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Prior voluntary wheel running attenuates neuropathic pain

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1. Introduction

Experimental evidence suggests that exercise may be therapeutic for pre-existing neuropathic pain of both peripheral and central origin.^{1,6,7,12–15,18,31,34,42,54,60} Several studies have reported that neuroimmune signaling, processes that subserve neuropathic pain after nerve injury,^{2,21} is suppressed in the neuraxis of rodents exercised after injury. For example, expression of activation markers for microglia (Iba1, CD11b) and astrocytes (GFAP) is decreased in the spinal dorsal horn,^{1,6,15,31,42} while pro-inflammatory cytokines tumor necrosis factor and interleukin-1 β (IL-1 β) are downregulated in the brainstem and sciatic nerve.^{7,12,13} Such pro-inflammatory cytokines are produced by reactive macrophages, microglia, and astrocytes, and increase neuroexcitability in nociceptive pathways (e.g. by downregulating astrocyte glutamate transporters).^{2,21,74} Brain derived neurotrophic factor (BDNF) expression is also attenuated in the dorsal root ganglia (DRG) and spinal dorsal horn, and potassium-chloride cotransporter (KCC2) expression is restored in the spinal dorsal horn in exercised rodents.^{1,14,42} This pro-nociceptive axis induces disinhibition following downregulation of KCC2 by P2X4 receptor-dependent BDNF release from microglia.^{17,66,67}

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Conflict of Interest

The authors have no conflicting financial interests.

The beneficial effects of exercise on the sequelae of neuropathic pain have only been explored in the context of reversal of pain after injury. However, as exercise has multiple effects on basal immune cell function and phenotype in the absence of injury, this intervention is potentially neuroprotective for neuropathic pain. For example, exercise alters the expression of receptors on immune cells (e.g. downregulates toll-like receptors),^{50–53,57} increases phagocytosis,⁶³ inhibits immune cell chemotaxis and infiltration into sites of inflammation,^{32,73} and promotes anti-inflammatory mediator synthesis by circulating leukocytes.^{47,48} Although clinical evidence indicates that exercise decreases the incidence of chronic pain within the general population,^{35,36} the protective role for exercise on subsequent neuropathic pain is experimentally under-investigated.^{15,19}

The aim of this study was to examine whether voluntary wheel running, performed either before or after peripheral nerve injury, would attenuate allodynia. This study also aimed to determine if attenuated allodynia was accompanied by suppressed neuroimmune signaling in the blood, sciatic nerve, DRG and lumbar dorsal horn. Given the prior focus on modulation of such signaling pathways by exercise beginning after nerve injury, noted above, we exclusively focused the analyses on rodents that had exercised prior to injury. Voluntary wheel running was chosen as the exercise paradigm, because: a) potential stress induced during forced protocols, such as treadmill running or swimming, may confound nociception,^{8,11,33} and; b) exercise occurs during the active phase, without the sleep disruption that can accompany forced exercise protocols. Here we report that voluntary wheel running both prevented and reversed allodynia after chronic constriction injury (CCI) of the sciatic nerve. We also report that the prevention of CCI-allodynia by voluntary wheel running ceasing prior to nerve injury was associated with decreased neuroimmune signaling in the lumbar spinal dorsal horn, attenuated macrophage infiltration into the injured sciatic nerve and DRGs, suppressed serum pro-inflammatory mediator levels at day 3 after CCI, and induced peripheral blood mononuclear cell (PBMC) anergy (suppressed reactivity) at day 14 post CCI.

2. Methods

2.1. Animals

Pathogen-free adult male Sprague Dawley rats (n = 6 rats/group for each experiment; 10–12 wks old on arrival; Harlan Labs, Indianapolis, IN) were used in all experiments. Rats were housed in temperature-controlled ($23 \pm 3^{\circ}\text{C}$) and light-controlled (12 h light:dark cycle; lights on at 07:00 h) rooms with standard rodent chow and water available *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Colorado Boulder.

2.2. Chronic Constriction Injury (CCI)

Neuropathic pain was induced using the CCI model of sciatic nerve injury.⁵ Surgery was performed at the mid-thigh level of the left hindleg as previously described.²² In brief, animals were anesthetized with isoflurane. The shaved skin was treated with Nolvasan and the surgery was performed aseptically. Four sterile chromic gut sutures (cuticular 4-0 WebGut, Patterson Veterinary, Devens, MA) were loosely tied around the gently isolated

sciatic nerve. For sham surgery, the sciatic nerve was isolated, but no chromic gut sutures were tied around the nerve. Animals were monitored post-operatively until fully ambulatory, prior to return to their home cage.

2.3. Voluntary wheel running

Rats were single-housed with in-cage running wheels, while sedentary control animals were single-housed with a locked wheel. Voluntary, unrestricted access to running or locked wheels occurred either: *i*) continuously for 6 weeks prior to surgery, and concluding on the day of surgery. This paradigm was selected as we have previously shown that it attenuates the pro-inflammatory response to endotoxin and stress;^{46,59} *ii*) continuously after surgery, beginning on the day of surgery; or *iii*) continuously after surgery, beginning 2 weeks after surgery. Daily wheel revolutions were recorded digitally using Vital View software (Mini Mitter, Bend, OR), and weekly distance traveled calculated by multiplying number of revolutions by wheel circumference (1.081 m).

2.4. Mechanical allodynia

Testing was conducted blind with respect to group assignment. Rats received at least three 60-minute habituations to the test environment prior to behavioral testing. The von Frey test¹⁰ was performed as previously described in detail.^{9,44} Assessments were made prior to running wheel exposure (baseline), prior to CCI or sham surgery (week 0), and then at weekly intervals. A logarithmic series of 10 calibrated Semmes-Weinstein monofilaments (von Frey hairs; Stoelting, Wood Dale, IL) were applied randomly to the left versus right hind paws to define the threshold stimulus intensity required to elicit a paw withdrawal response. Log stiffness of the hairs ranged from manufacturer-designated 3.61 (0.40 g) to 5.18 (15.14 g) filaments. The behavioral responses were used to calculate absolute threshold (the 50% probability of response) by fitting a Gaussian integral psychometric function using a maximum-likelihood fitting method,^{27,68} as described previously.^{43,44} This fitting method allowed parametric analyses that were not otherwise statistically appropriate.^{43,44}

2.5. Tissue analyses

Tissue analyses were performed only on rats that had been allowed access to running wheels or locked wheels continuously for 6 weeks prior to CCI, and concluding on the day of surgery. Three or 14 days after CCI or sham surgery, rats were deeply anesthetized with sodium pentobarbital. Either serum was collected (day 3) or blood was collected into tubes containing EDTA (day 14) via cardiac puncture. After transcardial perfusion with saline, ipsilateral L4-L5 dorsal quadrants and DRGs were flash frozen, while sciatic nerves were post fixed in 4% paraformaldehyde/0.1 M phosphate buffer (pH 7.4) for 24 h.

To prepare the frozen tissue for the assays, each tissue was added to 0.25–0.5 ml of Iscove's culture medium containing 5% fetal calf serum and an enzyme inhibitor mixture (100 mM amino-n-caproic acid, 10 mM EDTA, 5 benzamidine HCl, and 0.2 mM phenylmethylsulfonyl fluoride). Total protein was mechanically dissociated from tissue using a sonic dismembrator (Branson Ultrasonics Corp, Model 150E, Danbury, CT). Sonication consisted of 20 s of cell disruption at 52% amplitude. Sonicated samples were centrifuged at 14,000 rpm at 4°C for 10 min. Supernatants were removed and stored at

–80°C until Western blotting or ELISA was performed. Bradford protein assays were performed to determine total protein concentrations in sonication samples.

2.5.1. Ex vivo PBMC assay—PBMCs were isolated from blood collected at day 14 with Optiprep™ (Sigma, St. Louis, MO), as directed by the manufacturer for the mixer flotation method. Isolated cells were seeded into 96-well U-bottom plates, at a density of 1×10^5 cells per well. Cells were incubated in RPMI 1640 (5 % FBS) together with 10 ng/ml LPS (from *E. coli* O111:B4; Invivogen, San Diego, CA) or media control for 24 h, in a final volume of 200 μ l (n=3/group; each sample assayed in triplicate). The Greiss assay was performed on fresh supernatant, while remaining supernatant was frozen at –80°C.

2.5.2. Western blotting—Western blot analyses were performed on L4/5 spinal ipsilateral dorsal quadrants, as previously described.^{23,26} Primary antibodies and dilution ratios used were: rabbit P2X4R 1:400 (Alomone Labs, Jerusalem, Israel), rabbit p38 1:1000 (Cell Signaling, Danvers, MA), rabbit phospho-p38 1:1000 (Cell Signaling), rabbit p65/NF κ B 1:500 (Merck Millipore, Billerica, MA), rabbit NLRP3 1:500 (LifeSpan Biosciences, Seattle, WA), guinea pig GLT-1 1:5000 (Merck Millipore), goat Iba1 1:1000 (Abcam, Cambridge, MA), rabbit CD11b 1:500 (Abcam), rabbit ATF3 1:1000 (Santa Cruz Biotechnology, Dallas, TX). Mouse β actin 1:100,000 (Sigma) was used against loading control protein. Secondary antibodies used were: Goat anti-mouse IRDye 680RD 1:15,000 (LI-COR Biosciences, Lincoln, NE), goat anti-rabbit IRDye 800CW 1:15,000 (LI-COR Biosciences), donkey anti-guinea pig IRDye 800CW 1:15,000 (LI-COR Biosciences), and donkey anti-goat IRDye 800CW 1:15,000 (LI-COR Biosciences). Bands were quantified using Image Studio (LI-COR Biosciences).

2.5.3. Immunohistochemistry—After immersion postfixation, the sciatic nerves were cryoprotected in 30% sucrose with 0.1% azide at 4°C until slicing. Sections were freeze-mounted in OCT, and cut at 12 μ m frozen sections. Sections were post fixed with 4% paraformaldehyde for 8 mins, washed, permeabilized with 0.3% hydrogen peroxide, blocked for 1 h with 10% NGS, 0.3% Triton-X in PBS, and then incubated overnight at 4°C for 24 h in 2 % normal goat serum together with primary antibodies at the following dilution ratios: rabbit Iba1 1:500 (Abcam), rabbit CCL2 1:500 (Torrey Pines Biolabs, Secaucus, NJ), rabbit iNOS 1:400 (Merck Millipore), rabbit Arginase-1 1:50 (Santa Cruz Biotechnology). Slides were then washed, incubated in the secondary antibody at 1:200 (Goat anti rabbit or goat anti mouse biotin, Jackson Immuno Research, West Grove, PA) for 2 h. Sections were once again washed, incubated in ABC solution (Vector Laboratories, Burlingame, CA) for 2 h, washed, and incubated in inactive DAB (Sigma) for 10 min. DAB was then activated with B-D glucose (10mg/ml) and slides were incubated for 8 min, washed, and dried overnight. Slides were dehydrated in increasing concentrations of EtOH (50, 70, 95 and 100%), cleared in Citrisolv, dried and covered with DPX mountant (Sigma).

Images were acquired using an Olympus BX61 microscope (Olympus, Center Valley, PA) with Suite Cell Sens Dimension software (Olympus). All images were taken using the same exposure and settings, and captured at 10x magnification. Images were converted to 32 bit, corrected for threshold, and densitometry was conducted using NIH Image J software, while blinded to treatment conditions. Four images per animal were taken with 3 selections within

each image analyzed, resulting in 12 areas of analysis per animal. Data are expressed as total area positive for staining within the selected area.

2.5.4. ELISA—A total of 50 μ l of tissue sonicates, serum, or cell culture supernatants were used for the assays. Protein levels in serum samples were analyzed on multiplex ELISA cytokine (IFN γ , LOD: 25 pg/ml; IL-1 β , LOD: 6.25 pg/ml; IL-2, 3.125 pg/ml; IL-6, LOD: 4.165 pg/ml; IL-10, LOD: 3.125 pg/ml; CXCL1, LOD: 0.781 pg/ml; TNF, LOD: 25 pg/ml) and chemokine arrays (CCL2, LOD: 1.172 pg/ml; CCL3, LOD: 0.293 pg/ml; CXCL2, LOD: 0.391 pg/ml; CCL20, LOD: 6.25 pg/ml) (Aushon, CA, USA). Chemiluminescence was quantified on a Signature-PLUS CCD (Aushon) and analyzed using PROarray Analyst Software (Aushon). Protein levels in tissue sonicates and cell culture supernatants were determined using ELISA kits for rat IL-1 β (R&D Systems, Minneapolis, MN; LOD: 5 pg/ml), rat IL-10 (R&D Systems; LOD: 10 pg/ml), rat BDNF (Promega Corporation, Madison, WI; LOD: 15.6 pg/ml), and rat CCL2 (R&D Systems; LOD: 15.6 pg/ml). The assays were performed according to manufacturer's instructions. The analyte concentrations are presented as picograms per 100 μ g of total protein.

2.5.5. Greiss assay—Nitrite levels were determined using a commercially available Greiss reagent kit (Promega Corporation; LOD: 2.5 μ M). The assay was performed according to manufacturer's instructions. A total of 50 μ l of cell culture supernatant were used for the assay.

2.6. Statistics

Mechanical allodynia was analyzed as the interpolated 50% thresholds (absolute threshold). One-way ANOVA followed by Tukey's post hoc test was used to confirm that there were no baseline differences in absolute thresholds between treatment groups. Differences between treatment groups were determined using two-way ANOVA, followed by Sidak's post hoc test, with a correction for repeated measures for mechanical allodynia. A Pearson correlation was performed between weekly distance traveled and mechanical allodynia. $P < 0.05$ was considered significant, and all data are expressed as mean $\pm$ SEM.

3. Results

3.1. Effect of voluntary wheel running on CCI-induced allodynia

In the first experiment, rats were single-housed with an in-cage running wheel or a locked wheel continuously for 6 weeks prior to CCI or sham surgery, and concluding on the day of surgery. Von Frey thresholds were assessed prior to single housing and to surgery, and thereafter at weekly intervals until allodynia resolved in the sedentary CCI group (Figure 1A). Baseline thresholds were assessed on the day that voluntary wheel running began (−6), and the morning after voluntary wheel running ceased (BL). No differences in allodynia were observed between these timepoints, for any groups, demonstrating that any protective effects of voluntary wheel running could not be explained by increased basal thresholds. Prior voluntary wheel running did not affect mechanical thresholds in sham-operated rats, relative to sedentary controls. Prior voluntary wheel running persistently blunted CCI-allodynia for the duration of the injury, relative to sedentary CCI controls ($P < 0.05$).

There were no significant differences in weekly distance traveled between treatment groups (Figure 1B). In addition, there was no correlation between total distance traveled over 6 weeks and von Frey threshold at any timepoint after CCI (Table S1). Body mass significantly increased in sedentary rats with locked wheels, relative to rats with free access to running wheels, but there were no differences between sham and CCI groups submitted to the same activity procedure (Figure S1).

In the second experiment, rats were single-housed with an in-cage running wheel or a locked wheel continuously after surgery, beginning on the day of surgery. Von Frey thresholds were assessed prior to surgery, and thereafter at weekly intervals until allodynia resolved in the CCI and running wheel group (Figure 1C). No differences in allodynia were observed between groups prior to surgery. All rats developed maximal allodynia 2 weeks after CCI surgery, irrespective of voluntary wheel running. However, allodynia was progressively reversed from 4 weeks post CCI in the running wheel group, returning to sham thresholds by 9 weeks post CCI ($P < 0.05$). Voluntary wheel running did not affect mechanical thresholds in sham-operated rats, relative to sedentary controls. There were no significant differences in weekly distance traveled between treatment groups (Figure 1D). There was no correlation between weekly distance traveled and von Frey threshold at any timepoint after CCI (Table S2).

In the third experiment, rats were single-housed with an in-cage running wheel or a locked wheel continuously after surgery, beginning 2 weeks after surgery. Von Frey thresholds were assessed prior to surgery, and thereafter at weekly intervals until allodynia resolved in the CCI and running wheel group (Figure 1E). No differences in allodynia were observed between groups prior to surgery. Similar to those rats housed with running wheels beginning on the day of surgery, allodynia was progressively reversed from 4 weeks post CCI in the running wheel group ($P < 0.05$). Voluntary wheel running did not affect mechanical thresholds in sham-operated rats, relative to sedentary controls. There were no significant differences in weekly distance traveled between treatment groups (Figure 1F). There was no correlation between weekly distance traveled and von Frey threshold at any timepoint after CCI (Table S3).

3.2. Effect of prior voluntary wheel running on dorsal spinal neuroinflammation

Exercise after nerve injury has been reported to suppress spinal neuroimmune signaling induced by nerve injury.^{1,6,14,15,42} Therefore, we investigated whether prior running normalized the expression of two neuroimmune signaling pathways in the dorsal spinal cord at 3 and 14 days after CCI.

IL-1 β is a principal cytokine released by microglia (and other cell types) that induces neuroexcitation via several mechanisms, including downregulation of the glutamate transporter GLT-1.^{21,74} The transcription and activation of IL-1 β is respectively regulated by NF κ B, and NLRP3 inflammasomes.³⁷ We found that NLRP3, NF κ B p65, and IL-1 β were significantly upregulated, while GLT-1 was downregulated, 14 days ($P < 0.05$), but not 3 days after CCI in the locked wheel controls. However, 6 weeks of voluntary wheel running ceasing prior to CCI normalized the expression of these proteins 14 days after injury ($P < 0.05$; Fig. 2A-D).

BDNF release by microglia is responsible for disinhibition of second order projection neurons in the spinal dorsal horn, and is regulated by P2X4R and p38 MAPK signaling.^{17,67} We found that P2X4R, phosphorylated p38 (relative to total p38), and BDNF were significantly upregulated 14 days ($P < 0.05$), but not 3 days after CCI in the locked wheel controls. Notably, expression of these proteins at 14 days after injury was normalized by 6 weeks of voluntary wheel running ceasing prior to CCI ($P < 0.05$; Fig. 2E-G).

3.3. Effect of prior voluntary wheel running on DRG neuroinflammation

Macrophage activation in the DRGs is necessary for the development of nociceptive hypersensitivity after nerve injury.^{28,29,41,72} Therefore, we investigated whether prior running would attenuate the expression of the macrophage marker Iba1 and the chemokine CCL2 in the ipsilateral lumbar DRGs. Iba1, CD11b and CCL2 expression was significantly increased at 3 and 14 days after CCI in the locked wheel controls ($P < 0.05$), and decreased at both timepoints by 6 weeks of voluntary wheel running ceasing prior to CCI ($P < 0.05$; Fig. 3A,B).

Activating Transcription Factor 3 (ATF3) is a commonly used surrogate marker for neuronal damage after peripheral nerve injury.⁶⁹ Here, ATF3 was increased in the ipsilateral lumbar DRGs at both 3 and 14 days after CCI ($P < 0.001$). However, ATF3 expression was attenuated at both timepoints by 6 weeks of voluntary wheel running ceasing prior to CCI ($P < 0.05$; Fig. 3C).

3.4. Effect of prior voluntary wheel running on sciatic nerve neuroinflammation

Macrophage infiltration into the injured sciatic nerve contributes to nociceptive hypersensitivity after CCI.^{28,29,41,70,72} Therefore, we investigated whether prior running would attenuate the expression of macrophage markers in the sciatic nerve at 3 and 14 days after CCI. In the locked wheel controls, we found that CCI significantly increased expression of the macrophage marker Iba1 at day 14, but not day 3 ($P < 0.001$; Fig. 4A-C), and the chemokine CCL2 at days 3 and 14 ($P < 0.001$; Fig. 4D-F). In comparison, 6 weeks of voluntary wheel running ceasing prior to CCI decreased the expression of Iba1 at day 14 and CCL2 at days 3 and 14 ($P < 0.01$; Fig. 4A-F). However, the expression of Iba1 and CCL2 was still significantly elevated after 6 weeks of voluntary wheel running in the CCI groups at day 14, relative to the sham groups ($P < 0.001$; Fig. 4B,E). A similar expression pattern was found at proximal (Table 1) and distal (Table 2) sites.

Macrophages may be polarized towards either pro-inflammatory (M1) or anti-inflammatory (M2) phenotypes. A previous study demonstrated that 8 weeks of voluntary wheel running increases the ratio of gastrocnemius macrophages towards an M2 phenotype.³⁹ Therefore, we assessed the influence of prior running on macrophage phenotype in the sciatic nerve at 3 and 14 days after CCI. The expression of an M1 marker (iNOS) and an M2 marker (Arg-1) in the injured sciatic nerve were significantly increased at 3 and 14 days after CCI in the locked wheel controls ($P < 0.05$; Fig. 4G-L). We found that prior running attenuated the expression of iNOS due to CCI at both timepoints ($P < 0.01$; Fig. 4G,H). Compared to CCI locked wheel, Arg-1 expression was non-significantly increased at day 3 by prior running, but was significantly decreased at day 14 ($P < 0.01$; Fig. 4J,K). Notably, the expression of

iNOS and Arg-1 at day 14 was still elevated after 6 weeks of voluntary wheel running in the CCI groups, relative to the sham groups ($P < 0.001$). A similar expression pattern was found at proximal (Table 1) and distal (Table 2) sites, with the exception that Arg-1 was not elevated by voluntary wheel running at either site on day 3.

3.5. Effect of prior voluntary wheel running on serum cytokines and chemokines, and PBMC reactivity

Voluntary wheel running is posited to enhance anti-inflammatory signaling in circulating leukocytes.^{47,48} Therefore, we quantified the expression of cytokines and chemokines in serum collected 3 days after CCI (Fig. 5). CCL2, CCL3, and CXCL1 levels were significantly increased after CCI in the locked wheel controls ($P < 0.05$), while IL-10 levels were unchanged by CCI. The levels of CCL2, CCL3, and CXCL1 were attenuated by prior running ($P < 0.05$). Irrespective of injury, IL-10 levels were elevated by prior running ($P < 0.01$). IFN γ , IL-1 β , IL-2, IL-6, TNF, CXCL2 and CCL20 levels were below the limit of detection.

Next, we investigated whether prior running would attenuate the pro-inflammatory response, and enhance the anti-inflammatory response of PBMCs collected 14 days after CCI. LPS treatment induced a significant increase in IL-1 β ($P < 0.05$), and non-significant increases in nitrite and IL-10 from sedentary CCI controls (Fig. 6). In comparison, 6 weeks of running prior to CCI significantly decreased the production of IL-1 β , nitrite, and IL-10 in response to LPS ($P < 0.05$). Nitrite, IL-1 β , and IL-10 release by unstimulated PBMCs was not significantly altered by either voluntary wheel running or CCI (data not shown).

4. Discussion

We discovered that 6 weeks of voluntary wheel running, ceasing prior to sciatic injury, prevented the full development of CCI-induced neuropathic pain for the ~3 month duration of the injury. The prevention of CCI-allodynia by prior voluntary wheel running was associated with decreased neuroimmune signaling in the spinal dorsal horn at day 14, decreased DRG neuron injury at both day 3 and 14, attenuated macrophage infiltration into the DRGs and injured sciatic nerve at both day 3 and 14, suppressed pro-inflammatory mediator and increased anti-inflammatory mediator levels in the serum at day 3, and induced PBMC anergy (suppressed reactivity) at day 14. We also found that voluntary wheel running, beginning after CCI, progressively reversed established neuropathic pain. The potential for exercise to prevent the development of chronic pain is under-studied, though supported by clinical evidence that the prevalence of chronic pain is lower among individuals with a history of regular exercise.^{35,36} The strikingly persistent attenuation of allodynia by prior voluntary wheel running raises several questions, including how much exercise is required for protection. We found that distance travelled did not correlate with allodynia after CCI (Tables S1-3). Indeed, there was wide variability in the total distance run. For example, in the final week, rats varied in the distance run from 4 km to 23 km, yet all rats exhibited suppression of neuropathic pain. These data suggest that neuropathic pain protection is a function of regular exercise across time, rather than the amount of running *per se*. This is in accord with other studies that find that the total distance traveled in voluntary

wheel running does not predict the magnitude of effect on other endpoints, suggestive that even a small amount of voluntary exercise regularly performed over time can have dramatic effects.³ Other groups have investigated the impact of varying the overall duration of regular exercise. Two weeks of treadmill running or 3 weeks of voluntary wheel running prior to CCI or low thoracic contusion spinal cord injury, respectively, was not sufficient to attenuate the subsequent development of nociceptive hypersensitivity.^{15,19} However, it is not clear whether these differing results are due to the shorter duration of exercise, or the mechanistic differences between exercise paradigms or injury models. While 8 weeks of prior voluntary wheel running prevented initial hindpaw allodynia in an acidic saline model of chronic pain, the effect could not be recapitulated with 5 days of voluntary wheel running.⁵⁸ These results suggest that a threshold for protection exists.²⁵

A second question relates to the duration of protection after exercise ceases. In the only other study to report a protective influence of prior voluntary wheel running (here, 8 weeks of voluntary wheel running prior to intra-muscular acidic saline-induced chronic pain), the period of pain protection was limited to less a week.⁵⁸ In contrast, neuropathic pain was attenuated for the duration of the injury (~3 months) in the present study. This difference may be attributed to alternative mechanisms between the CCI and intramuscular acidic saline models. For example, chronic pain induced by the latter is dependent on local pro-inflammatory signaling,^{39,64} but unlike CCI, it is not dependent on spinal glia.^{24,38} This suggests that the long-term protective effects of voluntary wheel running could be mediated by attenuation of central immune signaling. Indeed, we discovered that prior voluntary wheel running normalized the expression of neuroexcitatory IL-1 β production and the attendant GLT-1 decrease, as well as expression of the disinhibitory P2X4R-BDNF axis in the dorsal spinal cord at day 14. That these markers were not significantly elevated in the spinal dorsal horn at day 3 post CCI, suggesting that the injury had not yet engaged spinal immune mechanisms, in contrast to previous reports.^{2,65} The 14-day data are consistent with a previous report that treadmill running after peripheral nerve injury attenuated BDNF expression and restored KCC2 expression in the spinal dorsal horn.⁴² Despite the common modulation of such neuroimmune pathways by exercise both before and after nerve injury, the mechanisms may ultimately diverge; exercise after injury must be maintained for reversal of nociceptive hypersensitivity to be sustained.⁶⁰

Another question concerns the mechanisms underlying the persistent protective effect of prior exercise. In addition to modulation of spinal neuroimmune pathways, we discovered that prior exercise, ending before nerve injury, suppressed infiltration of macrophages into the injured sciatic nerve and DRG, with attendant decreases in the potent macrophage chemoattractant CCL2 at both timepoints.⁷⁰ Macrophage activation in these tissues is necessary for the development of nociceptive hypersensitivity after nerve injury.^{28,29,41,72} CCL2 is also transported from the DRGs and released by primary afferents in the spinal dorsal horn to activate microglia after peripheral nerve injury.^{21,71} Despite modulation of such pro-nociceptive processes, it is not yet clear how prior exercise may influence the cascade of neuroimmunological and neuropathological events set into motion by later nerve injury. However, the direct acute effects of exercise (glucocorticoid release, sympathetic activation, hyperthermia, etc.) can be excluded, as they rapidly resolve after exercise, and thus are not present when peripheral nerve injury occurs. Prior voluntary wheel running

must create changes that mitigate injury and/or inflammation to prevent chronic pain. Several candidate mechanisms have been explored in the context of nerve injury. The first is promotion of neuronal survival and differentiation via neurotrophin-3 (NT-3), which is increased in the spinal cord and muscle after injury.^{20,30} Exercise-induced increases in NT-3 may be responsible for increased neurite outgrowth of injured DRG neurons in vitro, and increased conduction velocity in injured nerves.^{40,45} Induction of factors such as NT-3 may protect against CCI-induced damage, to attenuate a secondary neuroimmune response. A second potential mechanism involves induction of endogenous opioids after exercise.^{4,55,60} Aside from a direct analgesic effect, endogenous opioids are posited to be anti-inflammatory,⁶¹ and may suppress immune cell recruitment and immune-mediated neuroexcitation.

Our data suggest that alternative activation at the injury site of the sciatic nerve may be a neuroprotective mechanism underlying prior exercise early after CCI, but not at day 14. Macrophage infiltration into the sciatic nerve was generally elevated after CCI, and decreased by prior voluntary wheel running, though infiltration/activation was pronounced at day 14 relative to day 3, in contrast to previous reports.^{2,28,29,41,70,72} Notably, at day 3 immunoreactivity of Arg-1 was non-significantly elevated, while iNOS was decreased at the injury site by voluntary wheel running. This was accompanied by attenuated serum levels of pro-inflammatory chemokines, but elevation of the anti-inflammatory cytokine IL-10 at day. Arg-1 immunoreactivity was attenuated by voluntary wheel running in the proximal and distal regions at day 3, and all regions at day 14. The general suppression of immune signaling by prior voluntary wheel running at day 14 was paralleled in the ex vivo PBMC assay, in which production of all cytokines, including IL-10, was suppressed at this timepoint as well. Furthermore, IL-10 was not produced by unstimulated PBMCs following voluntary wheel running. That voluntary wheel running may have induced macrophage anergy might explain the profound suppression of allodynia after injury by voluntary wheel running, despite the fact that markers for macrophage infiltration/activation were still significantly elevated in this group relative to sham controls. As leukocyte function and polarization shifts temporally and anatomically in response to CCI and voluntary wheel running, further characterization is required to identify the contribution of these cells to the attenuation of neuropathic pain. Continuous voluntary wheel running after peripheral nerve injury hastened the recovery of nociceptive hypersensitivity. Whether running began immediately after injury or once neuropathic pain was established did not influence the rate of recovery. The reversal of peripheral neuropathic pain supports similar studies that have used forced exercise paradigms,^{1,7,12–15,34,42,54,60} but contrasts to a recent report wherein 2 weeks of voluntary wheel running did not reverse established allodynia.⁵⁶ This negative result may be due to the fact that this study terminated after 2 weeks of exercise, since reversal of allodynia was first emerging after 2 weeks of exercise in the present study.

Voluntary wheel running has recently been proposed as a non-reflexive measure of pain in inflammatory models.^{16,24,62} When voluntary wheel running was initiated after CCI in this study, distance travelled did not correlate with von Frey thresholds at any timepoint. This result suggests that unrestricted voluntary wheel running is not an alternative pain measure for CCI. Furthermore, the reversal of allodynia by voluntary wheel running is a major confound to any such attempt.

In conclusion, we discovered that prior voluntary exercise persistently attenuated the severity of allodynia in a rat model of neuropathic pain. Exercise decreased neuroimmune signaling in the spinal dorsal horn, the DRGs, and the injured sciatic nerve after injury; processes that are causal to the expression of nociceptive hypersensitivity.^{2,21} This study and others^{35,36,58} suggests that chronic pain should be considered a component of “the diseasome of physical inactivity”,⁴⁹ and that an active lifestyle may be an effective strategy to prevent severe neuropathic pain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Prior voluntary wheel running attenuates severity of CCI-induced allodynia (A) Rats were single-housed with an in-cage running wheel (RW) or a locked wheel (LW) continuously for 6 weeks prior to CCI or sham surgery, and concluding on the day of surgery. Von Frey thresholds were assessed prior to single housing and to CCI, and at weekly intervals thereafter. Prior voluntary wheel running significantly attenuated allodynia after CCI (time x treatment: $F_{42,280} = 6.00$, $P < 0.001$; time: $F_{14,280} = 18.4$, $P < 0.001$; treatment: $F_{3,20} = 47.6$, $P < 0.001$). (B) Distance travelled over the 6-week period prior to surgery. There were no significant differences in weekly distance traveled between treatment

groups (time x treatment: $F_{5,50} = 0.120$, $P = 1.0$; time: $F_{5,50} = 4.63$, $P < 0.01$; treatment: $F_{1,10} = 0.126$, $P = 0.7$) (C) Rats were single-housed with a RW or LW continuously after surgery, beginning immediately after surgery. Von Frey thresholds were assessed prior to surgery, and at weekly intervals thereafter. Voluntary wheel running significantly reversed allodynia after CCI (time x treatment: $F_{30,140} = 8.57$, $P < 0.001$; time: $F_{10,140} = 8.30$, $P < 0.001$; treatment: $F_{3,14} = 143.4$, $P < 0.001$). (D) Distance travelled over the 11-week period after surgery. There were no significant differences in weekly distance traveled between treatment groups (Figure 1D; time x treatment: $F_{10,100} = 0.206$, $P = 1.0$; time: $F_{10,100} = 1.33$, $P = 0.2$; treatment: $F_{1,10} = 0.0725$, $P = 0.7$). (E) Rats were single-housed with a RW or LW continuously after surgery, beginning 2 weeks after surgery. Von Frey thresholds were assessed prior to surgery, and at weekly intervals thereafter. Prior voluntary wheel running significantly attenuated allodynia after CCI (time x treatment: $F_{21,140} = 9.35$, $P < 0.001$; time: $F_{7,140} = 8.66$, $P < 0.001$; treatment: $F_{3,20} = 189.8$, $P < 0.001$). (F) Distance travelled over the 6-week period after surgery. There were no significant differences in weekly distance traveled between treatment groups (Figure 1F; time x treatment: $F_{5,50} = 0.323$, $P = 0.9$; time: $F_{5,50} = 15.5$, $P < 0.001$; treatment: $F_{1,10} = 0.394$, $P = 0.5$). ** $P < 0.01$, *** $P < 0.001$ relative to CCI+LW. Data are presented as mean $\pm$ SEM; $n = 6$ /group.

Figure 2. Prior voluntary wheel running attenuates CCI-induced lumbar dorsal spinal neuroinflammation

NLRP3 was (A) not significantly expressed at day 3 post CCI (CCI x exercise: $F_{1,20} = 2.62$, $P = 0.1$; CCI: $F_{1,20} = 0.62$, $P = 0.4$; exercise: $F_{1,20} = 0.48$, $P = 0.5$), but (B) elevated expression was normalized by 6 weeks of prior voluntary wheel running at 14 days after CCI (CCI x exercise: $F_{1,20} = 4.89$, $P < 0.05$; CCI: $F_{1,20} = 8.19$, $P < 0.01$; exercise: $F_{1,20} = 2.37$, $P = 0.1$). NFκB p65 was (C) not significantly expressed at day 3 post CCI (CCI x exercise: $F_{1,20} = 0.02$, $P = 0.9$; CCI: $F_{1,20} = 0.64$, $P = 0.4$; exercise: $F_{1,20} = 0.78$, $P = 0.4$), but (D) elevated expression was normalized by 6 weeks of prior voluntary wheel running at 14 days

after CCI (CCI x exercise: $F_{1,8} = 2.90$, $P = 0.1$; CCI: $F_{1,8} = 9.89$, $P < 0.05$; exercise $F_{1,8} = 20.21$, $P < 0.01$). As NF κ B and NLRP3 are responsible for transcription and activation of IL-1 β , (E) IL-1 β expression was not significantly increased at day 3 (3 days [CCI x exercise: $F_{1,20} = 2.84$, $P = 0.1$; CCI: $F_{1,20} = 1.28$, $P = 0.3$; exercise: $F_{1,20} = 0.0003$, $P = 0.9$), but (F) was accordingly normalized at day 14 (CCI x exercise: $F_{1,20} = 6.13$, $P < 0.05$; CCI: $F_{1,20} = 7.65$, $P < 0.05$; exercise $F_{1,20} = 3.84$, $P = 0.06$), and expression of the glutamate transporter GLT-1 (G) was not significantly affected at day 3 (CCI x exercise: $F_{1,20} = 0.97$, $P = 0.3$; CCI: $F_{1,20} = 0.08$, $P = 0.8$; exercise: $F_{1,20} = 5.39$, $P < 0.05$), but (H) was normalized at day 14 (CCI x exercise: $F_{1,20} = 7.72$, $P < 0.05$; CCI: $F_{1,20} = 2.97$, $P = 0.1$; exercise $F_{1,20} = 1.84$, $P = 0.2$). P2X4R expression was (I) not affected at 3 days post CCI (CCI x exercise: $F_{1,20} = 1.25$, $P = 0.3$; CCI: $F_{1,20} = 0.71$, $P = 0.4$; exercise: $F_{1,20} = 0.54$, $P = 0.5$), but (J) was normalized by prior voluntary wheel running at day 14 (CCI x exercise: $F_{1,20} = 7.16$, $P < 0.05$; CCI: $F_{1,20} = 4.45$, $P < 0.05$; exercise $F_{1,20} = 15.61$, $P < 0.001$). Phosphorylated p38 (relative to total p38) was (K) unaffected at 3 days post CCI (CCI x exercise: $F_{1,20} = 0.020$, $P = 0.9$; CCI: $F_{1,20} = 0.64$, $P = 0.4$; exercise: $F_{1,20} = 0.78$, $P = 0.4$), but (L) normalized at 14 days post CCI (CCI x exercise: $F_{1,20} = 16.34$, $P < 0.001$; CCI: $F_{1,20} = 21.14$, $P < 0.001$; exercise $F_{1,20} = 11.47$, $P < 0.01$). Levels of BDNF, produced downstream of P2X4R and p38 that disinhibits second order projection neurons in the spinal dorsal horn, was also (M) unchanged at day 3 (CCI x exercise: $F_{1,20} = 0.16$, $P = 0.7$; CCI: $F_{1,20} = 3.59$, $P = 0.07$; exercise: $F_{1,20} = 1.32$, $P = 0.3$), but (N) normalized 14 days after CCI (CCI x exercise: $F_{1,20} = 3.38$, $P = 0.08$; CCI: $F_{1,20} = 21.14$, $P < 0.001$; exercise $F_{1,20} = 11.47$, $P < 0.01$). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ relative to sham+LW; $^*P < 0.05$, $^{***}P < 0.001$ relative to CCI +LW. Data are presented as mean $\pm$ SEM; $n = 6$ /group.

Figure 3. Prior voluntary wheel running attenuates macrophage infiltration into the injured lumbar DRGs

When assessed at both 3 and 14 days after CCI/sham surgery, voluntary wheel running prior to CCI normalized expression of (A) the macrophage markers CD11b at day 3 (CCI x exercise: $F_{1,19} = 11.52$, $P < 0.01$; CCI: $F_{1,19} = 8.25$, $P < 0.01$; exercise: $F_{1,19} = 4.81$, $P < 0.05$), and (B) Iba1 at day 14 (CCI x exercise: $F_{1,8} = 7.72$, $P < 0.05$; CCI: $F_{1,8} = 5.08$, $P = 0.05$; exercise: $F_{1,8} = 3.90$, $P = 0.08$), and (C) the chemokine CCL2 at day 3 (CCI x exercise: $F_{1,20} = 11.28$, $P < 0.01$; CCI: $F_{1,20} = 1.85$, $P = 0.2$; exercise: $F_{1,20} = 2.13$, $P = 0.2$) and (D) day 14 (CCI x exercise: $F_{1,20} = 19.72$, $P < 0.001$; CCI: $F_{1,20} = 28.87$, $P < 0.001$; exercise:

$F_{1,20} = 20.66$, $P < 0.001$), suggesting attenuated macrophage infiltration into the injured DRGs after nerve injury. (E) The neuronal injury marker ATF3 was also decreased at day 3 (CCI x exercise: $F_{1,20} = 5.26$, $P < 0.05$; CCI: $F_{1,20} = 12.70$, $P < 0.01$; exercise: $F_{1,20} = 3.83$, $P = 0.06$), and (F) day 14 (CCI x exercise: $F_{1,20} = 25.48$, $P < 0.001$; CCI: $F_{1,20} = 27.65$, $P < 0.001$; exercise: $F_{1,20} = 22.17$, $P < 0.001$). $^{\#}P < 0.05$, $^{###}P < 0.001$ relative to sham+LW; $^{*}P < 0.05$, $^{***}P < 0.001$ relative to CCI+LW. Data are presented as mean $\pm$ SEM; $n = 6$ /group.

Figure 4. Prior voluntary wheel running attenuates macrophage infiltration into the injured sciatic nerve

The macrophage marker Iba1 was (A) elevated by CCI at day 3 (CCI x exercise: $F_{1,20} = 0.57$, $P = 0.4$; CCI: $F_{1,20} = 6.83$, $P < 0.01$; exercise: $F_{1,20} = 4.69$, $P < 0.05$), and (B) at 14 days (CCI x exercise: $F_{1,20} = 12.24$, $P < 0.01$; CCI: $F_{1,20} = 1951$, $P < 0.001$; exercise $F_{1,20} = 9.92$, $P < 0.01$), (C) with representative images from day 14. Expression of the chemokine CCL2 was increased at (D) day 3 (CCI x exercise: $F_{1,20} = 4.24$, $P = 0.052$; CCI: $F_{1,20} = 24.95$, $P < 0.001$; exercise: $F_{1,20} = 8.40$, $P < 0.01$), and (E) day 14 (CCI x exercise: $F_{1,20} = 5.84$, $P < 0.05$; CCI: $F_{1,20} = 247.4$, $P < 0.001$; exercise $F_{1,20} = 7.51$, $P < 0.05$), and attenuated by prior voluntary wheel running at both timepoints, with (F) representative images from day 14. In addition, voluntary wheel running prior to CCI attenuated expression of an M1 marker (iNOS) at (G) 3 days (CCI x exercise: $F_{1,20} = 1.60$, $P = 0.22$; CCI: $F_{1,20} = 49.36$, $P < 0.001$; exercise: $F_{1,20} = 17.51$, $P < 0.001$), and (H) 14 days (CCI x exercise: $F_{1,20} = 6.39$, $P < 0.05$; CCI: $F_{1,20} = 641.0$, $P < 0.001$; exercise $F_{1,20} = 10.74$, $P < 0.01$), with (I) representative images from day 14. An M2 marker (Arg-1) was (J) elevated by CCI and voluntary wheel running at 3 days (CCI x exercise: $F_{1,20} = 3.18$, $P = 0.08$; CCI: $F_{1,20} = 40.90$, $P < 0.001$; exercise: $F_{1,20} = 2.22$, $P = 0.15$), but (K) attenuated by voluntary wheel running at 14 days (CCI x exercise: $F_{1,20} = 12.33$, $P < 0.01$; CCI: $F_{1,20} = 658.8$, $P < 0.001$; exercise $F_{1,20} = 3.73$, $P = 0.07$), with (L) representative images from day 14. $###P < 0.001$

relative to sham+LW; ** $P < 0.01$, *** $P < 0.001$ relative to CCI+LW. Data are presented as mean $\pm$ SEM; $n = 6$ /group.

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Figure 5. Prior voluntary wheel running suppresses pro-inflammatory mediators in serum after CCI

Serum was collected from rats 3 days after CCI/sham surgery. Voluntary wheel running prior to CCI attenuated levels of (A) CCL2 (CCI x exercise: $F_{1,20} = 6.91$, $P < 0.05$; CCI: $F_{1,20} = 7.66$, $P < 0.05$; exercise $F_{1,20} = 19.32$, $P < 0.001$), (B) CCL3 (CCI x exercise: $F_{1,20} = 6.49$, $P < 0.05$; CCI: $F_{1,20} = 3.45$, $P = 0.07$; exercise $F_{1,20} = 7.79$, $P < 0.05$), and (C) CXCL1 (CCI x exercise: $F_{1,20} = 3.52$, $P = 0.07$; CCI: $F_{1,20} = 10.78$, $P < 0.01$; exercise $F_{1,20} = 11.02$, $P < 0.01$). Voluntary wheel running prior to CCI elevated levels of (D) IL-10 (CCI x exercise: $F_{1,20} = 1.18$, $P = 0.29$; CCI: $F_{1,20} = 0.20$, $P = 0.6$; exercise $F_{1,20} = 36.99$, $P < 0.001$). [#] $P < 0.05$, ^{##} $P < 0.01$, ^{###} $P < 0.001$ relative to sham+LW; ^{*} $P < 0.05$, ^{**} $P < 0.01$, relative to CCI+LW. Data are presented as mean $\pm$ SEM; $n = 6$ /group.

Figure 6. Prior voluntary wheel running induces PBMC anergy after CCI

PBMCs were collected from rats 14 days after CCI/sham surgery, and incubated with LPS (10 ng/ml) for 24 h. Voluntary wheel running prior to CCI attenuated expression of (A) IL-1 β (CCI x exercise: $F_{1,8} = 3.15$, $P = 0.1$; CCI: $F_{1,8} = 1.43$, $P = 0.3$; exercise $F_{1,20} = 15.04$, $P < 0.01$), (B) nitrite (CCI x exercise: $F_{1,8} = 4.92$, $P = 0.06$; CCI: $F_{1,8} = 0.45$, $P = 0.52$; exercise $F_{1,8} = 7.81$, $P < 0.05$), and (C) IL-10 (CCI x exercise: $F_{1,8} = 13.59$, $P < 0.01$;

CCI: $F_{1,8} = 0.05$, $P = 0.8$; exercise $F_{1,8} = 0.89$, $P < 0.4$). * $P < 0.05$ relative to CCI+LW. Data are presented as mean $\pm$ SEM; $n = 3/\text{group}$.

Table 1

Prior voluntary wheel running attenuates macrophage infiltration into the sciatic nerve, proximal to the injury site.

		Sham + LW	Sham + RW	CCI + LW	CCI + RW
Iba1	<i>Day 3</i>	2.3 ± 0.3	2.7 ± 0.3	5.0 ± 0.5 ^{###}	2.4 ± 0.5 ^{**}
	<i>Day 14</i>	0.5 ± 0.1	0.5 ± 0.1	18.7 ± 0.5 ^{###}	15.8 ± 0.6 ^{#####}
CCL2	<i>Day 3</i>	1.6 ± 0.2	1.4 ± 0.1	3.9 ± 0.6 [#]	3.0 ± 0.6
	<i>Day 14</i>	1.2 ± 0.2	1.9 ± 0.1	18.8 ± 1.3 ^{###}	11.8 ± 0.8 ^{#####}
iNOS	<i>Day 3</i>	2.1 ± 0.1	1.9 ± 0.1	5.4 ± 0.3 ^{###}	5.6 ± 0.4 ^{###}
	<i>Day 14</i>	2.9 ± 0.5	2.7 ± 0.3	21.9 ± 1.0 ^{###}	11.9 ± 0.6 ^{#####}
Arg1	<i>Day 3</i>	2.3 ± 0.2	2.2 ± 0.3	3.0 ± 0.2	2.6 ± 0.4
	<i>Day 14</i>	2.7 ± 0.3	6.8 ± 0.5	11.8 ± 0.7 ^{###}	9.3 ± 1.1 ^{####}

% area positive, assessed at 3 and 14 days after CCI/sham surgery.

^{###} $P < 0.001$ relative to sham + LW

^{*} $P < 0.05$

^{***} $P \pm 0.001$ relative to CCI+LW. Data are presented as mean ± SEM; $n = 6$ /group.

Table 2

Prior voluntary wheel running attenuates macrophage infiltration into the sciatic nerve, distal to the injury site.

		Sham + LW	Sham + RW	CCI + LW	CCI + RW
Iba1	<i>Day 3</i>	2.4 ± 0.3	2.2 ± 0.3	4.1 ± 0.5 [#]	3.0 ± 0.4
	<i>Day 14</i>	0.9 ± 0.1	0.4 ± 0.1	14.6 ± 0.6 ^{###}	13.5 ± 0.6 ^{###}
CCL2	<i>Day 3</i>	1.9 ± 0.3	1.3 ± 0.1	2.8 ± 0.4	2.5 ± 0.2
	<i>Day 14</i>	2.6 ± 0.2	3.6 ± 0.4	15.8 ± 0.5 ^{###}	12.5 ± 0.5 ^{***###}
iNOS	<i>Day 3</i>	2.8 ± 0.6	1.8 ± 0.8	2.9 ± 0.5	3.7 ± 0.7
	<i>Day 14</i>	5.1 ± 0.8	8.0 ± 1.1	13.0 ± 0.8 ^{###}	11.8 ± 0.8 ^{###}
Arg1	<i>Day 3</i>	2.1 ± 0.1	1.5 ± 0.1	3.6 ± 0.3 ^{###}	3.2 ± 0.3 [#]
	<i>Day 14</i>	6.4 ± 0.8	8.3 ± 0.7	18.0 ± 0.8 ^{###}	10.6 ± 0.5 ^{***###}

% area positive, assessed at 3 and 14 days after CCI/sham surgery.

^{###} $P < 0.001$ relative to sham + LW

^{***} $P \pm 0.001$ relative to CCI+LW. Data are presented as mean ± SEM; $n = 6$ /group.