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Exercise-Induced Changes to the Macrophage Response in the Dorsal Root Ganglia Prevent Neuropathic Pain after Spinal Cord Injury

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Abstract

Spinal cord injury (SCI) induces neuropathic pain that is refractory to treatment. Central and peripheral immune responses to SCI play critical roles in pain development. Although immune responses in the dorsal horn have been implicated in SCI-pain, immune mechanisms in the periphery, especially in the dorsal root ganglia (DRG), where nociceptor cell bodies reside, have not been well studied. Exercise is an immunomodulator, and we showed previously that early exercise after SCI reduces pain development. However, the mechanisms of exercise-mediated pain reduction are not understood. Therefore, we examined the 1) underlying immune differences in the spinal cord and DRG between rats with and without pain and 2) immunomodulatory effects of exercise in pain reduction. Rats were subjected to a unilateral contusion at C5 and tested for pain development using von Frey and mechanical conflict-avoidance paradigms. A subgroup of rats was exercised on forced running wheels starting at 5 days post-injury for 4 weeks. We observed greater microglial activation in the C7–C8 dorsal horn of rats with SCI-induced pain compared to rats with normal sensation, and early exercise reduced this activation independently of pain behavior. Further, abnormal pain sensation strongly correlated with an increased number of DRG macrophages. Importantly, exercise-treated rats that maintain normal sensation also have a lower number of macrophages in the DRG. Our data suggest that macrophage presence in the DRG may be an important effector of pain development, and early wheel walking exercise may mediate pain prevention by modulating the injury-induced macrophage response in the DRG. Further supportive evidence demonstrated that rats that developed pain despite exercise intervention still displayed a significantly elevated number of macrophages in the DRG. Collectively, these data suggest that macrophage presence in the DRG may be an amenable cellular target for future therapies.

Keywords: dorsal root ganglia; inflammation; macrophage; rehabilitation

Introduction

Spinal cord injury (SCI) affects ~500,000 people worldwide every year,[1] with the majority developing chronic neuropathic pain. Neuropathic pain after SCI can be attributed to a dysregulation and hyperexcitability of primary nociceptors in the dorsal root ganglia (DRG) and in neurons throughout the pain pathways in the central nervous system (CNS).[2–7] The limited pharmacological efficacy of current treatments may be because these drugs specifically target the activity of neuronal circuitry of the pain neuroaxis and not that of immune and inflammatory cells that interact with them. Indeed, neuropathic pain is now recognized as a neuroimmune phenomenon, with both central and peripheral contributing mechanisms.[8–14] Microglia are the resident immune cells of the CNS, and after SCI, pain is associated with activation of central microglia and astrocytes.[11,15–21] Microglia can interact with neurons by release of neuromodulators that affect synaptic transmission within the dorsal horn and supraspinal pain centers and, ultimately, pain behavior.[8,22–24] Interestingly, the SCI-induced immune and inflammatory response that occurs in the peripheral DRG, surrounding primary nociceptors, has not been well explored. Infiltration of peripheral bone-marrow–derived monocytes that differentiate into macrophages is a part of the innate immune response to injury,[25] and although they are recruited primarily for phagocytosis of debris, they, like microglia, can also release a plethora of neuromodulatory pro- and anti-inflammatory cytokines that can affect neuronal function.[22,26–28] Therefore, recruitment and persistence of macrophages and the resultant modulation of the inflammatory microenvironment and nociceptive transmission may be important in pain development and maintenance.

Exercise is a noninvasive, broad-spectrum, clinically useful intervention that modulates inflammation in addition to inducing activity-dependent neuronal plasticity after SCI.[29–35] Further,

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rehabilitative exercise is analgesic and modulates the immune response in peripheral neuropathic pain models.[36–39] Our lab has shown that exercise prevents pain development after SCI that corresponds to a reduction in aberrant sprouting of the central process of primary nociceptors into the dorsal horn.[40] Thus, it is possible that post-SCI exercise reduces pain development by acting directly on primary nociceptors or by modulating the peripheral immune response in the DRG.

The aim of the current work was to determine whether post-injury exercise prevents pain development by modulating the peripheral and central immune responses to SCI. In this article, we provide a better understanding of the cellular mechanisms of exercise-mediated pain prevention. We assessed pain development in rats using an established model of cervical SCI-induced neuropathic pain. SCI rats were exercised starting early post-SCI, and microglial and macrophage response to injury and exercise were assessed and compared in rats with and without pain. We report that rats with SCI-induced neuropathic pain have more macrophages in the DRG, along with increased microglial activation in the dorsal horn of the spinal cord, whereas rats that do not develop pain after SCI do not show this exaggerated immune response to SCI. Moreover, rats that receive acute exercise post-SCI are less likely to develop pain and have fewer macrophages in the DRG as well as reduced morphological signs of microglial activation in the spinal cord compared to naïve rats.

Methods

Subjects and surgeries

Adult, female Sprague-Dawley rats (225–250 g; Charles River Laboratories, Wilmington, MA) were housed 2–3 per cage in a controlled environment (12-h light-dark cycles) with food and water *ad libitum*. All experimental procedures were approved by the Drexel University Institutional Animal Care and Use Committee. Rats were randomly assigned to a naïve control ($n=5$) or SCI ($n=44$) group. All rats were handled and acclimated before baseline behavioral testing. Rats in the SCI group were subjected to a moderate spinal cord contusion injury at C5. A subset of rats ($n=15$) in the SCI group was subjected to an acute exercise paradigm for 4 weeks beginning at 5 days post-injury (dpi). After behavioral testing post-injury, SCI rats (Exercise and Non-Exercise) were further partitioned into two groups based on whether they developed pain (SCI No Pain [SCI NP; $n=12$] and SCI Pain [SCI P; $n=11$), and SCI Ex No Pain [Ex NP; $n=9$) and SCI Ex Pain [Ex P; $n=6$]). Groups of naive ($n=5$) and SCI ($n=6$) rats were sacrificed at 5 dpi to correspond to the time point of initiation of exercise.

The SCI was performed according to a clinically relevant and accepted model of SCI-induced neuropathic pain.[41] Briefly, rats were anesthetized using a ketamine (60 mg/kg)/xylazine (6 mg/kg)/acepromazine (6 mg/kg) cocktail and a hemilaminectomy was performed over C5, exposing the right dorsal surface of the spinal cord up to and partially over the midline. The spinal column was stabilized in the Infinite Horizons device (Precision Systems and Instrumentation, Lexington, KY),[42] and a custom probe was positioned 2 mm over the right dorsal surface of the C5 spinal cord close to the midline, centered between midline and the lateral edge. The spinal cord and surgical field were submerged in sterile saline, and a contusion was performed with 200-kdyne force, resulting in tissue displacement of 1500–1700 μm consistent across rats (see Table 1 for details). The incision was sutured in layers. Antibiotic (cefazolin, 160 mg/kg) was administered subcutaneously at the time of surgery to prevent infection. Lactated Ringer's solution (5 cc) was administered subcutaneously on the day of surgery and up to 5 days after surgery to prevent dehydration.

Table 1. Spinal Cord Injury Parameters for All Groups

Groups	n	*Force (kDyne)±SEM*	*Displacement (μm) ±SEM*	*Velocity (mm/s) ±SEM*
Naïve	5	Not applicable	Not applicable	Not applicable
SCI 5 days	6	224.66 ± 13.22	1711.1 ± 22.76	123.66 ± 1.17
SCI	23	239.78 ± 9.94	1643.06 ± 41.49	122.91 ± 0.62
SCI NP	12	227.37 ± 9.16	1662.52 ± 56.33	123.37 ± 1.13
SCI P	11	219.14 ± 9.05	1619.34 ± 72.89	120.43 ± 1.52
SCI Ex	15	234.54 ± 5.25	1598.66 ± 40.31	121.27 ± 0.63
Ex NP	9	235.69 ± 5.69	1612.15 ± 42.42	122.12 ± 0.73
Ex P	6	227.83 ± 13.99	1562.33 ± 96.73	119.667 ± 0.91

SCI spinal cord injury; SCI NP, SCI No Pain; SCI P, SCI Pain; SCI Ex, SCI Exercise; Ex NP, Exercise No Pain; Ex P, Exercise Pain; SEM, standard error of mean.

Forced exercise paradigm

Rats in the SCI Ex group were subjected to a 4-week forced exercise paradigm beginning at 5 dpi, 5 days a week for 20 min each day, as described previously.[40] Briefly, rats were placed in the forced exercise wheel walking system (Lafayette Instruments, Lafayette, IN) modified with a smooth vinyl surface added over rungs of the wheel to prevent slipping and compensate for the loss of function in the ipsilesional forepaw attributed to SCI. Rats were allowed to acclimate to the wheels before injury by being placed in the wheel walking system with the wheels locked in a stationary position. Starting at 5 dpi, rats were placed in the locked wheel walking system for 5 min before initiating exercise. The speed of running the wheels was gradually increased in 1-m/min increments, starting from 7 m/min to a maximum of 14 m/min as the forelimb capabilities of the rats allowed, and time spent at each speed was recorded. All rats walked at maximum speed by 10–12 dpi.

Behavioral testing

Rats were assessed for development of pain using von Frey monofilaments exerting varying forces on the rat forepaw with the up-down method.[43–45] All rats were acclimated to the apparatus, and behavioral parameters recorded preoperatively as the baseline response, and post-operatively at 28 dpi in a blinded experimental design. Ipsilesional (right) forepaws were tested to assess differences in pain perception attributed to the unilateral nature of our injury model. Operant testing for pain was performed 4 weeks post-SCI using the Mechanical Conflict-Avoidance Paradigm (MCAP; Noldus Technologies, Leesburg, VA).

von Frey testing

Rats were placed in individual Plexiglas chambers with a wire mesh bottom and allowed to acclimate for 10 min. Each paw was tested only when the weight of the rat was equally distributed on all four paws, or as much as possible post-SCI. Plastic monofilaments of varying bending forces (von Frey monofilaments; Stoelting Co., Wood Dale, IL) were applied to the plantar surface of the forepaw in the up-down method with an interval of 2–4 min between applications. A total of 10 von Frey monofilament stimulus applications were collected for each paw for each day of testing, beginning with the 5.18*g* force von Frey hair. Immediate forepaw withdrawal with evidence of supraspinal awareness (licking or looking at the paw, moving away from the stimulus, vocalization, etc.) was considered as a positive response, followed by application of von Frey hair of next lower force value. Absence of forepaw withdrawal was noted as a negative response, and the next higher force von

Frey hair was applied. Behavior indicating supraspinal awareness of tactile stimuli (glancing at paw, licking, and vocalization) was recorded. Paw withdrawal threshold (PWT) was determined as the lowest force (*g*) that produced a forepaw withdrawal and supraspinal behaviors in at least 50% of the applications at that force. Order of paw testing was randomized to minimize fatigue or an order effect. SCI rats that demonstrated a >50% reduction in PWT at 28 dpi compared to baseline were assigned to pain subgroups.[40,46,47]

Operant testing for cognitive perception of pain

The mechanical conflict-avoidance paradigm (MCAP) tests the cognitive perception of pain by presenting rats with a choice to remain in an aversive (brightly lit) environment to avoid painful mechanical stimulation, or subject themselves to the noxious stimulation to escape the aversive chamber and reach a preferred dark chamber. The experimental setup consists of a rectangular runway connecting the aversive (light) chamber with the preferred (dark) chamber (Fig. 1C). During 3 days of post-operative training (22–24 dpi), rats learned to spontaneously cross the runway to escape the aversive light stimulus. For testing, the runway floor was modified with an array of nociceptive probes (2 mm in height, 0.4 mm in diameter, spaced 10 mm apart), and latency to exit the aversive light chamber as well as time taken to cross the runway of nociceptive probes were recorded in seconds and averaged for each group. Rats that did not exit the lit chamber were assigned an escape latency of 30 sec for that trial, and those that failed to cross the runway in 60 sec were assigned a runway crossing time of 60 sec.[48]

Histology

Rats were sacrificed at 30 dpi (with a subset at 5 dpi) with an overdose of Euthasol (390 mg/kg of sodium pentobarbital and 50 mg/kg of phenytoin, intraperitoneally) followed by thoracotomy and perfused transcardially with 0.9% ice-cold saline followed by 4% paraformaldehyde (PFA). Eight millimeters of cervical spinal cord between C4 and C6 spanning the rostrocaudal extent of the lesion and a 4-mm block containing C7–C8 cord as well as ipsilesional C7 and C8 DRGs were dissected, post-fixed in PFA at 4°C overnight, then submersed in 30% sucrose for cryoprotection.

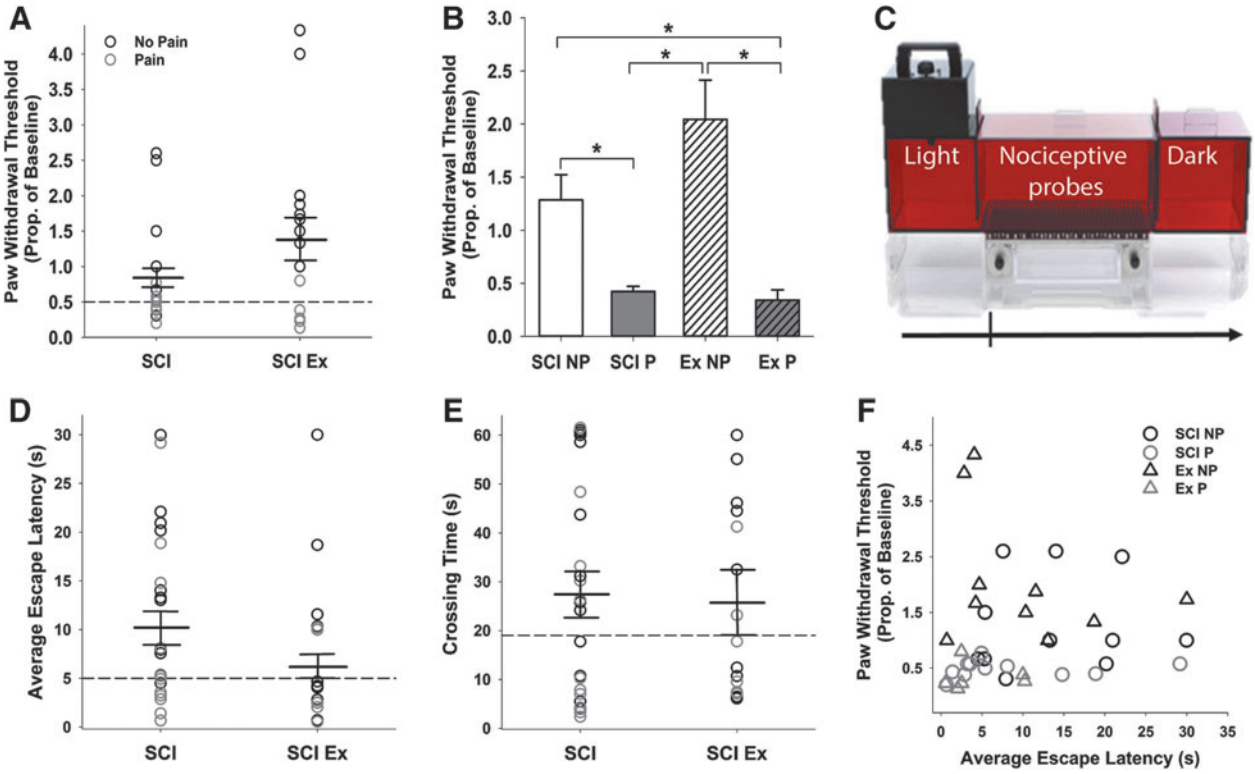


FIG. 1. Assessment of evoked and ongoing pain behavior after SCI in rats with and without exercise. PWTs were assessed preoperatively and at 28 days post-injury (dpi) in SCI rats with and without exercise, and proportional paw withdrawal thresholds relative to baseline were calculated. Rats were assigned to the SCI Pain (SCI P) group if their PWTs at 28 dpi were less than 50% of baseline. There were no overall differences between SCI and SCI Ex rats (**A**; gray circles indicate rats with PWT <50% baseline). A proportion of rats in the SCI only group as well as in the SCI Ex group developed pain. Analysis based on subgroups showed that SCI P rats had significantly lower proportional PWTs compared to SCI No Pain (**B**; SCI NP; $p = 0.02$) and Exercise No Pain (Ex NP; $p < 0.001$). Similarly, Ex Pain rats (Ex P) had significantly lower PWTs compared to SCI NP ($p = 0.017$) and Ex NP ($p < 0.001$). Importantly, SCI P and Ex P, and SCI NP and Ex NP did not differ significantly from one another (B). Cognitive perception of pain behavior was assessed using a mechanical conflict-avoidance paradigm depicted in (**C**). Rats were trained to cross a runway to escape a brightly lit aversive chamber and enter a safe, dark chamber. On testing day, the runway was inlaid with nociceptive probes and changes in latency to exit the bright chamber onto the nociceptive runway, as well as time taken to cross the runway into the dark chamber were measured. There was a nonsignificant tendency for the average latency to exit the brightly lit chamber to be lower in exercised rats than in unexercised rats (**D**). Whereas more rats in the SCI group (8 of 23) chose not to exit the chamber and cross the runway in one or more of the three trials compared to SCI Ex rats (2 of 15), there were no differences in crossing time between exercised and unexercised rats (**E**). There was no correlation between von Frey thresholds and escape latency in the MCAP (**F**; $r = 0.06$; $p > 0.05$). MCAP, Mechanical Conflict-Avoidance Paradigm; PWTs, paw withdrawal thresholds; SCI, spinal cord injury. Color image is available online.

Spinal cord blocks and DRGs were embedded in OCT compound (Fisher Scientific, Pittsburgh, PA) and sectioned at −20°C using a cryostat (Leica Microsystems, Wetzlar, Germany). Transverse 25-μm-thick sections 250 μm apart spanning across C4–C6 cord as well as 10-μm ipsilesional C7 DRG sections were mounted on gelatin-coated slides and 30-μm C7–C8 cord sections were floated in 0.1 M of phosphate-buffered saline (PBS) for immunohistochemical processing.

Analysis of lesion epicenter

Spinal cord sections (25 μm thick) 250 μm apart spanning the extent of the lesion from C4–C6 were stained with cresyl violet (Sigma-Aldrich, St. Louis, MO) for Nissl substance and eriochrome cyanine (Sigma-Aldrich) for myelin. Sections were cover-slipped with DPX (dibutylphthalate polystyrene xylene) mounting medium (Fisher Scientific). To determine the amount of spared tissue, the area of contralesional gray and white matter, spared gray and white matter on the ipsilesional side, and the extent of lesion cavity were measured using the Cavalieri estimator method (Stereo Investigator; MBF Bioscience, Burlington, VT) by an experimenter blinded to experimental groups. Proportion of spared tissue on the ipsilesional side compared to the tissue area on the contralesional side, as well as area of lesion cavity, was measured and compared across animals and groups.[40,41]

Analysis of microglia and macrophages

Spinal cord sections spanning the extent of the lesion from C4–C6 on slides, as well as floating sections of C7–C8, and ipsilesional C7 DRG sections on slides were washed three times with oxidized PBS with Tween 20 (OX-PBST; with thimerosal, T-8784; Sigma-Aldrich). Sections on slides (C4–C6 lesion, C7 R DRG) were treated with Dako Antigen Retrieval Solution (Agilent Pathology Solutions, Santa Clara, CA) at 95°C in a steamer for 20 min for heat-induced target retrieval. Slides and floating sections were incubated in blocking solution (5% normal serum, 1% fish gelatin [G7765; Sigma-Aldrich], 10% bovine serum albumin [BP1605; Fisher Scientific], 1% Triton X-100 [X-100; Sigma-Aldrich] in OX-PBS) at room temperature for 1 h, then incubated in primary antibodies (ED-1/mouse anti-rat ED-1/CD68 [MCA341R; Bio-Rad Laboratories, Hercules, CA] 1:1500, ionized calcium-binding adapter molecule 1 [Iba-1]-rabbit anti-rat Iba1 [1:6000; 019-19741; Wako Chemicals, Richmond, VA] overnight at room temperature. After three rinses in OX-PBST and a two hour incubation in secondary antibodies (biotinylated horse/anti-mouse immunoglobulin G [IgG]; H+L; #BA-2001; 1:200; Vector Laboratories, Burlingame, CA), biotin-SP–conjugated goat/anti-rabbit IgG H+L; #111-065-144; 1:200; The Jackson Laboratory, Bar Harbor, ME) sections were washed with 0.3% H_2O_2, 50% histology grade methanol in OX-PBS at room temperature for 30 min to quench endogenous peroxidases. Antibodies were visualized using the Vectastain Elite ABC reagent (Vector Laboratories) and 3,3′-diaminobenzidine (Vector Laboratories). DRG sections were counterstained with cresyl violet to visualize nuclear staining. Sections were dehydrated, cleared in Citrisolv (Fisher Scientific), and cover-slipped using DPX (Fisher Scientific).

ED-1 and Iba1 immunoreactivity was quantified using techniques adapted from Popovich and colleagues.[11,49] Optical density thresholds were manually selected using ImageJ (NIH, Bethesda, MD) for positively labeled tissue for either ED-1 or Iba1 and were quantified as the proportional area (PA) of positively stained tissue within a specific region. PA of ED-1 staining was measured from 5× images of whole cord from three representative sections 250 μm apart at the lesion epicenter (C4–C6) and C7–C8, and proportional area of Iba1 labeling in C7–C8 cord was measured from 40× images of medial and lateral ipsilesional dorsal horn from three representative sections at C7–C8 (Leica DM5500 B microscope; Leica Microsystems). Values for medical and dorsal horn images for three sections were averaged for each rat, and group averages are represented. For DRGs, ED-1–positive cells with phagocytic amoeboid morphology were identified as macrophages in methyl green counterstained sections and manually counted on 10-μm-thick sections 50 μm apart spanning through the entire ipsilesional C7 DRG and aggregated to represent total macrophage count for each DRG.

Statistical analysis

Statistical analysis was performed using SigmaPlot software (version 13; Systat Software, San Jose, CA). Wilks-Shapiro and Brown-Forsythe tests were used to assess normality and equal variance measures for all data before using parametric tests. One-way analysis of variance (ANOVA) and ANOVA on ranks were used to analyze differences between groups for PWTs, latency to exit and crossing time for MCAP, epicenter sparing, lesioned area, number of $ED1^+$ cells in the DRG and proportional area of $ED1^+$ and $Iba1^+$ tissue in the spinal cord, followed by Holm-Sidak (for one-way ANOVA) or Dunn's (for ANOVA on ranks) post-hoc comparisons. Chi-square tests were used to compare number of $ED1^+$ cells in DRG to tactile sensitivity, and Pearson correlations were used to compare proportional area of ED1 and Iba1 staining in the cord to tactile sensitivity. All graphs are reported as mean ± standard error of mean (SEM).

Results

Exercise therapy prevents the onset of neuropathic pain after spinal cord injury

Cohorts of rats in the SCI and SCI Ex groups demonstrated pain behavior (Fig. 1A; gray circles indicate rats with proportional paw withdrawal threshold <50% of baseline). There were no significant differences in the paw withdrawal thresholds between the SCI and SCI Ex groups overall (SCI, 0.85 ± 0.14; SCI Ex, 1.405 ± 0.3; $p > 0.05$). The baseline forepaw withdrawal threshold for all rats before SCI was 16.82 ± 3.66*g* force (averaged for all groups). By 28 dpi, rats within the SCI P cohort demonstrated a significantly decreased paw withdrawal threshold proportional to baseline (0.42 ± 0.04) compared to SCI No Pain (SCI NP, 1.28 ± 0.23; $p = 0.023$) and SCI Ex No Pain (Ex NP, 2.04 ± 0.36; $p < 0.001$; Fig. 1B). Rats in the Ex NP group had normal paw withdrawal thresholds (2.04 ± 0.36) whereas Ex P rats had a decreased paw withdrawal threshold at 28 dpi (0.34 ± 0.09) compared to SCI NP ($p = 0.017$) and Ex NP (Fig. 1B; $p < 0.001$). Importantly, positive paw withdrawal responses to von Frey monofilaments were accompanied by behavior indicative of supraspinal awareness of pain, such as glancing or licking at the paw, guarding paw from further stimulation, and nocifensive behavior. Rats in the Ex NP group, and some rats in the SCI NP group, tended to demonstrate hypoalgesic behavior, two rats had thresholds 4 times that of baseline, but most rats maintained near normal thresholds (Fig. 1A).

Figure 1D and 1E reports the data acquired in the MCAP (depicted in Fig. 1C). Gray circles indicate individual animals that exhibited pain-like behavior on the von Frey test. Rats within the SCI group demonstrated a nonsignificant trend toward an increased latency to exit the aversive light chamber to cross to the dark chamber over a surface with nocifensive probes compared to exercised SCI rats (Fig. 1D; $p > 0.05$). More rats within the unexercised SCI group chose not to cross the runway and instead remained in the light chamber (assigned a maximum crossing time of 60 sec; Fig. 1D). However, rats in both unexercised or exercised groups crossed the runway in similar times (Fig. 1E; $p > 0.05$), confirming that the divergent behavior was not a result of a motor deficiency or

inability to cross the runway with probes. Exercise appears to decrease the demonstrated pain behavior. Further, Figure 1F demonstrates that there is no correlation between paw withdrawal threshold and escape latency ($p > 0.05$). The lack of a correlation is not surprising, given that the MCAP examines ongoing pain in all four paws of the rat and does not identify hypersensitive paws. Rigorous standardization and validation of this test will help determine whether the MCAP will be effective at revealing differences in behavior between SCI rats with and without pain regardless of exercise.

Importantly, these exercise-induced behavioral effects are not an effect of the severity of the spinal cord lesion. Lateralized, contusive SCI produced a core lesion centered at C5 spinal cord extending from C4 through to C6, consisting of a cystic cavity surrounded by a thin rim of white matter in the lateral and ventral funiculi on the injured side (Fig. 2A–E). Quantitatively, the proportion of spared tissue of the ipsi- versus contralesional side of the spinal cord at the lesion epicenter was not different between SCI rats with and without exercise (Fig. 2F; SCI, 0.42 ± 0.031; SCI Ex, 0.429 ± 0.04; $F_{(1,33)} = 0.008$; $p = 0.927$) or between SCI rats at 5 days and rats with and without pain at 28 dpi (Fig. 2G; SCI 5 days, 0.40 ± 0.05; SCI NP, 0.46 ± 0.04; SCI P, 0.388 ± 0.04). $F_{(4,36)} = 0.389$; $p = 0.815$. Early wheel walking exercise had no effect on tissue sparing at the injury epicenter (Fig. 2G; Ex NP, 0.41 ± 0.06; Ex P, 0.44 ± 0.05; $F_{(4,36)} = 0.389$; $p = 0.815$). There were no significant differences in the contusive force, displacement of cord, and velocity of impact probe between groups ($p > 0.05$; Table 1).

Spinal cord microglia, but not macrophage response, is modulated by post–spinal cord injury exercise

As elucidated by our lab and others, microglial activation within the dorsal horn at and below the level of SCI is critical to pain development after SCI.[10,11,28,50–52] In naïve rats, microglia in their resting state display a ramified morphology, with small cell bodies and long, elaborate processes (Fig. 3A). Comparisons between naïve, SCI, and SCI Ex groups did not reveal any differences in microglial activation in the C7–C8 dorsal horn ($p > 0.05$; Fig. 3G). More-detailed analysis of Iba1 immunocytochemical staining of microglia in the C7–C8 spinal cord, which corresponds to forepaw dermatomes, revealed a significant increase in the proportional area of Iba1$^+$ tissue, indicating microglial activation, by 5 dpi (Fig. 3B) compared to naïve (Fig. 3A) tissue ($10.41 \pm 0.4\%$; $p = 0.006$). By 30 dpi, SCI NP rats (Fig. 3C) had microglia predominantly in a resting state within the dorsal horn ($5.49 \pm 0.8\%$; $p = 0.005$ compared to SCI 5 dpi; $p > 0.05$ compared to naïve). However, robust microglial activation was observed in the ipsilesional dorsal horn of SCI P rats (Fig. 3D; $10.70 \pm 1.24\%$; $p = 0.011$ vs. naïve; $p = 0.012$ vs. SCI NP). Iba1$^+$ cells in the dorsal horn of SCI P rats demonstrated hypertrophic cell bodies and retracted process typical of phagocytic cell morphology adopted by activated microglia (Fig. 3D, inset) that was distinct from the ramified, resting state observed in microglia in SCI NP rats (Fig. 3C, inset) and naïve rats (Fig. 3A). Importantly, rats in the Ex NP group demonstrated Iba$^+$ cell morphology similar to SCI NP rats (Fig. 3E, inset), pointing to a role of exercise in modulation of microglial/macrophage recruitment, proliferation, and activation within the spinal cord ($6.55 \pm 0.4\%$; $p > 0.05$ compared to naïve). Rats in the Ex P group also exhibited little microglial activation in the dorsal horn (Fig. 3F, inset; $6.78 \pm 1.02\%$; $p > 0.05$ vs. naive), suggesting that exercise modulates microglial activation, but that this may not be sufficient to reduce pain development after SCI (quantified in Fig. 3H). Degree of microglial activation in the superficial dorsal horn was positively associated with the severity of allodynia (%baseline paw withdrawal threshold) after SCI (Pearson coefficient $r = -0.403$; $p = 0.0163$; Fig. 3I).

There is robust hematogenous macrophage recruitment and phagocytic cell activity within and around the lesion created by a spinal cord injury.[26,49,53–55] Interestingly, there were no differences

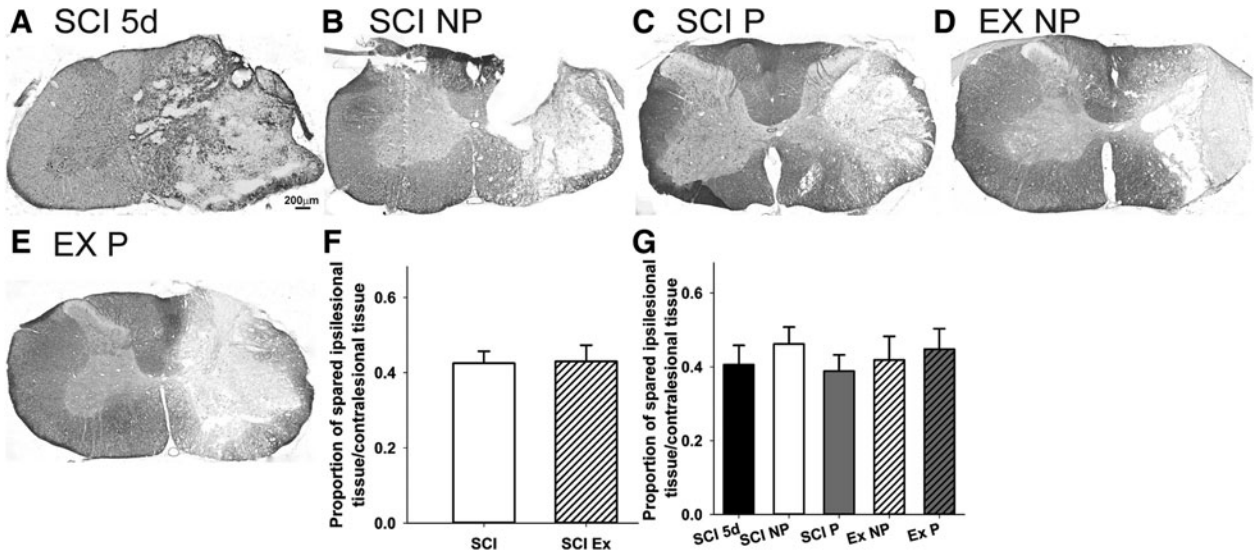


FIG. 2. Assessment of SCI lesion severity. Differences in pain behavior were not dependent on the degree of tissue sparing at the lesion epicenter. The 200-kdyn impact produced a moderate, unilateral lesion with degeneration of gray matter and a spared rim of white matter on the side of the injury (representative images for all groups; **A–E**). Importantly, gray and white matter of the contralesional spinal cord remained intact. There were no differences between SCI rats with and without exercise in the proportion of tissue spared on the ipsilesional side compared to the contralesional intact side (**F**; $F_{(1,33)} = 0.008$; $p = 0.927$) or between SCI 5 days rats and SCI and SCI Ex rats with and without pain (**G**; $F_{(4,36)} = 0.389$; $p = 0.815$). SCI, spinal cord injury.

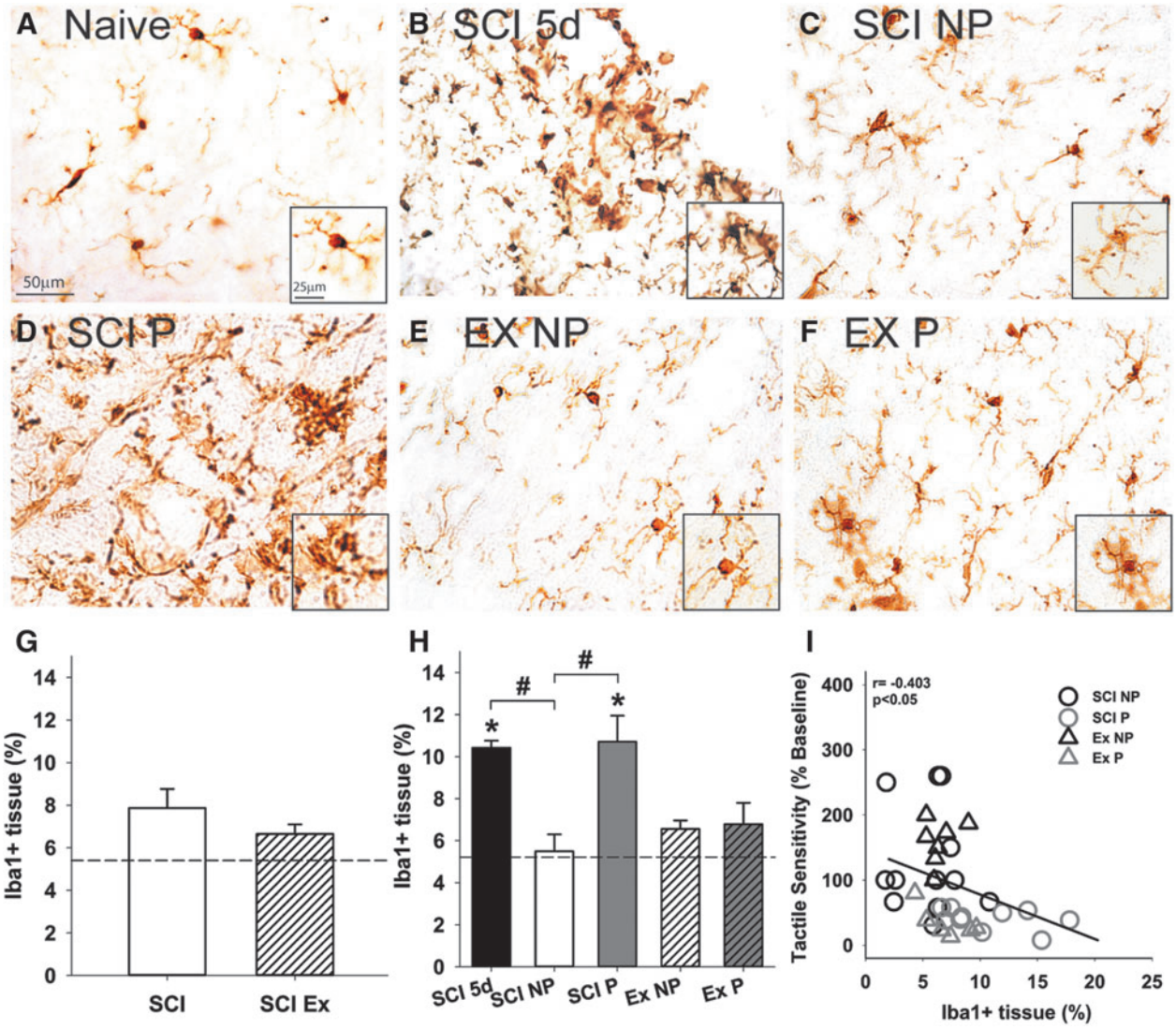
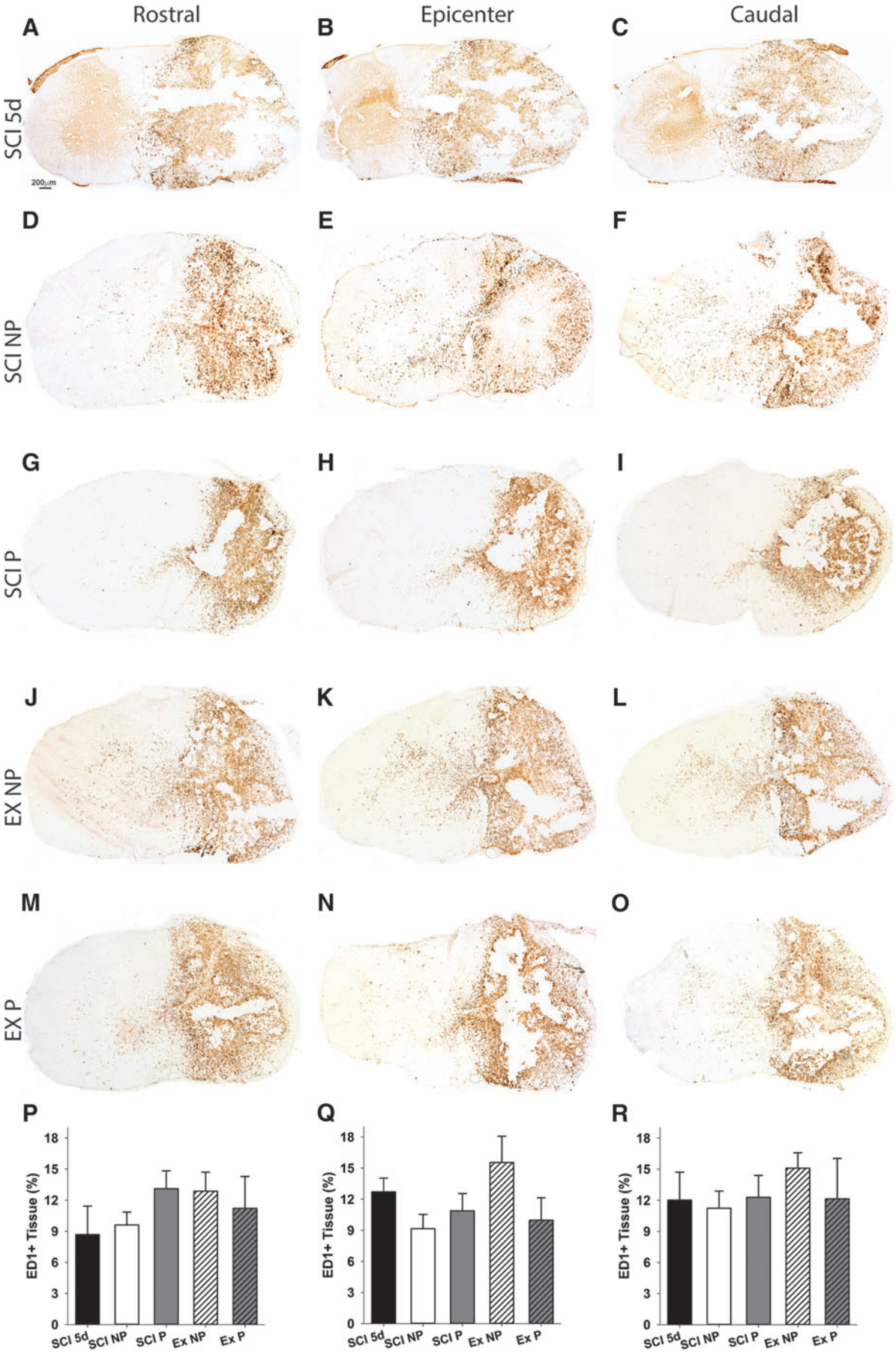


FIG. 3. Microglial activation in the C7–C8 dorsal horn corresponding to forepaw dermatomes. Microglial activation in ipsilateral dorsal horn measured as proportional area of Iba1$^+$ tissue in the medial and lateral dorsal horn of the C7–C8 spinal cord corresponding to forepaw dermatomes, from images at 40× magnification. There were no significant differences in microglial activation between SCI rats with and without exercise (**G**; dashed line indicates naive). Further partition of SCI and SCI Ex groups revealed that there was pronounced microglial activation by 5 dpi (**B**; $n=6$; $p=0.006$) compared to naïve (**A**; $n=5$), that is, significantly lower by 30 dpi in SCI NP rats (**C**; $n=12$; $p=0.005$ vs. 5 dpi, $p>0.05$ vs. naïve). Rats in the SCI P group have a significantly higher proportional area of Iba1$^+$ staining in the C7–C8 dorsal horn (**D**; $n=10$) at 30 dpi compared to naïve ($p=0.011$) and SCI NP ($p=0.012$). Microglia in the SCI P group demonstrated an activated phagocytic phenotype (inset, **D**) whereas those in the naïve and SCI NP groups appeared to be ramified/resting (insets A, C, and **E**). SCI Ex NP (E; $n=9$) and SCI Ex P (**F**, $n=6$) did not display significantly different Iba1$^+$ staining compared to the naïve dorsal horn, (**H**; dashed line indicates naive). Correlational analysis revealed larger proportional area of Iba1$^+$ stained tissue in the dorsal horn corresponding to lower paw withdrawal thresholds expressed as a percentage of baseline thresholds (**I**; $r=-0.403$; $p<0.05$). dpi, days post-injury; Iba1, ionized calcium-binding adapter molecule 1; SCI, spinal cord injury. Color image is available online.

in the proportional area of ED-1$^+$ staining at the lesion epicenter (Fig. 4) or in C7–C8 spinal cord (Fig. 5). Macrophages are concentrated in and around the lesion cavity (Fig. 4A–O), presumably to clear away debris. They are present by 5 dpi, and this secondary injury response does not appear to be related to the development of pain, nor is it modulated by acute exercise. Within the lesion, the proportional area of ED-1, when calculated as percent of total area of whole spinal cord (ipsi- and contralesional) showed no significant differences between groups at 1) 250 μm rostral to epicenter (Fig. 4P; SCI 5 days, 8.68 ± 2.73%; SCI NP, 9.60 ± 1.25%; SCI P,

FIG. 4. Phagocytic immune cell accumulation at the lesion. The area occupied by tissue stained positively for ED1 as a proportion of the cross-sectional area of lesioned spinal cord was calculated at the lesion epicenter (**B, E, H, K, N**) and 250 μm rostral (**A, D, G, J, M**) and caudal (**C, F, I, L, O**) to the epicenter for all rats in the SCI 5d (A–C; $n=6$), SCI NP (D–F; $n=11$), SCI P (G–I; $n=10$), Ex NP (J–L; $n=9$), and Ex P (M–O; $n=5$) groups. Group averages ± SEM are represented for percent area of ED1$^+$ staining. There was no significant difference between groups at each cross-section (**P**; rostral to epicenter, $F_{(3,31)}=0.733$; $p=0.54$; **Q**; epicenter, $F_{(3,30)}=2.308$; $p=0.097$; **R**; caudal to epicenter, $F_{(3,31)}=0.775$; $p=0.517$). SCI, spinal cord injury; SEM standard error of mean. Color image is available online.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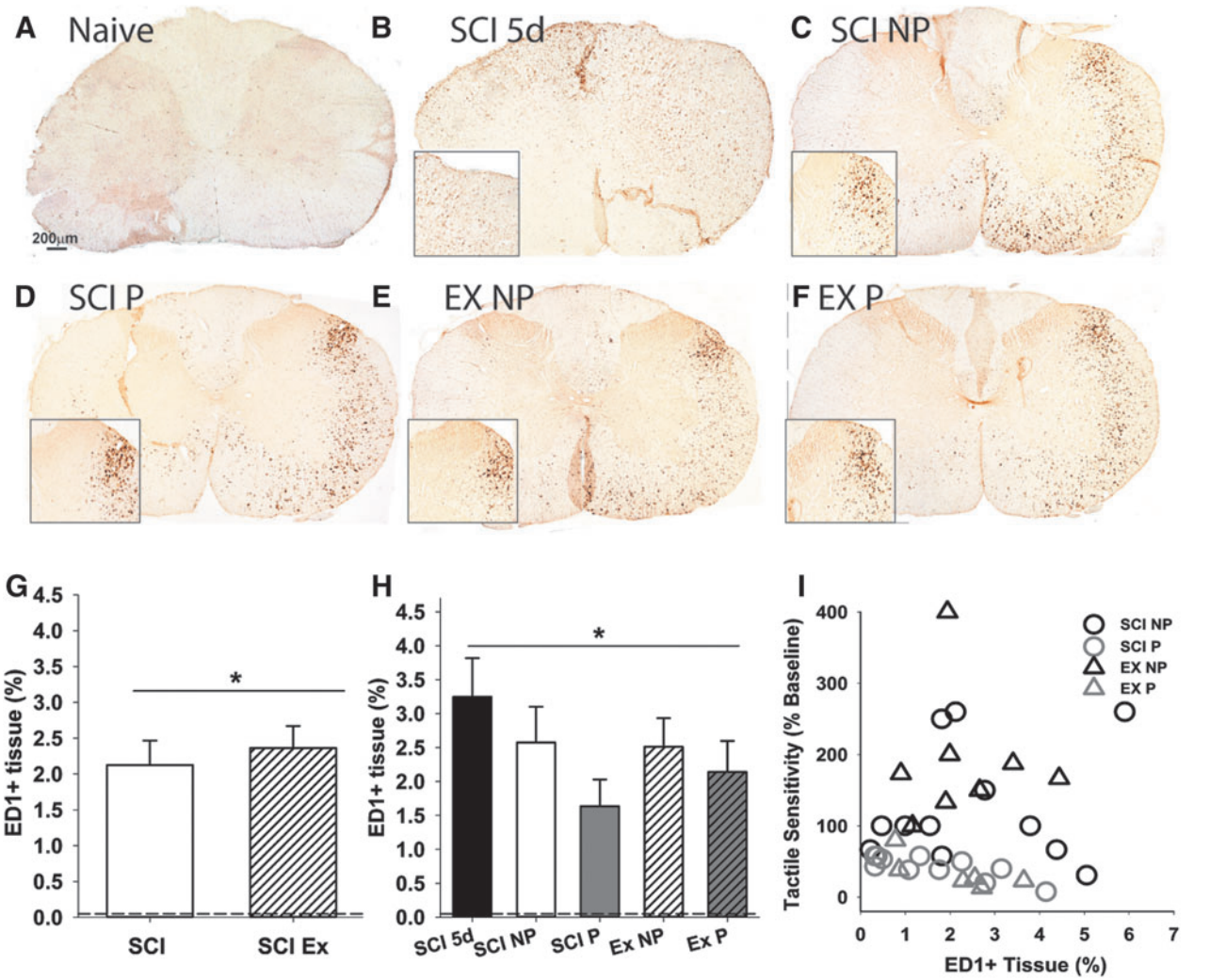

FIG. 5. Phagocytic immune cell accumulation in the C7–C8 spinal cord. The area occupied by tissue stained positively for ED1 as a proportion of the cross-sectional area of representative sections from C7–C8 spinal cord was calculated for all rats in the Naïve (**A**; $n=5$), SCI 5 days (**B**; $n=6$), SCI NP (**C**; $n=12$), SCI P (**D**; $n=11$), Ex NP (**E**; $n=9$), and Ex P (**F**; $n=6$) groups. Three representative sections per rat were analyzed and averaged. Group mean averages ± SEM were represented for the percent area of $ED1^+$ staining. There was no positive ED1 staining in naïve C7–C8 spinal cord sections (A). Dashed line indicates naïve $ED1^+$ tissue. $ED1^+$ staining was predominantly in the ipsilesional dorsolateral quadrants (inset within A–F for each group). Whereas both SCI and SCI Ex groups exhibited significantly increased degree of staining vs naïve ($p<0.05$), there was no significant difference between SCI and SCI Ex groups (**G**; dashed line indicates naïve $ED1^+$ tissue; $F_{(1,26)}=0.232$; $p=0.634$). Pain subgroups were significantly different from naïve, but not from one another (**H**; dashed line indicates naïve; $F_{(5,43)}=3.741$; $p=0.007$). There was no correlation between ED1 labeling and tactile sensitivity (**I**; $r=0.221$; $p=0.183$). SCI, spinal cord injury; SEM standard error of mean. Color image is available online.

13.10 ± 1.72%; Ex NP, 12.86 ± 1.83%; Ex P, 11.20 ± 3.07%; $F_{(3,31)}=0.733$; $p=0.54$); 2) the epicenter (Fig. 4Q; SCI 5 days, 12.7 ± 1.33%; SCI NP, 9.15 ± 1.37%; SCI P, 10.87 ± 1.66%; Ex NP, 15.53 ± 2.52%; Ex P, 9.968 ± 2.17%; $F_{(3,30)}=2.308$; $p=0.097$); and 3) 250 μm caudal to the epicenter (Fig. 4R; SCI 5 days, 12.01 ± 2.69%; SCI NP, 11.22 ± 1.66%; SCI P, 12.28 ± 2.11%; Ex NP, 15.09 ± 1.48%; Ex P, 12.13 ± 3.89%; $F_{(3,31)}=0.775$; $p=0.517$).

In the C7–C8 spinal cord, ED-1–positive macrophages accumulated by 5 dpi (Fig. 5B) and were present at least up to 30 dpi in the ipsilesional white matter (Fig. 5C–F). They were concentrated in regions where Wallerian degeneration was presumably occurring, anterogradely in damaged cortico-, rubro-, and vestibulospinal tracts of all groups (Fig. 5B–F, inset of the ipsilesional dorsolateral quadrants). They were also present in the contralesional ventrolateral white matter, where fibers from the ascending spinothalamic tract reside. Whereas both SCI and SCI Ex groups were significantly different from naïve (Fig. 5A,G; $p<0.05$), there was no significant difference between SCI (2.12 ± 0.34%) and SCI Ex (2.36 ± 0.30%) groups (Fig. 5G; $F_{(1,26)}=0.232$; $p=0.634$). Pain subgroups were significantly different from naïve, but not from one another (Fig. 5H; Naïve, 0.02 ± 0.007%; SCI NP, 2.57 ± 0.52%; SCI P, 1.63 ± 0.39%; Ex NP, 2.51 ± 0.42%; Ex P, 2.13 ± 0.45%; $F_{(5,43)}=3.741$; $p=0.007$). There was no correlation with tactile sensitivity (Fig. 5I; Pearson's $r=0.221$; $p=0.183$).

Exercise mediated reduction in dorsal root ganglia macrophages reduces incidence of spinal cord injury pain

We quantified post-SCI macrophage invasion into, or proliferation in, the C7–C8 DRGs containing cell bodies of primary

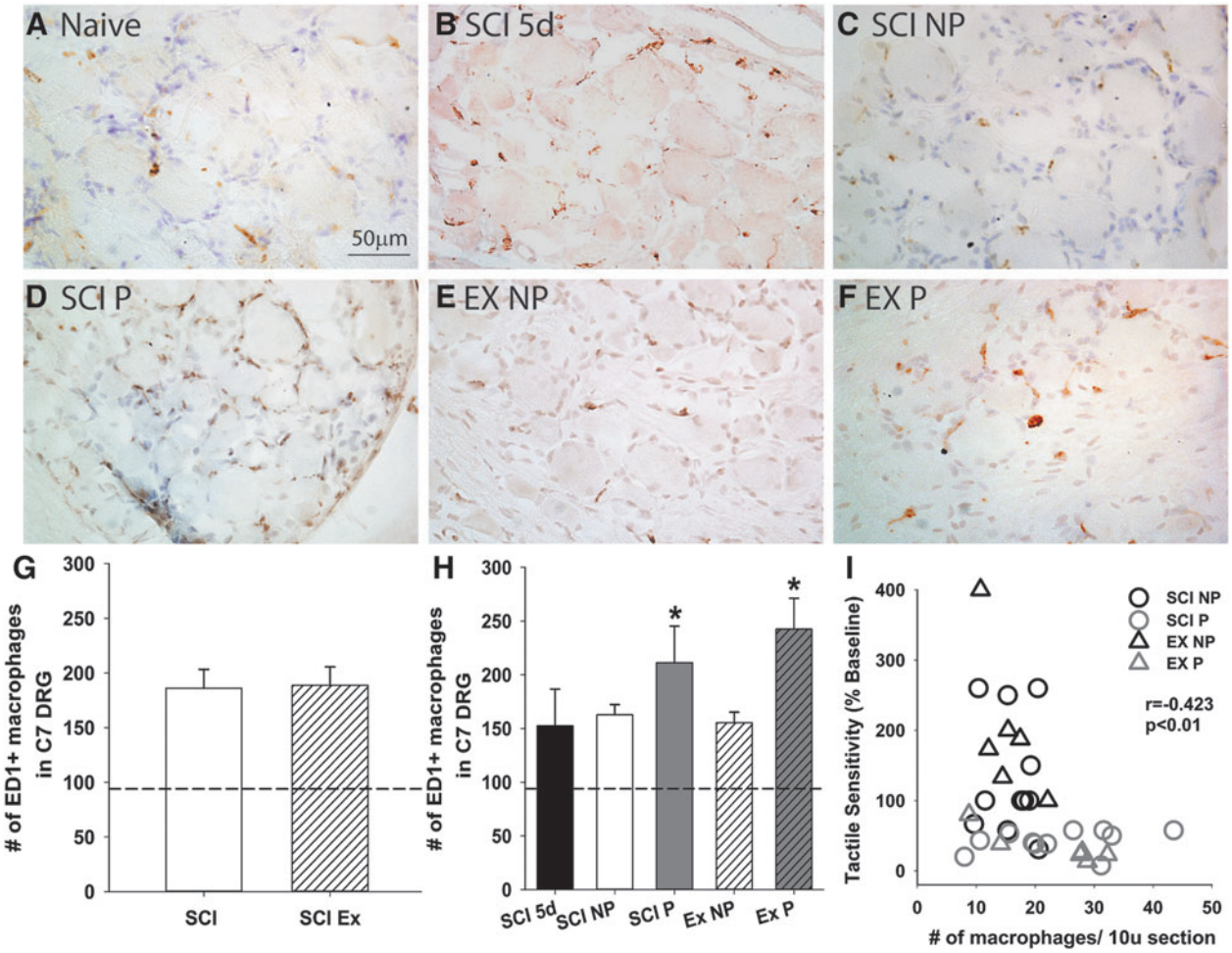

FIG. 6. Macrophage response to SCI and exercise in the C7–C8 DRG. The total number of ED1$^+$ cells in C7–C8 ipsilesional DRG were counted from 10-μ-thick sections 50 μm apart throughout the entire DRG as representative of whole DRG. Macrophages were present in the DRG at 5 dpi (**B**, $p > 0.05$ compared to naïve; **A**, $n = 5$). The total number of macrophages in SCI ($n = 23$) and SCI Ex rats ($n = 15$) at 30 dpi were not significantly different from each other or naïve (**G**, dashed line indicates naïve; $p > 0.05$). However, ED1$^+$ cell numbers at 30 dpi were significantly higher in SCI P rats (**D**; $n = 10$) compared to naïve rats (A; $n = 5$; $p = 0.042$), while ED1$^+$ cell count for SCI NP (**C**, $n = 11$) was not significantly different from naïve. Only Ex P (**F**; $n = 6$) had significantly higher numbers of macrophages (242.5 ± 28.4; $p < 0.01$ compared to naive), while ED1$^+$ cell count for Ex NP rats (**E**; $n = 9$) was not significantly different from naïve (dashed line indicates naïve, all data quantified in **H**). The number of elevated macrophages averaged per section in the C7–C8 ipsilesional DRGs was significantly correlated with lower paw withdrawal thresholds expressed as a percentage of baseline thresholds (**I**; $r = 0.423$; $p = 0.0141$). dpi, days post-injury; DRG, dorsal root ganglia; SCI, spinal cord injury. Color image is available online.

sensory neurons corresponding to forepaw dermatomes (C7 and C8) at 5 and 30 dpi. After SCI, we observed robust macrophage recruitment to, or proliferation in, the C7 and C8 DRGs by 5 dpi, the time point when early exercise was initiated, that persisted at 30 dpi (Fig 6B, 6C–F, 6G,H, Naïve 6A, indicated by dashed line in bar graph; SCI 5 days, 152.5 ± 34.1). Total numbers of macrophages in SCI and SCI Ex rats at 30 dpi were not significantly different from one another or naïve (Fig. 6G; $p > 0.05$; dashed line indicates the number of macrophages in naïve rats). Partitioning the data based on the presence or absence of pain revealed that the number of ED-1$^+$ cells in rats within the SCI NP group was not significantly different from naïve DRGs (Fig 6A,C,H; naïve, 94 ± 19.59; SCI NP, 162.75 ± 9.55). However, rats in the SCI P group had a significantly larger number of ED-1$^+$ cells in C7–C8 DRGs at 30 dpi compared to naïve (Fig 6D,H; SCI P, 211.36 ± 33.89; $p = 0.042$).

Analysis based on the presence or absence of pain behavior in the SCI Ex group revealed similar findings. The Ex NP subgroup did not have more ED-1$^+$ cells compared to naive (Fig. 6E; Ex NP, 152.77 ± 9.03; $p > 0.05$), but the Ex P subgroup had a significantly higher number of macrophages compared to naïve controls (Fig. 6F; Ex P, 242.5 ± 28.4; $p < 0.01$). This suggests that although exercise may be able to modulate peripheral macrophage response in the DRG after SCI to an extent, presence of pain is strongly correlated with presence of macrophages in the DRG, with or without exercise. Indeed, the number of ED-1$^+$ cells in the DRG across groups was significantly inversely correlated with the normalized PWTs; rats with more macrophages had a lower pain threshold (Fig. 6I; Pearson's r = –0.380; $p < 0.05$).

Discussion

It is now understood that exercise can reduce neuropathic pain development contributing to improved functional recovery after SCI; early aerobic exercise interventions, as initiated in this study

and others, are sufficient to reduce SCI-induced pain (Fig. 1).[40,56–58] The results presented here extend these findings by characterizing both the microglial response in the spinal cord and the macrophage response in the DRG in response to injury and exercise. In the current experiments, we generated multiple cohorts of exercised rats in order to have sufficient statistical power to use exercise as a tool to understand critical mechanisms of pain. We have previously shown that early wheel walking reduces the incidence of SCI-induced pain by 83%.[40] In our previous studies, 7% of animals developed pain despite exercise therapy. Our objective in this study was to use both pain and no pain subgroups in exercised rats to identify mechanisms that underlie the persistence of pain in subgroups where therapeutic intervention is ineffective. We observed microglial activation in the dorsal horn of the spinal cord of rats with SCI-induced pain compared to rats with normal sensation, and early exercise reduced this activation independent of pain behavior. We observed an elevated presence of macrophages in the DRGs of rats subjected to a C5 spinal cord contusion injury that develop neuropathic pain compared to SCI rats that maintain normal sensation. Abnormal pain sensation is strongly correlated with an increased number of macrophages in the DRG. Importantly, exercise-treated rats that maintain normal sensation (Ex NP) also have similar number of macrophages in the DRG as naïve and SCI NP. Collectively, these data suggest that whereas microglial activation in the dorsal horn of the spinal cord correlates with pain behavior, it is likely that macrophage presence in the DRG is critical to pain, and modulating this response (here, by exercise) may play a key role in mechanisms that prevent pain development. Our data suggest that macrophage presence in the DRG may be an important effector of pain development, and early wheel walking exercise may mediate pain prevention by modulating the injury-induced macrophage response in the DRG. That SCI rats that developed pain despite exercise intervention displayed a significantly elevated number of macrophages in the DRG provides further supportive evidence for this relationship. It also suggests that a generalized therapy may not be sufficient to attenuate pain in a heterogenous population in the clinical setting and points to the need for individualized approaches in physical rehabilitation efforts.

We demonstrate that macrophages have already been recruited to the DRG when exercise is initiated at 5 dpi (Fig. 6B), and suggest that exercise is acting to alter their proliferation, clearance, or secretome. It is plausible that macrophage response to exercise may affect nociceptor anatomical and physiological plasticity.[40,59] Further experiments to determine whether macrophage infiltration to the DRG alter neuroimmune interactions leading to pain behavior are underway in the lab.

Microglial activation and spinal cord injury pain

Resident microglial activation in the dorsal horn has been implicated and well studied in the context of pain development after SCI.[11,15,16,51,52,60,61] Here, we observed pain-specific increases in microglial activation in the dorsal horn of SCI rats. Like macrophages, activated microglia release proinflammatory and nociceptive mediators that interact with receptors on dorsal horn pain projection neurons and interneurons to induce their depolarization.[62,63] Our data reinforces the hypothesis that activated microglia in the dorsal horn of the spinal cord may be involved in the development of neuropathic pain after SCI (Fig. 3). However, it may be not necessary to maintain pain. In Figure 3G, early post-injury exercise reduced dorsal horn microglial activation without a concomitant reduction in pain behavior. This is contrary to studies showing reduction in pain upon manipulation of microglia.[11,51] However, these data support the hypothesis that early inflammatory/immune cell responses can induce prolonged sensitization of dorsal horn processing of painful stimuli.

Peripheral macrophage infiltration and spinal cord injury pain

In addition to resident CNS microglia that respond to injury, macrophages from the circulation are recruited to the lesion site to assist with wound healing and clearance of debris.[50,64–69] We observe that macrophages are present at the lesion site[26,49,55,70] (Fig. 4) and at the C7–C8 level of the spinal cord (Fig. 5), where somatosensory information from the forepaw is processed, in similar numbers regardless of pain profile or exercise. At C7–C8, macrophages are found exclusively within degenerating white matter tracts. Their absence in the gray matter of the dorsal horn at this level suggests that although macrophage infiltration to, or proliferation in, the spinal cord is a critical component of the immune response at the injury epicenter (C4–C6), it is unlikely that these cells are mediating aberrant excitability of dorsal horn pain circuits.

Resident macrophages are present in all peripheral tissues, and macrophages from the bloodstream rapidly infiltrate the DRG in response to neuronal injury, infection, as well as paracrine and circulating inflammatory signals.[28,71–74] Presence of macrophages in the DRG is strongly associated with presence of pain after SCI (Fig. 6), and this has been established in chemotherapy-induced pain as well as peripheral nerve injury models of pain.[75–80] In the SCI groups that do not develop pain, there is a marked absence of a macrophage response, whereas in groups with pain, macrophages persist in the DRG. It is well established that peripheral macrophages can undergo morphological and functional changes depending on the environmental cues they receive.[8,9,81–84] Thus, they can play a diverse role in pain modulation by adopting a spectrum of pro- and anti-inflammatory phenotypes and secreting a myriad of pro- or anti-inflammatory cytokines that modulate expression of ion channels, such as transient receptor potential ankyrin 1, transient receptor potential vanilloid 1, and voltage-gated sodium channels Nav1.7-1.9, to mitigate excitability of primary nociceptors.[8,9,83,84] Whether macrophages that persist chronically in the DRG after central injury take on an inflammatory or anti-inflammatory phenotype is important and warrants further study.

Further, this heightened peripheral immune response to SCI may be at the crux of promoting aberrant anatomical and functional plasticity of nociceptors.[84–86] *In vitro* and *in vivo* studies within and outside the context of neuropathic pain show the effect of macrophages on neurite outgrowth of sensory neurons with or without damage to the afferents.[87–90] The macrophage secretome may be driving this outgrowth by way of stimulating synthesis of regeneration-associated genes (such as growth-associated protein 43, activating transcription factor 3, and signal transducer and activator of transcription 3) that are known to induce sprouting of primary sensory afferents terminating in the dorsal horn.[91,92]

The signals that initiate macrophage recruitment in the DRG (e.g., damage-associated molecular patterns, adenosine triphosphate, and chemokines) are likely released because of direct damage to primary afferent fibers. van Steenwinckel and colleagues[93] showed that axonal damage can induce synthesis and retrograde transport of macrophage chemoattractants to the neuron soma and subsequent release in the DRG, inducing neurogenic inflammation.[75,94,95] SCI damages primary afferent fibers that travel in the dorsal columns

and Lissauer's tract as well as those that terminate in the dorsal horn of the spinal cord, introducing the possibility that SCI induces retrograde transport of chemoattractant molecules to the DRG that lead to macrophage recruitment or proliferation and macrophage-mediated pain. Moreover, these chemoattractants can act on neurons themselves to induce both nociceptor hyperexcitability by activating ion channels as well sprouting of sensory afferents.[75–78,90,93] Interruption of chemokine (c-c motif) ligand 2 (CCL2) signaling (a key macrophage chemoatrractant) by inhibiting its receptor, C-C chemokine receptor type 2, pharmacologically or by genetic knockdown prevents neuropathic pain development.[77,79] Future studies will assess whether macrophage chemoattractants promote neuroimmune interactions and pain development after SCI independently or by induction of macrophage-laden, proinflammatory DRG microenvironment.[80,86] This bidirectional communication between neurons and immune cells makes neuroinflammation a persistent phenomenon in the development and maintenance of chronic pain within the DRG after SCI and merits further exploration.

Exercise as a tool to dissect mechanisms underlying spinal cord injury pain

Exercise is a multi-potent therapy with myriad effects on the central and peripheral nervous systems as well as the immune response, and to dissect the individual mechanisms of the beneficial effect of exercise on the reduction of pain is challenging. Different modalities of rehabilitative exercise have been shown to prevent or reduce neuropathic pain in peripheral and central injury models.[37,39,40,59,96–98] The effect of exercise is systemic, and its modulation of neuropathic pain is likely attributed to its cumulative effects along the sensory neuroaxis. Exercise has been shown to directly modulate immune and glial cell recruitment as well as their function by reducing the levels of chemoattractant proteins like CCL2 or CCL5 and altering the inflammatory balance from a pro- to an anti-inflammatory state.[38,99–102]

Exercise as a therapy for spinal cord injury pain

It is important to note that the timing of exercise induction after SCI can be critical. Early exercise (starting at 5 dpi) appears to help normalize the peripheral immune response without affecting the acute inflammatory response needed to assist in wound healing at the injury epicenter as the severity of the SCI was not different between exercised and unexercised rats (Fig. 2). Interestingly, there are conflicting reports about the efficacy of exercise on the reduction of established SCI-induced pain. We have previously shown[103] that delayed aerobic exercise was ineffective at reducing established pain, whereas others show some attenuation.[56,58] One possibility for this discrepancy could be related to the timing of the intervention. If exercise was initiated after bidirectional neuroinflammatory cascades that maintain pain were established and pain set in, it may no longer be able to modulate inflammation and aberrant sensation. Similarly, the intensity of exercise is also an important factor to consider when establishing rehabilitative interventions for pain, given that intense exercise can promote a pro-inflammatory response, and cause or increase pain in experimental animal models and humans.[104–106]

In summary, the studies conducted here identify a marked increase in macrophages within the DRGs specifically as predictors of pain after SCI. Post-injury exercise reduces the number of macrophages in the DRG that corresponds to reduced pain behavior, whereas exercise reduces microglial activation in the dorsal horn of the spinal cord that does not correspond to alterations in pain behavior. These data suggest that the immune response in the DRG is integral to pain development, and maintenance and macrophages may be an attractive target for future therapies.

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Author Disclosure Statement

No competing financial interests exist.

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