Peripheral Inflammation Accelerates the Onset of Mechanical Hypersensitivity after Spinal Cord Injury and Engages Tumor Necrosis Factor α Signaling Mechanisms

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Abstract

Previously, we showed that noxious stimulation of the tail produces numerous detrimental effects after spinal cord injury (SCI), including an earlier onset and increased magnitude of mechanical hypersensitivity. Expanding on these observations, this study sought to determine whether localized peripheral inflammation similarly impacts the expression of mechanical hypersensitivity after SCI. Adult rats received a moderate contusion injury at the thoracic level (T10) or sham surgery, and were administered complete Freund's adjuvant (CFA) or vehicle in one hindpaw 24 hours later. Examination of locomotor recovery (Basso, Beattie, and Bresnahan [BBB] score) showed no adverse effect of CFA. Mechanical testing with von Frey hairs was done at time-points ranging from 1 h to 28 days after CFA or vehicle treatment, and rats were sacrificed at 1, 7, or 28 days for cellular assessment. Unlike vehicle-treated SCI rats where mechanical hypersensitivity emerged at 14 days, CFA-treated SCI rats showed mechanical hypersensitivity as early as 1 h after CFA administration, which lasted at least 28 days. CFA-treated sham subjects also showed an early onset of mechanical hypersensitivity, but this was maintained up to 7 days after treatment. Cellular assessments revealed congruent findings. Expression levels of c-fos, tumor necrosis factor α (TNF α), TNF receptors, and members of the TNF α signaling pathway such as caspase 8 and phosphorylated extracellular related kinase (pERK) were preferentially upregulated in the lumbar spinal cord of SCI-CFA rats. Meanwhile, c-jun was significantly increased in both CFA-treated groups. Overall, these results together with our previous reports, suggest that peripheral noxious input after SCI facilitates the development of pain by mechanisms that may require TNF α signaling.

Keywords: central sensitization; chronic pain; mechanical hypersensitivity; peripheral inflammation; TNF α

Introduction

ALTHOUGH CHRONIC NEUROPATHIC PAIN is a frequent consequence of spinal cord injury (SCI),^{1,2} only limited progress has been made in the development of effective treatments. The processes that underlie SCI-induced neuropathic pain are complex and involve interactions that extend beyond the primary insult.^{3,4} Consistent with these previous reports, we recently showed that the development and maintenance of mechanical hypersensitivity after SCI is exacerbated by noxious tailshock, delivered shortly after SCI.⁵ Earlier studies showed that electrical stimulation of the tail or leg, or capsaicin administration to the paw induces a variety of negative effects after SCI, such as impairing recovery of locomotor functions^{6–8} and inhibiting adaptive spinal plasticity.^{9–11} Together, these observations suggest stimulation that produces central sensitization can augment maladaptive nociceptive plasticity after SCI.

The neural mediators of pain are not fully known. Among the many likely candidates, studies have implicated the inflammatory cytokines, neurotrophins and protein kinases. Interestingly, noxious stimulation that induces detrimental behavioral consequences has been shown to reduce the spinal expression of the growth-enhancing neurotrophin, brain-derived neurotrophic factor (BDNF),⁷ but significantly enhance expression of the inflammatory cytokine tumor necrosis factor α (TNF α) and several of its downstream signaling components.⁵ These findings suggest that TNF α is likely involved in the cellular processes that produce pain after SCI. Although TNF α has been shown to influence neural survival, exerting both neuroprotective and neurodegenerative actions,^{12–14} it has been mostly linked to neurodegenerative effects and maladaptive plasticity after SCI, and is associated with many secondary effects of SCI. For example, TNF α produces excitotoxic and apoptotic cell death by engaging both inflammatory and caspase-dependent

apoptotic pathways.¹⁵ TNF α also plays a role in microglial activation following injury,¹⁶ an action that might be self-potentiating. The spinal learning deficit that is induced by noxious stimulation after complete SCI is mediated by TNF α .¹⁷ Finally, TNF α might be critical to the development and expression of inflammatory,^{18,19} peripheral injury-induced,^{20–22} and SCI-induced^{5,18,23} pain.

Although stimulation of the tail at intensities that engage C-fibers induces maladaptive cellular and behavioral plasticity after SCI, it can be argued that noxious tailshock does not accurately represent physiological events occurring at the time of injury. In the current study, we examined whether stimulation that evokes local peripheral inflammatory pain similarly exerts cellular and behavioral plasticity consistent with exaggerated pain states after SCI. Complete Freund's adjuvant (CFA) is routinely used to induce inflammatory pain. Also, CFA induces cellular changes associated with nociceptive plasticity, such as increased glutamate receptor phosphorylation and efficiency,^{24,25} activation of extracellular signal-regulated kinases (ERK)^{26,27} and increased TNF α expression.²⁸ In adult rats with a moderate thoracic contusion SCI, we examined the effect of a single administration of CFA on recovery of locomotor function and on hindlimb withdrawal threshold that may reflect mechanical allodynia. Also, we assessed the temporal expression of TNF α and some of its downstream targets in the lumbar spinal cord. We show that CFA administration caused an early emergence of mechanical hypersensitivity after SCI. Moreover, CFA increased TNF α expression and that of several key components of the TNF α signaling cascade.

Methods

All experiments were conducted in male Sprague Dawley rats obtained from Charles River Laboratories (Wilmington, MA). Rats were approximately 9 weeks old and weighed between 275 and 325 g at the time of surgery. They were paired-housed and maintained on a 12-h light/dark cycle, with all behavioral testing performed during the light cycle. Food and water were available *ad libitum*. All experiments were carried out in accordance with National Institutes of Health (NIH) standards for the care and use of laboratory animals (NIH publication No. 80-23), and were approved by Emory University School of Medicine, Division of Animal Resources. Every effort was made to minimize suffering and limit the number of animals used.

Surgery and spinal contusion injury

Rats were anesthetized with isoflurane (5%, gas). Once a stable level of anesthesia was achieved, the concentration of isoflurane was lowered to 2–3%. A midline incision was made to expose the spinal column at vertebral level T8–T11. The T9 vertebra was carefully removed to expose the underlying T10 spinal cord, whereas the vertebral bodies of T8 and T10–T11 were clamped to stabilize the spinal column. Contusion injury at T10 was induced using the Infinite Horizon Impactor (Precision Systems, Kentucky, IL) with a force of 150 kdyn and zero dwell time. Bilateral bruising of the dorsal spinal cord was verified by examination under the dissecting microscope. The overlying muscles were sutured and the skin closed with sterile 9-mm EZ-clips (Stoelting Co., Wood Dale, IL). The animals were treated with triple antibiotic ointment (bacitracin-neomycin-polymyxin B) topically. To compensate for fluid loss, rats were given a 3-mL intraperitoneal injection of 0.9% saline after surgery, and thereafter only if needed. Bladders were expressed twice daily (morning and evening) until the animals had empty bladders for 3 consecutive days at the times of expression. Animals were weighed daily for 1 week after surgery and moni-

tored for infection or signs of unexplained discomfort. Rats used as sham controls underwent the same surgical procedure, but did not receive a contusion SCI.

Experimental design

These experiments were designed to investigate the effect of peripheral sensitization induced by CFA following SCI on hindlimb mechanical withdrawal responses and on the expression of the pro-inflammatory cytokine, TNF α , its receptors, and several downstream targets, as well as the expression of phosphorylated extracellular related kinase (pERK)1/2. All animals received an SCI or sham procedure as described above. One day after the surgical procedure, rats in each group were treated with 100 μ L of CFA (1 mg of *Mycobacterium tuberculosis*, heat killed and suspended in 0.85 mL paraffin oil and 0.15 mL mannide monooleate; Sigma-Aldrich, St. Louis, MO) or saline (Sal), which was administered subcutaneously to the dorsal surface of one hindpaw in a counter-balanced manner. Subsequently, animals from all four experimental groups (Sham-Sal, Sham-CFA, SCI-Sal, and SCI-CFA) were assigned to one of three behavioral/cellular groups.

Experimental group 1 (28 day): This first experiment was undertaken to assess the effect of CFA on maintenance of pain hypersensitivity after SCI. Behavioral assessment of hindpaw sensitivity was done at 7, 14, 21, and 28 days after CFA or saline treatment. Animals in this group were sacrificed for cellular assays immediately after the last behavioral assessment.

Experimental group 2 (7 day): This experiment assessed the effect peripheral inflammation has on the onset or early expression of pain after SCI. Rats were evaluated for hindpaw mechanical sensitivity at 1 h, 1 day, and 7 days after CFA or saline treatment. The rats were sacrificed immediately after day 7 behavioral tests for cellular assays.

Experimental group 3 (1 day): A third group of animals was sacrificed 1 day after CFA or saline treatment for cellular assays.

Behavioral locomotor assessment

The day after surgery, and before CFA or saline administration, locomotor behavior was assessed using the Basso, Beattie, and Bresnahan (BBB) scale²⁹ in an open enclosure for all subjects to ensure the effectiveness of the contusion injury (for details, refer to Grau and associates⁶). Sham control rats with a baseline BBB score of 21 (no locomotor deficit) and SCI rats with a baseline BBB score <8 were included in the study ($n = 105$; 50 sham and 55 SCI subjects). Following baseline evaluation, the subjects were assigned to a treatment group (CFA or Sal) in a manner that ensured injury severity was balanced across groups before treatment. BBB evaluation was then undertaken daily at 1–6 days post-CFA or saline treatment, in both 7-day and 28-day treatment groups, and at 14, 21, and 28 days after treatment for rats in the 28-day treatment group.

Assessment of mechanical withdrawal responses

To assess the effect of noxious stimulation on mechanical sensitivity, we tested tactile thresholds using Semmes-Weinstein von Frey filaments (Stoelting, Wood Dale, IL). Before surgical manipulation, all rats were acclimated to the room used for behavioral tests and testing apparatuses for at least 3 days. Prior to surgery (pre-surgery baseline) and at different post-surgery time-points, withdrawal response threshold to tactile stimulation was evaluated. For mechanical assessment, rats were individually placed in testing chambers with metal mesh floors at least 20 min before testing for acclimation. Using calibrated von Frey hairs, the 50% g withdrawal threshold was determined using the up-down method of Dixon.³⁰ Starting with filament #4.31 (2 g), the filaments were applied perpendicularly against the midplantar surface of each hindpaw in a sequential ascending or descending order. Even at

acute time-points, when SCI rats are unable to adequately plantar place the hindpaws, every effort was taken to ensure the von Frey filament was consistently applied to the midplantar surface. For each rat, the mean withdrawal threshold was computed by averaging the withdrawal thresholds of both right and left hindpaws. A threshold value of 15 g was considered a maximum cutoff. The establishment of mechanical hypersensitivity (demonstrated as a significant decrease in paw withdrawal threshold compared with pre-surgery baselines) was assessed in SCI and sham subjects after CFA or saline treatment. To minimize experimental bias, mechanical assessment was performed by two investigators. However, because CFA produces noticeable paw edema, it was impossible for investigators to be completely blind to the subject's treatment group.

RNA extraction and qRT-PCR

We also investigated the effect SCI and CFA have on the messenger RNA (mRNA) expression of TNF α , the TNF α receptor 1 (TNFR1) and TNFR2, c-jun, caspase 8, and caspase 3 in the lumbar spinal cord. The mRNA expression of c-fos, an immediate early gene and marker of neuronal activity, was also assessed. All rats from the individual experimental groups were sacrificed at 1 day ($n = 20$), 7 days ($n = 46$), or 28 days ($n = 39$) following CFA or saline treatment. The subjects were deeply anesthetized with urethane (1.2 g/kg) and 1 cm of spinal cord encompassing L3–L5 was rapidly removed. The spinal cord was processed for the extraction of both total RNA (RNeasy Mini Kit; Qiagen, Valencia, CA) and total protein (see below). Total RNA (100 ng) was converted into cDNA using TaqMan EZ RT-PCR Core Reagents (Applied Biosystems, Carlsbad, CA) and the mRNA levels of the genes of interest were measured by TaqMan quantitative real-time (qRT)-PCR using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA). β -actin served as a control gene. The sequences of probes, and forward and reverse primers for all targets were obtained from Thermo Fisher Scientific, Waltham, MA. The mRNA expression for each gene of interest was normalized to β -actin expression and presented as a fold-change increase or decrease in experimental groups compared with the Sham-Sal control (normalized to 1).

Protein extraction and western blot

Western blotting was used for quantification of the protein expression of TNF α , c-fos, c-jun, caspase 8, caspase 3, and pERK1/2 in the lumbar spinal cord. Subsequent to RNA extraction, total protein was extracted from the organic layer containing protein and DNA using the QIAzolTM lysis reagent protocol for isolation of genomic DNA and/or proteins from fatty tissue (Qiagen, Valencia, CA). Details on this procedure have been previously reported^{31,32} (also see articles by Garraway and colleagues^{5,7}). Following determination of the protein concentration using the Bradford Assay (BioRad, Hercules, CA), protein samples were diluted in Laemmli sample buffer and stored at -80°C at known concentrations of total protein (typically 2–5 $\mu\text{g}/\mu\text{L}$). Equal amounts (30 μg) of total protein were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Following transfer onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA), the blots for non-phosphorylated proteins were blocked for 1 h in 5% blotting grade milk (BioRad, Hercules, CA) in tris-buffered saline and Tween-20 (TBST), whereas blots for pERK1/2 were blocked in 5% bovine serum albumin in TBST. After blocking, the blots were incubated overnight at 4°C in primary antibody diluted in blocking solution as follows: TNF α (1:500; #ARC3012, Invitrogen, Camarillo, CA); c-fos (1:250; #SC-52, Santa Cruz Biotech, Dallas, TX); c-jun (1:2000; #NB110-55569), caspase 8 (1:1000; #NB100-56116), and caspase 3 (1:1500; #NB100-56113) from Novus Biological, Littleton, CO; and pERK1/2 (1:500; #07-

467, Millipore, Temecula, CA). β -tubulin (1:1000; #05-661, Upstate Cell Signaling, Lake Placid, NY) served as a control.

The following day, blots were washed in TBST (3×10 min) at room temperature, then were incubated in horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse secondary antibodies (1:5,000; #31460 or #31430, respectively; Pierce, Rockford, IL) for 1 h at room temperature. Following wash (3×10 min), the blots were developed with standard enhanced chemiluminescence (Thermo Fisher Scientific, Waltham, MA) and imaged with SynGene Gel Imaging System. Ratios of the integrated densitometry of each protein of interest to the loading control (β -tubulin) were calculated, normalized to sham controls, and averaged for animals within each group. The data are presented as a percentage change compared with Sham-Sal (100%).

Statistical analysis

Behavioral data were analyzed using repeated measures (RM) analysis of variance (ANOVA) with time-point as the within-subjects factor (one-way ANOVA or two-way ANOVA), treatment/injury group as the between-subjects factor (two-way ANOVA), and an *a priori* alpha value of 0.05 or below being considered significant. These were followed by post hoc tests corrected for multiple comparisons. In all cases, the choice of multiple comparisons test used (Tukey, Dunnett, or Sidak) was based on GraphPad Prism recommendation as being most appropriate for the particular dataset. Mean group differences were also evaluated with independent *t* tests, whenever applicable. Comparisons of cellular changes among the groups were analyzed by one-way ANOVA, followed by appropriate post hoc comparisons. In text and figures, all data are presented as mean $\pm$ standard error of the mean (SEM), and in figures, * and # indicate $p < 0.05$, ** indicates $p < 0.01$, and ^ indicates $p < 0.001$. The statistical analyses were conducted with GraphPad Prism 6 (GraphPad Prism, La Jolla, CA).

Results

This study assessed the effect of noxious stimulation on withdrawal responses to mechanical stimulation, and on the temporal expression of TNF α and its receptors, TNFR1 and TNFR2, in the lumbar spinal cord at acute and chronic periods following SCI. The mRNA and protein expression levels of c-fos, c-jun, caspase 8 and caspase 3, and pERK1/2 protein levels in the lumbar spinal cord were also examined.

Inclusion-exclusion criteria and group sizes

The study consisted of three experimental groups with terminal end-points at 28 days, 7 days, and 1 day after CFA or saline treatment. In each group, rats that died before the final behavioral assessment ($n = 5$) were excluded from the study. The total numbers of subjects in the study groups were as follows: Group 1 (28 days): Sham-Sal ($n = 10$), Sham-CFA ($n = 10$), SCI-Sal ($n = 9$), and SCI-CFA ($n = 10$); Group 2 (7 days): Sham-Sal ($n = 12$), Sham-CFA ($n = 10$), SCI-Sal ($n = 12$), and SCI-CFA ($n = 12$); and Group 3 (1 day): Sham-Sal ($n = 4$), Sham-CFA ($n = 4$), SCI-Sal ($n = 6$), and SCI-CFA ($n = 6$). All subjects in each experimental group were used for cellular assessment of mRNA and protein expressions.

BBB Locomotor scores

An analysis of the baseline BBB scores confirmed that the groups did not differ prior to CFA/vehicle treatment, at both 7 days ($F_{[1, 21]} < 1.0, p > 0.05$) and 28 day ($F_{[1, 16]} < 1.0, p > 0.05$). One day after surgery, the average BBB score for all contused animals in the 7- and 28-day groups was 1.1 ± 0.3 (SCI-Sal: 1.1 ± 0.5 and SCI-

FIG. 1. BBB locomotor scores. CFA treatment had no effect on the BBB locomotor scores of SCI rats in the 7-day (A) or 28-day (B) groups. There were no significant differences in the BBB scores of SCI-Sal (open circles) and SCI-CFA (open rectangles) rats at any time-point. BBB, Basso, Beattie, and Bresnahan; CFA, complete Freund's adjuvant; Sal, saline; SCI, spinal cord injury.

CFA: 1.1 ± 0.4). As shown in Figure 1A, CFA administration had no effect on BBB locomotor scores during the first week post-surgery of 7-day subjects ($F_{[1, 26]} = 0.3$, $p > 0.05$; two-way ANOVA). In the 28-day group (Fig. 1B), when BBB locomotor scores were also assessed at 14, 21, and 28 days after treatment, there were no differences between the two groups ($F_{[1, 14]} = 2.1$, $p > 0.05$; two-way ANOVA). However, a significant CFA $\times$ Day interaction emerged later in the 28-day group ($F_{[8, 128]} = 3.10$, $p < 0.01$). The two groups exhibited opposing trends over days, with vehicle-treated rats showing faster recovery relative to the CFA-treated group after day 6. All sham subjects had a day 1 BBB score of 21 (not shown). As seen with SCI subjects, CFA did not impair locomotor scores in sham controls.

Effect of CFA treatment on paw diameter

CFA is known to induce a pronounced local edema. Paw diameters were measured using a calibrated caliper, which was applied midpoint across the dorsal to plantar surface of both hindpaws. Measurements were made before CFA or saline administration, and at 1, 7, 14, 21, and 28 days after treatment. Figure 2 shows changes in the ipsilateral paw diameter before and after CFA treatment in the 28-day group of rats. RM-ANOVA revealed an overall effect of CFA treatment in both sham ($F_{[2, 9]} = 12.5$, $p < 0.01$) and SCI ($F_{[2, 12]} = 12.2$, $p < 0.001$) rats. As shown in Figure 2, the diameter of the CFA-treated paw remained significantly increased compared with pre-treatment values up to 21 days in sham subjects and 28 days in SCI subjects. Also, at post-treatment times ranging from 1 to 28 days, CFA-treated rats had significantly larger paw diameters than their saline-treated counterparts (p values ranged from $p < 0.05$ to $p < 0.0001$). There were no group differences in the contralateral hindpaws at any time-point (not shown). In the 7-day group, the ipsilateral paw diameter was similarly increased in CFA-treated sham and SCI subjects at 1 h, 1 day, and 7 days after CFA, compared with pre-treatment values (p values ranged from $p < 0.01$ to 0.001 [not shown]).

CFA treatment increased the development of mechanical sensitivity after SCI

The effects of SCI and CFA on mechanical withdrawal responses are illustrated in Figure 3. From prior observations, we noted that rats do not exhibit enhanced sensitivity to von Frey stimulation 1 day after a moderate to severe spinal cord injury⁵ (also reported by Lindsey and associates³³). Here, we investigated

the effect CFA administration shortly after SCI has on the development of hindpaw mechanical hypersensitivity in adult rats.

Effect of CFA treatment in sham-operated rats

Administration of saline to sham subjects (Sham-Sal; filled circles) had no effect on mechanical thresholds in the 7-day (Fig. 3A) or 28-day (Fig. 3B) experimental groups, confirmed by RM-ANOVA ($F_{[2, 26]} = 2.1$, $p > 0.05$) and ($F_{[3, 24]} = 0.7$, $p > 0.05$), respectively. In contrast, sham subjects that received CFA (Sham-CFA, filled triangles) showed a significant reduction in their withdrawal threshold compared with pre-surgery baseline values, in both the 7-day group ($F_{[2, 32]} = 5.1$, $p < 0.05$) and the 28-day group ($F_{[3, 25]} = 8.4$, $p < 0.001$). In the 28-day group, a significant effect of CFA was observed at 7 days (** $p < 0.01$), but waned by 14 days. At 21 days after treatment, the withdrawal threshold completely returned to baseline level (Fig. 3B). When the effect of CFA was assessed at earlier time-points, a significant reduction in withdrawal threshold was observed in sham subjects at 1 h (* $p < 0.05$) and 7 days (** $p < 0.01$) after CFA (Fig. 3A), compared

FIG. 2. Ipsilateral paw diameter. CFA produced significant paw edema in both sham and SCI subjects, which was observed from 1 day after CFA treatment and lasted up to 21 days in sham and 28 days in SCI rats. Also, at all time-points, the paw sizes of the CFA-treated subjects were significantly increased compared with the saline-treated rats (* $p < 0.05$, ** $p < 0.01$, and $^{\wedge}p < 0.001$) compared with pre-treatment values. CFA, complete Freund's adjuvant; Sal, saline; SCI, spinal cord injury.

FIG. 3. Hindpaw mechanical sensitivity. CFA treatment in both sham and SCI rats caused an increase in mechanical sensitivity (reduced withdrawal threshold) starting at 1 h in the 7-day group (**A**) and at 7 days in the 28-day group (**B**), compared with their pre-surgery baseline values. In Sham-CFA subjects (filled triangles), mechanical hypersensitivity was observed at 7 days after CFA. Sham-Sal rats (filled circles) did not exhibit increased mechanical sensitivity at any time-point. SCI-Sal rats (open circles) showed pain hypersensitivity at all time-points (1 h to 28 days after CFA). SCI-CFA rats (open rectangles) developed mechanical sensitivity at 14 days after treatment and remained sensitive up to 28 days. At 1 h and 1 day in the 7-day group and at 7 days in the 28-day group there was a significant decrease in the withdrawal thresholds of SCI-CFA rats compared with SCI-Sal rats. * $p < 0.05$, ** $p < 0.01$, and $\phi p < 0.001$ compared with pre-surgery baselines; # $p < 0.05$; and $\phi p = 0.050$ compared with saline-treated counterpart. CFA, complete Freund's adjuvant; Sal, saline; SCI, spinal cord injury.

with pre-surgery baseline. There were no differences in the withdrawal thresholds of the ipsilateral and contralateral hindpaws of Sham-CFA rats at any time-point. RM-ANOVAs showed that as early as 1 h post-CFA, both treated and untreated paws showed significantly reduced withdrawal threshold compared with their baseline values (ipsilateral paw, 6.2 ± 1.6 g [$F_{(2, 19)} = 5.0$, $p < 0.05$] and contralateral paw, 8.1 ± 1.6 g [$F_{(3, 25)} = 3.4$, $p < 0.05$]). Overall, these results illustrate that the inflammatory agent CFA induces short-lasting bilateral mechanical hypersensitivity.

Effect of CFA treatment in SCI rats

Next, we compared the effects of CFA versus saline treatment after SCI on hindpaw withdrawal threshold (Fig. 3). There were no differences in withdrawal threshold in saline-treated SCI rats (SCI-Sal; open circles) in the 7-day group over time ($F_{[2, 25]} = 0.9$, $p > 0.05$). However, in the 28-day group, SCI-Sal rats developed mechanical hypersensitivity at time-points ranging from 14 to 28 days ($F_{[3, 26]} = 11.2$, $p < 0.0001$). Although mechanical hypersensitivity was first observed at 14 days (* $p < 0.05$), the greatest effect was seen at 21 days ($\phi p < 0.001$). Unlike treatment of SCI rats with vehicle (SCI-Sal), RM-ANOVA revealed that CFA treatment (SCI-CFA; open rectangles) significantly altered withdrawal thresholds in both 7-day ($F_{[2, 24]} = 5.5$, $p < 0.01$) and 28-day ($F_{[3, 27]} = 10.3$, $p < 0.001$) experimental groups. Specifically, in these rats, CFA treatment produced mechanical hypersensitivity as early as 1 h after treatment in the 7-day group (7.0 ± 1.2 g), which persisted to 28 days (4.2 ± 1.0 g). In SCI-CFA subjects, mechanical threshold was significantly reduced only in the ipsilateral hind paw at 1 h (4.8 ± 1.2 g) and 1 day (5.7 ± 1.3 g) after treatment ($F_{[2, 20]} = 6.9$, $p < 0.01$; RM-ANOVA) compared with baseline, even though the sensitivity was not significantly different than that in the untreated hindpaws. RM-ANOVA showed that from 7 days and onward, mechanical sensitivity was similarly increased in both ipsilateral ($F_{[3, 24]} = 9.1$, $p < 0.001$) and contralateral ($F_{[3, 30]} = 8.0$, $p < 0.001$) hindpaws.

Additional analyses comparing the mean differences in withdrawal threshold of the SCI rats treated with saline versus CFA

showed significant differences at 1 h and 1 day post-treatment in the 7-day group, and at 7 days in the 28-day group (Fig. 3A,B, respectively; # $p < 0.05$, t tests). Although there was an apparent difference in withdrawal thresholds of SCI-Sal and SCI-CFA subjects at 28 days, the difference was not significant ($\phi p = 0.050$, unpaired t test). Overall, CFA caused a significant reduction in withdrawal threshold in sham-operated rats that lasted up to 7 days, whereas in SCI subjects, CFA induced mechanical hypersensitivity at 1 h and this effect lasted up to 28 days post-treatment. SCI-Sal rats did not exhibit a significant reduction in mechanical sensitivity until day 14, which also persisted until 28 days. These results show that peripheral inflammation significantly accelerated the onset of hypersensitivity after SCI.

CFA treatment increases c-fos mRNA expression in the lumbar spinal cord

The effect SCI and CFA have on the mRNA expression of c-fos, an immediate early gene and marker for neuronal activity, was also examined. C-fos has also been linked to nociceptive activity and the expression of pain.^{34,35} We previously reported elevated c-fos expression in the lesioned spinal cord 1 h, but not 1 day after SCI and noxious tailshock.⁷ Here, we found that c-fos mRNA levels in the lumbar spinal cord were altered by SCI and CFA (Fig. 4A). In the 1-day group, one-way ANOVA revealed an effect of treatment ($F_{[3, 16]} = 3.8$, $p < 0.05$). C-fos mRNA was elevated only in Sham-CFA rats (1.9 ± 0.3 , $p < 0.05$), compared with the Sham-Sal control group. Similarly, there was a main effect of treatment in the 7-day ($F_{[3, 26]} = 3.3$, $p < 0.05$) and 28-day ($F_{[3, 41]} = 3.3$, $p < 0.05$) groups. At both time-points, c-fos mRNA was elevated in the lumbar spinal cord of SCI-CFA subjects only (1.5 ± 0.2 and 1.8 ± 0.3 , respectively), compared with the Sham-Sal control groups. Western blot analysis was used to examine c-fos protein in the lumbar spinal cord (Fig. 4B). There were no group differences in c-fos expression in the 1-day group ($F_{[3, 14]} = 2.8$, $p > 0.05$, one-way ANOVA). However, one-way ANOVA revealed significant group differences in both 7-day ($F_{[3, 25]} = 5.1$, $p < 0.01$) and 28-day ($F_{[3, 19]} = 5.0$, $p < 0.05$) groups. Specifically, SCI-

FIG. 4. C-fos mRNA and protein expression. (A) C-fos mRNA was upregulated in the lumbar spinal cord of Sham-CFA rats 1 day after treatment. In SCI-CFA-treated subjects, c-fos mRNA expression was significantly elevated in the lumbar spinal cord at 7 and 28 days after treatment ($*p < 0.05$, compared with Sham-Sal). (B) Although there were no differences in c-fos protein expression at 1 day, there were robust increases in its expression in the SCI-CFA rats at both 7 and 28 days. In both group, SCI-CFA rats had significantly higher c-fos protein than all other groups. The increase was significantly larger than all other groups. Images of c-fos western blot at 28 days, and β -tubulin (western blot loading control) are shown at bottom. $*p < 0.05$, compared with Sham-Sal and Sham-CFA, and $\#p < 0.05$ compared with SCI-Sal. CFA, complete Freund's adjuvant; mRNA, messenger RNA; Sal, saline; SCI, spinal cord injury.

CFA subjects had robustly elevated c-fos protein expression compared with all other groups (7-day, $346 \pm 89\%$ and 28-day, $347 \pm 92\%$; $*p < 0.05$, compared with Sham-Sal and Sham-CFA, and $\#p < 0.05$ compared with SCI-Sal subjects).

CFA elevates TNF α and its receptors' expression in the lumbar spinal cord

A secondary objective of this study was to examine changes in TNF α expression and several downstream TNF α -induced mediators in the lumbar spinal cord induced by CFA treatment following SCI. As shown in Figure 5, a one-way ANOVA showed a main effect of treatment on TNF α mRNA levels 1 day after treatment ($F_{[3, 14]} = 5.6$, $p < 0.05$). Compared with Sham-Sal, TNF α mRNA was increased in the lumbar spinal cord of Sham-CFA (1.9 ± 0.1 ; $*p < 0.05$), SCI-Sal (1.9 ± 0.3 ; $*p < 0.05$), and SCI-CFA (2.3 ± 0.2 ; $**p < 0.01$) subjects. There were no group differences in TNF α mRNA levels at 7 or 28 days after treatment. There was also an effect of treatment on the expression of soluble TNF α (sTNF α)

FIG. 5. TNF α and the TNF receptors 1 and 2 expression. (A) TNF α mRNA was increased in all three treatment groups 1 day after treatment, compared with Sham-Sal rats. (B) At 7 days after treatment, TNF α protein levels were significantly increased in the lumbar spinal cord of SCI-CFA subjects, compared with Sham-Sal controls and saline-treated SCI subjects. An example of a representative western blot image for TNF α , at 7 days, is shown at bottom of (B). β -tubulin served as a loading control protein. (C) Both TNFR1 and TNFR2 mRNA expressions were robustly increased in the lumbar spinal cord of SCI-CFA rats at 1 day after treatment. $*p < 0.05$ and $**p < 0.01$ compared with Sham-Sal; and $\#p < 0.05$ compared with SCI-Sal. CFA, complete Freund's adjuvant; mRNA, messenger RNA; Sal, saline; SCI, spinal cord injury; TNF α , tumor necrosis factor alpha; TNFR1, TNF receptor 1; TNFR2, TNF receptor 2.

protein, which was examined by western blot ($F_{[3, 36]} = 5.3$, $p < 0.01$). Specifically, at 7 days post-treatment, sTNF α was robustly increased in the lumbar spinal cord of SCI-CFA rats ($158 \pm 22\%$), compared with the shams ($*p < 0.05$) and SCI-Sal ($\#p < 0.05$) groups (Fig. 5B).

The mRNA expression of TNFR1 and TNFR2 was also examined. One day after treatment, both TNFR1 and TNFR2 levels were significantly elevated in the lumbar spinal cord of SCI-CFA rats as revealed by ANOVA ($F_{[3, 16]} = 4.4$, $p < 0.05$) and ($F_{[3, 16]} = 4.8$, $p < 0.05$), respectively, and shown in Figure 5C. There were no differences in the expression of TNFR1 and TNFR2 in the spinal cord at 7 or 28 days among the different groups.

Effect of CFA on key elements of the TNF α signaling pathway

The activation of the TNFRs by TNF α engages several signaling cascades including kinases- and caspases-dependent mechanisms. Thus, we assessed the mRNA and protein expression of c-jun, caspase 8 and caspase 3, and the protein expression of pERK1/2. C-jun is an immediate early gene that can be induced by TNF α signaling and is associated with apoptotic pathways in neurons.³⁶ Elevation in c-jun is seen in other forms of injury, including peripheral nerve^{37,38} and brain³⁹ injury. Similarly, caspase 8 and the apoptotic effector caspase 3 are implicated in deleterious effects of SCI, particularly apoptosis, and pERK1/2 is a critical mediator of inflammatory and neuropathic pain.

C-jun mRNA was significantly elevated in both CFA-treated groups ($F_{[3, 15]}=5.2, p<0.05$) 1 day after treatment (Fig. 6Ai). Western blotting revealed a significant increase in c-jun protein in both CFA-treated groups ($F_{[3, 20]}=4.2, p<0.05$) at 7 days (Fig. 6Aii). At 1 day after treatment, caspase 8 mRNA was upregulated in SCI-CFA ($F_{[3, 15]}=4.2, p<0.05$) rats (Fig. 6B). There was also a significant group effect in caspase 3 mRNA levels at 1 day after treatment ($F_{[3, 16]}=4.2, p<0.05$), although it was upregulated in the spinal cord of Sham-CFA rats only. Western blotting showed no changes in the expression of caspase 8 protein (55 kd), the inactive caspase 3 (32 kd) and cleaved caspase 3 (17 kd;

not shown). The active subunits of caspase 8 and caspase 3 (~12 kd) were not detected. pERK1/2 protein levels were examined by western blots. There was a significant effect of treatment 1 day after treatment in that both pERK1 ($F_{[3, 16]}=5.3, p<0.05$) and pERK2 ($F_{[3, 16]}=4.3, p<0.05$) were elevated in the SCI-CFA group compared with Sham-Sal. There were no other differences in pERK1/2 expression (Fig. 7).

Discussion

Previously, we showed that nociceptive stimulation of the tail shortly after SCI profoundly impacts recovery of locomotor functions,⁷ and the expression of mechanical sensitivity,⁵ a potential behavioral correlate of central sensitization.^{40,41} Also, capsaicin, which activates C-fibers, acutely perturbs spinal learning and induces hindpaw hypersensitivity after SCI,¹¹ and promotes apoptotic cell death.⁸ Here, we expand on these findings by exploring whether peripheral inflammation of the hindpaw similarly impacts the development and long-term expression of pain. Further, as TNF α signaling appears to play a critical role in the devastating effects of SCI, including shock-induced mechanical hypersensitivity,⁵ we also assessed expression profiles of TNF α signaling. Overall, we report that (1) CFA had no substantial effect on the recovery of locomotion, but hastened the onset of mechanical hypersensitivity after SCI from 2 weeks to 1 h, (2) mechanical hypersensitivity persisted to 28 days after treatment, and (3) CFA treatment increased the expression of TNF α and components of its downstream signaling pathway, in the lumbar spinal cord. The observation that CFA did not worsen locomotor function was contrary to our prior results,^{5,7,8} which suggest intense C-fiber activity impairs locomotor recovery following SCI. This conflicting result raises the possibility that the duration of C-fiber activity might be a critical factor in determining locomotor outcomes after SCI. Specifically, it mildly suggests that prolonged activation of C-fiber, as elicited by CFA,^{42,43} might preserve locomotor function after SCI rather than undermine it. However, future studies are needed to elucidate the mechanisms underlying these opposing outcomes.

The neural mechanisms including the specific factors that are pivotal to the development of chronic neuropathic pain after SCI are not fully elucidated. Moreover, very little is known about the effects aberrant nociceptive input has on pain hypersensitivity after SCI. Whereas over 70% of SCIs are part of a multi-trauma event,^{44,45} experimental models most often do not depict the "other" events that occur at the time of injury. Therefore, our studies are guided by the need to explore this important facet of SCI. Clearly, noxious stimulation in the immediate aftermath of SCI reliably exerts a variety of detrimental actions including maladaptive spinal plasticity and impairment of locomotor and bladder functions.⁴⁶ Also, it appears that these actions converge on spinal mechanisms that are implicated in nociception such as, although not limited to, altered BDNF-TrkB signaling^{47,48} and increased TNF α expression¹⁷ (reviewed by Ferguson and colleagues⁴⁹ and Grau and associates⁴⁶). Collectively, these findings show that both motor and sensory functions after SCI are vulnerable to painful input arising from peripheral sites below the level of injury. After SCI, CFA-induced peripheral sensitization exerted more modest behavioral effects than noxious tailshock.⁵ Specifically CFA increased the onset of mechanical hypersensitivity after SCI, but had negligible effects on its overall magnitude compared with SCI alone. Nonetheless, several important observations, which are discussed below, were made in this study.

FIG. 6. C-jun and caspases expression. **(A)** C-jun mRNA and protein expression were robustly elevated in the lumbar spinal cord of CFA-treated sham and SCI rat. Images of c-jun western blot at 7 days, and β -tubulin (western blot loading control) (shown at bottom of [A]). **(B)** One day after CFA, caspase 8 and caspase 3 mRNA levels were increased in SCI and sham rats, respectively. * $p<0.05$ and ** $p<0.01$ compared with Sham-Sal controls. CFA, complete Freund's adjuvant; mRNA, messenger RNA; Sal, saline; SCI, spinal cord injury.

FIG. 7. pERK expression. One day after treatment, pERK 1 and 2 protein levels were significantly increased in the lumbar spinal cord of SCI-CFA rats (* $p < 0.05$ compared with Sham-Sal). Examples of representative western blot images for pERK1 and 2 are shown at bottom. β -tubulin served as a loading control. CFA, complete Freund's adjuvant; pERK, phosphorylated extracellular related kinase; Sal, saline; SCI, spinal cord injury.

First, the presence of peripherally derived noxious input after SCI profoundly influences when mechanical hypersensitivity is first observed. Thus, it can be postulated that the development of pain after SCI is somewhat predicated on the presence of noxious input. Moreover, the emergence of pain after SCI is impacted by the presence of aberrant noxious input irrespective of the type or source, as this outcome was observed following both peripheral inflammation and electrical stimulation.⁵ SCI-CFA rats displayed a phenotype that paralleled those of Sham-CFA plus SCI-Sal rats, suggesting that unlike noxious shock, CFA effects after SCI could be simply additive. Further, because both CFA-treated sham and SCI rats exhibited mechanical hypersensitivity from 1 h to 7 days post-treatment, it can be assumed that although CFA might influence behavioral or cellular responses after SCI, CFA-induced hypersensitivity is not altered by SCI. Our current study fails to identify the exact interaction between CFA-induced inflammation and SCI in the development of pain hypersensitivity. Nonetheless, this does not negate the important overall observation that despite the modality, noxious input exacerbates pain responses after SCI. That peripheral hypersensitivity is critical to pain after SCI has been previously demonstrated. Bedi and colleagues⁵⁰ showed that increased spontaneous activity generated in or near small-diameter dorsal root ganglion (DRG) somata drives hyperactive pain states after SCI, and a more recent report from the same laboratory directly related persistent pain after SCI to activity in primary afferents.⁵¹ Electrophysiological studies assessing primary neuronal activity were not undertaken here. However, because CFA increases spontaneous discharge in C-fiber and A δ -fiber primary afferent neurons,⁵² it is probable that CFA-induced persistent activity in primary afferents stimulates early onset pain hypersensitivity after SCI.

Second, peripheral inflammation after SCI engages critical central sensitization-like mechanisms. SCI subjects who received

CFA consistently expressed higher spinal levels of key inflammatory and nociceptive mediators, thereby suggesting that CFA induces persistent neuronal activity centrally. These cellular changes cannot be attributed exclusively to CFA administration, because although peripheral edema was consistent between both CFA-treated groups, equivalent cellular plasticity was not observed in Sham-CFA subjects. Instead, they reflect a convergence of peripheral nociceptive activity and SCI-induced spinal mechanisms. Several central mediators of inflammatory and neuropathic pain⁵³ are also implicated in the detrimental effects of SCI, events such as activation of kinases,^{54,55} and glial activation^{56,57} with subsequent increases in inflammatory cytokines. Both SCI and CFA-induced pain have been associated with increased levels of c-fos,^{27,58,59} TNF α ,^{5,60,61} c-jun signaling,^{5,62,63} and pERK,^{26,27,55,64} which further implies the likelihood that these nociceptive genes are critically involved in producing pain hypersensitivity after SCI. We found that concurrent SCI and CFA treatment had the most robust effect on TNF α protein expression in the lumbar spinal cord. Similarly, c-fos, a marker of central sensitization⁶⁵ that is induced by TNF α ⁶⁶ remained elevated in the lumbar spinal cord of SCI-CFA rats, into the chronic stage of injury (Fig. 4). An increase in c-fos protein expression in rats that received both SCI and CFA, but not those that received SCI or CFA only, strengthens the overall view that peripheral inflammation exacerbates maladaptive nociceptive plasticity after SCI, even in the absence of robust behavioral deficits.

In our previous study, TNF α levels were assessed in the lesioned spinal cord.⁵ The mRNA and protein levels were elevated in the dorsal spinal cord up to 7 days after shock. Here, we did not observe increases in TNF α protein at an earlier time-point (Fig. 5). Detloff and colleagues⁶⁷ reported similar temporal changes in TNF α expression in the lumbar spinal cord after a thoracic-level moderate contusion injury. Unlike our previous study where dorsal and ventral parts of the injured spinal cord were individually assessed, here we examined the entire L3–L5 spinal cord segment. Therefore, it is likely that moderate to large increases in TNF α expression in the dorsal spinal cord might be offset by the inclusion of the ventral spinal cord, where substantially smaller increases are expected. It is also possible that spatially, TNF α expression is robustly upregulated in the lesion epicenter, but to a lesser extent in distal spinal cord segments. An alternate explanation for the modest expression changes in TNF α may be related to the modality of peripheral stimulation. As was previously stated, noxious tailshock exerted more robust behavioral effects than peripheral inflammation. Therefore, it is conceivable that the expression of behavioral or cellular plasticity is linearly determined by the intensity of the peripheral stimulus and that cellular changes are commensurate with the behavioral effects.

Third, the observation that cellular plasticity is more robust at earlier time-points is consistent with the overall behavioral outcome that CFA accelerated the onset of mechanical hypersensitivity after SCI. Like TNF α , pERK and c-jun are implicated in both inflammatory and neuropathic pain, and pERK is considered an effective marker of central sensitization.⁶⁵ These downstream mediators of TNF α signaling, together with the TNFRs and caspase 8 were elevated mainly at the acute time-points after SCI and CFA (Figs. 6 and 7). It can be surmised from these results that TNF α -mediated signaling is a possible mechanism that underlies the early onset of neuropathic pain after SCI. A similar conclusion was made by Detloff and colleagues,⁶⁷ who showed a relationship between the onset of pain after SCI and elevated spinal levels of microglial activation and TNF α expression. However, that TNF α levels did

not remain elevated into the chronic time-points cannot be interpreted as an absence of TNF α signaling in the maintenance of neuropathic pain after SCI. Although an increase in TNF expression in the spinal cord was reported shortly after SCI in a human study,⁶⁸ studies in rodents have shown maintained increases in TNF α expression after SCI.^{67,69,70}

Like pain, SCI-induced apoptosis is mediated in part by TNF α -dependent mechanisms.^{71,72} As seen with TNF α , the spinal expression of caspase 8 mRNA was increased only acutely, whereas there were no changes in caspase 3 expression after SCI. Because caspase 8 and caspase 3 are an initiator and executor of TNF α -induced apoptosis, respectively, it appears that apoptosis after SCI is somewhat restricted to the lesion epicenter^{5,8} and does not extend to the uninjured lumbar spinal cord. Currently, there is no convincing evidence that apoptosis directly leads to pain hypersensitivity after SCI. However, the notion that apoptosis may serve as a central mediator of chronic neuropathic pain has received some attention. Sekiguchi and associates⁷³ showed injury-induced pain hypersensitivity associates with increased apoptosis in DRG neurons, and Joseph and Levine⁷⁴ suggested that apoptotic genes might contribute to acute pain hypersensitivity.

This study is an extension of our investigation into the impact peripheral nociceptive processes have on pain after SCI. The main outcome of this work is a demonstration that the development or expression of pain after SCI depends on a confluence of events, many of which extend beyond the borders of the primary insult. In addition to the array of secondary processes that accompany the primary insult and are implicated in the detrimental effects of injury, our combined studies demonstrate that peripheral nociceptive input indiscriminately exerts influence on the consequences of SCI, including pain. Clearly, pain after SCI is a complex phenomenon that results from a convergence of neural processes. Importantly, these observations lead us to conclude that the emergence and subsequent augmentation of pain after SCI can involve interactions between peripherally derived noxious input and “central sensitization-like” neuroplastic mechanisms.

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Author Disclosure Statement

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