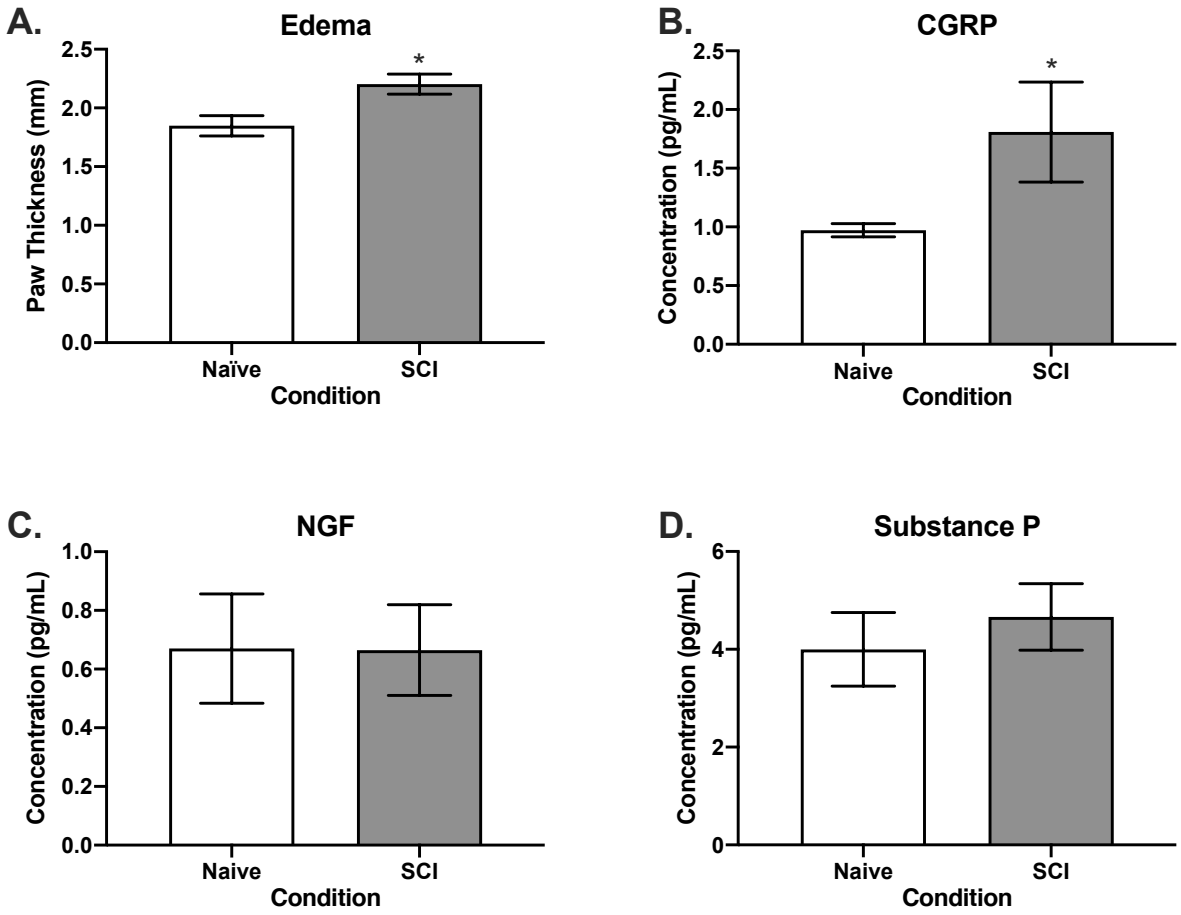

Figure 3. Analysis of hindpaw edema and concentrations of cutaneous CGRP, NGF, and SP. Edema (**A**) was determined by increased paw thickness (mm) in SCI relative to naïve mice. Average concentrations of CGRP (**B**), NGF (**C**), and SP (**D**) found in hindpaw skin for 7 days following SCI. We found that SCI increased paw thickness and concentration of CGRP present in the skin. * = significant difference from naïve, $p<.05$.

Figure 4

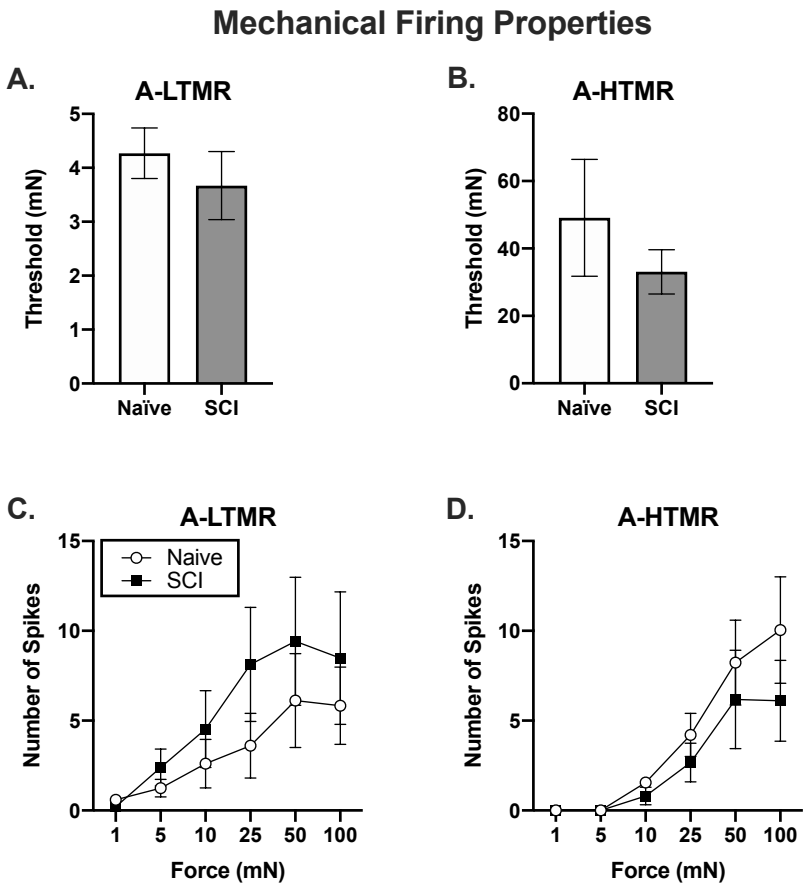

Figure 4. Analysis of mechanically evoked response thresholds and force-dependent firing rates from A-LTMR (**A, C**) and A-HTMR (**B,D**) 24 hr following SCI. No significant differences in average threshold or firing rates emerged, all $p>.05$.

Figure 5

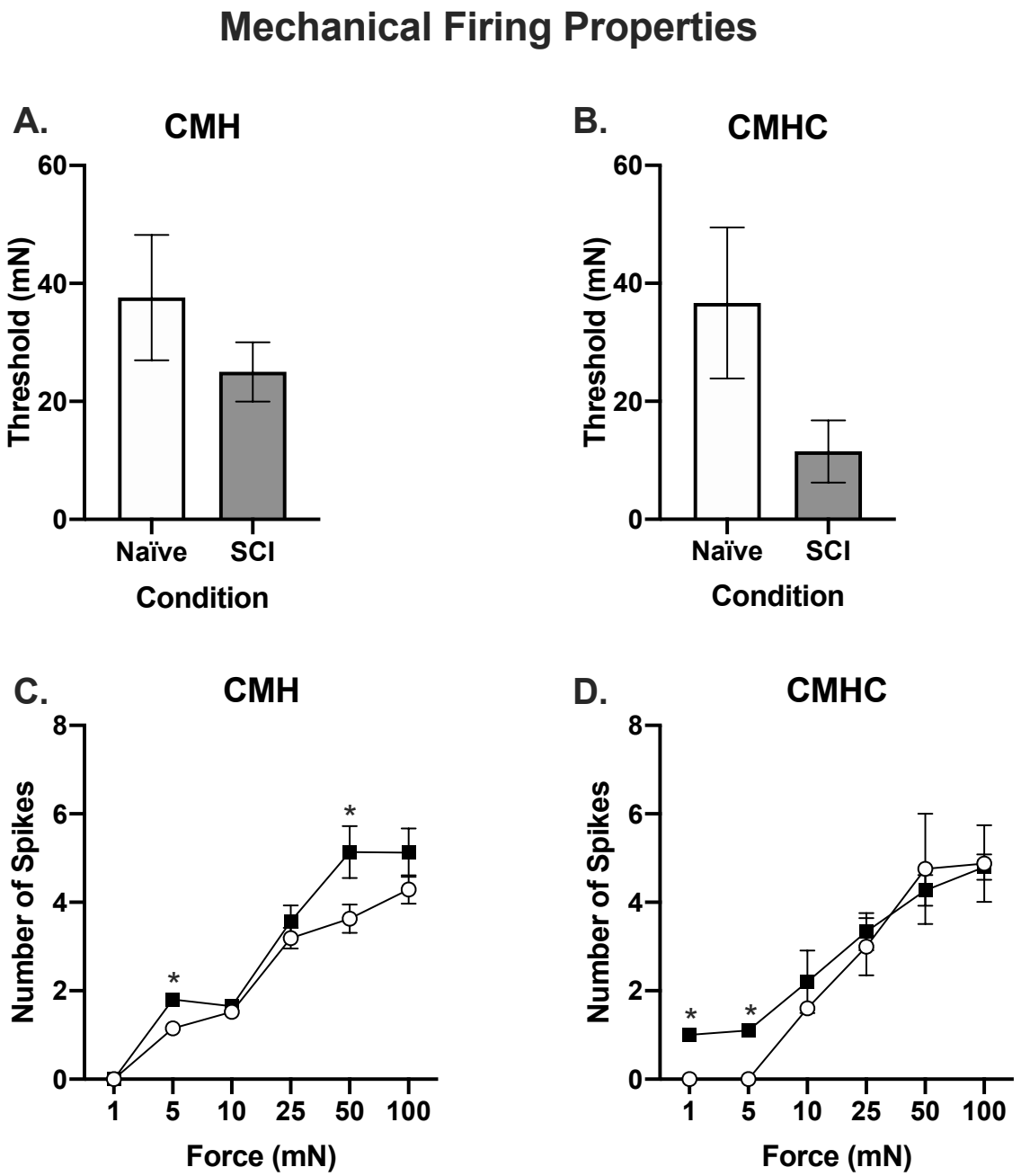

Figure 5. C-fiber mechanical response thresholds and force-dependent firing rates. Response thresholds and firing rates are shown for CMH (**A, C**) and CMHC (**B, D**) nociceptors 24 hr following SCI. While mechanical thresholds were not statistically different between naïve and SCI conditions, we observed a general increase in firing rates as greater force was applied to the skin. We also observed significantly increased firing rates in the lower range of stimulation in SCI relative to naïve neurons. *=significantly different from naïve, $p<.05$.

Figure 6

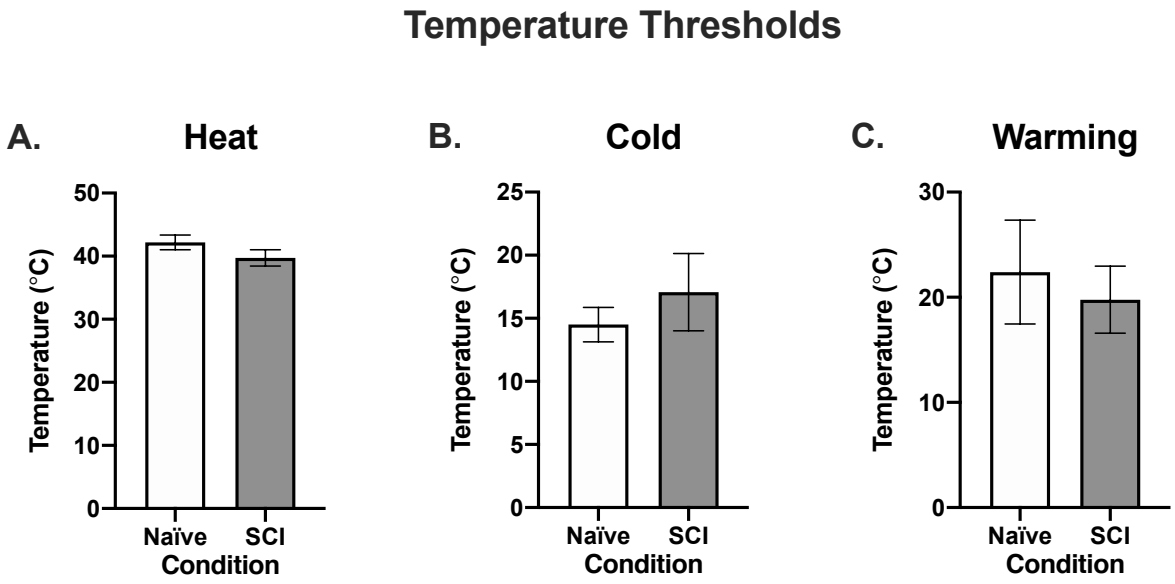

Figure 6. C-fiber temperature response thresholds. Average response thresholds following heat (**A**), cold (**B**), and warming (**C**) stimulation were assessed 24 hr following SCI. No stimulus-specific differences were observed when comparing naïve and SCI neurons, all $p>.05$.

Figure 7

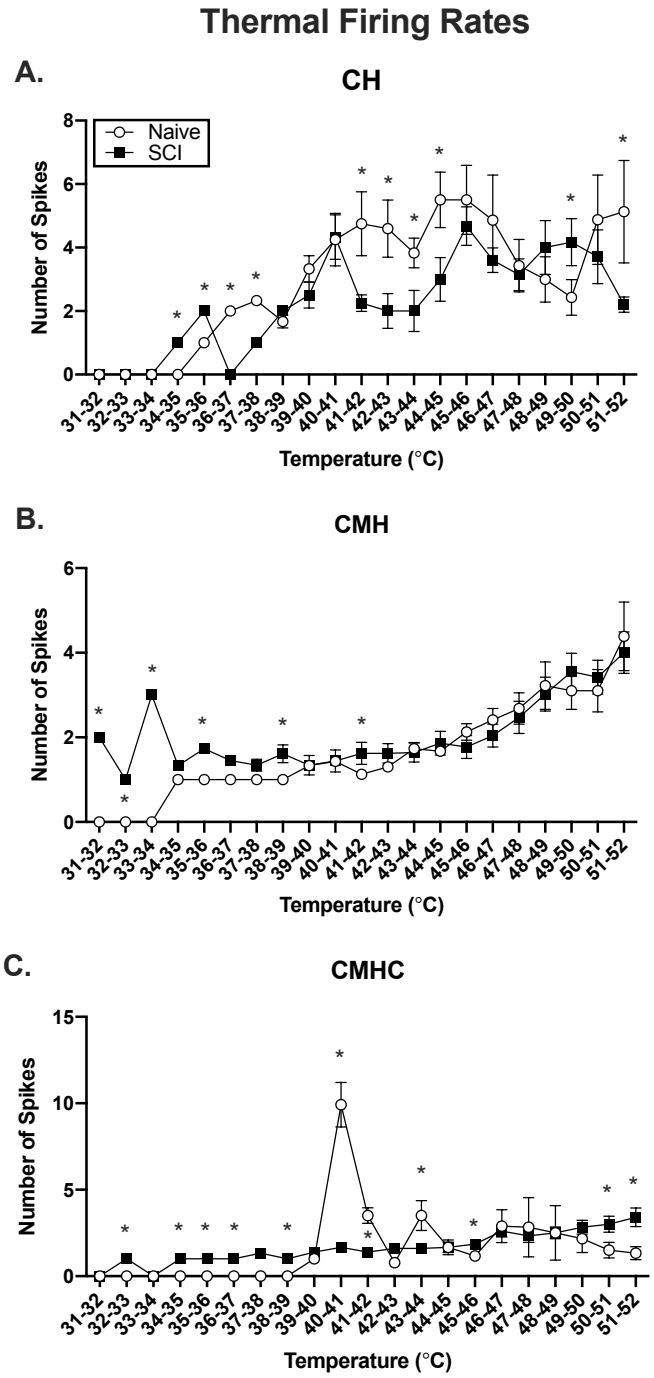

Figure 7. Firing rates for thermally-sensitive nociceptors in response to increasing temperature. Firing rates are depicted for CH (**A**), CMH (**B**), and CMHC (**C**) neurons in response to a 12 s thermal ramp ranging from 31-52°C. In general, we found that SCI increased thermal sensitivity, and SCI neurons fired in response to lower stimulation than naïve nociceptors. *=significantly different from naïve, all $p<.01$.

Figure 8

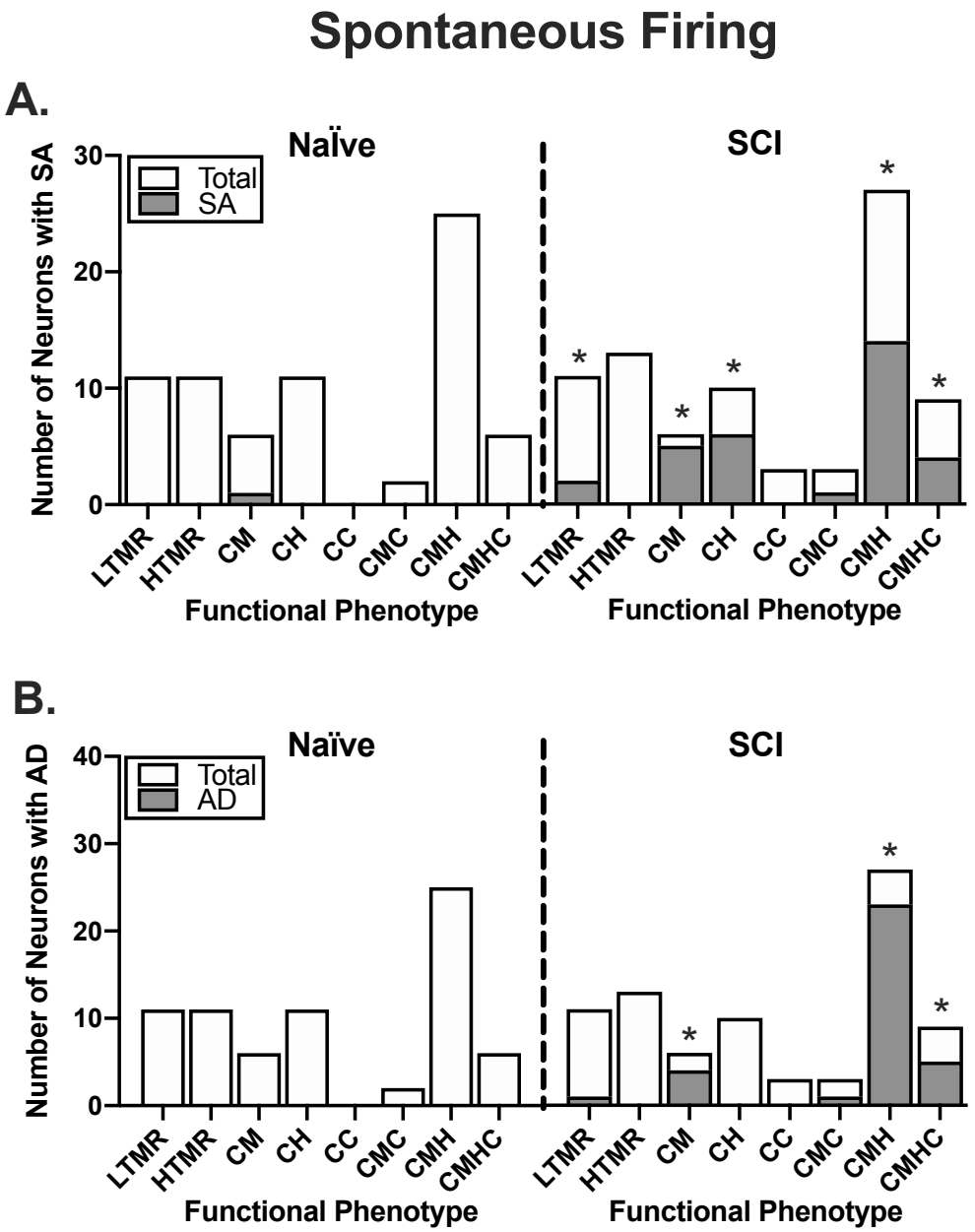

Figure 8. Spontaneous activity (SA) and afterdischarge (AD) in functional subtypes of afferents. **A**) incidence of SA in characterized naïve and SCI afferents. Data are depicted as number of neurons that exhibited SA (gray bars) relative to total of characterized neurons (white bars). **B**) incidence of AD in characterized naïve and SCI neurons depicted as number of neurons that exhibited AD (gray bars) relative to total of characterized neurons (white bars).