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Original Article

Comparing effects of rest with or without a NK1RA on fibrosis and sensorimotor declines induced by a voluntary moderate demand task

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Abstract

Objectives: Fibrosis is one contributing factor in motor dysfunction and discomfort in patients with overuse musculoskeletal disorders. We pharmacologically targeted the primary receptor for Substance P, neurokinin-1, using a specific antagonist (NK1RA) in a rat model of overuse with the goal of improving tissue fibrosis and discomfort. **Methods:** Female rats performed a low repetition, high force (LRHF) grasping task for 12 weeks, or performed the task for 12 weeks before being placed on a four week rest break, with or without simultaneous NK1RA treatment. Results were compared to control rats (untreated, or treated 4 weeks with NK1RA or vehicle). **Results:** Rest improved LRHF-induced declines in grip strength, although rest plus NK1RA treatment (Rest /NK1RA) rescued it. Both treatments improved LRHF-induced increases in muscle TGFβ1 and collagen type 1 levels, forepaw mechanical hypersensitivity (Rest/NK1RA more effectively), macrophage influx into median nerves, and enhanced collagen deposition in forepaw dermis. Only Rest/NK1RA reduced muscle hypercellularity. However, LRHF+4wk Rest /NK1RA rats showed hyposensitivity to noxious hot temperatures. **Conclusions:** While the NK1RA induced hot temperature hyposensitivity should be taken into consideration if this or related drug were used long-term, the NK1RA more effectively reduced muscle hypercellularity and improved grip strength and forepaw mechanical hypersensitivity.

Keywords: Cummulative Trauma Disorders, Muscle, Nerve, TGFbeta 1, Collagen

Introduction

Overuse-induced musculoskeletal disorders (MSDs) are a leading cause of pain and physical disability worldwide. They are an enormous burden to society in terms of human suffering and lost productivity[1]. Some disorders even advance to the stage that affected persons cannot perform simple personal tasks, e.g., buttoning their clothes. These injuries result from cumulative small amplitude forces occurring with overexertion, repetitive activities or forceful actions[2,3]. In humans, fibrosis has been implicated as a key mechanism in the pathology and symptoms associated with chronic overuse-MSDs[4,5]. Yet, prevention of these disorders is hampered by many problems[6], raising the need for effective treatments.

Substance P (SubP) and its primary receptor, neurokinin-1 (NK1R) are involved in pain behaviors associated with carpal tunnel syndrome, for example, and increase in muscles with overuse[7,8]. The Substance P-NK1R pathway has also been implicated as an inducer of collagen production and hypercellularity in over-use induced fibrotic tissue changes[9,10]. Use of a neurokin-1 receptor antagonist (NK1RA) has been proposed as a therapeutic target for the treatment of over-use induced fibrosis[10,11], although this hypothesis has yet to be tested *in vivo*.

We have a clinically relevant model in which rats perform an operant grasping and isometric lever pulling task for a food reward that we use to examine the etiology and treatment of upper extremity overuse MSDs[12]. We have shown greater changes induced by long-term performance of higher

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demand than in lower demand tasks[12]. Rats performing a high repetition high force task (4 reaches/min at 53% of rats' maximal voluntary pulling force) for 12 weeks show greater increases in inflammatory cytokines and macrophages in muscles and median nerves, than in rats performing a low repetition (2 reaches/min) high force task (LRHF) for 12 weeks[12]. However, similar declines in grip strength and forepaw mechanical hypersensitivity were induced by each task. In rats performing a high repetition high force task for 6 to 18 weeks, there was increased muscle fibrosis and Substance P immunoexpression in muscles and peripheral nerves in parallel with sensorimotor declines[8,13]. We have yet to examine contributions of fibrogenic and peripheral Substance P responses to functional declines in rats performing a LRHF task.

Muscle fibrogenic responses are enhanced by inflammatory cytokines in rats performing a high repetition high force task for 6-12 weeks[13], matching others' findings suggesting that inflammatory responses can affect muscle fibrosis[14,15]. Yet, performance of a LRHF task leads to only a low induction of inflammatory cytokines in muscles[12]. Examination of muscles of LRHF task rats provides as a unique opportunity to separate out mechanical activity-induced from inflammation-induced changes. We sought here to determine if long-term performance of a LRHF task induces muscle fibrosis, and then if cessation of the task reduces it. Since muscle fibrosis, once established can be difficult to reverse[16,17], we also blocked signaling in the SubP-NK1R pathway using a NK1RA, hypothesizing that it would aid in reducing overuse-induced fibrogenic changes as well as LRHF task induced sensorimotor declines.

Methods

(More detailed methods are available in Supplemental materials, Animal overview and repetitive overuse task)

Experiments were approved by the Institutional Animal Care and Use Committee in compliance with NIH guidelines for humane care and use of laboratory animals. Young adult Sprague-Dawley rats were used. Female rats were used since human females have a higher incidence of work-related musculoskeletal disorders than males[18]. Rats used were food restricted to 5% less than weights of age-matched rats with free-access-to-food (used for weight comparison purposes only), and allowed to gain weight over the experiment.

Experimental task rats first underwent an initial shaping period for 5 weeks to learn a high force lever pulling task, as previously described[19], for 10 min/day, 5 days/week. After this period, a point equal to task week 0, shaped rats began the task. Task rats performed a low repetition high force task (LRHF) at a low reach rate (1.15±0.27 reaches/min, mean±SEM) that required extending their forearm forward into a portal, grasping a force lever bar, and then exerting a fairly isometric pull for at least 90 msec at 47% of their maximum pulling force, for 2 hrs/day, in 30 min intervals, with 1.5 hr rest breaks between, 3 days/wk, for 12 weeks. Their mean grasp force was 1.39±0.01 Newtons (N), and mean grasp duration was 146±4.92 msec. The rats had a preferred reach limb, which was noted during each task session.

At the end of task week 12, LRHF rats were divided into three cohorts. One group was euthanized and tissues collected (LRHF), a second group ceased task performance and rested for 4 weeks (LRHF+Rest), while a third group rested for 4 weeks while simultaneously receiving NK1R antagonist treatment (LRHF+Rest/NK1RA). Reach limbs were collected from these rats (n=19, 10 and 10 per group, respectively); 5-7 limbs per group were processed for histological and immunohistochemical analyses and the remaining for ELISA.

Additional rats were served as food-restricted controls (FRC) rats. These animals rested in their cages with daily handling. FRC rats were euthanized at matched time points as LRHF task rats as: untreated FRC rats (FRC, n=19, bilateral tissue collection); FRC rats treated in their final four weeks with vehicle (FRC+Vehicle; 95% ethanol in saline, n=10, bilateral tissue collection); and FRC rats treated in their final four weeks with NK1RA (FRC+NK1RA, n=10, bilateral tissue collection). One limb per FRC rat was processed for histological analyses and remaining for ELISA.

Pharmacological treatments of animals

A subcohort of LRHF task rats was treated for four weeks while resting with a selective NK1RA (L732,138, Tocris Bioscience, R&D, Minneapolis, MN) at 5 mg/kg body weight in 50 µl of 95% ethanol in saline (dose determined from[20,21]). This dose was injected, i.p., 3x/week during the four week rest period. Subcohorts of FRC rats were either left untreated, or were treated for four weeks with the NK1RA drug or its vehicle (50 µl of 95% ethanol, i.p.) at matched time points as treated LRHF rats.

Behavioral assays

Individuals carrying out these tests were blinded to group assignment. LRHF and FRC groups were assayed for reflexive grip strength using a rat grip strength meter (1027SR-D58, Columbus Instruments, Columbus, Ohio). Grip strength was assayed after onset of food restriction (baseline), after task week 12, and after the four week treatment period. The test was repeated 5 times per limb. Maximum grip strength per trial is reported. Forepaw sensitivity to mechanical probing was assayed at similar time points from all rats using nylon monofilaments (Semmes-Weinstein monofilaments, Stoelting, Woodale, IL) and described methods[22]. Number of limb withdrawal responses out of 10 reported for each monofilament is reported. A two-choice temperature place preference test was used to determine temperature aversion using a temperature testing apparatus (T2CT, BioSeb, France), and described methods[8].

ELISAs

Animals were anesthetized and tissues for ELISA removed prior to perfusion of rats with fixative. Blood and muscle

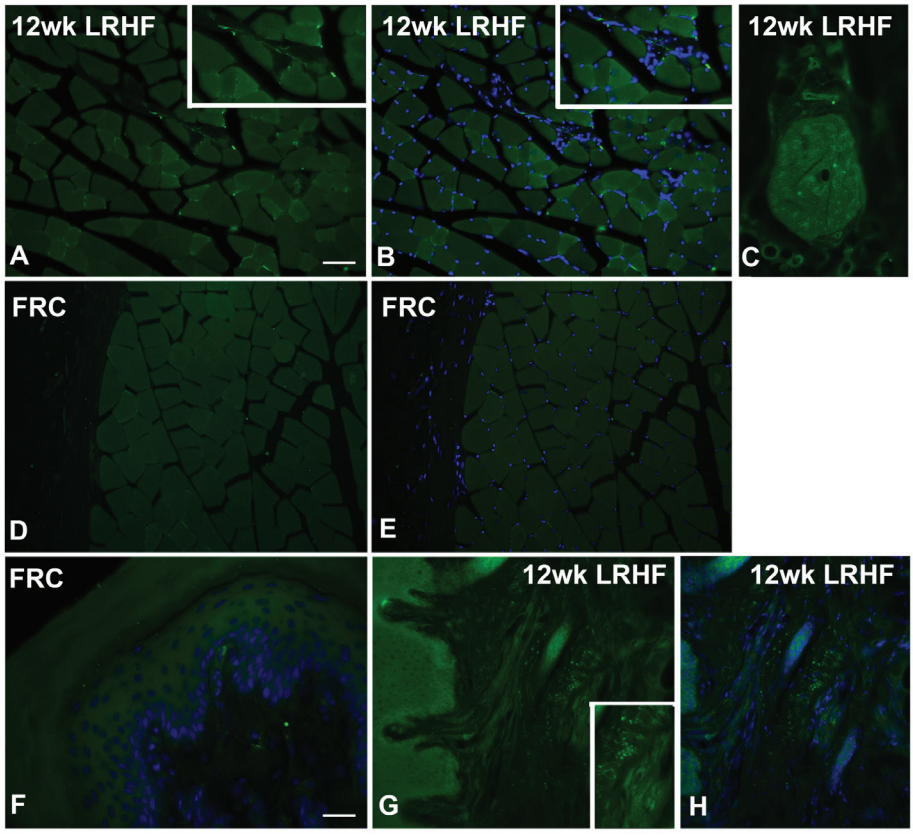

Figure 1. Substance P immunostaining in flexor digitorum muscles and upper dermis of forepaw skin. **A)** Substance P immunostaining (green) in 12 week LRHF rat muscle, with Substance P immunostaining around myofibers and in nerve axons (example enlarged in inset). **B)** Same image with DAPI staining (blue). **C)** Substance P immunostaining in a larger nerve in muscle of LRHF rat. **D)** Substance P immunostaining in muscle of FRC rat. **E)** Same image with DAPI staining. **F)** Substance P immunostaining and DAPI in dermis of a forepaw pad of FRC rat. **G)** Substance P immunostaining in dermis of forepaw pad of LRHF rat. Inset shows enlarged image of an area with axons. **H)** Same image with DAPI staining. Scale bars in A and F=50 μm, and is applicable to the other panels.

tissues were collected and prepared for ELISA as described in Supplemental Methods. Serum and muscle lysates (n=5-12/gp) were assayed for Collagen type 1 (LS-F5638, LifeSpan BioSciences, Seattle, WA) and transforming growth factor beta 1 (TGFβ1; ADI-900-155, Enzo, Farmingdale, NY). Muscle lysates (n=6-11/group) were also assayed for IL-1alpha, IL-1beta, CXCL1, CCL2, TNFα (tumor necrosis factor alpha) and VEGF (vascular endothelial growth factor) (RV1MAG-26K, Merk Millipore; LXSARM, R&D). All samples were run in duplicate. Total protein content of muscles was determined using BCA Protein Assays, and muscle data is reported as pg of protein per mg of total protein. Serum data is reported as pg of protein per ml of serum.

Histology and Immunohistochemistry

Five to seven reach limbs per LRHF group and ten per FRC group were collected for histological and immunohistological analyses. Animals were deeply anesthetized and euthanized, serum collected and contralateral limbs for ELISAs removed from FRC rats, before undergoing transcardial perfusion with 4% paraformaldehyde (pH 7.4). Perfused limbs were postfixed for 24 hours, cryoprotected in sucrose, and cryosectioned into 15 μm thick sections. Muscle cross-sections were immunostained for collagen type 1 (C2456, 1:500 dilution, Sigma-Aldrich, Inc., St. Louis, MO) using described antibodies and methods[8,13], before coverslipping with 80% glycerol in PBS with DAPI. Preabsorption methods were performed to demonstrate that the antibody bound specifically to collagen type 1 using a purified rat protein (C7661, Sigma). Adjacent sections were stained with hematoxylin. Numbers of hematoxylin stained nuclei per flexor digitorum cross section were counted by an individual blinded to group assignment in 5-10 rats/group.

Cross-sectional and longitudinal cryosectioned forelimb soft tissues were also immunostained for Substance P, degraded myelin basic protein, and $CD68^-$ immunopositive macrophages, as previously described[8,23,24]. Numbers of

CD68+ cells in median nerve branches from the wrist to mid-forepaw were counted in longitudinally cut cryosections by an individual blinded to group assignment, in 5-10 fields per nerve branch, in 5 rats/group. CD68+ cells were counted similarly in cross-sectional slices of flexor digitorum muscles (n=5 rats/group).

Forepaws with digits were removed after fixation, paraffin embedded, and sectioned longitudinally into 5 µm thick sections, before undergoing Masson's trichrome staining. Collagen staining (blue) in upper dermis of skin of forepaws and digits was quantified (n=5-7/group) using published methods[25].

Statistical analyses

All data are expressed as the mean ± SEM. Grip strength differences were first assayed between FRC groups to examine for treatment-induced differences (pre- versus post-injection). Then, grip strength differences were assayed using a two-way ANOVA and factors: group and treatment, as were differences in muscle and serum collagen and TGFβ1 ELISA data, and percent area with collagen staining. Forepaw mechanical sensitivity differences between FRC or LRHF groups were examined using two-way repeated measures ANOVAs and factors: treatment group and monofilament size. Temperature aversions, assessed only a few days prior to euthanasia to avoid learning confounds, was analyzed using two-way repeated measures ANOVAs and factors: treatment treatment group and temperature. with a Geisser-Greenhouse correction as sphericity was not assumed. One-way ANOVAs were used to compare inflammatory cytokine/chemokines, cell density and CD68 cell counts, across groups. Sidak or Holm-Sidak's multiple comparison tests (the latter for the repeated measures ANOVAs) were used for post hoc assays, and adjusted p values are reported. Adjusted p values of < 0.05 were considered significant.

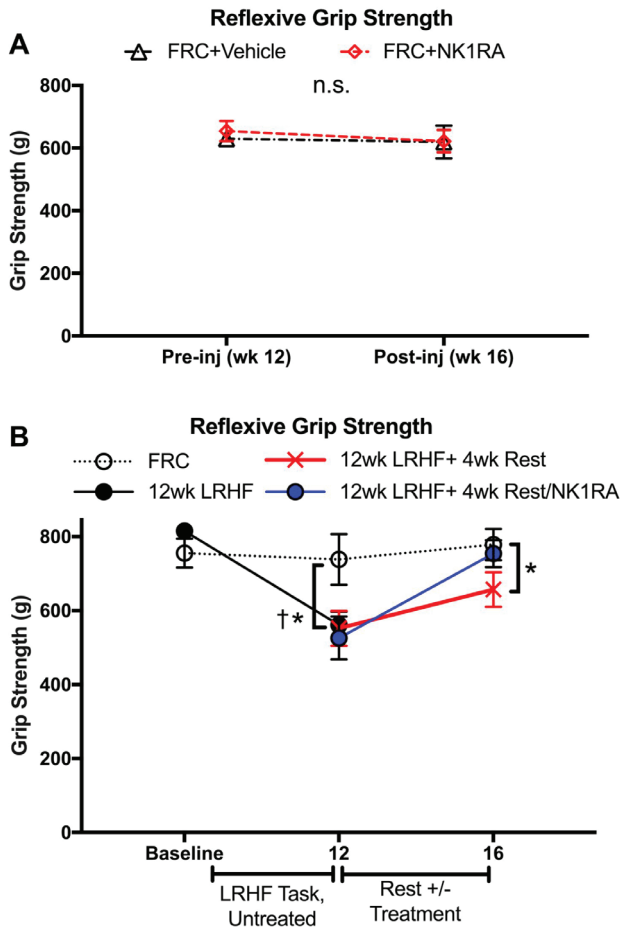

Figure 2. Motor abilities. (A & B) Grip strength in FRC and LRHF groups. **A)** FRC rats before and after 4 weeks of NK1RA (n=10) or vehicle (n=10). **B)** LRHF rats were tested at baseline and after performing the LRHF task for 12 weeks (n=19), and after receiving 4 week Rest or Rest+NK1RA treatments (n=8 each). *: p<0.05, compared to age-matched FRC rats (n=8-9 per time point in panel B); †: p<0.05, compared to baseline; n.s.= not significant.

Results

LRHF task increased Substance P immunostaining in muscles and upper dermis

We observed increased Substance P immunostaining in flexor digitorum muscles of 12 week LRHF rats, relative to FRC rats (Figures 1A-E). This immunostaining was localized to small cells on periphery of myofibers and nerves in the muscle (Figures 1A-C). Only a few Substance P immunopositive cells were observed in FRC rats (Figures 1D,E). More Substance P immunostained axons were visible in upper dermis of forepaw skin of LRHF rats, than in FRC rats (Figures 1F-H).

LRHF-induced grip strength declines improved with rest and rescued with NK1RA treatment

Reflexive grip strength was similar in FRC+ Vehicle versus FRC+NK1RA rats at pre- versus post-treatment (Figure 2A). Grip strength declined in LRHF rats performing the task for 12 weeks, compared to baseline levels and age-matched FRC rats (Figure 2B). The 4-week Rest treatment improved grip strength, while the Rest/NK1RA treatment rescued it to control levels (Figure 2B).

Inflammatory responses were not elevated in flexor digitorum muscles of any group

Grip strength declines could be due to increased muscle inflammatory cytokines[26]. Therefore, we examined levels of several cytokines and chemokines in flexor digitorum muscles of 12 week LRHF and FRC rats. No group

Table 1. Cytokine/Chemokine levels in flexor digitorum muscles of 12-week LRHF rats versus age-matched control rats.

Analyte	FRC	12-week LRHF	p values
IL-1α	0.13 ± 0.02	0.08 ± 0.009	p=0.06
IL-1β	0.05 ± 0.009	0.04 ± 0.004	p=0.79
CXCL1	0.009 ± 0.0004	0.009 ± 0.0005	p=0.55
CCL2	0.0009 ± 0.00003	0.0008 ± 0.00003	p=0.21
VEGF	0.005 ± 0.0005	0.004 ± 0.0004	p=0.09
Mean ± SEM pg/ml serum is shown; n=5-11/group.			

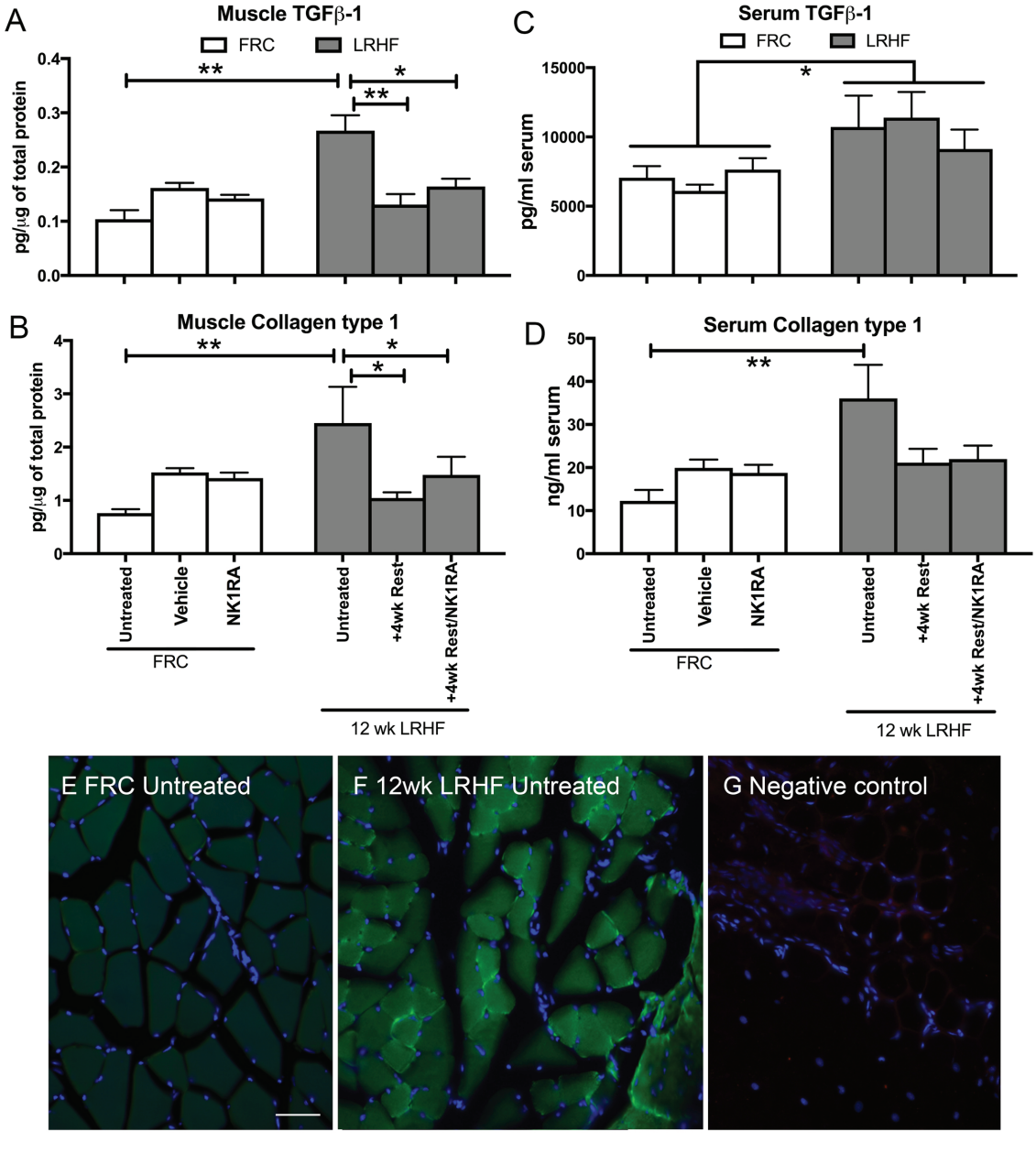

Figure 3. TGFβ1 and Collagen type 1 in flexor digitorum muscles and serum. **A & B)** TGFβ1 and Collagen type 1 levels in muscles (FRC, n=9; FRC+Vehicle, n=10; FRC+NK1RA, n=10; LRHF, n=12; LRHF+Rest, n=5; LRHF+Rest/NK1RA, n=5). **C & D)** TGFβ1 and Collagen type 1 levels in serum (FRC, n=12; FRC+Vehicle, n=10; FRC+NK1RA, n=10; LRHF, n=9; LRHF+Rest, n=5; LRHF+Rest/NK1RA, n=5). **E & F)** Collagen type 1 immunoreactivity (green) and DAPI staining in muscle crosssections of FRC and LRHF rats. Increased collagen type I deposition is present around many individual myofibers in LRHF muscle. **G)** Negative control staining in which antibody was incubated with purified protein prior to use. *:p<0.05 and **:p<0.01, as shown. Scale bar in E=50 μm, and applicable to other panels.

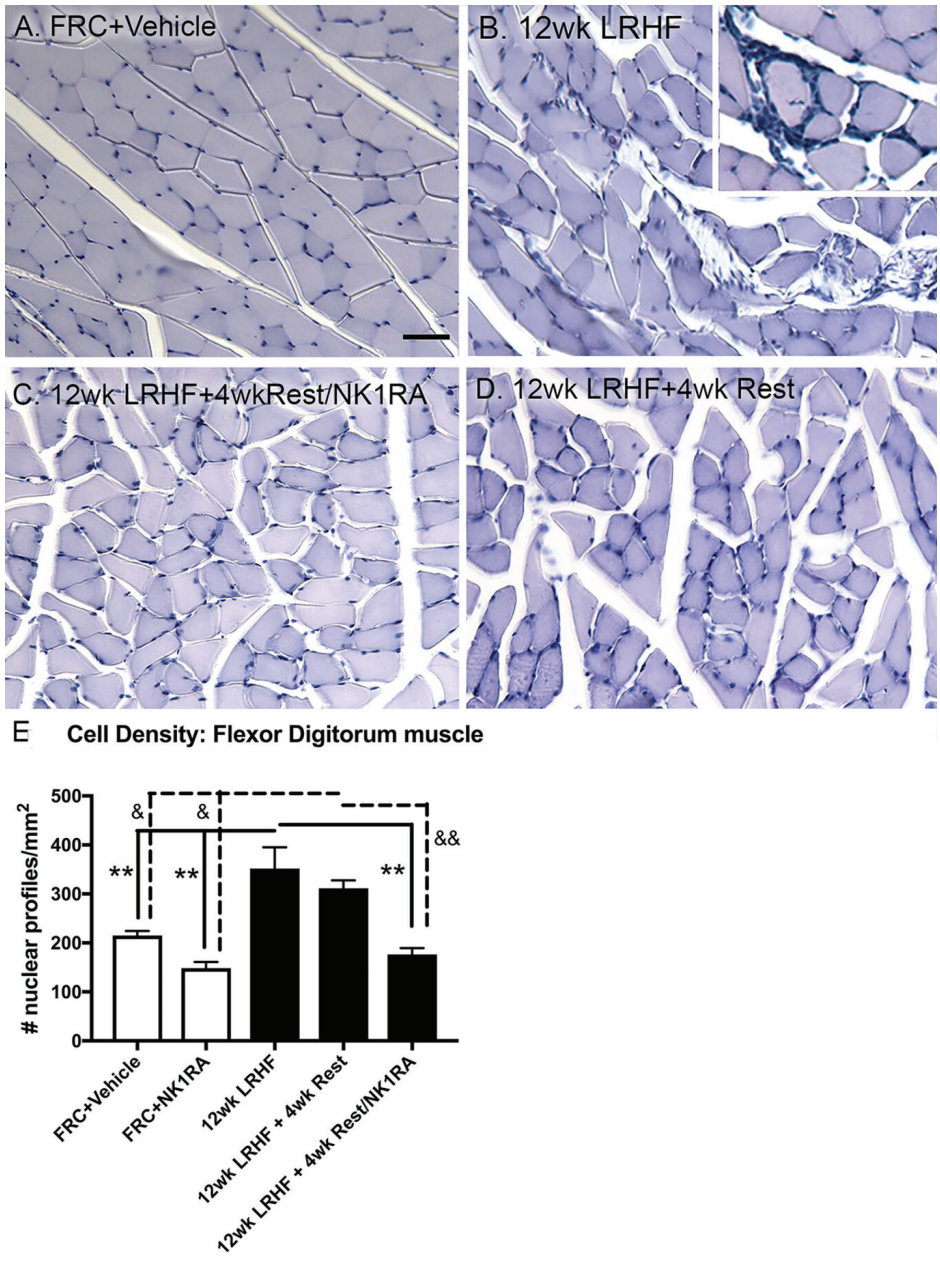

Figure 4. Cell nuclear density in flexor digitorum muscles after hematoxylin staining. **(A-D)** Representative images of cellularity. Panels B and D show increased nuclei around myofibers and endomysium in muscle crosssections. Inset shows an area with even higher nuclear density. **D)** Number of nuclear profiles per mm^2 in FRC+Vehicle (n=10), FRC+NK1RA (n=5), LRHF (n=7), LRHF+Rest/NK1RA (n=5), and LRHF+Rest (n=5) rat muscles. * and **: p<0.05 and <0.01, compared to LRHF, and $^{\&\&}$: p<0.01, compared to LRHF+Rest. Scale bars in A = 50 µm; applicable to other panels.

differences were observed for IL-1α, IL-1β, CCL2, CXCL1 or VEGF (Table 1). TNFα levels were below detectable levels in both groups. We examined muscles for CD68-immunopositive macrophages and observed no group differences (data not shown). These findings ruled out inflammation as a contributor to grip strength declines at the time of tissue collection.

LRHF-induced muscle fibrosis improved with rest and NK1RA treatments

Since declines in muscle strength can result from muscle fibrosis[8,13,27], muscles were examined for TGFβ1 and collagen type 1. Untreated LRHF rats showed increased muscle levels of TGFβ1 and collagen type 1, compared to

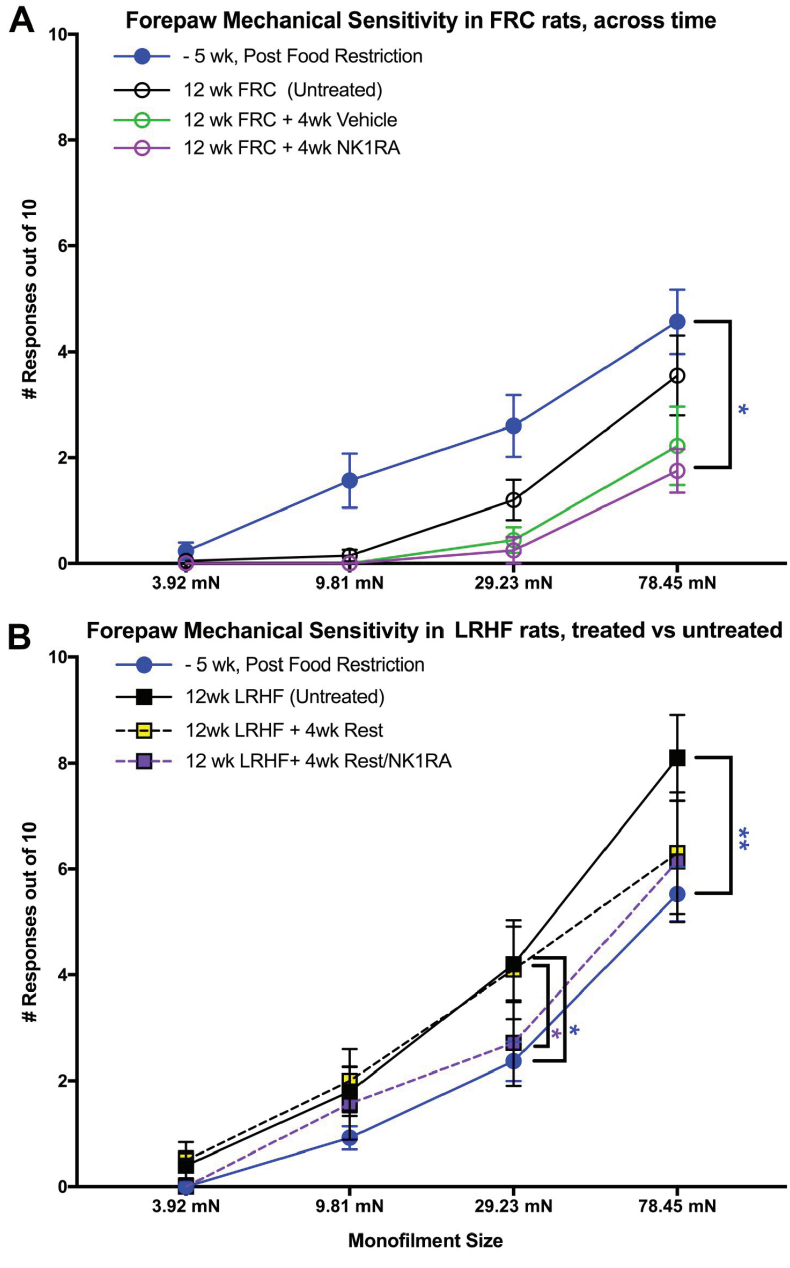

Figure 5. Mechanical Sensitivity, assayed using monofilaments of four different milliNewton (mN) sizes. **A)** FRC rats at baseline (after onset of food restriction, n= 29), 17 weeks later (equivalent to 12wk task time point, n=20), and after 4 week treatments with NK1RA (n=10) or vehicle (n=10). **B)** LRHF rats tested at baseline (after food restriction and prior to 5 week training period, n=29), after performing the LRHF task for 12 weeks (n=10), and after receiving Rest or Rest+NK1RA treatments for 4 weeks (10/group). *: p<0.05 and **: p<0.01, compared to baseline levels.

untreated FRC, LRHF + 4wk Rest and LRHF +4wk Rest/NK1RA rats (Figures 3A,B). Serum levels of TGFβ1 were higher in all LRHF groups, compared to FRC groups (Figure 3C). Serum levels of collagen type 1 increased in LRHF rats, compared to FRC rats (Figure 3D). Increased collagen type I immunoexpression in LRHF rat muscles was observed around myofibers, relative to FRC rats (Figures 3E,F). No labeling was observed in tissues incubated with pre-absorbed anti-collagen type 1 antibody (Figure 3G).

LRHF-induced muscle hypercellularity declined with NK1RA treatment

Overuse can induce hypercellularity[28]. Therefore, we examined hematoxylin-stained sections of flexor digitorum muscles and observed increased numbers of nuclei around myofibers in LRHF and LRHF + 4wk Rest/NK1RA rat muscles (Figure 4B,D), relative to FRC and LRHF+Rest/NK1RA rat muscles (Figure 4A,C). Quantification confirmed this finding (Figure 4E).

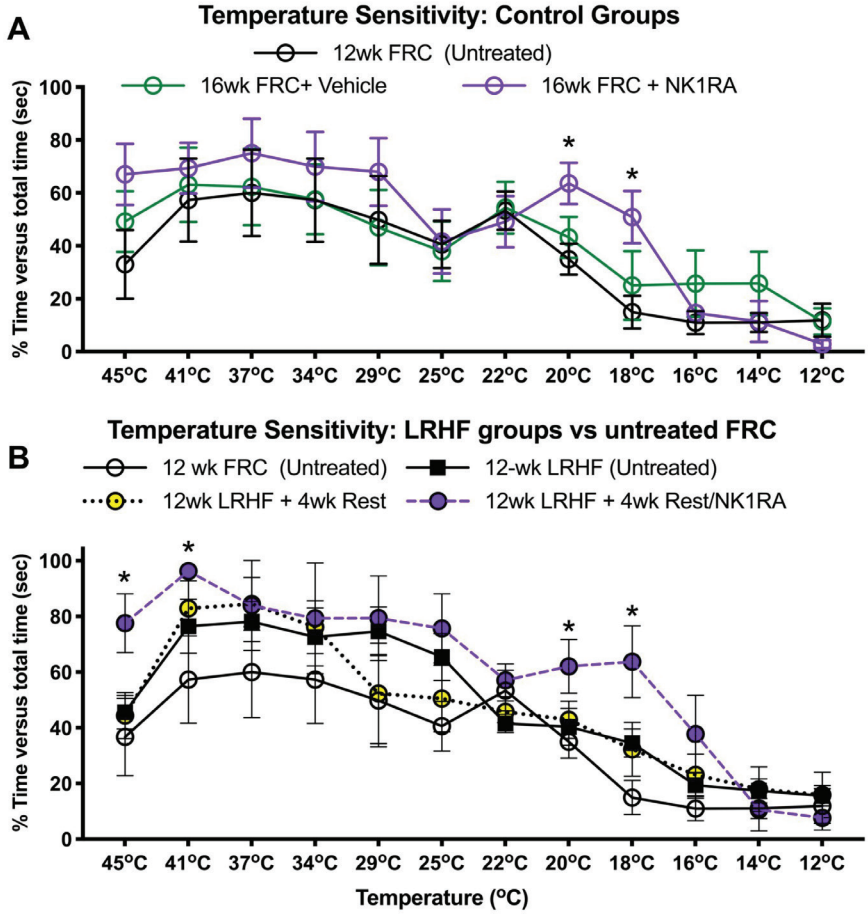

Figure 6. Place preference testing for temperature aversion. Time spent on a variable plate cooled one day from 22°C to 12°C, and on a different day from 22°C to 45°C, versus a plate maintained at 22°C, is presented for FRC groups (A) and LRHF groups versus untreated FRC rats (B). N=10/group. *: p<0.05, compared to untreated FRC rats.

LRHF-induced mechanical hypersensitivity improved with both treatments, and more with NK1RA treatment

Forepaw mechanical sensitivity declined in FRC rats across time (Figure 5A), likely due to acclimation to repeated testing. Interestingly, forepaw mechanical sensitivity to probing with the 78.45 mN sized monofilament was lower in FRC + 4wk NK1RA rats, compared to baseline (p=0.03; Figure 5A). In contrast, forepaw mechanical hypersensitivity increased to 29.23 and 78.45 mN sized monofilaments in LRHF rats, compared to baseline (Figure 5B). Both four weeks of Rest and Rest/NK1RA similarly improved the task-induced forepaw mechanical hypersensitivity to 78.45 mN sized monofilament. Only Rest/NK1RA treatment ameliorated forepaw mechanical hypersensitivity to the 29.23 mN sized monofilament (Figure 5B).

NK1RA treatment resulted in hyposensitivity to noxious hot temperatures

Temperature place preference testing to hot to cold temperatures was assayed, relative to a room temperature plate. Significant differences s were seen between the FRC+NK1RA and untreated FRC groups indicative of a NK1RA treatment induced hyposensitivity to 20°C and 18°C temperatures (Figure 6A; results of two-way repeated measures ANOVA showed different outcomes to the temperature tested (p<0.0001), yet no differences between the three FRC treatment groups (p=0.53)). Also, temperature sensitivity did not differ between untreated LRHF versus FRC rats (Figure 6B). However, NK1RA treatment induced a hyposensitivity to 45°C, 41°C, 20°C and 18°C in LRHF+NK1RA/Rest rats, compared to untreated FRC rats (Figure 6B; results of two-way repeated measures ANOVA showed different outcomes to the temperature tested (p<0.0001), and significant treatment group differences (p<0.048).

LRHF-induced median nerve inflammation and increased dermal collagen, but not axonal die-back

One cause of forepaw mechanical hypersensitivity can be neuroinflammation[24]. Therefore, we examined median nerve

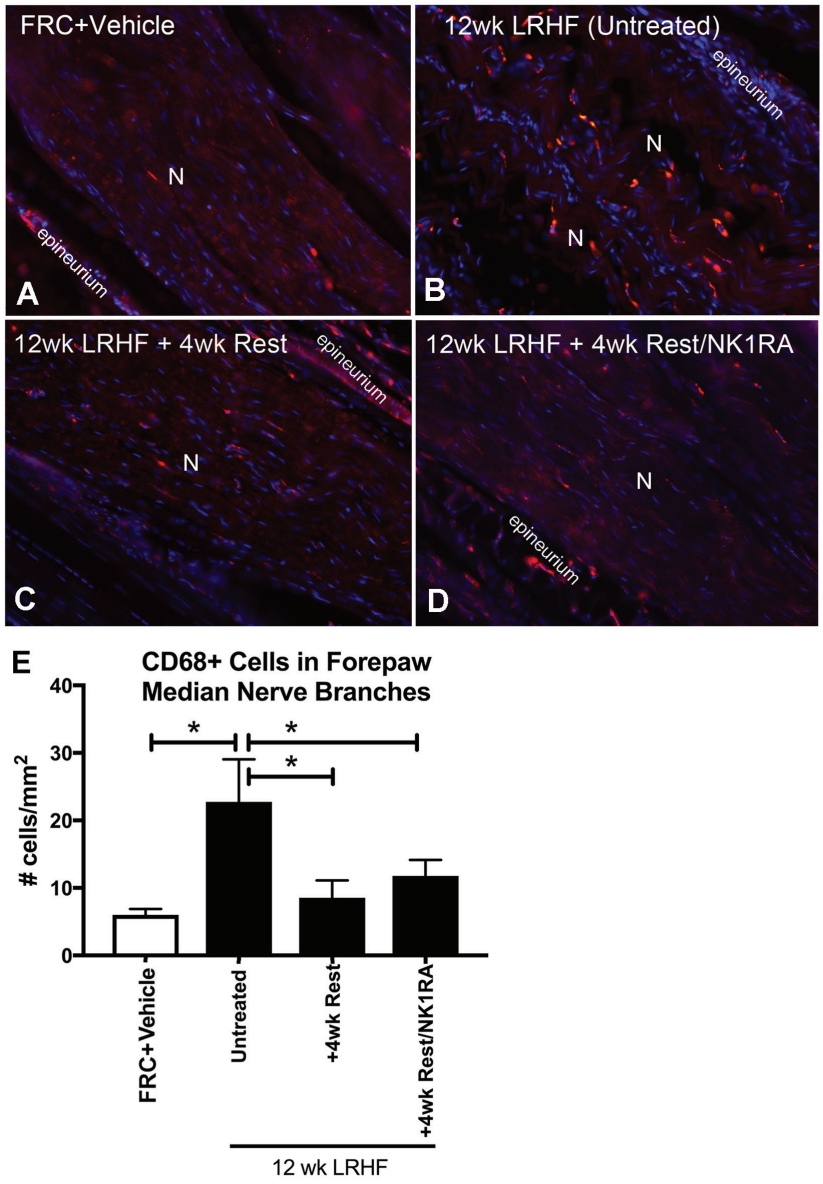

Figure 7. CD68-immunopositive macrophages in median nerve branches at wrist and proximal forepaw. **A-D)** CD68-immunopositive macrophages in median nerve branches of FRC+Vehicle, LRHF, LRHF+Rest, and LRHF+ Rest/NK1RA rats. **E)** Numbers of CD68+ macrophages in median nerves (n=5/group). *: p<0.05, compared to groups as shown; N=nerve. Scale bars in panel A = 50 µm; applicable to other panels.

branches in the forepaw and wrist for CD68 immunopositive macrophages. We observed increased CD68$^+$ macrophages in nerves of LRHF rats, compared to FRC rats (Figures 7A,B,E; ANOVA: p=0.02). These increases were ameliorated in LRHF+Rest and LRHF+Rest/NK1RA rats (Figures 7C,D,E). We also examined the upper dermis of forepaws and digits for nerve morphological changes and collagen deposition (this region contains many nerve branches innervating skin). Collagen deposition was increased in the upper dermis of untreated LRHF rats (Figures 8C,F), compared to the other groups (Figures 8A,B-F). Yet, no nerve die-back was observed (representative nerve branches are indicated by arrows in Figures 8A-E). Median nerve branches were examined for increased degraded myelin basic protein that would be indicative of nerve damage; none was observed (data not shown). These data combined are suggestive an early stage irritative neuritis to this moderate demand task.

Discussion

Increased Substance P immunoreactive cells and axons and fibrogenic responses were induced by performance of this

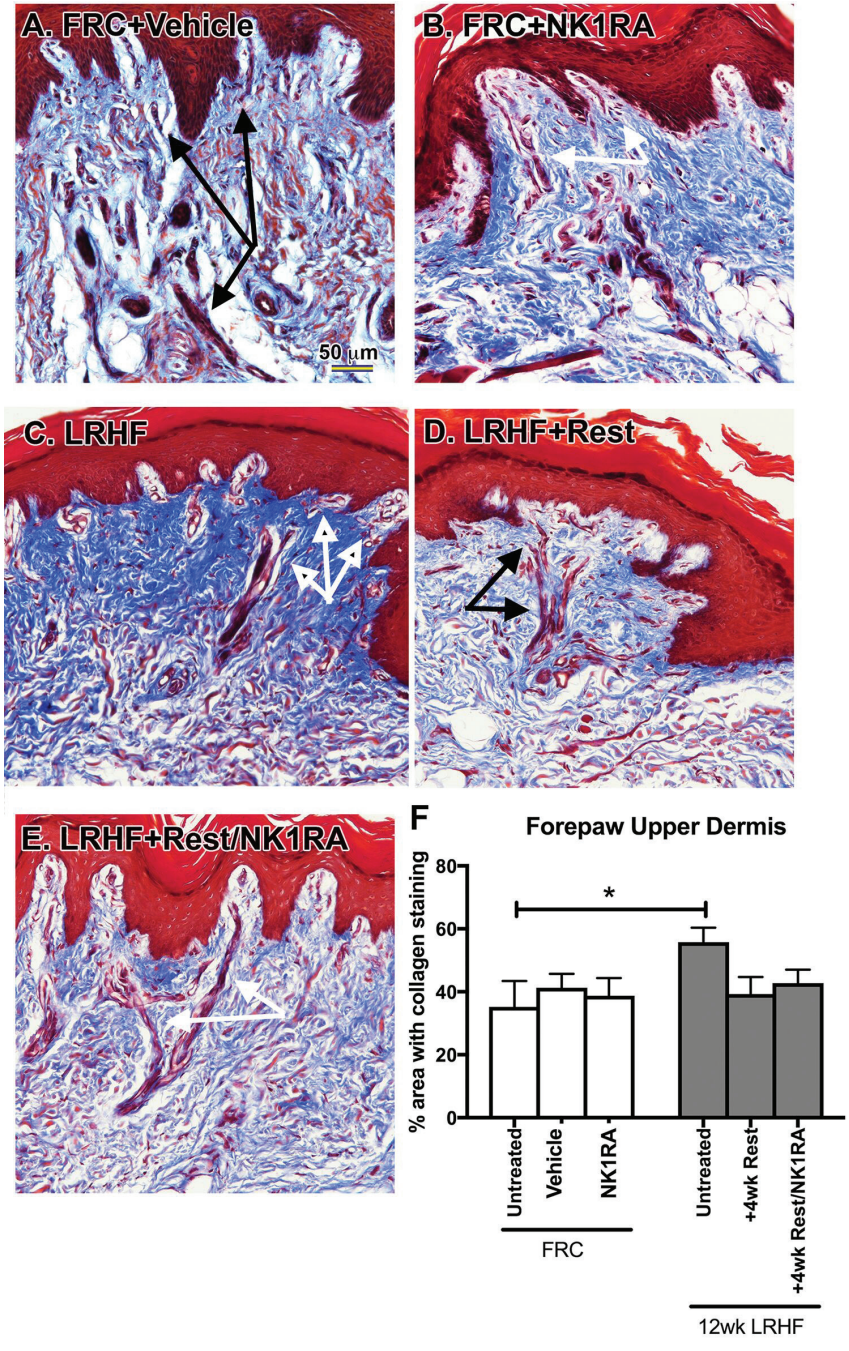

Figure 8. Representative images of Masson's Trichrome staining in upper dermis of forepaw digits of: **A)** FRC+Vehicle, **B)** FRC+NK1RA, **C)** LRHF, **D)** LRHF+Rest, and **E)** LRHF+Rest/NK1RA rats. Arrows indicate median nerve branches. **F)** Quantification of percent area with collagen staining (blue) in upper dermis of digits (n=5-7/group). *: $p<0.05$, compared to untreated FRC rats. Scale bars in A= 50 µm; applicable to other panels.

moderate demand LRHF task in flexor digitorum muscles and upper dermis of forepaws. These changes were accompanied by declines in grip strength, forepaw mechanical allodynia, and neural inflammation. Four weeks of rest improved LRHF-induced declines in grip strength, although rest combined with NK1RA rescued grip strength to control levels. Both Rest and Rest/NK1RA improved all but the hypercellularity, which was rescued by Rest/NK1RA treatment. Both Rest and Rest/NK1RA improved LRHF-induced forepaw mechanical hypersensitivity and neuroinflammation, although Rest/

NK1RA was more effective at reducing forepaw mechanical sensitivity to 29.23 mN sized monofilaments. NK1RA treated rats showed hyposensitivity (significant in LRHF+4wk Rest/NK1RA rats) to noxious hot temperatures, suggesting the drug has anti-nociceptive properties.

Substance P is a tachykinin involved in nociception, inflammatory processes (e.g., myositis), muscle derangement, fibrotic tendinosis and postoperative peritoneal adhesion formation[9,10,29]. Local administration of Substance P accelerates hypercellularity in an experimental tendinopathy model[10]. Substance P has high affinity for the neurokinin-1 receptor (NK-1R)[30]. Marked increases in NK1R immunoreactivity has been observed on nerve fascicles, white blood cells, blood vessel walls, and as fine point-like structures on myofibers in muscles subjected to overuse[11,28]. Antagonists to this receptor have been used to block or reduce cardiac fibrosis, fibrogenic factors in colitis-induced fibrosis, and TGFβ1 production by lung epithelial cells in lung fibrosis[21,31,32]. One goal here was to determine if blocking Substance P signaling reduced TGFβ1 and collagen type 1 production in muscles after long term LRHF task performance. The effectiveness of the accompanying rest prevented us from answering this question, although we did see evidence of Substance P's role in muscle hypercellularity, similar to findings showing Substance P is a mechanoresponsive regulator of tenocyte proliferation[33].

Rest is an effective treatment for initial shaping-only induced sensorimotor declines in this model[22,34]. In one study, forepaw mechanical hypersensitivity was evident immediately after shaping to high force (55% of the rats' maximum pulling force)[22]. Recovery from this mechanical hypersensitivity was evident by the next time point assessed, the sixth week of rest[22]. In a second study examining the effects of a high repetition low force task in older rats (14-18 months of age), both forepaw mechanical hypersensitivity and grip strength declines were evident post-shaping[34]. Recovery from mechanical hypersensitivity in these older rats was evident by the third week of rest; however, grip strength did not recover with 12 weeks of rest. Contributing factors included persistently increased TNFalpha levels in the muscles of the older rats, as well as persistent median nerve dysfunction, neither of which recovered with 12 weeks of rest, changes not present in these current young adult LRHF rats.

Similar to past studies examining the effects of moderate demand tasks on grip strength[12,35], LRHF rats examined here show reduced grip strength at 12 weeks of task performance. The four week Rest and Rest/NK1RA treatments improved grip strength, although Rest/NK1RA returned grip strength to control levels. Grip strength declines can result from both increased muscle inflammatory cytokines[26] and fibrosis[8,13,27]. Muscle inflammation was ruled out as a potential contributor in this study. Instead, since increased intramuscular collagen deposition can decrease muscle strength[27], the improved grip strength with both treatments may be the result of reduced collagen deposition around myofibers, although reduced nerve inflammation cannot be ruled out since both treatments reduced this as well. The further improvement in Rest/NK1RA rats could be due to reduced muscle hypercellularity or anti-nociceptive properties of NK1RA drugs[30,36].

Muscle levels of TGFβ1 and collagen type 1 were increased by the LRHF task, matching past findings showing that performance of repetitive tasks ranging in demand from high repetition low force to high repetition high force increases these fibrosis related proteins in flexor digitorum muscles[8,13,37]. Collagen type 1 was a fairly good serum biomarker of underlying muscle fibrogenic changes, similar to past findings that serum levels of hydroxyproline (a major component of collagen) increase in parallel with muscle and tendon levels of collagen[37]. In contrast, serum TGFβ1 levels were increased in all LRHF animals, with or without treatment. The sustained serum levels of TGFβ1 may be the consequence of ongoing tendinosis[37] or bone reparative changes, tissues not examined in this study.

We have previously shown forepaw mechanical hypersensitivity as a consequence of this LRHF task in parallel with increased median nerve inflammation and extraneural fibrosis[12,35]. The sensory declines were replicated in this current study. Regions of extraneural fibrosis in 12 week LRHF rats were extended here to include forepaw regions containing distal ends of the median nerve. We further extended past findings to show that four weeks of rest with or without the NK1RA treatment improved forepaw mechanical hypersensitivity to 78.45 mN size monofilaments, although the NK1RA treatment also ameliorated forepaw mechanical hypersensitivity to 29.23 mN size monofilaments. One cause of forepaw mechanical hypersensitivity can be inflammatory neuritis[24], which the NK1RA drug reduced. Blocking NK1R signaling may also reduce substance P nociceptor signaling, suggestive of anti-nociceptor abilities by the NK1RA used here, similar to other studies in which various NK1R antagonists reduce visceral hyperalgesia, acid-induced muscle pain, and neuropathic pain occurring after peripheral nerve damage[36]. However, the anti-nociceptor ability of the NK1RA used here was perhaps too effective in that it induced a hyposensitivity to noxious hot temperatures (45°C and 41°C) in LRHF + 4wk Rest/NK1RA rats.

With regard to a potential clinical use for this drug in humans, use of a neurokin-1 receptor antagonist (NK1RA) has also been proposed as a therapeutic target for the treatment of over-use induced fibrosis, such as for tendon and muscle hypercellularity[10,11]. This hypothesis has been shown to have validity in cultured tenocytes collected from rat and human tendons[8,33], although this study is the first time this hypothesis has been tested *in vivo*. NK1RAs have been used to reduce cardiac and colitis-induced in animal models[21,31], as well as to reduce pain and pain-related behaviors in several animal models (reviewed in reference 30). However, few clinical trials have shown that they have similar analgesic properties in humans (reviewed in reference 30). Perhaps instead, its anti-proliferative and fibrogenic properties should be explored in future clinical trials. We suggest though that the negative side effect of noxious temperature hyposensitivity observed in this study should be kept in mind when designing those trials.

This study has several limitations. Only female animals were used. This should be considered when assessing the generality of the study outcomes. Substance P has been shown to increase angiogenesis. Histological evidence of angiogenesis was not examined within the muscles. However, we found that VEGF, an angiogenic-related cytokine, did not increase in the muscles, encouraging us to look at other potential contributors to observed changes. Our investigations were focused on muscle, nerve and behavioral changes. Future studies could focus on tendons or bones to determine the effect of the LRHF task and treatments on these tissues.

In conclusion, the 4 week rest treatment reduced LRHF-induced fibrotic tissue changes and improved sensorimotor declines, although not always to control levels. The finding that rest was fairly effective confirms overexertion theories of chronic overuse, that maladaptive changes occur in tissues with continued demands, yet if demands are low (in this case, low repetition high force task demands), and if sufficient rest time is allowed after the overuse, complete recovery may be possible[38]. Additionally, the present study adds information on multiple roles for Substance P in overuse musculoskeletal disorders *in vivo*, including muscle hypercellularity and perhaps nociception. While the NK1RA induced hot temperature hyposensitivity should be taken into consideration if this or related drug were used long-term, the NK1RA more effectively reduced muscle hypercellularity and improved grip strength and forepaw mechanical hypersensitivity.

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Supplemental material

Detailed methods

Animal overview and repetitive overuse task

Experiments were approved by the Temple University Institutional Animal Care and Use Committee in compliance with NIH guidelines for the humane care and use of laboratory animals. Young adult female Sprague-Dawley rats (3 months of age at onset of experiments) from Charles Rivers (Wilmington, MA) were used. Female Sprague–Dawley rats were used since human females have a higher incidence of work-related musculoskeletal disorders than males[29-32]. Rats were housed individually in a central animal facility in a 12-hour light: dark cycle with free access to water, gently handled at least twice per week and provided cage enrichment toys. Rats used in the study were food restricted to 5% less than weights of age-matched control rats with free-access-to-food (used for weight comparison purposes only), and allowed to gain weight over the course of the experiment. In addition to food reward pellets (a mix of 45 mg purified chocolate flavored grain and banana flavored pellets; Bio-Serv, NJ, USA), all rats received Purina rat chow (Woodstock, Ontario, Canada) in their cages daily.

Experimental task rats first underwent an initial shaping period for 5 weeks to learn a high force lever pulling task (described further below), before going on to perform the task for 12 weeks. Steps of the five-week shaping period were as previously described[33] and occurred for 10 min/day, 5 days/week. After this shaping period, a point equal to week 0 of the task, the shaped rats began the reaching and lever pulling task regimen.

Experimental task rats performed a low repetition high force task (LRHF) at a low reach rate (1.15±0.27 reaches/min, mean ± SEM) that required extending their forearm forward into a portal, grasping a force lever bar, and then exerting a fairly isometric pull for at least 90 milliseconds (msec) at approximately 47% of their maximum pulling force, for 2 hrs/day, in 30 min intervals, with 1.5 hr rest breaks between, 3 days/wk, for 12 weeks. The rats' mean grasp force was 1.39±0.01 Newtons (N; mean ± SEM), and mean grasp duration on the lever bar was 146±4.92 msec. The rats had a preferred reach limb that they used to pull on the lever bar, which was noted during the shaping sessions and during each task session. Additional details are as previously published[33].

At the end of 12 weeks, LRHF rats were divided into three cohorts. One group was euthanized and tissues collected after this 12 week task period (LRHF), a second group ceased task performance and rested for 4 wks (LRHF+4wk Rest), while the third group rested for 4 weeks while simultaneously receiving NK1R antagonist treatment (LRHF+4wk Rest/NK1RA). Reach limbs were collected from these rats (n=19, 10 and 10 per group, respectively); 6-7 limbs per group were processed for histological or immunohistochemical analyses and the remaining for ELISA.

Additional rats were served as food-restricted controls (FRC) rats that did not undergo shaping or perform the task. These animals rested in their cages with daily handling until the end of the experiment. FRC rats received similar amounts of rat chow and food reward pellets as LRHF rats. FRC rats were euthanized at matched time points as LRHF task rats as either: untreated FRC rats (FRC, n=19, bilateral tissue collection); FRC rats treated in their final four weeks with vehicle (FRC+Vehicle; 95% ethanol in saline, n=10, bilateral tissue collection); and FRC rats treated in their final four weeks with NK1RA (FRC+NK1RA, n=10, bilateral tissue collection). Ten limbs per FRC group were processed for histological or immunohistochemical analyses and the remaining 10 per group for ELISA.

Pharmacological treatments of animals

As indicated above, a subcohort of LRHF task rats was treated for four weeks while resting with a selective NK1RA (L732,138, catalog number 0868, Tocris Bioscience, R&D, Minneapolis, MN). This drug was delivered dehydrated and resuspended as recommended by the company in 100% ethanol into a 100mM stock solution (50 mg of powder in 1.058 ml of 100% ethanol). Stock solution was aliquoted (30 µl each) and frozen at -80oC until use, at which time the aliquots were further diluted in saline for an end dose of 5 mg of NK1RA/kg body weight in 50 µl of 95% ethanol in saline (dose determined from [34-36]). This dose was injected, i.p., 3x/week (Monday, Wednesday and Friday) during the four week rest period. Subcohorts of FRC rats were either left untreated, or were treated for four weeks with the NK1RA drug or its vehicle (50 µl of 95% ethanol in saline, i.p.) at matched time points as treated LRHF rats.

Behavioral assays

The individuals carrying out these tests were blinded to group assignment or study outcome. Behavioral procedures were conducted at the same time per day to minimize diurnal-related factors. All task and FRC rats were assayed for reflexive grip strength using a rat grip strength meter (1027SR-D58, Columbus Instruments, Columbus, Ohio). Grip strength was assayed after onset of food restriction (baseline), after task week 12 and again after the four week treatment period. The test was repeated 5 times per limb and per timepoint. Maximum grip strength per trial is reported. Forepaw sensitivity to mechanical probing was assayed at similar time points from all rats using nylon monofilaments (Semmes-Weinstein monofilaments, Stoelting, Woodale, IL) and described methods[37]. This test was repeated 10 times per limb per trial, and the number of limb withdrawal responses out of 10 reported for each monofilament is reported. A two-choice temperature place preference test was used to determine temperature aversion using a temperature testing apparatus, T2CT, BioSeb, France, and described methods[13]. Briefly, the reference plate was 22°C, while the variable test plate was altered one day

from 22°C to 12°C across a 35 minute testing period with 300 seconds per temperature to test cold temperature aversion, and on a different day from 22°C to 45°C, to test hot temperature aversion. Rats were free to choose their preferred position, which was tracked by a motion sensitive video camera. Manufacturer's software calculated the time spent on each plate per temperature. Data is presented as percent time spent on the variable plate versus total time per temperature (300 sec). Temperature place preference was tested only once per range of temperatures at 2-3 days before euthanasia to avoid confounds of learning preference.

ELISAs

For *in vivo* experiments, animals were anesthetized with 5% isoflurane in oxygen and euthanized by cardiac exsanguination. Tissues for ELISA were removed prior to perfusion of rats with fixative for histological assays described below. For the ELISAs, blood was collected, centrifuged and serum harvested and frozen at -80°C until assayed. Flexor digitorum muscle samples were flash frozen and stored at -80°C until thawed and homogenized in sterile, ice-cold, phosphate-buffered saline (PBS) containing proteinase inhibitors (#5056489001, Sigma-Aldrich), before being centrifuged (12000 rpm, 15 min, 4°C). Supernatants were stored at -80°C until assayed. Serum (n=5-12/gp) and muscle lysates (n=5-12/group) were assayed using commercially available ELISA kits for Collagen type 1 (LS-F5638, LifeSpan BioSciences, Seattle, WA) and transforming growth factor beta 1 (TGFβ1; ADI-900-155, Enzo, Farmingdale, NY). Untreated FRC and LRHF muscle lysates (n=6-11/group) were also assayed using magnetic multiplex ELISA kits for IL-1alpha, IL-1beta, CXCL1 (also known as GRO/KC and neutrophil activating protein 3), CCL2 (also known as macrophage chemotactic protein 1), TNFα (tumor necrosis factor alpha), and VEGF (vascular endothelial growth factor) (# RV1MAG-26K, Merk Millipore; # LXSARM, R&D Luminex). ELISAs were conducted using manufacturers' protocols. All samples were run in duplicate. Total protein content of muscles was determined using BCA Protein Assays, and muscle data is reported as pg of protein of interset per mg of total protein. Serum data is reported as pg of protein per ml of serum.

Histology and Immunohistochemistry

Five to seven reach limbs per LRHF group and ten per FRC group were collected, cryosectioned and underwent histological and immunohistological analyses. For this, animals were deeply anesthetized with 5% isoflurane in oxygen and euthanized by cardiac exsanguination, serum collected and limbs to be used for ELISAs removed as described above, before undergoing transcardial perfusion with 250 ml of saline and then with 350 ml of 4% paraformaldehyde in phosphate buffer (pH 7.4). Perfused limbs were postfixed for 24 hours. Flexor digitorum muscles, associated connective tissues and nerves were removed from bones using a scapel as an *en bloc* mass from elbow to mid-forepaw. This soft tissue was cryoprotected in 10% and then 30% sucrose in phosphate buffer (48 hours each). A 2.5 mm piece of each muscle was removed using a scalpel from the mid-to-proximal muscle mass, cryosectioned into 15 µm thick crosssections, placed onto charged slides and dried overnight at room temperature, before storage at -80°C until being immunostained or histological stained. Remaining forelimb soft tissue mass was treated similarly except for being cryosectioned longitudinally into 15 µm thick sections.

Muscle crosssectional cryosections were immunostained in batched subsets for collagen type 1 (#C2456, diluted 1:500 in phosphate buffer with 4% goat serum, Sigma-Aldrich, Inc., St. Louis, MO) using previously described antibodies and methods[13,19,38], before coverslipping with 80% glycerol in PBS with DAPI included as a nuclear counterstain. Briefly, after a 0.5 % pepsin antigen retrieval step for 15 min at room temperature, sections were incubated for 20 min in 4% goat serum, and then with anti-collagen type 1 antibody overnight at 4°C. This was followed by incubation with an appropriate secondary antibody conjugated to a green fluorescent tag (DyLight 488; Jackson ImmunoResearch, West Grove, PA). Preabsorption controls were performed to demonstrate if the antibody bound specifically to collagen type 1 using a purified rat protein (C7661, Sigma). A two fold excess of purified protein was pre-incubated with the antibody overnight at 4°C, the mixture centrifuged, and then the pre-absorbed antibody supernatant incubated with the tissues (after pepsin and goat serum treatment) similar to that described above before washing and incubation with the secondary antibody. Adjacent sections were stained with hematoxylin and coverslipped with 80% glycerol in phosphate buffer to reduce tissue shrinking. Numbers of hematoxylin stained nuclei per flexor digitorum cross section were counted by an individual blinded to group assignment in 5-10 rats/group, using a 20x objective and an image analysis system (Bioquant Osteo II, Bioquant Image Analysis Corporation, Nashville, TN)

Cross-sections and longitudinal cryosections of forelimb soft tissues were immunostained for Substance P and degraded myelin basic protein, using previously described methods and antibodies[13,39,40]. Substance P and degraded myelin basic protein immunostaining was examined in sections using an epifluorescent microscope (Nikon E800). Cross-sections and longitudinal cryosections of forelimb soft tissues were immunostained for CD68-immunopositive macrophages using previously described methods[39] and antibody from Abcam (#AB125212, Abcam, Cambridge, MA), diluted 1:300 in phosphate buffer with 4% goat serum. Fluorescence immunostained images were collected using an upright microscope with bright field and epifluorescent features (E800, Nikon, Melville, NY) interfaced with digital camera (*Retiga 4000R QImaging Firewire Camera, Surry, BC Canada*) and PC computer. CD68 cell immunostaining was examined in flexor digitorum muscles and median nerve

branches. Numbers of $CD68^+$ cells within median nerve branches from the wrist to mid-forepaw were counted in longitudinally cut cryosections by an individual blinded to group assignment and study outcome, in 5-10 fields per nerve branch, in 5 rats/group, using a 20x objective and an image analysis system (Bioquant Osteo II). Only regions within the nerves sections were quantified (regions external to the epineurium were excluded from the counts). Numbers of $CD68^+$ cells were counted similarly in cross-sectional slices of flexor digitorum muscles (n=5 rats/group). Cell count data is presented as numbers of $CD68^+$ cells per mm2.

Forepaws with digits were not included in the above flexor digitorum soft tissue mass. Forepaws with digits were removed after fixation, processed *en bloc* for paraffin embedding, and then after this embedding were sectioned longitudinally using a microtome into 5 µm thick sections, before storage at room temperature until undergoing Masson's trichrome staining. Collagen staining (stained blue) in the upper dermis of skin of forepaws and digits was quantified using a previously described thresholded pixel count method and is presented as percent area with collagen staining[41].

Statistical analyses

GraphPad PRISM v.7 was used for all statistical analyses. All data are expressed as the mean ± SEM. Grip strength differences were first assayed between FRC groups to examine for treatment-induced differences (pre- versus post-injection). Then, grip strength differences were assayed using a two-way ANOVA and the factors: group and treatment, with post-hoc assays comparing results to baseline levels and LRHF groups to FRC groups. Two-way ANOVAs were used to compare muscle and serum collagen type 1 and TGFβ1 ELISA data, and percent area with collagen staining, using the factors: group and treatment. Forepaw mechanical sensitivity differences between FRC or LRHF treatment groups were examined using two-way repeated measures ANOVAs and the factors: treatment group and monofilament size. Temperature aversions, assessed only a few days prior to euthanasia to avoid learning confounds, was analyzed using two-way repeated measures ANOVAs and the factors: treatment group and temperature. One-way ANOVAs were used to compare inflammatory cytokine levels, cell density in flexor digitorum muscles, and CD68 cell counts, across groups. Sidak multiple comparison tests were used for post hoc assays, and adjusted p values are reported. An adjusted p value of <0.05 was considered significant.