Exercise Reverses Nociceptive Sensitization, Upregulated Neuropeptide Signaling, Inflammatory Changes, Anxiety, and Memory Impairment in a Mouse Tibia Fracture Model

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ABSTRACT

Background: This study tested the hypothesis that ad lib running wheel exercise in a tibia fracture model of complex regional pain syndrome can reverse hindlimb nociceptive sensitization and inflammation in mice.

Methods: Three weeks after tibia fracture, the cast was removed and hindlimb von Frey thresholds and unweighting were tested; the mice were then randomized to either ad lib access to a running wheel for 4 weeks or no wheel access. After 4 weeks the behavioral testing was repeated and then skin, sciatic nerve, and spinal cord tissues collected for polymerase chain reaction and enzyme immunoassay measurements of neuropeptide and inflammatory mediator levels. A similar protocol was used in fracture mice treated with exercise for 4 weeks, and then the running wheel was removed for 2 weeks. Memory and anxiety were measured in both groups with use of open-field, zero-maze, and novel-objects recognition assays.

Results: At 7 weeks postfracture the mice with no wheel access exhibited hindlimb allodynia and unweighting, anxiety, memory loss, upregulated spinal neuropeptide signaling, and increased hind paw and spinal inflammatory mediator expression, but the postfracture mice allowed to exercise for 4 weeks exhibited none of these changes (n = 12/cohort). When exercise was stopped for 2 weeks after 4 weeks of running, hindlimb allodynia and unweighting were rekindled, and this nociceptive sensitization was associated with increased sciatic nerve neuropeptide levels and hind paw skin interleukin 6 and nerve growth factor expression (n = 12/cohort).

Conclusions: Daily exercise reversed nociceptive sensitization, inflammation, anxiety, and memory loss after tibia fracture.
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CHRONIC pain after surgery and trauma is being increasingly scrutinized in regards to its frequency, severity, and costs and will be given its own diagnostic category in the upcoming International Classification of Diseases, Eleventh Revision (ICD-11).[1] Estimates of chronic pain after surgery vary enormously, affecting from 5 to 85% of patients, with some of the highest rates observed among patients after amputation, herniorrhaphy, thoracotomy, and breast surgery.[2,3] One specific form of chronic limb pain observed after trauma and surgery is complex regional pain syndrome. Complex regional pain syndrome can develop after a variety of upper- and lower-extremity surgical procedures.[4,5]

The mechanisms mediating complex regional pain syndrome are unknown, but limb immobilization is probably a factor. The traumatized limb is usually immobilized in casts, splints, or fixators before the development of complex regional pain syndrome,[6,7] and patients guard the affected

What We Already Know about This Topic

- Tibial fracture is often accompanied by allodynia, unweighting, and local and systemic inflammation. Whether injury- or pain-induced, relative immobility contributes to the development of these symptoms, and inflammatory changes are not clear.
- In a mouse model of tibial fracture, the effects of increased exercise and mobility, 4 weeks after the fracture, on allodynia, unweighting, inflammation, and behavioral consequences was evaluated.

What This Article Tells Us That Is New

- Exercise significantly improved mobility, reduced allodynia and unweighting, and attenuated behavioral consequences of anxiety and memory loss. Exercise also had a salutary effect on systemic inflammation. Of interest, suspension of exercise recapitulated allodynia, unweighting of the fractured limb, and inflammation.
- The results suggest that exercise can improve long-term outcomes after tibial injury in experimental models.

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limb to prevent movement-induced pain.[8] Furthermore, aggressive mobilization of the limb has been reported to alleviate complex regional pain syndrome symptoms,[8] but a recent review noted a lack of high-quality clinical trial data supporting exercise therapy for complex regional pain syndrome.[9] Contrariwise, 4 weeks of forearm cast immobilization in normal individuals caused skin warmth, hyperalgesia, and movement-evoked pain, symptoms partially mimicking complex regional pain syndrome.[10] These data support the hypothesis that prolonged immobilization contributes to the development of complex regional pain syndrome and that exercise and early mobilization is beneficial.

Distal limb fracture is the most common cause of complex regional pain syndrome,[11,12] and a rodent distal tibia fracture model recapitulates many of the nociceptive, vascular, trophic, and cognitive features of complex regional pain syndrome.[13,14] Using the tibia fracture model, we previously demonstrated that immobilization contributed to the development of postfracture nociceptive and inflammatory changes and that early mobilization reversed these changes.[15] Tibia fracture with 4 weeks of cast immobilization in rats resulted in hind paw allodynia, unweighting, warmth, edema, increased sciatic nerve substance P and calcitonin gene-related peptide protein, increased skin substance P neurokinin 1 receptors, and increased inflammatory mediator protein expression in the hind paw skin (tumor necrosis factor, interleukin 1, interleukin 6, nerve growth factor) and cord (interleukin 1, nerve growth factor).[15] After 4 weeks of cast immobilization alone, these same changes occurred, except spinal interleukin 1 levels were not elevated.[15] Treating cast-only rats with a substance P neurokinin 1 receptor antagonist inhibited development of nociceptive and inflammatory changes, similar to the neurokinin 1 receptor antagonist effects observed in the fracture cast rats.[15] Complex regional pain syndrome–like symptoms such as warmth and mechanical allodynia resolved much earlier in the cast-immobilized (no fracture) rats than in the fracture-casted rats.[16,17] When tibia fracture rats were treated with intramedullary pinning instead of casting, they began weight bearing within days and by 4 weeks postfracture nociceptive sensitization resolved and neuropeptide signaling and inflammatory mediator expression returned to normal.[15] These data indicate that immobilization alone caused changes in nociception, neuropeptide signaling, and inflammatory mediator expression similar to, but less robust than, the changes observed after fracture and casting, and early mobilization after fracture inhibited these changes. The current study used the mouse tibia fracture model to determine whether daily running exercise for 4 weeks can reverse postfracture complex regional pain syndrome–like changes, including nociceptive sensitization, exaggerated substance P and calcitonin gene-related peptide signaling, inflammatory changes in the hind limb and lumbar cord, anxiety, and memory loss.

Materials and Methods

Animals and Drugs

These experiments were approved by the Veterans Affairs Palo Alto Health Care System Institutional Animal Care and Use Committee (Palo Alto, California) and followed the animal subjects guidelines laid out in the Guide for the Care and Use of Laboratory Animals of the National Academy of Sciences. Three-month-old male C57BL/6J mice (No. 000664, Jackson Laboratory, USA) were used in these experiments. The mice were housed individually under pathogen-free conditions with soft bedding and were given food and water *ad libitum*, with a 12:12 light:dark cycle. During the experimental period the animals were fed Teklad lab rodent diet 2018 (Harlan Laboratories, USA), which contains 1.0% calcium, 0.7% phosphorus, and 1.5 U/g vitamin D3, and were kept under standard conditions with a 12-h light–dark cycle. Data collection was conducted blind to group assignment.

To assess if interleukin 6 supported allodynia in 9-week fracture mice that had or had not been previously exercised between weeks 3 and 7 postfracture, both groups of fracture mice were treated with TB-2–081 (2 mg/kg, subcutaneous, provided by Kenner Rice, Ph.D., National Institute on Drug Abuse, Bethesda, Maryland). TB-2–081 is an orally active small molecule interleukin 6 receptor antagonist originally isolated from the skin of a toad. Hind paw von Frey testing was performed before and 15 min after TB-2–081 injection. In another experiment, additional cohorts of 9-week fracture mice that had or had not been previously exercised were injected with anti–nerve growth factor (muMab 911, 10 mg/kg intraperitoneal, provided by David Shelton, Ph.D., Rinat/Pfizer, San Francisco, California) and 7 days later underwent von Frey testing. This anti–nerve growth factor antibody is a tropomyosin-related kinase A–immunoglobulin G fusion molecule that binds to the nerve growth factor molecule, thus blocking the binding of nerve growth factor to the tropomyosin-related kinase A and p75 nerve growth factor receptors and inhibiting tropomyosin-related kinase A autophosphorylation.

Surgery

The tibia fracture model was performed in 3-month-old male mice as previously described.[13] During isoflurane anesthesia, a hemostat was used to make a closed fracture of the right tibia just distal to the middle of the tibia. The hind limb was then wrapped in casting tape (Delta-Lite, BSN Medical, Germany) so the hip, knee, and ankle were all fixed. The cast extended from the metatarsals of the hind paw up to a spica formed around the abdomen. A window was left open over the dorsal paw and ankle to prevent constriction when postfracture edema developed. After fracture and casting, the mice were given subcutaneously 2 days of buprenorphine (0.05 mg/kg) and baytril (5 mg/kg) as well as 1.0 ml of isotonic sodium chloride solution. At 3 weeks after surgery, the mice were anesthetized with isoflurane and the cast removed. All mice had union at the fracture site by manual inspection.

Hind Paw Nociceptive Testing

To measure mechanical allodynia in the mice, an up-down von Frey testing paradigm was used, as we have previously described.[13] Briefly, mice were placed on wire mesh platforms in clear cylindrical plastic enclosures 10 cm in diameter and 40 cm in height; after 15 min of acclimation, von Frey fibers of sequentially increasing stiffness were applied against the hind paw plantar skin at approximately midsole, with care taken to avoid the tori pads, and pressed upward to cause a slight bend in the fiber and left in place for 5 s. Withdrawal of or licking the hind paw after fiber application was scored as a response. When no response was obtained, the next stiffest fiber in the series was applied to the same paw; if a response was obtained, a less-stiff fiber was applied. Testing proceeded in this manner until four fibers had been applied. Estimation of the mechanical withdrawal threshold by data-fitting algorithm permitted the use of parametric statistics for analysis.[18] Hind paw mechanical nociceptive thresholds were analyzed as the difference between the fracture side and the contralateral untreated side.

An incapacitance device (IITC Inc., Life Science, USA) was used to measure hind paw unweighting. The mice were manually held in a vertical position over the apparatus with the hind paws resting on separate metal scale plates, and the entire weight of the rat was supported on the hind paws. The duration of each measurement was 6 s, and six consecutive measurements were taken at 10-s intervals. All six readings were averaged to calculate the bilateral hind paw weight-bearing values.[13] Right hind paw weight-bearing data were analyzed as a ratio between the right hind paw weight and the sum of right (R) and left (L) hind paw values ([2R/{R + L}] × 100%).

Hind Paw Temperature Testing

The temperature of the hind paw was measured with a fine-wire thermocouple (Omega Engineering, USA) applied to the paw skin, as described previously.[13] The investigator held the wire with an insulating Styrofoam block. Three sites were tested over the dorsum of the hind paw: the space between the first and second metatarsals (medial), the second and third metatarsals (central), and the fourth and fifth metatarsals (lateral). After a site was tested in one hind paw, the same site was immediately tested in the contralateral hind paw. The testing protocol was medial dorsum right then left, central dorsum right then left, lateral dorsum right then left, medial dorsum left then right, central dorsum left then right, and lateral dorsum left then right. The six measurements for each hind paw were averaged for the mean temperature. Hind paw temperature data were analyzed as the difference between the fractured side and the contralateral unfractured side.

Hind Paw Volume Testing

A laser sensor technique was used to determine the dorsal-ventral thickness of the hind paw, as we have previously described.[13] The measurement sensor device used in these experiments (Limab, Sweden) has a measurement range of 200 mm, with a 0.01-mm resolution. Hind paw volume data were analyzed as absolute value or the difference between the fracture side and the contralateral untreated side.

Running Wheel Exercise Protocols

Mice underwent behavioral testing for allodynia, unweighting, warmth, and edema at baseline, and then underwent distal tibia fracture and casting for 3 weeks. On the day after cast removal, the mice underwent repeat behavioral testing and then were randomized into two cohorts; one group had running wheels placed in their cages, and the other group had no wheels in their cages. The mice were individually housed, and the exercise group had ad lib access to the running wheels 24 h/day, 7 days a week. Behavioral testing was repeated at 4, 5, 6, and 7 weeks after fracture; then, the wheels were removed and behavioral testing was repeated at 9 weeks postfracture. A computerized activity wheel (AWM software, version 6.9.2057.18763; Lafayette Instrument, USA) allowed monitoring of the daily distances the mice ran and in one experiment was used to restrict the running distance to 0.5 km/daily.

Enzyme Immunoassay

After 4 weeks of wheel running exercise (7 weeks postfracture) or at 2 weeks after removing the wheels from the cages (9 weeks postfracture), the mice were euthanized by carbon dioxide inhalation and cervical dislocation; the mouse hind paw dorsal skin and sciatic nerve were collected and frozen immediately on dry ice. All tissues were cut into fine pieces in ice-cold phosphate-buffered saline, pH 7.4, containing a cocktail of protease inhibitors (Roche Applied Science, USA) and then homogenized with a Bio-Gen PRO200 homogenizer (PRO Scientific, USA). The homogenates were centrifuged for 15 min at 12,000*g*, 4°C. The supernatants were aliquoted and stored at −80°C until required for enzyme-linked immunosorbent assay performance. Total protein contents in all tissue extracts were measured by the DC Protein Assay kit (Bio-Rad Laboratories, USA) to normalize mediator levels. Mouse interleukin 1 beta, interleukin 6, tumor necrosis factor alpha, nerve growth factor, C-C motif chemokine ligand 2, substance P (specificity protein), and calcitonin gene-related peptide protein levels were measured in duplicate by use of mouse interleukin 1, interleukin 6, tumor necrosis factor, and C-C motif chemokine ligand 2 (R&D Systems, USA), nerve growth factor (Millipore Merck, Germany), substance P (MyBioSource, USA), and calcitonin gene-related peptide (Peninsula Laboratories, USA) enzyme-linked immunosorbent assay kits in accordance with the manufacturer's instructions. The results of all assays were confirmed by repeating the experiment twice.

Quantitative Real-time Polymerase Chain Reaction

After 4 weeks of wheel-running exercise (7 weeks postfracture) or at 2 weeks after removing the running wheels from

the cages (9 weeks postfracture), the mice were euthanized by carbon dioxide inhalation and cervical dislocation; the fracture limb hind paw skin, dorsal root ganglia (L3 to S1), and corresponding spinal cord (L4 and L5 lumbar enlargement) were collected. Controls tissues were collected from control nonfracture mice and from fracture mice that did not have wheels placed in their cages, at 7 and 9 weeks postfracture. Total RNA was extracted by use of the RNeasy Mini Kit (Qiagen, Germany), and the purity and concentration were determined spectrophotometrically. Then, complementary DNA was synthesized from 1-μg RNA with an iScript complementary DNA Synthesis Kit (Bio-Rad Laboratories). Real-time polymerase chain reactions were conducted with use of the SYBR Green polymerase chain reaction master mix (Applied Biosystems, USA). Real-time polymerase chain reaction amplification of interleukin 1, interleukin 6, tumor necrosis factor, nerve growth factor, C-C motif chemokine ligand 2, tachykinin precursor 1, tachykinin receptor 1, receptor activity-modifying protein 1, calcitonin-related polypeptide α, calcitonin-related polypeptide β, calcitonin receptor-like receptor, and 18S was performed on an ABI 7900HT sequencing detection system (Applied Biosystems). To validate the primer sets used (table 1), we performed dissociation curves to document single-product formation, and agarose gel analysis was conducted to confirm the size. The data from real-time polymerase chain reaction experiments were analyzed as described in the manufacturer's manual for the ABI 7900HT sequencing detection systems. All results were confirmed by repeating the experiment three times.

Open-field, Zero-maze, and Novel-object Recognition Assays

The open-field arena measured 40 × 40 × 40 cm and was made of opaque plastic material. Luminosity inside the arena was measured to be 50 lux. The mice were placed into the arena and allowed to explore for 10 min. Total locomotor activity (distance traveled) and the time spent in the central portion (11% of total area) were determined for each mouse. All recordings were automatically analyzed in real time by TopScan software (Clever Sys Inc., USA). The time spent in the center area was used as an index of thigmotaxis, a measure often used for evaluating general anxiety levels in rodents.[19]

The zero maze was used to measure anxiety, in accordance with previously published methods.[20] The maze had an outer diameter of 61 cm and inner diameter of 51 cm; it was situated 61 cm above the floor, and the closed quadrants had walls 15 cm tall. Luminosity inside the open quadrant was measured to be 50 lux, whereas that inside the closed quadrant was measured to be 20 lux. Mice were placed facing one of the closed quadrants of the maze at the beginning of testing, and the number of entries into the open quadrants over a 5-min test period was recorded with TopScan software.

Working memory was assessed by novel-object recognition testing in the same arena as the one used for the open-field test.[21] Exploration behavior was used to assess object recognition. Mice were placed into the arena and presented with two identical objects for 10 min (habituation). Then, during a 5-min trial, one of the objects was moved to a new location and exploratory behavior (investigation time) was recorded with TopScan software. Subsequently, the mice were returned to their home cages for a 5-min period and were then returned to the arena after one of the previous identical objects had been replaced with a novel one. The novel object had a distinct shape and size different from the original object pairs. Exploration behavior during the first 5-min was recorded. These experiments were performed under 50-lux luminosity.

Statistical Analysis

Statistical analysis was done with Prism 4.02 (GraphPad Software, USA). Sample sizes were based on a power

Table 1. Primers Used for Real-time Polymerase Chain Reaction

Gene	GenBank Accession #	Forward Primer	Reverse Primer	Product Size (bp)
IL-1β	NM_031512	agtctgcacagttccccaac	agacctgacttggcagagga	230
IL-6	NM_012589	cacaagtccggagaggagac	acagtgcatcatcgctgttc	168
TNF-α	NM_012675	ctcccagaaaagcaagcaac	cgagcaggaatgagaagagg	210
NGF	XM_227525	acctcttcggacactctgga	gtccgtggctgtggtcttat	168
CCL2	NM_031530	tcccacttcctgctgcttctctta	agcaaaggctgctggtctcatagt	86
TAC1	NM_012666	tttgcagaggaaatcggtgccaac	ggcattgcctccttgatttggtca	83
TACR1	NM_012667	ctggaaagaggagccttgtg	ctgagacggaaaggaacagc	205
RAMP1	NM_031645	ggcaaacaagattggctgtt	aatggggagcacaatgaaag	154
CALCA	NM_017338	agaagagatcctgcaacactgcca	ggcacaaagttgtccttcaccaca	94
CALCB	NM_138513	cccagaagagatcctgcaac	agttcctcagacccgaaggt	158
CALCRL	NM_012717	tcattgtggtggctgtgttt	aatgggaccatggatgatgt	176
18S	NR_046237	tcaactttcgatggtagtcgccgt	tccttggatgtggtagccgtttct	108

bp = base pair; CALCA = calcitonin-related polypeptide α; CALCB = calcitonin-related polypeptide β; CALCRL = calcitonin receptor-like receptor; CCL2 = C-C motif chemokine 2; CGRP = calcitonin gene-related peptide; IL-1β = interleukin 1β; IL-6 = interleukin 6; NGF = nerve growth factor; RAMP1 = receptor activity-modifying protein 1; TAC1 = tachykinin precursor 1; TACR1 = tachykinin 1 receptor; TNF-α = tumor necrosis factor α.

analysis of preliminary and previously published data generated from each of the proposed assays in fracture animals. On the basis of this analysis, we calculated that the proposed experiments would require eight animals per cohort to provide 80% power to detect a 25% difference between group means (using a two-tailed test with a 0.05 significance level). Animals were randomized to experimental groups with computer-generated random numbers, and all testing was performed in a blinded fashion, when possible. In response to reviewer concerns, an additional exercise nonfracture control group was added to the cognitive studies; thus, the cognitive behavioral experiments performed in these mice were not performed in a blinded fashion.

No animals were excluded after enrollment into the experimental cohorts, and no data were excluded from statistical analysis. Normal distribution of the data was confirmed with the D'Agostino-Pearson omnibus normality test, and two-tailed test assumptions were used in all analyses with a 0.05 significance level. Figure 1 data were analyzed with a two-way repeated-measures ANOVA followed by Bonferroni *post hoc* multiple-comparison testing. All other data were evaluated with a one-way ANOVA followed by Bonferroni *post hoc* multiple-comparison testing. Data are presented as the mean ± SD.

The hind paw von Frey mechanical nociceptive threshold, temperature, and thickness were analyzed as the difference between the fracture side (right, R) and the contralateral untreated side (left, L). Right hind paw weight-bearing data were analyzed as a ratio between twice the right hind paw weight bearing and the sum of the right and left hind paw weight-bearing values ([2R/{R + L}] × 100%).

Results

Exercise Reversed Pain Behaviors, Warmth, and Edema in the Fractured Hind Paw

When the cast was removed at 3 weeks postfracture, the mice exhibited ipsilateral hind paw von Frey allodynia (−1.3 ± 0.4 *vs.* 0.1 ± 0.2 Δg, $P < 0.001$), unweighting (63 ± 4 *vs.* 100 ± 1%, $P < 0.001$), warmth (1.2 ± 1.3 *vs.* 0.1± 0.2 Δ°C, $P = 0.010$), and edema (0.17 ± 0.21 *vs.* 0.001 ± 0.01 Δmm, $P = 0.017$), compared to baseline (fig. 1A–D). When exercise wheels were placed in cages of the 3-week postfracture mice for 4 weeks (between postfracture weeks 3 to 7), the mice quickly began using the exercise wheels at night, and after 4 weeks there were no significant differences in the average distances run by the fracture mice *versus* nonfracture control mice (5.4 ± 4.2 *vs.* 6.9 ± 1.9 km/day, $P = 0.481$; fig. 1E). Hind paw allodynia (week 5, −0.8 ± 0.2 *vs.* −1.4 ± 0.2 Δg, $P < 0.001$; week 7, −0.6 ± 0.4 *vs.* −1.3 ± 0.2 Δg, $P < 0.001$) and unweighting (week 5, 81 ± 4 *vs.* 67 ± 3%, $P < 0.001$; week 7, 93 ± 2% *vs.* 71 ± 4%, $P < 0.001$) were progressively reversed with running exercise, compared to fracture mice not provided running wheel access. When the running wheel was removed from the cages of the fracture mice at 7 weeks postfracture, the hind paw allodynia (week 9, −0.8 ± 0.3 *vs.* −1.1 ± 0.3 Δg, $P = 1.000$) and unweighting (week 9, 81 ± 4 *vs.* 67 ± 3%, $P = 0.804$) were rekindled within 2 weeks, compared to fracture mice not provided with running wheel access (fig. 1, A and B). When 3-week postfracture mice were given exercise wheels for 4 weeks that were computer controlled to lock after running 0.5 km/day, their hind paw allodynia and unweighting were progressively reversed, similar to the effects seen with ad lib wheel running (fig. 1A–D).

Exercise Reversed Postfracture Increases in Interleukin 1 and C-C Motif Chemokine Ligand 2 mRNA and Protein in the Hind Paw Skin

At 7 weeks postfracture, neuropeptide and inflammatory mediator gene expression in the fracture limb hind paw skin was measured by real-time polymerase chain reaction (fig. 2). Compared to nonfracture mice, messenger RNA (mRNA) expression, interleukin 1, and C-C motif chemokine ligand 2 mRNA levels were elevated at 7 weeks postfracture in nonexercised mice (110% and 310%, respectively), and 4 weeks of wheel-running exercise reversed this increase (fig. 2, A and E). Hind paw skin mRNA levels for interleukin 6, tumor necrosis factor, nerve growth factor, tachykinin precursor 1, tachykinin receptor 1, receptor activity-modifying protein 1, calcitonin-related polypeptide α, calcitonin-related polypeptide β, and calcitonin receptor-like receptor were unchanged at 7 weeks postfracture, *versus* control nonfracture mice, and wheel running had no effect on the expression of these mediators (fig. 2, B–D, F–K). Protein levels for inflammatory mediators in the hind paw skin were also evaluated by enzyme immunoassay (fig. 3). Similar to gene expression, interleukin 1 (control, 29.6 ± 5.3, *vs.* fracture + no wheel, 40.2 ± 10.8, $P = 0.040$) and C-C motif chemokine ligand 2 (control, 42.8 ± 9.8, *vs.* fracture + no wheel, 58.4 ± 21.5, $P = 0.038$) protein levels were increased at 7 weeks postfracture, when compared to nonfracture mouse protein levels, and wheel running reversed this increase (interleukin 1: fracture + no wheel, 40.2 ± 10.8 *vs.* fracture + wheel, 30.0 ± 10.3 pg/mg, $P = 0.021$; C-C motif chemokine ligand 2: fracture + no wheel, 58.4 ± 21.5, *vs.* fracture + wheel, 42.2 ± 9.2 pg/mg, $P = 0.034$). Hind paw skin protein levels for interleukin 6, tumor necrosis factor, nerve growth factor, substance P, and calcitonin gene-related peptide were unchanged at 7 weeks postfracture, *versus* control nonfracture mice, and wheel running had no effect on the expression of these mediators (fig. 2, B–D, F–K).

Exercise Had No Effect on Sciatic Nerve Neuropeptide Expression

At 7 weeks postfracture, substance P and calcitonin gene-related peptide gene (tachykinin precursor 1, calcitonin-related polypeptide α, and calcitonin-related polypeptide β) expression in the fracture limb lumbar dorsal root ganglia were measured by real-time polymerase chain reaction, and substance P and calcitonin gene-related peptide protein levels in the sciatic nerve were measured by enzyme

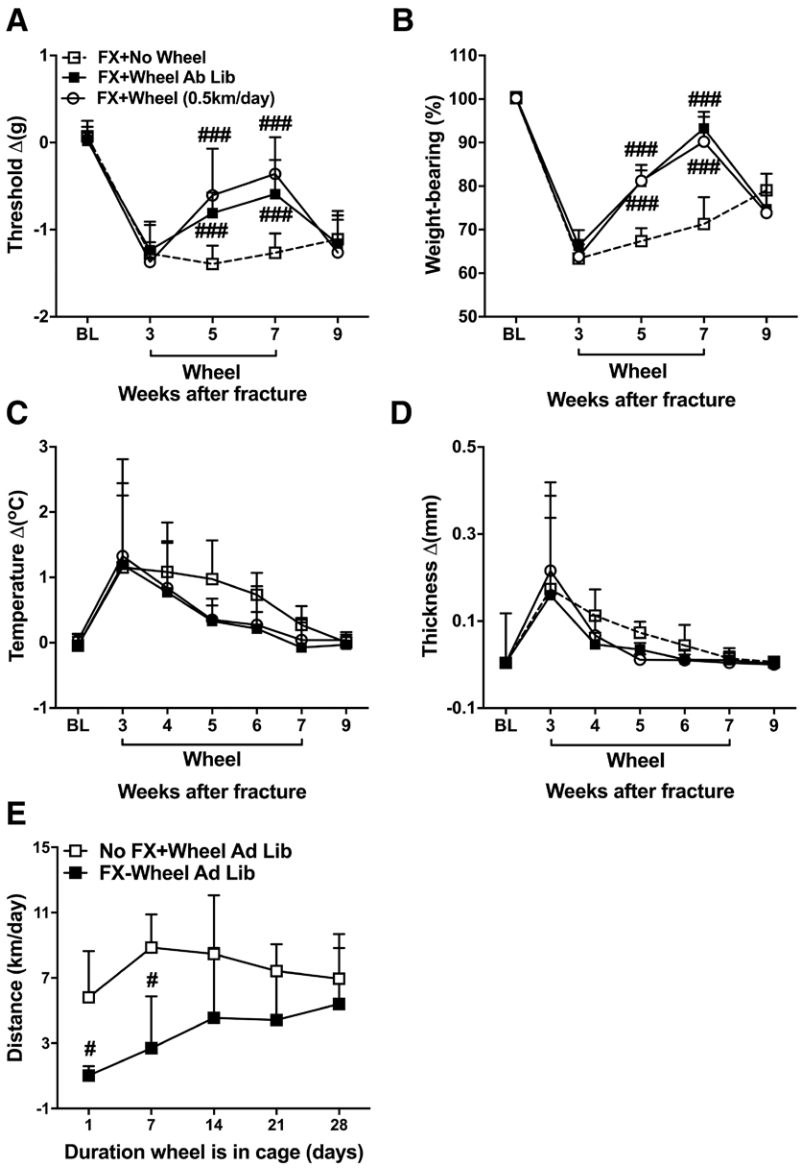

Fig. 1. Effects of running exercise on postfracture nociception and vascular changes. Mouse hind paw von Frey fiber withdrawal thresholds (*A*), weight bearing (*B*), temperature (*C*), and paw thickness (*D*) were measured at baseline (BL); the mice underwent right tibia fracture and hindlimb casting for 3 weeks, and then the cast was removed and the mice retested the following day. Then, one cohort of mice had a running wheel placed in their cage for 4 weeks. There were three experimental groups, fracture with no wheel (FX + no wheel, n = 12), fracture with ad lib access to wheel (FX + wheel ad lib, n = 12), and fracture with only 0.5 km/day wheel access (FX + wheel 0.5 km/day, n = 7). Allodynia (*A*), unweighting (*B*), warmth (*C*), and edema (*D*) were observed at 3 weeks postfracture. The mice with access to the running wheel had reduced hind paw allodynia and unweighting over the duration of the exercise interval (postfracture weeks 3 to 7). When the running wheel was removed from the cage for 2 weeks (postfracture weeks 7 to 9), the hind paw allodynia and unweighting were exacerbated, returning to the same levels as seen in fracture mice not treated with running wheel access. Limited access (0.5 km/day) to the running wheel between postfracture weeks 3 to 7 resulted in the same beneficial effects as ad lib access to the wheel. Another experiment measured the distance that mice ran at 1, 7, 14, 21, and 28 days after the running wheel was placed in their cages and ad lib access to the wheel was allowed (*E*). The fracture mice ran 1.0 km/day the first day of ad lib wheel access (FX + wheel ad lib, n = 8), and this gradually increased to 5.4 km/day for more than 4 weeks. After 2 weeks of ad lib wheel access, there was no significant difference between the distances the fracture mice were running daily and the distances that nonfracture control mice (no FX + wheel ad lib, n = 8) were running. The hind paw von Frey mechanical nociceptive threshold, temperature, and thickness were analyzed as the difference between the fracture side and the contralateral untreated side; thus, a negative value for von Frey testing indicates allodynia and a positive value for temperature and thickness represents warmth and edema. Right hind paw weight-bearing data were analyzed as a ratio between twice the right hind paw weight bearing and the sum of the right (R) and left (L) hind paw weight-bearing values ([2R/{R+L}]×100%); thus, a percentage less than 100% indicates unweighting. Values are means ± SD. Two-way ANOVA with Bonferroni *post hoc* testing. #$P < 0.05$, ##$P < 0.01$, and ###$P < 0.001$ for fracture + wheel ad lib or fracture + wheel (0.5 km/day) *versus* fracture + no wheel.

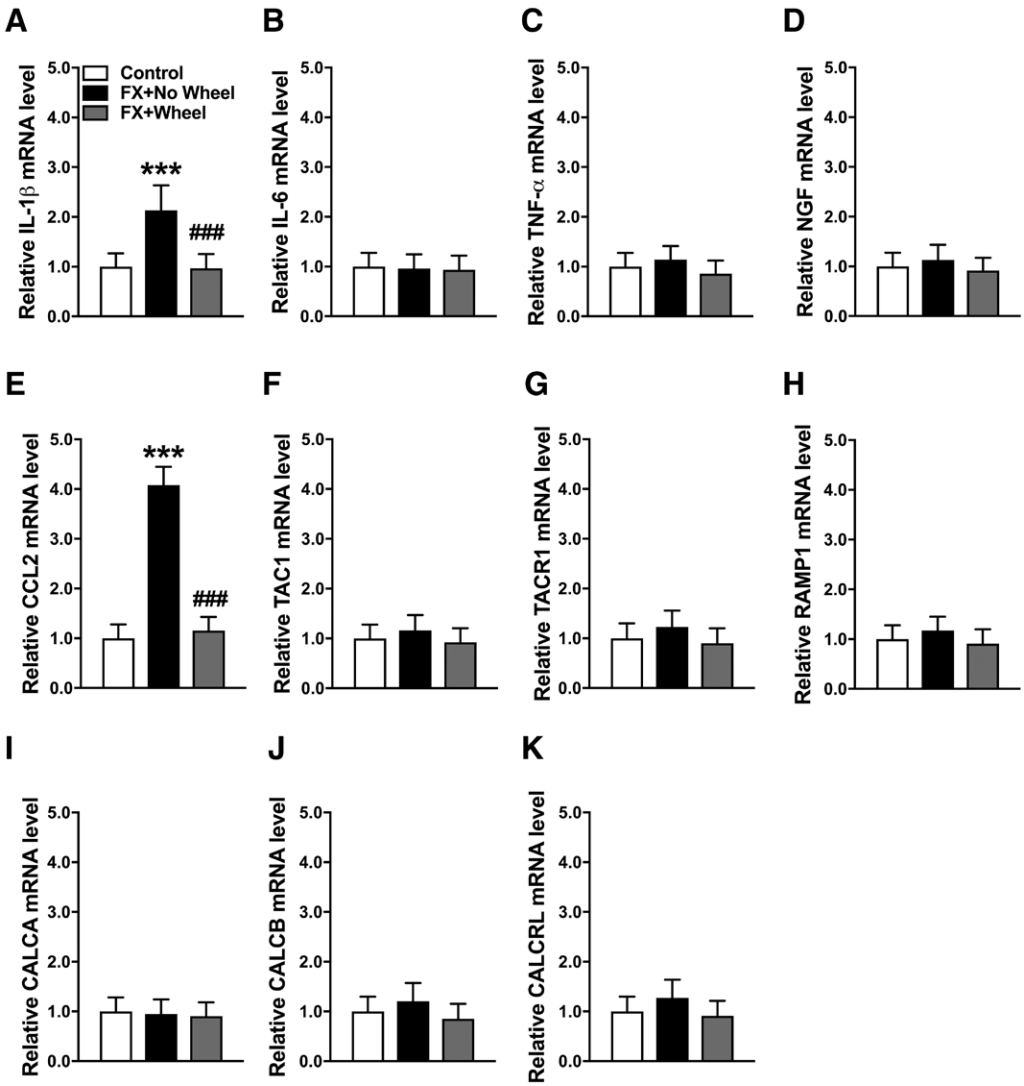

Fig. 2. The effects of exercise on postfracture gene expression of cutaneous inflammatory mediators (*A–K*). Expression of inflammatory mediators in the fracture limb hind paw skin were measured by real-time polymerase chain reaction. Interleukin 1β (*A*) and C-C motif chemokine ligand 2 (*E*) gene expression were upregulated at 7 weeks postfracture (FX + no wheel), compared to nonfractured control mice (control); this increase was reversed in fracture mice provided with 4 weeks of ad lib access to a running wheel (FX + wheel), starting at 3 weeks postfracture. There were no changes in the hind paw skin expression of interleukin 6 (IL-6, *B*), tumor necrosis factor-α (TNF-α, *C*), nerve growth factor (NGF, *D*), substance P/tachykinin precursor 1 (TAC1, *F*), substance P/tachykinin receptor 1 (TACR1, *G*), the calcitonin gene-related peptide (CGRP) receptor activity-modifying protein 1 (RAMP1, *H*), calcitonin-related polypeptide α (CALCA, *I*), calcitonin-related polypeptide β (CALCB, *J*), and calcitonin receptor-like receptor (CALCRL, *K*) at 7 weeks postfracture (FX + no wheel), compared to control nonfracture mice. Exercise had no effects on the postfracture expression of IL-6, TNF-α, NGF, TAC1, TACR1, RAMP1, CALCA, CALCB, or CALCRL. Values are means ± SD, n = 8. One-way ANOVA with Bonferroni *post hoc* testing. ***$P < 0.001$ for fracture + no wheel or fracture + wheel *versus* control. ###$P < 0.001$ for fracture + wheel *versus* fracture + no wheel.

immunoassay (fig. 4). There were no increases in dorsal root ganglia tachykinin precursor 1, calcitonin-related polypeptide α, and calcitonin-related polypeptide β mRNA levels and no increases in sciatic nerve substance P and calcitonin gene-related peptide protein levels at 7 weeks postfracture. Four weeks of wheel-running exercise had no effect on the expression of tachykinin precursor 1, calcitonin-related polypeptide α, calcitonin-related polypeptide β, substance P, and calcitonin gene-related peptide.

Exercise Reversed Postfracture Increases in Interleukin 1, Interleukin 6, Tumor Necrosis Factor, C-C Motif Chemokine Ligand 2, Tachykinin Precursor 1, Receptor Activity-modifying Protein 1, Calcitonin-related Polypeptide α, and Calcitonin-related Polypeptide β mRNA in the Ipsilateral Lumbar Cord

At 7 weeks postfracture, neuropeptide and inflammatory mediator gene expression in the lumbar cord innervating the fracture limb was measured by real-time polymerase chain

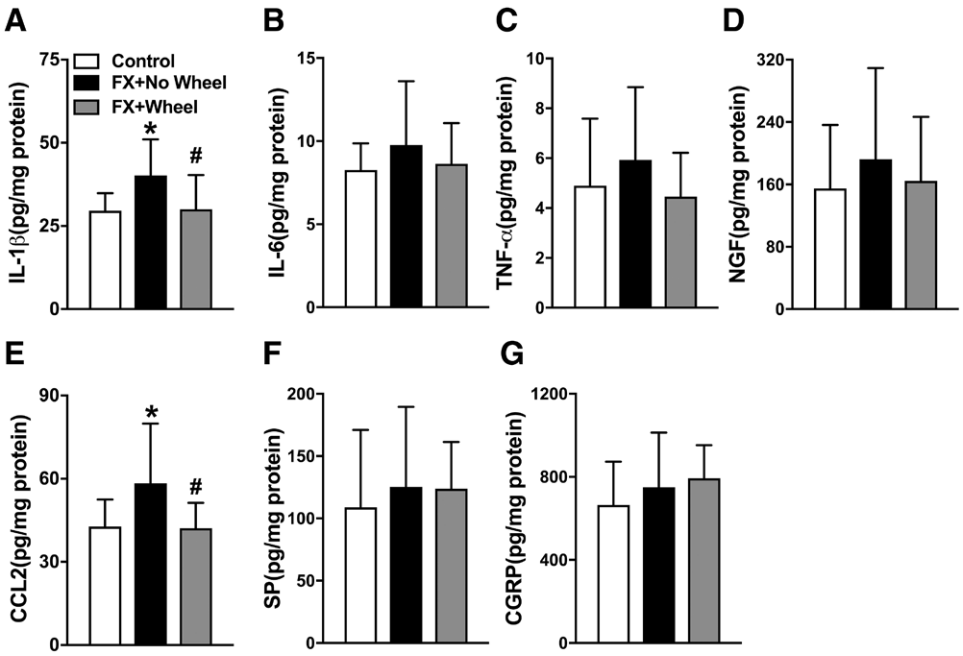

Fig. 3. The effects of exercise on postfracture protein expression of cutaneous inflammatory mediators (*A–G*). Protein levels of interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), nerve growth factor (NGF), C-C motif chemokine ligand 2 (CCL2), substance P (SP), and calcitonin gene-related peptide (CGRP) in the fracture limb hind paw skin were determined by enzyme immunoassay. Similar to the changes observed in gene expression (fig. 2), IL-1β (*A*) and CCL2 (*E*) protein expression were upregulated at 7 weeks postfracture (FX + no wheel), compared to nonfractured control mice (control), but returned to normal levels after 4 weeks of wheel running, starting at 3 weeks postfracture (FX + wheel). Hind paw skin protein levels for IL-6, TNF-α, NGF, SP, and CGRP (*B–D*, *F*, *G*) were not elevated at 7 weeks postfracture, compared to nonfractured control mice; 4 weeks of wheel running in fracture mice had no effect. Values are means ± SD, n = 8 per cohort. One-way ANOVA with Bonferroni *post hoc* testing. **P* < 0.05 for fracture *versus* control. #*P* < 0.05 for fracture + wheel *versus* fracture.

reaction (fig. 5). Compared to nonfracture mice, interleukin 1 (110%), interleukin 6 (120%), tumor necrosis factor (100%), C-C motif chemokine ligand 2 (50%), tachykinin precursor 1 (90%), receptor activity-modifying protein 1 (50%), calcitonin-related polypeptide α (140%), and calcitonin-related polypeptide β (100%) mRNA levels were elevated at 7 weeks postfracture, and 4 weeks of wheel-running exercise reversed this increase (fig. 5, A–C, E, F, H–J). Spinal cord mRNA levels for nerve growth factor, tachykinin receptor 1, and calcitonin receptor-like receptor were unchanged at 7 weeks postfracture, *versus* control nonfracture mice, and wheel running had no effect on the expression of these mediators (fig. 5, D, G, K). Lumbar cord protein levels for these inflammatory mediators were also evaluated by enzyme immunoassay, but levels were below the sensitivity thresholds for the assay kits used in this study.

Stopping Exercise Induced the Upregulation of Substance P and Calcitonin Gene-related Peptide in the Sciatic Nerve and Interleukin 1 and Nerve Growth Factor in the Hind Paw Skin

A cohort of fracture mice were treated with 4 weeks ad lib running wheel exercise (between postfracture weeks 3 to 7), and then the running wheels were removed from the cages for 2 weeks; the mice were retested and then euthanized (9 weeks after fracture). Control fracture mice had no access to running wheels and were also euthanized at 9 weeks after fracture. At 9 weeks postfracture, neuropeptide and inflammatory mediator gene expression in the fracture limb hind paw skin was measured by real-time polymerase chain reaction (fig. 6). Compared to the nonfracture control mice, only C-C motif chemokine ligand 2 (200%) mRNA levels were elevated at 9 weeks postfracture in the nonexercised fracture mice, and 4 weeks of wheel-running exercise persistently reversed this increase even after exercise had been stopped for 2 weeks (fig. 6E). Interestingly, hind paw skin mRNA levels for interleukin 6, tumor necrosis factor, nerve growth factor, tachykinin precursor 1, tachykinin receptor 1, receptor activity-modifying protein 1, calcitonin-related polypeptide α, calcitonin-related polypeptide β, and calcitonin receptor-like receptor were unchanged at 9 weeks postfracture in control mice that had no running wheel treatment, *versus* control nonfracture mice, but mice that had 4 weeks running wheel treatment and then 2 weeks no running wheel access had increased interleukin 6 (240%) and nerve growth factor (90%) mRNA levels in the hind paw skin, relative to nonfracture control mice (fig. 6, B and D). Wheel running for 4 weeks and then no exercise for 2 weeks did not affect the expression of tumor necrosis factor, tachykinin precursor 1, tachykinin receptor 1, receptor activity-modifying protein 1, calcitonin-related polypeptide α, calcitonin-related

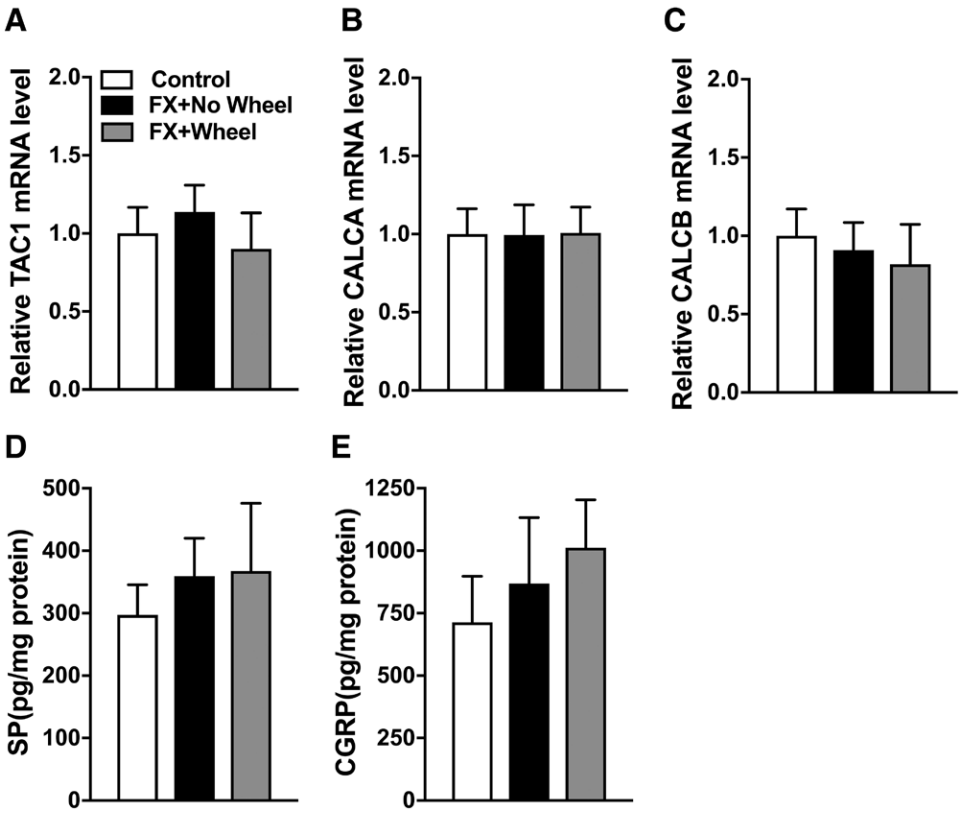

Fig. 4. Exercise had no effects on postfracture neuropeptide expression in the sciatic nerve. Gene expression levels for substance P/tachykinin precursor 1 (TAC1, *A*), calcitonin-related polypeptide α (CALCA, *B*), and calcitonin-related polypeptide β (CALCB, *C*) were determined in the lumbar dorsal root ganglia by real-time polymerase chain reaction; substance P (*D*) and calcitonin gene-related peptide (*E*) protein levels in the sciatic nerve were determined by enzyme immunoassay. No changes in dorsal root ganglia substance P and calcitonin gene-related peptide gene expression or sciatic nerve protein expression were observed in the fracture limb at 7 weeks postfracture (FX + no wheel), compared to nonfractured control mice (control); 4 weeks of wheel running in fracture mice had no effect (FX + wheel). Values are means ± SD, n = 8 per cohort. One-way ANOVA with Bonferroni *post hoc* testing.

polypeptide β, and calcitonin receptor-like receptor mRNA in hind paw skin (fig. 6, C, F–K).

Protein levels for substance P and calcitonin gene-related peptide in the sciatic nerve and for interleukin 6 and nerve growth factor in the hind paw skin were also evaluated by enzyme immunoassay. At 9 weeks postfracture, sciatic nerve substance P levels were increased *versus* controls (control, 285 ± 47, *vs.* fracture + no wheel, 350 ± 22, $P = 0.013$), but calcitonin gene-related peptide (control, 805 ± 199, *vs.* fracture + no wheel, 949 ± 248, $P = 0.529$) levels were not increased at 9 weeks postfracture *versus* controls (fig. 7). When fracture mice were allowed to run for 4 weeks and then stopped exercising for 2 weeks, there was an increase in sciatic nerve substance P (control, 285 ± 47, *vs.* fracture + wheel, 386 ± 47 pg/mg, $P < 0.001$) and calcitonin gene-related peptide protein levels (control, 805 ± 199, *vs.* fracture + wheel, 1,177 ± 164 pg/mg, $P = 0.005$) *versus* controls (fig. 7, A and B). Similar to gene expression, interleukin 6 and nerve growth factor protein levels were not increased at 9 weeks postfracture *versus* control levels (fig. 7, C, E), but in the fracture mice who ran for 4 weeks and then stopped exercising for 2 weeks there was an increase in interleukin 6 (control, 9.6 ± 2.4, *vs.* fracture + wheel, 19.0 ± 2.8 pg/mg, $P = 0.001$; fracture + no wheel, 9.8 ± 1.9, *vs.* fracture + wheel, 19.0 ± 2.8 pg/mg, $P < 0.001$) and nerve growth factor (control, 171 ± 56, *vs.* fracture + wheel, 260 ± 66 pg/mg, $P = 0.001$; fracture + no wheel, 175 ± 3.8, *vs.* fracture + wheel, 260 ± 66 pg/mg, $P = 0.002$) protein levels in the skin. Postulating that interleukin 6 and nerve growth factor upregulation in the fracture hind paw contributed to the rekindling of nociceptive sensitization after stopping exercise, we tested the effects of a single injection of an interleukin 6 receptor antagonist (TB-2–081) or an antinerve growth factor antibody in the 9-week fracture mice. The 9-week postfracture mice not treated with wheel exercise had no change in hind paw allodynia after TB-2–081 or anti–nerve growth factor antibody injections, but the 9-week postfracture wheel-treated mice with rekindled nociceptive sensitivity after stopping exercise had reduced allodynia after TB-2–081 (fracture + wheel baseline, −1.0 ± 0.2, *vs.* fracture + wheel+TB-2–081, −0.4 ± 0.4 Δg, $P < 0.001$) or anti–nerve growth factor antibody (fracture + wheel baseline, −0.7 ± 0.4, *vs.* fracture + wheel + antinerve growth factor, −0.2 ± 0.3 Δg, $P = 0.027$) injections (fig. 7, D, F).

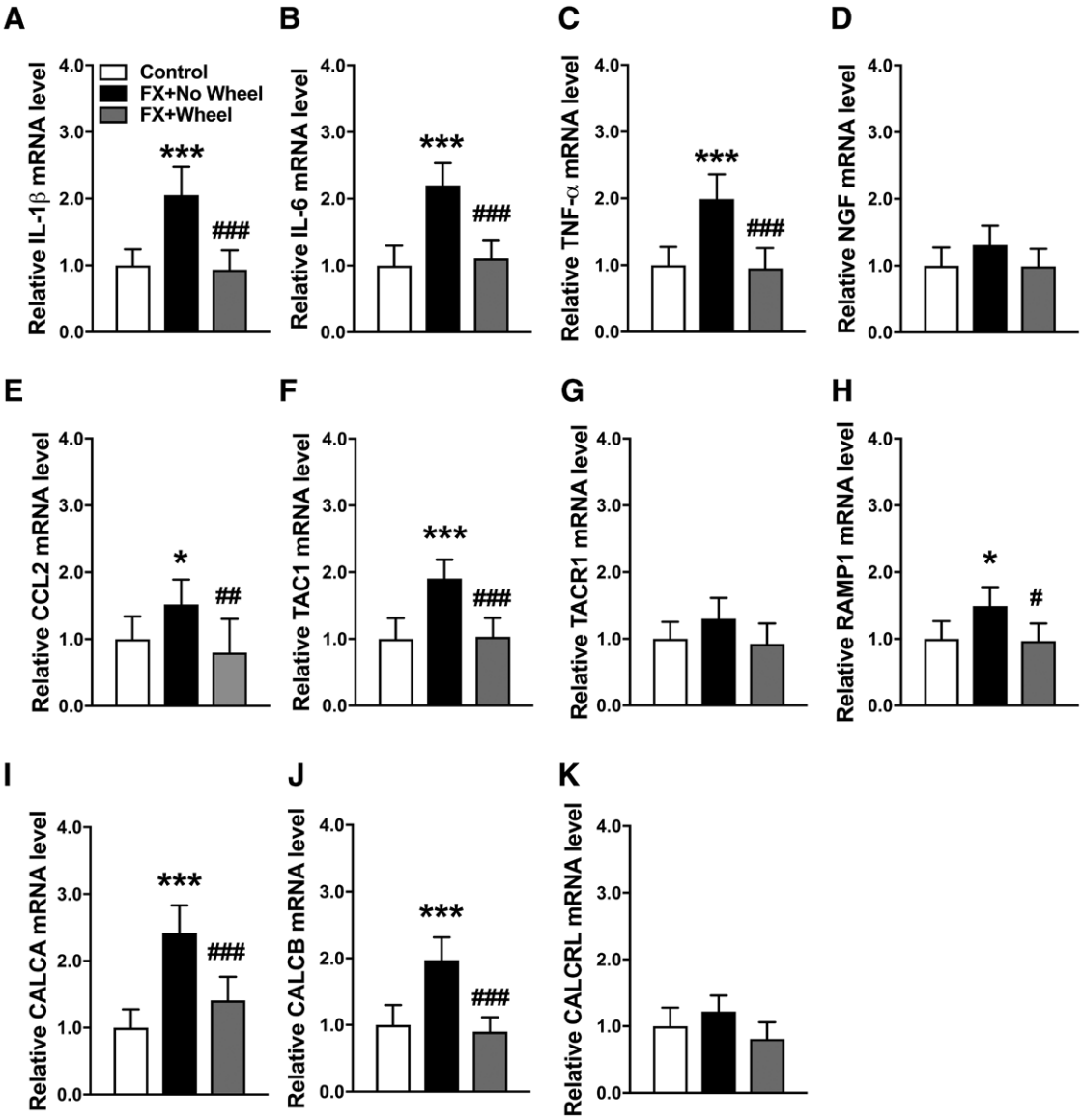

Fig. 5. The effects of exercise on postfracture gene expression of spinal cord inflammatory mediators (*A–K*). Inflammatory mediator expression in the lumbar cord innervating the fracture limb was measured by real-time polymerase chain reaction. Interleukin 1β (IL-1β), interleukin 6 (IL-6), tumor necrosis factor α (TNF-α), C-C motif chemokine ligand 2 (CCL2), tachykinin precursor 1 (TAC1), receptor activity-modifying protein 1 (RAMP1), calcitonin-related polypeptide α (CALCA), and calcitonin-related polypeptide β (CALCB, *A–C*, *E–F*, *H–J*) gene expression were upregulated at 7 weeks postfracture (FX + no wheel), compared to nonfractured control mice (control). All these increases were reversed by voluntary wheel running for 4 weeks starting at day 21 after fracture (FX + wheel). There were no changes in the hind paw skin expression of nerve growth factor (NGF, *D*), tachykinin receptor 1 (TACR1, *G*), and calcitonin receptor-like receptor (CALCRL, *K*) at 7 weeks postfracture, compared to nonfracture control mice, and exercise had no effects on the postfracture expression of NGF, TACR1, or CALCRL. Values are means ± SD, n = 8 per cohort. One-way ANOVA with Bonferroni *post hoc* testing. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 for fracture + no wheel or fracture + wheel *versus* control; # *P* < 0.05, ##*P* < 0.01, and ###*P* < 0.001 for fracture + wheel *versus* fracture + no wheel. CGRP = calcitonin gene-related peptide.

Exercise-induced Inhibition of Postfracture Upregulated Interleukin 6, Tachykinin Precursor 1, Tachykinin Receptor 1, Calcitonin-related Polypeptide α, Calcitonin-related Polypeptide β, and Calcitonin Receptor-like Receptor mRNA Expression in the Spinal Cord Persisted after Stopping Exercise

At 9 weeks postfracture, neuropeptide and inflammatory mediator gene expression in the lumbar cord innervating the fracture limb was measured by real-time polymerase chain reaction (fig. 8). Interleukin 6 (60%), tachykinin precursor 1 (100%), tachykinin receptor 1 (70%), calcitonin-related polypeptide α (70%), calcitonin-related polypeptide β (70%), and calcitonin receptor-like receptor (60%) mRNA levels were elevated at 9 weeks postfracture in mice that had no running wheel treatment, *versus* control nonfracture mice, but the fracture mice who ran for 4 weeks and then

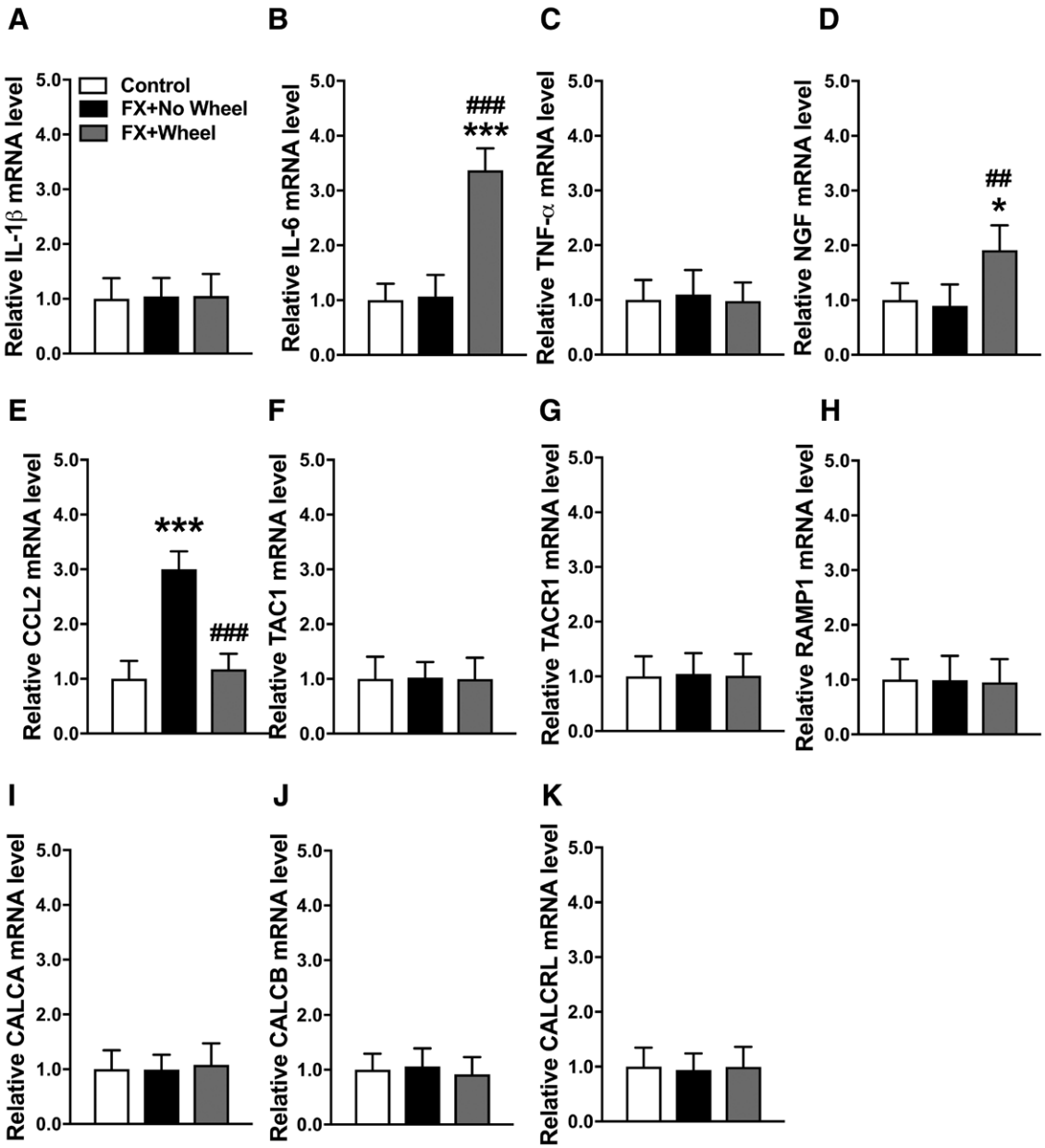

Fig. 6. The effects of stopping exercise on cutaneous gene expression of interleukin 6 (IL-6) and nerve growth factor (NGF; *A–K*). Figure 1 illustrates the reoccurrence of allodynia and unweighting in exercised mice after stopping exercise for 2 weeks. In this experiment a cohort of fracture mice (FX + wheel) were treated with 4 weeks ad lib access to a running wheel, starting at 3 weeks postfracture, and then the wheel was removed for 2 weeks; the animals were euthanized at 9 weeks postfracture and the skin collected. Control fracture mice not provided access to a running wheel (FX + no wheel) were also euthanized at 9 weeks postfracture and the skin collected. The expression levels of inflammatory mediators in the fracture limb hind paw skin were measured by real-time polymerase chain reaction. Only C-C motif chemokine ligand 2 (CCL2, *E*) gene expression was upregulated at 9 weeks postfracture (FX + no wheel), compared to nonfractured control mice (control); this increase was reversed in fracture mice provided with 4 weeks of ad lib access to a running wheel (FX + wheel), starting at 3 weeks postfracture, and this effect persisted after stopping exercise for 2 weeks (week 9 postfracture). At 9 weeks postfracture (FX + no wheel) hind paw skin interleukin 1β (IL-1β, *A*), IL-6 (*B*), tumor necrosis factor-α (TNF-α, *C*), NGF (*D*), tachykinin precursor 1 (TAC1, *F*), tachykinin receptor 1 (TACR1, *G*), receptor activity-modifying protein 1 (RAMP1, *H*), calcitonin-related polypeptide α (CALCA, *I*), calcitonin-related polypeptide β (CALCB, *J*), and calcitonin receptor-like receptor (CALCRL, *K*) messenger RNA (mRNA) levels did not significantly differ from control nonfracture mice (control). Stopping exercise for 2 weeks resulted in increased cutaneous IL-6 (*B*) and NGF (*D*) mRNA levels at 9 weeks postfracture but did not affect the expression of IL-1β, IL-6, TNF-α, NGF, TAC1, TACR1, RAMP1, CALCA, CALCB, or CALCRL mRNA. Values are means ± SD, n = 8. One-way ANOVA with Bonferroni *post hoc* testing. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 for fracture + no wheel or fracture + wheel *versus* control; #*P* < 0.05, ##*P* < 0.01, and ###*P* < 0.001 for fracture + wheel *versus* fracture + no wheel.

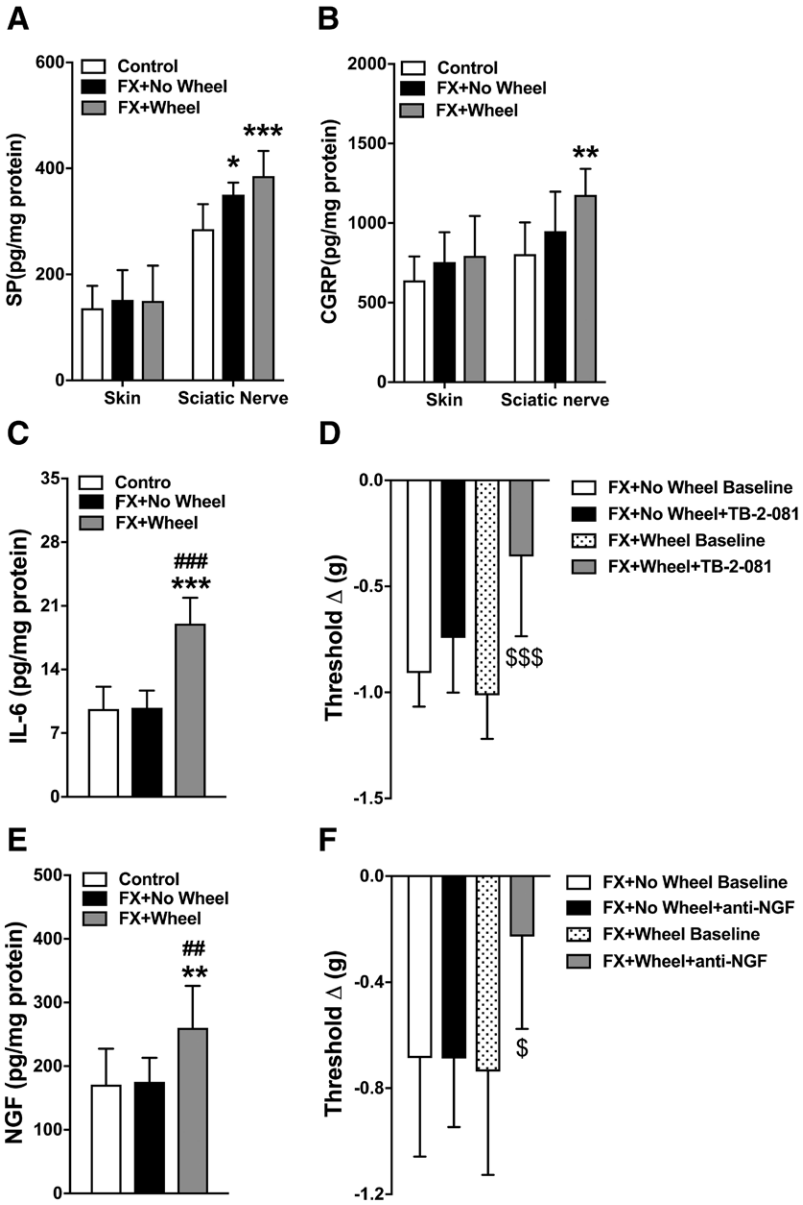

Fig. 7. The effects of stopping exercise on sciatic nerve expression of substance P (SP) and calcitonin gene-related peptide (CGRP) and the cutaneous pronociceptive mediators interleukin 6 (IL-6) and nerve growth factor (NGF; *A–F*). Figure 1 illustrates the reoccurrence of allodynia and unweighting in exercised mice after stopping exercise for 2 weeks. In these experiments a cohort of fracture mice (FX + wheel) were treated with 4 weeks ad lib access to a running wheel, starting at 3 weeks postfracture, and then the wheel was removed for 2 weeks; the animals were euthanized at 9 weeks postfracture and the sciatic nerve and skin collected. Control fracture mice not provided access to a running wheel (FX + no wheel) were euthanized (at 9 weeks postfracture) and the tissues collected. Sciatic nerve SP and CGRP proteins and hind paw skin IL-6 and NGF proteins were measured by enzyme immunoassay. SP (*A*), CGRP (*B*), IL-6 (*C*), or NGF (*E*) protein were not upregulated at 9 weeks postfracture in the untreated fracture mice (FX + no wheel), compared to nonfractured control mice (control). Stopping exercise for 2 weeks in the wheel-treated fracture mice caused an increased expression of SP (*A*), CGRP (*B*), IL-6 (*C*), and NGF (*E*) proteins at 9 weeks postfracture. At 9 weeks postfracture there were no differences in baseline hind paw mechanical allodynia (von Frey thresholds) between fracture mice that were treated with wheel exercise for 4 weeks and then the wheel was removed for 2 weeks and fracture mice that were never given wheel access (*D*, *F*). When either an IL-6 receptor antagonist (TB-2–081) or an anti-NGF antibody was injected into the fracture mice at week 9, there was no effect in the fracture mice that had not previously been given wheel access, but both drugs reversed mechanical allodynia in fracture mice that had been given 4 weeks running exercise and then the exercise was discontinued for 2 weeks. Values are means ± SD, n = 8. One-way ANOVA with Bonferroni *post hoc* testing. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 for fracture + no wheel or fracture + wheel *versus* control; #*P* < 0.05, ##*P* < 0.01, and ###*P* < 0.001 for fracture + wheel *versus* fracture + no wheel; $*P* < 0.05 and $$$*P* < 0.001 for fracture + wheel baseline *versus* fracture + wheel+TB-2–081.

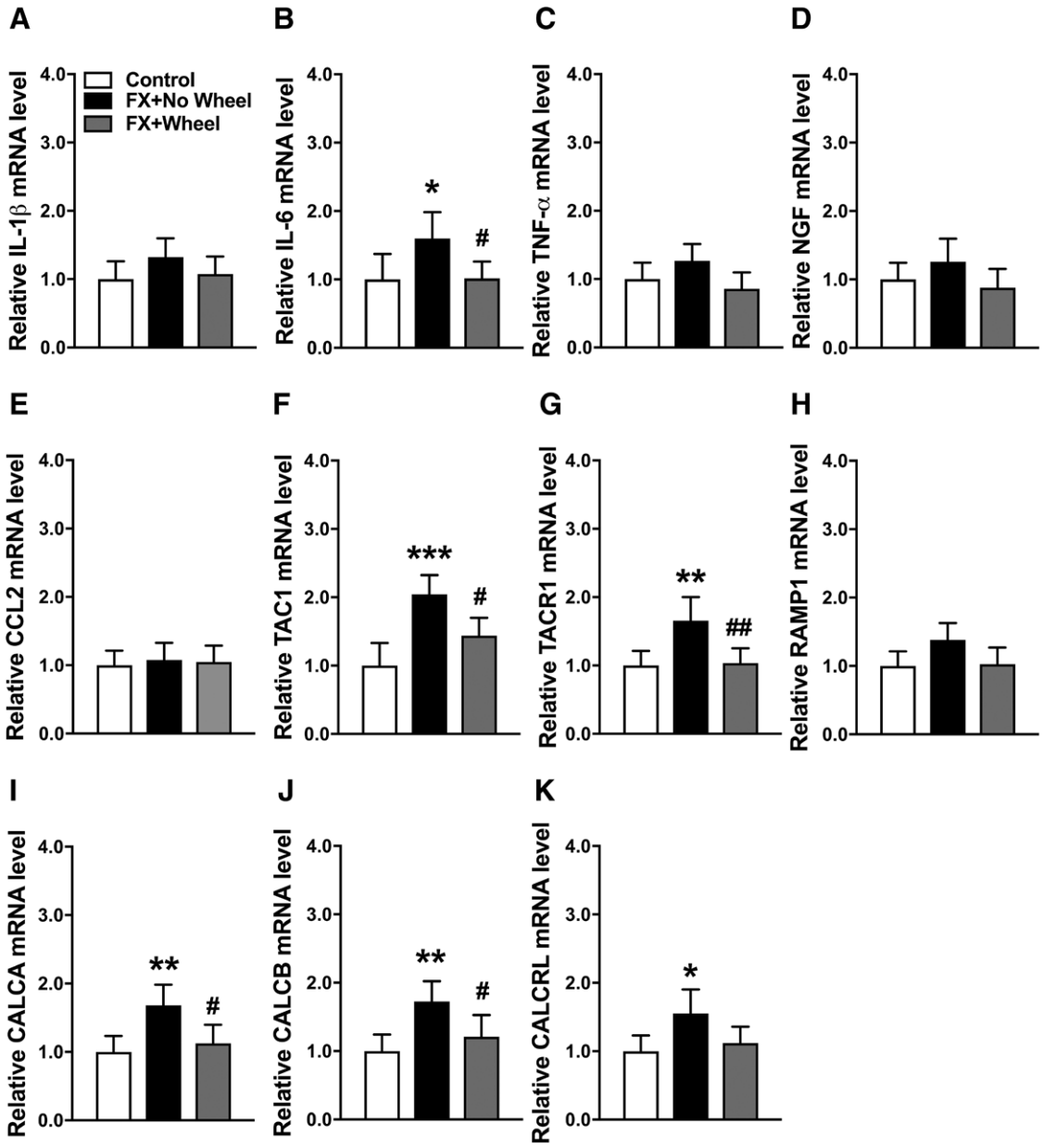

Fig. 8. The effects of stopping exercise on postfracture gene expression of spinal cord inflammatory mediators (*A–K*). Figure 1 illustrates the reoccurrence of allodynia and unweighting in exercised mice after stopping exercise for 2 weeks. In this experiment a cohort of fracture mice (FX + wheel) were treated with 4 weeks ad lib access to a running wheel, starting at 3 weeks postfracture, and then the wheel was removed for 2 weeks; the animals were euthanized (9 weeks postfracture) and the skin collected. Control fracture mice not provided access to a running wheel (FX + no wheel) were euthanized (9 weeks postfracture) and the skin collected. Inflammatory mediator expression in the lumbar cord innervating the fracture limb was measured by real-time polymerase chain reaction. Interleukin 6 (IL-6, *B*), tachykinin precursor 1 (TAC1, *F*), tachykinin receptor 1 (TACR1, *G*), calcitonin-related polypeptide α (CALCA, *I*), calcitonin-related polypeptide β (CALCB, *J*), and calcitonin receptor-like receptor (CALCRL, *K*) messenger RNA (mRNA) levels were upregulated at 9 weeks postfracture, compared to nonfractured control mice. All these increases in inflammatory mediator gene express were reversed by 4 weeks wheel running (fig. 5), and this reversal persisted after stopping wheel running. There were no changes in the hind paw skin expression of interleukin 1β (IL-1β, *A*), tumor necrosis factor α (TNF-α, *C*), nerve growth factor (NGF, *D*), C-C motif chemokine ligand 2 (CCL2, *E*), and receptor activity-modifying protein 1 (RAMP1, *H*) at 9 weeks postfracture (no wheel), compared to nonfracture control mice, and exercise had no effects on the 9-weeks postfracture expression of these genes. Values are means ± SD, n = 8 per cohort. One-way ANOVA with Bonferroni *post hoc* testing. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 for fracture + no wheel or fracture + wheel *versus* control; #*P* < 0.05, ##*P* < 0.01, and ###*P* < 0.001 for fracture + wheel *versus* fracture + no wheel. CGRP = calcitonin gene-related peptide.

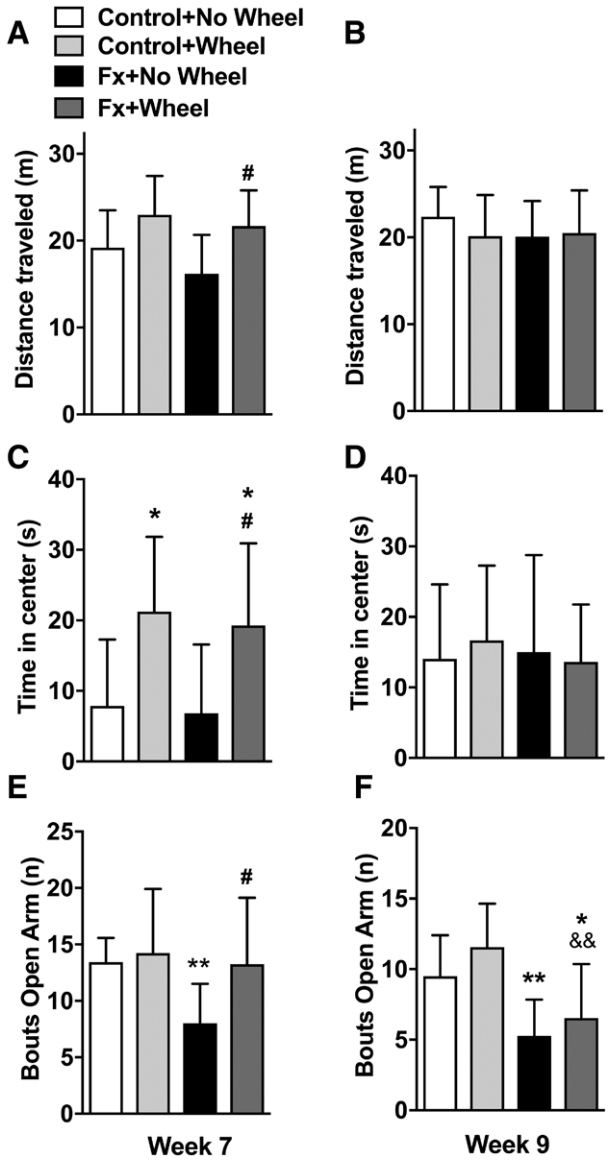

Fig. 9. Exercise improved postfracture locomotor activity and reduced anxiety. Fracture mice were treated with 4 weeks of wheel running (FX + wheel, week 7) or no wheel access (FX + no wheel, week 7); nonfracture mice with and without access to running wheels were used as controls (control + wheel, control + no wheel, week 7). In an additional set of experiments, fracture and control mice were given 4 weeks of wheel access and then the wheel was removed for 2 weeks (FX + wheel, control + wheel, week 9) or were not given access to a running wheel and were tested at 9 weeks postfracture (FX + no wheel, week 9). In the open-field test, compared to nonfracture controls, there was no reduction in the distance traveled by the fracture + no wheel cohort at 7 (*A*) or 9 (*B*) weeks postfracture. Wheel exercise (FX + wheel) did increase the distance traveled, compared to fracture + no wheel (*A*), but this effect was lost after stopping exercise for 2 weeks (*B*). The exercise fracture mice spent increased time in the center of the open-field assay, compared to no exercise fracture mice or controls (*C*), but this effect was lost after stopping exercise for 2 weeks (*D*). After 4 weeks of wheel exercise, control

(*Continued*)

stopped exercise for 2 weeks had persistent reversal of postfracture upregulated neuropeptide and inflammatory mediator expression (fig. 8, B, F, G, I–K).

Exercise Reversed Postfracture Anxiety and Working Memory Impairment

Consistent with our previous results in fracture mice,[14] the distance traveled in the open-field assay did not significantly differ between the nonexercise fracture mice and the nonfracture controls at 7 or 9 weeks postfracture (fig. 9, A and B). However, fracture mice having access to the running wheel for 4 weeks displayed significant increases in distance traveled compared to their nonexercise fracture counterparts (week 7: 21.7 ± 4.1 *vs.* 16.2 ± 4.5 m, $P = 0.011$). At 2 weeks after stopping exercise, locomotor activity in the exercised fracture mice returned to levels similar to those observed in the control or nonexercise fracture groups (fig. 9, A and B). Similarly, there were no significant differences between the nonexercise fracture mice and controls in the time spent in the center of the open-field assay at 7 or 9 weeks (fig. 9, C and D). The exercised nonfracture controls (week 7: 21.2 ± 10.6 *vs.* 7.8 ± 9.4 s, $P = 0.036$) and the exercised fracture mice (week 7: 19.3.2 ± 11.7 *vs.* 6.8 ± 11.7 s, $P = 0.017$) displayed increased center time compared to the nonexercise fracture mice or controls, but after stopping exercise for 2 weeks this effect resolved (fig. 9, C and D).

Previously, we noted decreased risk-taking behavior in fracture mice with the zero-maze assay,[14] and we observed similar results in the nonexercise fracture mice. At 7 and 9 weeks after fracture, the nonexercise fracture mice were less likely to enter the open arms of the maze compared to nonfractured controls (week 7: 8.0 ± 3.5 *vs.* 13.4 ± 2.1 bouts, $P = 0.004$; week 9: 5.3 ± 2.6 *vs.* 9.5 ± 2.9 bouts, $P = 0.004$), consistent with neophobic anxiety (fig. 9, E and F). Exercising the fracture mice for 4 weeks increased the number of entries into the open arms of the maze (week 7: 13.3 ± 5.9 *vs.* 8.0 ± 3.5 bouts, $P = 0.048$), but after stopping exercise for 2 weeks this effect resolved.

Fig. 9. (*Continued*) nonfracture mice also spent increased time in the center of the open-field assay, compared to controls lacking wheel access, and this effect was also lost after stopping exercise for 2 weeks (*D*). Compared to no fracture controls, the fracture + no wheel mice were less likely to enter the open arms of the zero-maze assay; this behavior signifies increased anxiety at 7 (*E*) and 9 (*F*) weeks postfracture. Four weeks of wheel exercise reversed anxiety behavior in the zero-maze test in fracture mice (FX + wheel) but had no effect on controls (*E*); this anxiolytic effect was lost after stopping exercise for 2 weeks (*F*). Exercising the fracture mice caused an increase in the time spent in the open arms of the zero maze (*E*), but this effect was lost after stopping exercise for 2 weeks (*F*). Values are means ± SD, n = 11 to 15 per cohort. One-way ANOVA with Bonferroni *post hoc* testing. **P* < 0.05 and ***P* < 0.01 for fracture + no wheel or fracture + wheel *versus* control; #*P* < 0.05 and ##*P* < 0.01 for fracture + wheel *versus* fracture + no wheel; &&*P* < 0.01 for fracture + wheel *versus* control + wheel.

Similar to our previous findings in postfracture mice,[14] the nonexercise fracture mice had impaired object recognition working memory at both 7 weeks (fracture + no wheel: 39 ± 12% familiar *vs.* 60 ± 12% novel, $P = 1.000$) and 9 weeks (fracture + no wheel: 43 ± 17% familiar *vs.* 57 ± 17% novel, $P = 0.109$) after fracture (fig. 10, A and B), and this impairment was reversed after wheel exercise at both 7 weeks (fracture + wheel: 35 ± 20% familiar *vs.* 65 ± 20% novel, $P = 0.001$) and 9 weeks (fracture + wheel: 25 ± 16% familiar *vs.* 75 ± 16% novel, $P < 0.001$) after fracture. Unlike the transient effects of exercise on reducing anxiety in fracture mice, working memory improvements were still present 2 weeks after stopping exercise (fig. 10, A and B).

Discussion

The tibia fracture mice exhibited hind paw allodynia, unweighting, warmth, and edema (fig. 1); these pain behaviors spontaneously resolve over a 4-month period, and the inflammatory symptoms recover within 6 weeks.[13] Four weeks of ad lib wheel running, starting at the time of cast removal, accelerated the resolution of allodynia, unweighting, warmth, and edema, but when exercise was stopped for 2 weeks the pain behaviors were rekindled (fig. 1). Four weeks of ad lib wheel running (gradually increasing to 5.6 km/day) had the same effect as running 0.5 km/day for 4 weeks (fig. 1). Rodent exercise studies almost uniformly show analgesic effects in a variety of pain models using various types and intensities of exercise.[22–27] Some studies have reported reoccurrence and others observed no reoccurrence of neuropathic pain after stopping exercise.[25,26] No previous exercise studies have used the tibia fracture model or examined exercise effects on skin, nerve, and spinal cord neuropeptide or cytokine expression.

Neurogenic inflammation is mediated by neuronal release of substance P and calcitonin gene-related peptide, neurotransmitters that activate their vascular receptors to induce extravasation and vasodilatation. Electrically evoked extravasation and vasodilatation responses are enhanced in complex regional pain syndrome patients, and when substance P is microdialyzed in complex regional pain syndrome skin there is an exaggerated extravasation response.[28,29] Furthermore, serum levels of substance P and calcitonin gene-related peptide are elevated in complex regional pain syndrome patients.[30–32] Similarly, in the tibia fracture model there is increased substance P and calcitonin gene-related peptide expression in the sciatic nerve, spinal cord, and serum, upregulated substance P neurokinin 1 receptor expression in endothelial cells and keratinocytes in hind paw skin and in spinal cord homogenates, and enhanced substance P–evoked extravasation and edema responses in the fracture limb.[33,34] Systemic treatment with an substance P receptor antagonist reduced hind paw allodynia, warmth, and edema in fracture rats, and intrathecal treatment with a substance P or calcitonin gene-related peptide receptor antagonist reduced nociceptive sensitization.[16,34,35] Additionally, at 3 weeks postfracture, wild-type mice exhibited hind paw allodynia, unweighting, warmth, and edema, but in substance P–deficient fracture mice allodynia and unweighting were attenuated, and there was no warmth and edema.[13] Fracture mice lacking the calcitonin gene-related peptide receptor activity-modifying protein 1 receptor had a similar presentation. These data support the hypothesis that neuropeptide signaling is amplified in the affected skin, vasculature, and spinal cord after fracture, as well as in complex regional pain syndrome patients, and that this facilitated signaling contributes to the development of complex regional pain syndrome–like changes.

Exercise effects on neuropeptide signaling in the fracture hind limb and spinal cord were evaluated. There was no postfracture change in the expression of the tachykinin precursor 1 gene, the substance P/tachykinin receptor 1 gene, the

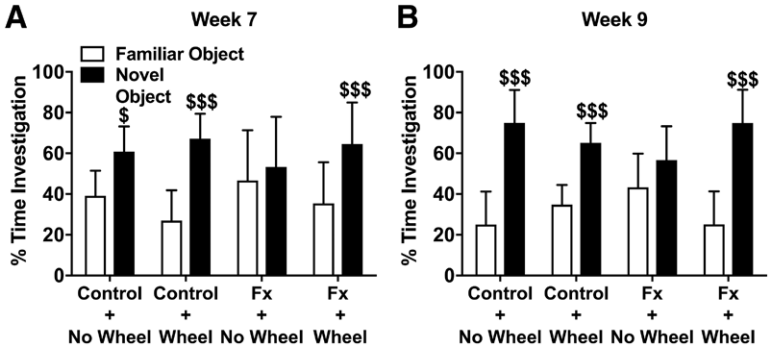

Fig. 10. Exercise improved postfracture memory impairment. Object-recognition memory testing was performed with 5-min intervals between acquisition and retrieval trials. Fracture mice (FX + no wheel) exhibited reduced object-recognition memory compared to nonfracture control + no wheel mice (*A*), and 4 weeks of exercise reversed this memory impairment in fracture mice (FX + wheel). After discontinuing exercise for 2 weeks the beneficial effects of previous exercise were still present (*B*). Exercise in nonfracture control + wheel mice had no effect on object-recognition memory (*A*). All recordings were analyzed in real time by automated software. Values are means ± SD, n = 11 to 15 per cohort. One-way ANOVA with Bonferroni *post hoc* testing. $$P < 0.05$ and $$$P < 0.001$ for novel *versus* familiar object.

calcitonin-related polypeptide α gene, and calcitonin-related polypeptide β gene, the calcitonin receptor-like receptor gene, and the receptor activity-modifying protein gene in the ipsilateral hind paw skin and dorsal root ganglia of unexercised 7-week fracture mice (figs. 2, 3, 4). The spontaneous resolution of facilitated neuropeptide signaling in the injured hind limb at 7 weeks postfracture is consistent with the temporal resolution of upregulated neuropeptide signaling in the injured limb observed between 4 and 16 weeks after fracture in rats.[17,33] The spinal expression of tachykinin precursor 1, calcitonin-related polypeptide α, calcitonin-related polypeptide β, and receptor activity-modifying protein 1 was upregulated at 7 weeks postfracture (fig. 5); exercise inhibited this upregulation (fig. 5), and this inhibitory effect persisted even after stopping exercise for 2 weeks (fig. 8).

When substance P or calcitonin gene-related peptide is microdialyzed into human skin, there is no immediate pain; this result supports the premise that substance P and calcitonin gene-related peptide act as intermediate mediators in the development of inflammatory pain.[36] When applied to keratinocytes *in vitro*, substance P and calcitonin gene-related peptide stimulated expression of tumor necrosis factor, interleukin 1, interleukin 6, and nerve growth factor.[37] Plantar injection of substance P induced a sequential increase in keratinocyte expression of tumor necrosis factor, interleukin 1, interleukin 6, and nerve growth factor in rat skin *in vivo* that resolved after 48 h, temporally correlating with the development and resolution of allodynia.[38] Furthermore, at 3 weeks postfracture, mice expressed elevated levels of tumor necrosis factor, interleukin 1, interleukin 6, C-C motif chemokine ligand 2, and nerve growth factor in the hind paw skin and spinal cord, but in substance P and calcitonin gene-related peptide receptor activity-modifying protein 1 receptor–deficient mice the postfracture pain behaviors were attenuated and only interleukin 6 levels were increased in the hind paw skin.[13,34] These results demonstrate that neuropeptide signaling can evoke keratinocyte and spinal cord expression of inflammatory mediators *in vitro* and *in vivo*.

At 3 to 4 weeks after fracture in mice and rats, there is increased expression of tumor necrosis factor, interleukin 1, interleukin 6, nerve growth factor, and C-C motif chemokine ligand 2 mRNA and protein in the ipsilateral skin and spinal cord.[13,15,17,33,34,39,40] Keratinocytes are the primary cellular source of these inflammatory mediators in the fracture hind paw skin[41] and in complex regional pain syndrome–affected skin.[42] Spinal cord microglia and astrocytes have been identified as the primary source of spinal inflammatory mediators in a variety of chronic pain models,[43] and both microglia and astrocytes are chronically activated in the tibia fracture model.[35] Furthermore, intraplantar injection of each of these inflammatory mediators into normal paw skin rapidly induced prolonged sensitization.[41,44] Systemic or intrathecal treatment with inhibitors or antagonists for tumor necrosis factor, interleukin 1, interleukin 6, nerve growth factor, or C-C motif chemokine ligand 2 all reduced hind paw allodynia and unweighting in 4-week fracture rats; these changes indicate a role for cytokine and growth factor signaling in postfracture sensitization.[34,40,44,45] Collectively, these data suggest that inflammatory mediators can act at the cutaneous nociceptor and spinal cord levels to induce postfracture nociceptive sensitization.

Previously we observed that tumor necrosis factor, interleukin 1, interleukin 6, and nerve growth factor protein levels in the hind paw skin and spinal cord were elevated at 4 weeks, but by 16 weeks postfracture cutaneous inflammatory mediator levels returned to baseline, and spinal levels of tumor necrosis factor, interleukin 1, and nerve growth factor were persistently elevated.[17] In addition, intrathecal, but not systemic injection of an interleukin 1 receptor antagonist or anti–nerve growth factor antibody, reduced nociceptive behaviors at 16 weeks. These results indicate that fracture caused increased peripheral and central inflammatory mediator production acutely, but that spinal inflammation may be more important for persistent nociceptive sensitization in the tibia fracture model.

The current study examined the effects of 4 weeks ad lib wheel running on inflammatory mediator expression in the ipsilateral hind paw skin and lumbar spinal cord of fracture mice. The 7-week postfracture mice that were not exercised had increased interleukin 1 and C-C motif chemokine ligand 2 mRNA and protein levels in the hind paw skin (figs. 2 and 3) and increased interleukin 1, interleukin 6, tumor necrosis factor, and C-C motif chemokine ligand 2 mRNA levels in the spinal cord (fig. 5). Four weeks of daily wheel running completely reversed the postfracture increases in skin and cord inflammatory mediators (figs. 2–4) and resolved hind paw allodynia and unweighting (fig. 1); these results indicate that daily exercise can effectively alleviate inflammation and pain behaviors in this complex regional pain syndrome model. Other investigators have observed exercise inhibition of microglia and astrocyte proliferation in mouse neuropathic pain models,[23,26,46] and we postulate that exercise-induced glial inhibitory effects in the tibia fracture model may mediate the reversal of postfracture spinal inflammatory mediator expression.

Stopping exercise for 2 weeks induced increased sciatic nerve substance P and calcitonin gene-related peptide protein levels, upregulated expression of interleukin 6 and nerve growth factor mRNA and protein in the hind paw skin, and triggered the reoccurrence of allodynia and unweighting in the fracture limb (figs. 1, 6, 7), without any change in spinal cord inflammatory mediator expression (fig. 8). When either an interleukin 6 receptor antagonist (TB-2–081) or an anti–nerve growth factor antibody was injected into exercised fracture mice that had stopped exercising, their hind paw allodynia was reversed (fig. 7). These results suggest that after stopping exercise hind paw sciatic nerve neuropeptide signaling was upregulated and cutaneous inflammation was rekindled, resulting in nociceptive sensitization. The fracture

Table 2. Exercise Effects on Postfracture Upregulation of Neuropeptide-signaling Proteins and Inflammatory Mediators in Skin, Nerve, and Spinal Cord

	Neuropeptide Signaling		Cytokines and NGF	
	7 Weeks after Fracture	9 Weeks after Fracture	7 Weeks after Fracture	9 Weeks after Fracture
Skin	Skin mRNA/**protein** levels after fracture: No change in TAC1/**SP**, TACR1, RAMP1, CALCA/**CGRP**, CALCB/**CGRP**, CALCRL levels. Exercise (weeks 3–7) had no effect on TAC1/**SP**, TACR1, RAMP1, CALCA/**CGRP**, CALCB/**CGRP**, CALCRL levels.	Skin mRNA levels after fracture: No change in TAC1, TACR1, RAMP1, CALCA, CALCB, CALCRL levels. Exercise (weeks 3–7) had no effect on TAC1, TACR1, RAMP1, CALCA, CALCB, CALCRL levels.	Skin mRNA/**protein** levels after fracture: ↑IL-1/*IL-1*, ↑CCL2/*CCL2*, and no change in IL-6/*IL-6*, TNF/*TNF*, NGF/*NGF* levels. Exercise (weeks 3–7) reversed the increase in IL-1/*IL-1* and CCL2/*CCL2* levels.	Skin mRNA/**protein** levels after fracture: ↑CCL2 and no change in IL-1, IL-6/**IL-6,** TNF, NGF/**NGF** levels. Exercise (weeks 3–7) caused an increase in IL-1/**IL-1** and NGF/**NGF** levels.
Sciatic nerve	Sciatic nerve **protein** levels after fracture: No change in **SP** or **CGRP** levels. Exercise (weeks 3–7) had no effect on **SP** and **CGRP** levels.	Sciatic nerve **protein** levels after fracture: No change in **SP** or **CGRP** levels. Exercise (weeks 3–7) caused increased **SP** and **CGRP** levels.		
Dorsal root ganglia	Dorsal root ganglia mRNA levels after fracture: No change in TAC1, CALCA, or CALCB levels. Exercise had no effect on TAC1, CALCA, or CALCB levels.			
Spinal cord	Cord mRNA levels after fracture: ↑TAC1, ↑RAMP1, ↑CALCA, ↑CALCB, and no change in TACR1 or CALCRL levels. Exercise (weeks 3–7) reversed the increase in TAC1, RAMP1, CALCA, and CALCB levels.	Cord mRNA levels after fracture: ↑TAC1, ↑TACR1, ↑CALCA, ↑CALCB, ↑CALCRL, and no change in RAMP1 levels. Exercise (weeks 3–7) reversed the increase in TAC1, TACR1, CALCA, CALCB, and CALCRL levels.	Cord mRNA levels after fracture: ↑IL-1, ↑IL-6, ↑TNF, and ↑CCL2, no change in NGF levels. Exercise (weeks 3–7) reversed the increase in IL-1, IL-6, TNF, and CCL2 levels.	Cord mRNA levels after fracture: ↑IL-6, no change in IL-1, TNF, NGF, or CCL2 levels. Exercise (weeks 3–7) reversed the increase in IL-6 levels.

Proteins indicated with boldface.

CALCA = calcitonin-related polypeptide α; CALCB = calcitonin-related polypeptide β; CALCRL = calcitonin receptor-like receptor; CCL2 = C-C motif chemokine 2; CGRP = calcitonin gene-related peptide; IL-1 = interleukin 1β; IL-6 = interleukin 6; mRNA = messenger RNA; NGF = nerve growth factor; RAMP1 = receptor activity-modifying protein 1; SP = substance P; TAC1 = tachykinin precursor 1; TACR1 = tachykinin receptor 1; TNF = tumor necrosis factor α.

mice that were not exercised also had nociceptive sensitization at 9 weeks postfracture, but there was no upregulation of cutaneous or spinal inflammatory mediators and nerve growth factor and interleukin 6 inhibitors or antagonists had no effect on this sensitization (figs. 1 and 7). We postulate that this reflects the spontaneous resolution of innate immune pronociceptive mechanisms by 9 weeks postfracture in nonexercised mice and that from 9 to 20 weeks postfracture the only pronociceptive mechanisms mediating sensitization in nonexercised mice are the adaptive autoimmune mechanisms recently identified in this model.[47]

Exercise also reduced anxiety on the open-field and zero-maze assays, but these effects were lost after stopping exercise for 2 weeks (fig. 9). Postfracture object-recognition memory impairment was also improved with exercise, and this effect persisted after stopping exercise (fig. 10). Previously we observed similar anxiety-related behaviors and impairment in novel-object recognition in 7- to 9-week postfracture mice, as well as structural changes and synaptic plasticity in the brain.[14] Correspondingly, decreased memory, global cognitive impairments, and increased anxiety frequently occur in complex regional pain syndrome patients.[48,49] Collectively, these results suggest that daily exercise could potentially ameliorate the complex regional pain syndrome pain experience by modifying its associated cognitive and emotional comorbidities.

In conclusion, daily exercise reversed the upregulation of neuropeptide (table 2) and inflammatory mediator expression in skin and spinal cord, as well as the pain behaviors, anxiety, and memory impairments observed in the tibia fracture mouse model of complex regional pain syndrome. The current study has several limitations, including those inherent to using animal models of pain and the translational value of the data acquired. Clinical investigations are required to determine whether exercise can inhibit upregulated neuropeptide signaling and inflammatory mediator expression in skin and spinal cord and reverse nociceptive sensitization, anxiety, and memory impairment in complex regional pain syndrome patients.

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Competing Interests

The authors declare no competing interests.

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