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RESEARCH ARTICLE

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Blocking CTGF/CCN2 reverses neural fibrosis and sensorimotor declines in a rat model of overuse-induced median mononeuropathy

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Abstract

Encapsulation of median nerves is a hallmark of overuse-induced median mononeuropathy and contributes to functional declines. We tested if an antibody against CTGF/CCN2 (termed FG-3019 or Pamrevlumab) reduces established neural fibrosis and sensorimotor declines in a clinically relevant rodent model of overuse in which median mononeuropathy develops. Young adult female rats performed a high repetition high force (HRHF) lever-pulling task for 18 weeks. Rats were then euthanised at 18 weeks (HRHF untreated), or rested and systemically treated for 6 weeks with either an anti-CCN2 monoclonal antibody (HRHF-Rest/FG-3019) or IgG (HRHF-Rest/IgG), with results compared with nontask control rats. Neuropathology was evident in HRHF-untreated and HRHF-Rest/IgG rats as increased perineural collagen deposition and degraded myelin basic protein (dMBP) in median nerves, and increased substance P in lower cervical dorsal root ganglia (DRG), compared with controls. Both groups showed functional declines, specifically, decreased sensory conduction velocity in median nerves, noxious cold temperature hypersensitivity, and grip strength declines, compared with controls. There were also increases of ATF3-immunopositive nuclei in ventral horn neurons in HRHF-untreated rats, compared with controls (which showed none). FG-3019-treated rats showed no increase above control levels of perineural collagen or dMBP in median nerves, Substance P in lower cervical DRGs, or ATF3-immunopositive nuclei in ventral horns, and similar median nerve conduction velocities and thermal sensitivity, compared with controls. We hypothesize that neural fibrotic processes underpin the sensorimotor declines by compressing or impeding median nerves during movement, and that inhibiting fibrosis using an anti-CCN2 treatment reverses these effects.

KEYWORDS

dysfunction, median nerve, overuse injury, work-related musculoskeletal disorders

1 | INTRODUCTION

Median nerve encapsulation is a hallmark of fibrotic overuse-induced median mononeuropathy, which is also termed compressive median mononeuropathy, and contributes to functional declines.[1,2] This disorder constitutes 90% of entrapment neuropathies diagnosed in the United States,[3] and affects ~1 in 1000 individuals in the general population.[4] Such entrapment is thought to play key roles in associated motor weakness and disability, and increased discomfort, likely due to the distortion of dynamic properties of encapsulated neural tissues and adherence of adjacent structures,[2] in addition to the overall compressive forces that irritate the nerve.[5]

There is an increasing body of evidence linking fibrotic median mononeuropathy and occupational activities.[6,7] These studies and others indicate that occupational tasks characterized by prolonged repetitive and forceful movements of the hand and wrist, such as prolonged repetitive forceful gripping or work with flexed or extended wrists, are strongly associated with an increased risk for development of this type of median mononeuropathy.[7] This musculoskeletal disorder is highly prevalent among workers worldwide, with an incidence rate of ~6.3 per 10 000 full-time equivalence.[6]

Treatments for median mononeuropathy range from rest, splinting, nonsteroidal anti-inflammatory, and corticosteroid drug (all conservative treatments) to surgical release interventions. Surgical release provides the most effective long-term outcome, but fails in 8% to 25% of patients.[8] Splinting is less effective than surgical release or injections of steroids around the nerve, which are also not always effective.[9]

The establishment of tissue fibrosis is the main cause of long-term discomfort and functional decline. Although treatments aimed at reducing tissue fibrosis have proved largely ineffective, the recent development of monoclonal antibodies that block cellular communication network factor 2, formally known as connective tissue growth factor (CCN2/CTGF) signaling holds new promise. CCN2 is a secreted matricellular protein with four modular domains that independently interact with different molecules, including collagen and proteoglycans in the extracellular matrix.[10] In Mdx mice, a model of Duchenne muscular dystrophy and significant muscle fibrosis,[11] treatment with a neutralizing monoclonal antibody that targets the von Willebrand Factor type C domain of CCN2 substantially reduced muscle fibrosis and improved muscle.[11] This monoclonal antibody, known as FG-3019 and Pamrevlumab, shows the ability to attenuate fibrosis in idiopathic pulmonary fibrosis[12] (ClinicalTrials.gov Identifier: NCT01890265), and Duchenne muscular dystrophy (NCT02606136), and locally advanced, unresectable pancreatic cancer[13] (NCT02210559). No adverse events have been found to date. Although several groups, have shown enhanced presence of CCN2 in perineural tissues that are adjacent to or surrounding encapsulated median nerves in animal models and patients with compressive median mononeuropathy symptoms,[1,14,15] FG-3019 has yet to be explored as a treatment for nerve encapsulation (ie, perineural fibrosis) in humans.

We have an established clinically relevant model of occupational activities in which rats learn and then perform an operant grasping and isometric lever-pulling task for a food reward for extended time periods.[5,16,17] In this model, reach rates and force levels were determined from studies on risk exposure to humans,[18] and functional outcomes assayed are similar to those tested in patients. Rats develop duration- and exposure-dependent declines in median nerve conduction velocity, declines in grip strength and voluntary task performance, neuritis, elevated behavioral indices of discomfort, and increased perineural fibrosis.[15,19,20] For example, after 12 to 18 weeks of performance of a high repetition high force task (HRHF task of 4 reaches/minute of reaching and grasping a lever bar, at 60% of the rats' maximum pulling force), rats develop significant sensorimotor behavioral declines, perineural fibrosis, and a 16% to 20% decline in mean median nerve conduction velocity.[15]

Using this operant model, we recently found that CCN2 is critical to the development and early progression of chronic overuse-induced nerve and muscle fibrosis and functional declines in rats that performed the HRHF task for 3 weeks.[14] Two weeks of systemic injections of the FG-3019 agent reduced the early progression of musculotendinous and nerve fibrosis, and improved sensorimotor declines.[14] Also, 6 weeks of systemic injections of FG-3019 reversed established muscle fibrosis in 18-week HRHF rats.[21] We extended these studies to now examine if use of this antibody also reverses established perineural fibrosis in 18-week HRHF rats. We hypothesized that the FG-3019 agent would reverse perineural fibrosis and associated neuropathology, and improve sensorimotor declines that occur concomitant with the fibrosis, compared with rest alone as a treatment.

2 | METHODS

Detailed methods, including enzyme-linked immunosorbent assay (ELISA) and antibody specifics, are in Supporting Information (See Supplemental Materials) and Table S1.

2.1 | Animals

Experiments were approved by Institutional Animal Care and Use Committee in compliance with National Institutes of Health guidelines. Studies were conducted on 31 young adult, female, Sprague-Dawley rats (3 month of age at onset). Rats were randomly divided into one of four groups: (1) age- and weight-matched food restricted controls (FRC, n = 10); (2) task rats that were shaped and then performed a HRHF task for 18 weeks (HRHF-untreated, n = 10; 3, and 4) two groups of task rats that performed the HRHF task for 18 weeks before cessation of the task for 6 weeks with simultaneous systemic treatment with an anti-CCN2 antibody (HRHF-Rest/FG-3019, n = 6), or with a human immunoglobulin (HRHF-Rest/IgG, n = 5) before euthanasia (Figure 1). As described previously, rats in this model are food restricted to motivate participation, yet are allowed to gain

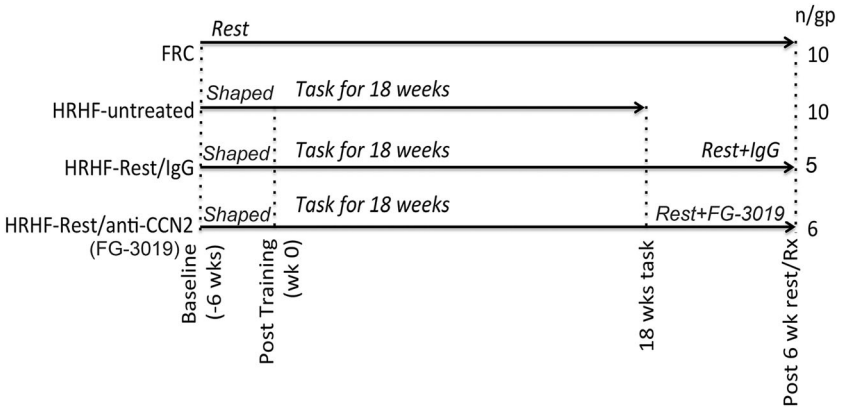

FIGURE 1 Design of experiment. Young adult female Sprague-Dawley rats were randomly divided into one of four groups: age- and weight-matched food restricted controls (FRC); task rats that were handled for 1 week and then shaped for 6 weeks, before going on to perform a HRHF task for 18 weeks (HRHF-untreated); and two groups of task rats that performed the HRHF task for 18 weeks before cessation of the task for 6 weeks with simultaneous systemic treatment with a human immunoglobulin (HRHF-Rest/IgG) or an anti-CCN2 antibody called FG-3019 and Pamrevlumab (HRHF-Rest/FG-3019, hereafter), before euthanasia. CCN2, cellular communication network factor 2; HRHF, high repetition high force

weight over time.[14] Rats were 3 months of age at the beginning of the experiments and 7.5 to 9 months of age at their completion.

2.2 | HRHF task

Task rats were acclimated for 1 week and then shaped for 6 weeks to learn a reaching and lever-pulling task at high force loads at no specified reach rate (ramping upwards from naïve, 10 min/d and 5 d/wk). These task rats then went on to perform a HRHF reaching and lever-pulling task for 18 weeks for a food reward. The specifics of this task were pulling the lever bar at 48% of the rats' maximum pulling force and a reach rate of 4 reaches/min, for 2 h/d, in four 30 minute intervals per task day (with a 1.5 hour break between session), for 3 d/wk (Monday, Wednesday, and Friday), and for 18 weeks (Figure 1). The animals used in this study were ambidextrous from the onset (ie, beginning during training) and utilized either forearm to pull the lever bar interchangeably across each session, day and week.

2.3 | Pharmacological treatments

As shown in Figure 1, the HRHF-Rest/FG-3019 rats performed the HRHF task for 18 weeks, before then resting for 6 weeks while being treated 2×/wk with a human anti-CCN2 monoclonal antibody (FG-3019, FibroGen, Inc, San Francisco, CA; 40 mg/kg body wt, intraperitoneal [IP], 50 µL/injection, with the dose and route of administration according to manufacturer's suggestions for rats). The HRHF-Rest/IgG rats performed the HRHF task for 18 weeks, before then resting for 6 weeks while being treated 2×/wk for 6 wks with a human immunoglobulin (IgG; FibroGen; 50 µL/injection, IP) as the vehicle control. Effects of FG-3019 and IgG in control rats have been previously reported (no negative side effects were observed).[14]

2.4 | Behavioral assays

All rats in the study underwent behavioral assays for grip strength, bilaterally, and cold temperature sensitivity. Behavioral assays took place on Tuesdays or Thursdays only between 10 AM and 2 PM. Reflexive grip strength was tested using a rat grip strength meter (1027SR-D58, Columbus Instruments, Columbus, OH) after onset of food restriction, after cessation of the HRHF task (HRHF week 18), and after 3 and 6 weeks of rest with treatments. The test was repeated five times/limb during each testing session. Maximum grip strength per trial is reported. All rats pulled on the lever bar with both hands, that is, were bilateral. Therefore, data from each limb were included in the statistics as replicate data. Temperature sensitivity was assessed using previously described methods,[22] once, a few days before euthanasia (to avoid the confounds of learning the test) using a two-temperature choice apparatus (T2T, Bioseb, Marseille, France). On this instrument, rats were timed for how long they preferred to stand on a thermal plate set at room temperature (22°C), as opposed to a second plate of increasing or decreasing temperature, in 2°C steps (3 minute per step). Preference/aversion to increasing temperature (22°C-45°C) vs decreasing temperature (22°C-12°C) were assayed on separate days.

2.5 | Electrophysiology

Median nerve electrophysiological studies were performed immediately prior to euthanasia only (to avoid any effects on other behavioral assays). Animals (8 FRC, 10 HRHF, 5 HRHF-Rest/IgG, and

6 HRHF-Rest/FG-3019) were anesthetized in a chamber with 5% isoflurane in oxygen, before being moved to a nose cone with a continuous dose of 2.5% isoflurane in oxygen. Animals were kept warm on a rat warming pad during this procedure. An orthodromic technique was used in which the median nerve was stimulated distally in the thenar eminence and sensory nerve action potentials (SNAPs) recorded proximally at the elbow. The SNAP parameter studied was nerve conduction velocity (m/s), derived from the peak of latency (ms). Measurements were recorded at least five times per nerve and the accepted result was the average. Since each forelimb of the task rats was used to reach, data from each forelimb were included as replicate data.

2.6 | Immunohistochemistry

All animals were deeply anesthetized with 5% isoflurane in oxygen at either 18 weeks or 18-week + 6 weeks rest/treatment after onset of the HRHF task. Controls were euthanized at an age-matched time point as treated rats. After anesthesia, rats were euthanized by performing a thoracotomy and cardiac puncture for blood collection using a 23-gauge needle. Then, all rats underwent intracardial perfusion with first saline and then buffered 4% paraformaldehyde. Afterwards, the skin was removed and the forelimbs were further postfixed for 2 days in the same fixative. The forelimb muscle-nerve mass was removed using a scalpel from the elbow into the distal forepaw. This mass was cyropreserved by immersion first in 10% and then in 30% sucrose in phosphate buffer for 2 days each, before being frozen in OCT compound (Fisher Healthcare Tissue-Plus embedding medium for frozen tissue specimens) on dry ice, and then longitudinally cryosectioned (14 μm). Sections were placed onto charged slides and stored at −80°C until use.

Antibodies were first validated using several methods as previously described,[22] and immunohistochemically (see Supplemental Methods and Figure S1).

Then, tissue sections were immunostained in batched sets for each antibody, by the same individual. Longitudinally cut sections containing nerves at the level of the wrist were chosen and underwent antigen retrieval steps (Supplemental Methods), before incubation overnight with specific antibodies against: CCN2 (SC-14939, Santa Cruz, Dallas, TX; 1:300 dilution in phosphate buffered saline [PBS]), collagen type I (AB6308, AbCam, Cambridge, MA; 1:300 dilution in PBS), degraded myelin basic protein (dMBP, ab5864, Millipore, Burlington, MA; 1:500 dilution in PBS), and protein gene product 9.5 (PGP 9.5, a pan neuronal marker; ab8189, AbCam; 1:50 dilution in PBS). See Supplemental Methods and Table S1 for more details. Sections with median nerves were also stained with an anti-human IgG tagged with Dylight 650 to detect presence of the FG-3019 monoclonal antibody (see Supplemental Methods and Figure 2) and as previously described.[5,14,22] Following washing and incubation with appropriate secondary antibodies, and 4′,6-diamidino-2-phenylindole (DAPI) nuclear counterstaining, sections were coverslipped with 80% glycerol in PBS.

Cervical (C) 6 and 7, and thoracic (T) 1 level dorsal root ganglia (DRG) and spinal cord segments were also collected from rats after intracardial perfusion with fixative. These tissues were processed and cryosectioned, as previously described.[23] DRG and spinal cord sections underwent antigen retrieval steps (Supplemental Methods) before incubation with specific antibodies against activating transcription factor 3 (ATF3, a stress inducible protein found in injured yet regeneration competent neurons[24]) using SC-81189, Santa Cruz, 1:200 dilution in phosphate buffer, with an overnight incubation. Sections were also stained with NeuN (a neuronal cell body marker; ab177487, Abcam; 1:200 dilution in PBS), substance P (AB1566, Millipore, 1:500 dilution in PBS), and/or PGP9.5 (as above for nerves). See Supplemental Methods and Table S1 for more details. Following washing and incubation with appropriate secondary antibodies, and DAPI nuclear counterstaining, sections were coverslipped with 80% glycerol in PBS.

2.7 | Histomorphometry

Quantification was performed in tissues from all rats in the study, in three fields/tissue/rat by individuals blinded to group assignment, using computerized image analysis systems (Bioquant Corporation, Nashville, TN) that were interfaced with Nikon epifluorescent microscopes via digital cameras (QImaging). Longitudinal sections that included the median nerve at the level of the wrist were used to quantify the amount of collagen immunostaining around the median nerve as well as the amount of dMBP within the median nerve. For each, a thresholded pixel count and an irregular region of interest method were used. The total number of NeuN immunostained neuronal cells bodies and the number that also contained ATF3+/immunostaining in their nuclei were counted in lower cervical DRG and spinal cord ventral horns, bilaterally. Two independent individuals assessed ATF3 immunostaining. The total number of PGP 9.5 immunostained neuronal cells bodies that were also substance P+ immunostained was counted in lower cervical DRG, bilaterally. Data from right and left sides were combined as replicate data in the statistics, since task rats were bilateral.

2.8 | Serum ELISA

After the deep anesthesia and blood collection described above (for all rats in the study), the collected blood was placed into uncoated 15 mL tubes and allowed to clot for 1 hour before being centrifuged at 12 000 rpm for 20 minute at 4°C. Serum was harvested and frozen at −80°C until assayed. Estradiol levels were analyzed in serum using ELISA (ES180S-100, Calbiotech SKU)

2.9 | Statistical analyses

A power analysis from past work was first performed and showed a minimum of five/group was needed for the various assays.[5,14]

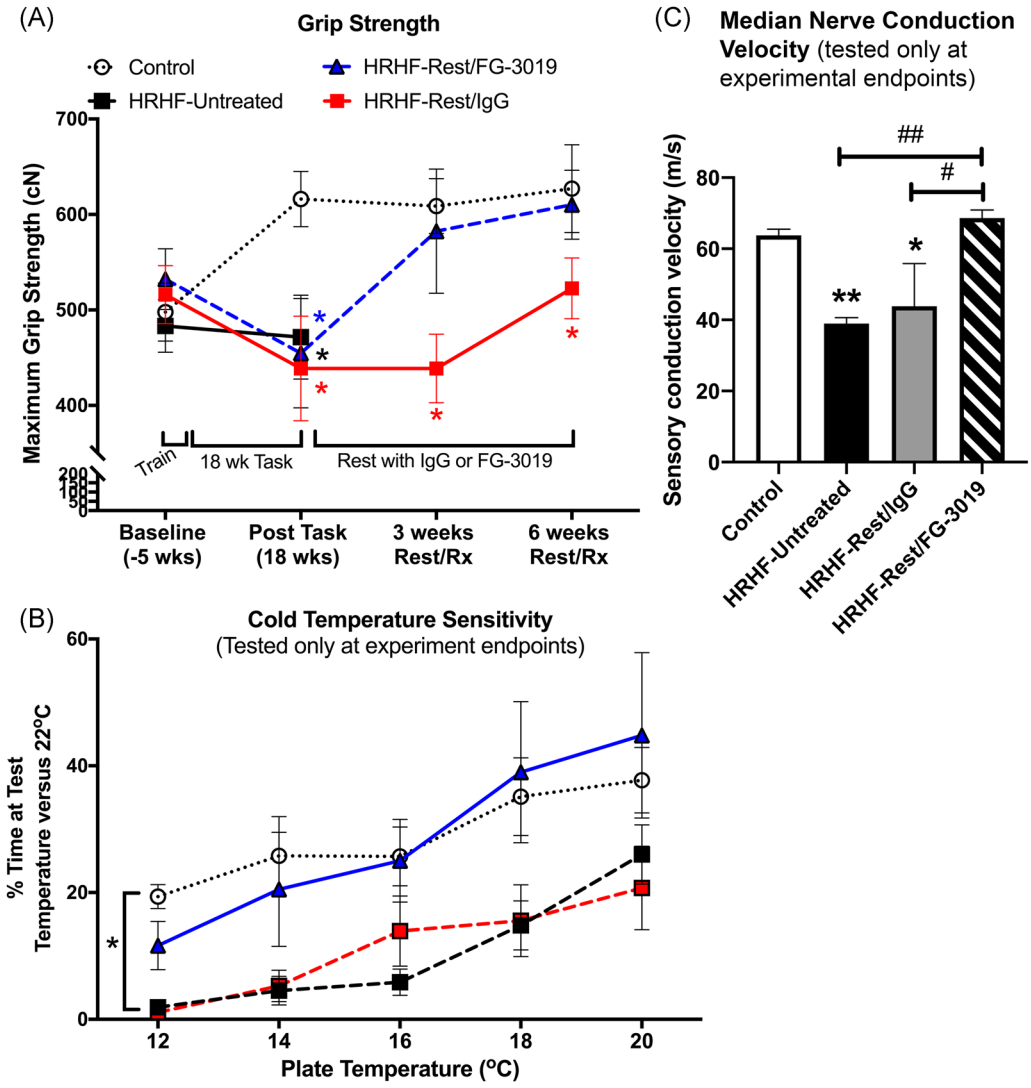

FIGURE 2 Sensorimotor functional changes showing improved function in FG-3019 treated animals. A, Grip strength shown at baseline, 18 weeks after beginning the high repetition high force (HRHF) task, and after 3 and 6 weeks of rest with treatments. Rest was provided to HRHF-Rest/IgG and HRHF-Rest/FG-3019 groups during the 6-week treatment period. B, Cold temperature sensitivity results prior to euthanasia. C, Median nerve sensory conduction velocity results tested a few days prior to euthanasia. $^{*}P < .05$ and $^{**}P < .01$ compared with age-matched FRC rats; $^{\#}P < .05$ and $^{\#\#}P < .01$ compared with HRHF-untreated or HRHF-Rest/IgG rats as shown. Number (n)/group: n = 8-10 FRC (8 for NCV and 10 for the other assays), n = 10 HRHF, n = 5 HRHF-Rest/IgG, and n = 6 HRHF-Rest/FG-3019 rats. FRC, food restricted control [Color figure can be viewed at wileyonlinelibrary.com]

Therefore, at least five per group and per assay were utilized. Immunostaining and nerve conduction data were compared using one-way analysis of variances (ANOVAs). Behaviors outcomes were compared using two-way repeated measures ANOVAs (group × week [grip] and group × temperature [temperature sensitivity]). ANOVAs were followed by Tukey multiple comparison post hoc tests, with adjusted *P* values used. Pearson correlations were used to compare final grip strength and cold sensitivity to 12°C outcomes to serum estradiol levels. An alpha level of less than .05 was used, after adjustment for multiple comparisons. All data are expressed as mean ± SEM.

3 | RESULTS

3.1 | Improved functional outcomes after FG-3019 treatment

After 18 weeks of HRHF task performance, each HRHF task group showed lower forearm grip strength compared with levels in age-matched FRC rats (Figure 2A). HRHF-Rest/IgG group rats had slightly improved grip strength levels after 6 weeks of rest plus IgG treatment, although it still remained lower than FRC rats. In contrast, grip strength levels improved in HRHF-Rest/FG-3019 rats by the third

week of treatment and remained improved. Hypersensitivity to the noxious cold temperature of 12°C was evident in HRHF-untreated and HRHF-Rest/IgG rats, compared with FRC rats (Figure 2B). The FG-3019 treatment improved cold sensitivity in HRHF-Rest/FG-3019 rats to FRC rat levels. Heat sensitivity did not differ between any group (data not shown), matching prior 18-week HRHF rat findings.[22] Median nerves between the elbow and wrist showed reduced sensory conduction velocity in HRHF-untreated and HRHF-Rest/IgG rats, compared with FRC rats (Figure 2C). HRHF-Rest/FG-3019 rats showed improved median nerve conduction velocity, compared with HRHF-untreated and HRHF-Rest/IgG rats.

3.2 | Reduced perineural fibrosis and degraded myelin in median nerves after FG-3019 treatment

Since the combined behavioral changes could be due to median mononeuropathy, we examined median nerves for enhanced CCN2 and collagen type 1 deposition that would indicate perineural fibrosis. Untreated HRHF rats showed increased CCN2 and collagen type I immunoexpression in perineural regions (Figure 3A), as did HFHF-Rest/IgG rats (Figure 3B), relative to FRC rats (Figure 3D). However, the deposition of each protein was similar to FRC rat levels in HRHF-Rest/FG-3019 rats (Figure 3C). Quantification of the collagen type I immunoexpression confirmed the collagen type I findings (Figure 3E).

Increased dMBP immunostaining was also evident in median nerves of HRHF-untreated and HRHF-Rest/IgG rats, compared with FRC rats (Figures 4A,B vs 4D). In contrast, dMBP immunostaining was at FRC rat levels in the HRHF-Rest/FG-3019 nerves (Figure 4C vs 4D). Quantification of the dMBP immunoexpression confirmed these findings (Figure 4E).

3.3 | FG-3019 agent can be detected in median nerves

Using an anti-human IgG antibody, the presence of the human anti-CCN2 agent (FG-3019) was detected in the perineural regions surrounding of median nerve and its branches of HRHF-Rest/FG-3019 rats (Figure 5A). No FG-3019 staining was observed in untreated HRHF rats (Figure 5B). Thus, the FG-3019 can be detected in perineural tissues immediately surrounding the median nerve.

3.4 | Task induced changes in DRG and ventral horn neurons improve with FG-3019 treatment

The reduced grip strength was suggestive of dysfunction in motor neuron axons. Thus, we next examined ventral horns of lower cervical spinal cord segments for increased immunoexpression of ATF3, a stress inducible protein found in injured yet regeneration competent neurons.[24] We observed higher numbers of neurons (NeuN+) with ATF3+ immunostaining in their nuclei in HRHF-untreated and HRHF-Rest/IgG rats (Figure 6A,B), compared with FRC rats (Figure 6D,E). The percentage of neurons with ATF3+ nuclear immunostaining in ventral horns of HRHF-Rest/FG-3019 rats was similar to FRC rat levels and lower than in HRHF-untreated and HRHF-Rest/IgG rats (Figure 6C,E). In contrast, cervical level DRG neurons showed no evidence of ATF3+ nuclear immunostaining in any rat or group (Figure S2).

Yet, since HRHF-untreated and HRHF-Rest/IgG rats showed cold temperature sensitivity, we next examined DRG that receive sensory axons from the median nerve for indices of sensitization. We observed that neurons in lower cervical level DRGs of HRHF-untreated and HRHF-Rest/IgG rats contained increased numbers of neurons (PGP 9.5+) that coexpress substance P (Figure 7C-F) compared with FRC rats (Figure 7A,B,I). In contrast, lower cervical level DRGs of HRHF-Rest/FG-3019 rats contained similar numbers of PGP 9.5+/substance P+ neurons as FRC rats (Figure 7G-I), and lower numbers than in HRHF-untreated and HRHF-Rest/IgG rats (Figure 7I).

3.5 | Terminal serum estradiol levels did not correlate with final behavioral outcomes

Serum estradiol levels are known to play a role in pain sensitivity.[25] Therefore, we tested estradiol levels in serum collected from the 18-week HRHF-untreated, HRHF-untreated and HRHF-Rest/IgG, and age-matched controls during euthanasia in order to determine if circulating estradiol levels were contributing to the observed behavioral declines. No significant changes in serum estradiol levels were evident between the groups (Figure 8). The serum estradiol levels also did not correlate with final grip strength outcomes ($r = .19$, $r^2 = .04$, $P = .27$) or cold temperature sensitivity outcomes ($r = -.09$, $r^2 = -.01$, $P = .60$).

4 | DISCUSSION

We have previously shown perineural fibrosis and sensorimotor declines in rats performing this same high demand reaching and grasping task for 3 to 18 weeks (with greater perineural fibrosis with longer task performance).[14,22] We have also shown that the early use of this same anti-CCN2 (FG-3019) agent for 2 weeks reduces the early development and progression of these changes in 3-week HRHF rats,[14] and that use of the FG-3019 agent for 6 weeks could reverse established muscle fibrosis that developed in 18-week HRHF rats.[21] The current study extends on those initial studies by showing that systemic FG-3019 reverses perineural fibrosis, improves sensorimotor function, reduces central effects of such compression (ie, ventral horn motor neuron stress) and improves nerve conduction velocity to control levels after 18 weeks of performance of a high demand reaching and pulling task. However, 6 weeks of rest with an IgG vehicle treatment failed to reduce fibrosis or improve function.

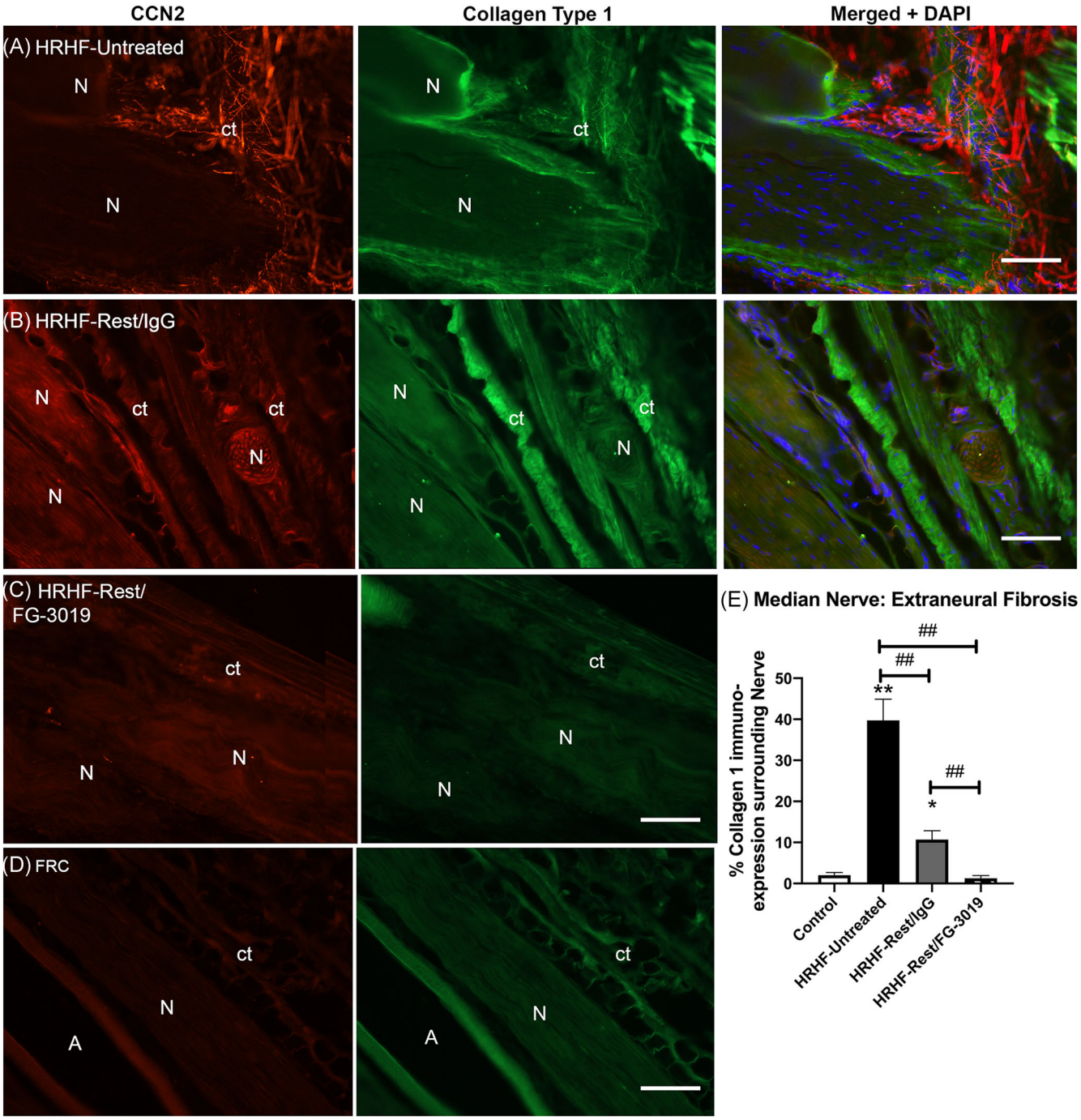

FIGURE 3 CCN2 and collagen type I immunostaining of median nerves, at the level of the wrist, in longitudinal cryosections, showing reduced staining of each to control levels in FG-3019 treated rats. A-D, Representative images of CCN2 (red) and collagen type I (green) immunoexpression in median nerves at the level of the wrist, is shown for each group. At far right, DAPI was used as a nuclear counterstain in merged images of panels A and B. E, Quantification of the percent of collagen immunoexpression in a 30 μm region of interest surrounding the median nerve at the level of the wrist. Number (n) limbs assessed/group for panel E: n = 10 FRC, n = 10 HRHF, n = 5 HRHF-Rest/IgG, and n = 6 HRHF-Rest/FG-3019 rats. $^{*}P < .05$ and $^{**}P < .01$ compared with FRC rats; $^{\#\#}P < .01$ compared with HRHF-untreated or HRHF-Rest/IgG rats as shown. Scale bar = 50 μm. A, artery; CCN2, cellular communication network factor 2; DAPI, 4′,6-diamidino-2-phenylindole; FRC, food restricted control; HRHF, high repetition high force; N, nerve; ct, dense perineural connective tissue [Color figure can be viewed at wileyonlinelibrary.com]

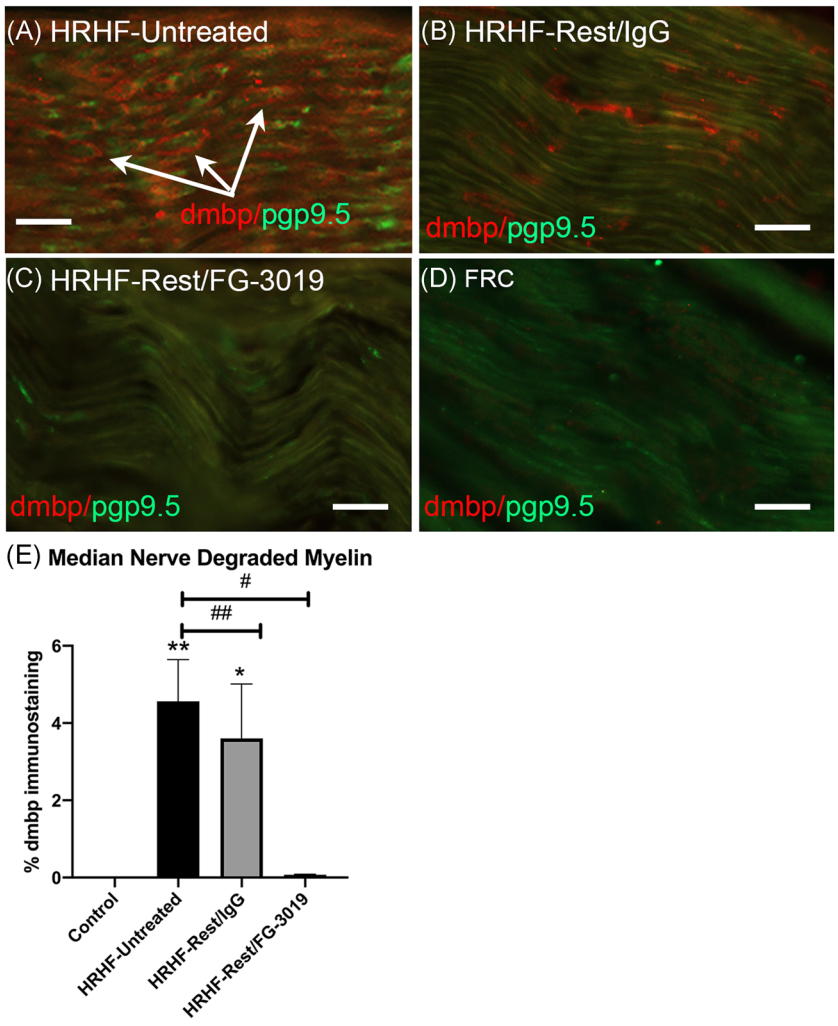

FIGURE 4 Degraded myelin basic protein immunostaining in median nerves, at the level of the wrist, in longitudinal cryosections, showing reduced staining to control levels in FG-3019 treated rats. A-D, Representative images of degraded myelin basic protein (dMBP, red) and protein gene product 9.5 (PGP 9.5, green, a pan neuronal/axon marker) immunoexpression in median nerves of each group. Arrows indicate examples of degraded myelin basic protein, which was highest in HRHF-untreated rats. E, Quantification of the percent degraded myelin basic protein immunoexpression in median nerves, at the level of the wrist. Same number of limbs/gp as in legend of Figure 3. $^{*}P < .05$ and $^{**}P < .01$ compared with FRC rats; $^{\#}P < .05$ and $^{\#\#}P < 0.01$ compared with HRHF-untreated or HRHF-Rest/IgG rats as shown. Scale bar = 25 μm. FRC, food restricted control; HRHF, high repetition high force [Color figure can be viewed at wileyonlinelibrary.com]

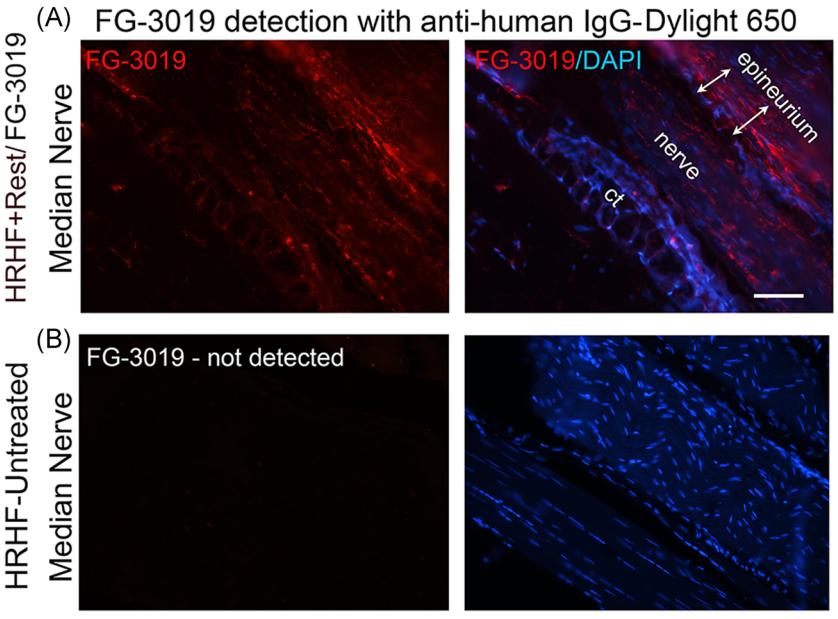

FIGURE 5 Anti-human IgG detection of the FG-3019 agent in median nerves. A, FG-3019 was detected in nerves of HRHF-FG-3019 rats using an anti-human IgG antibody. B, This staining was not detected in untreated HRHF rat nerves, as they lacked FG-3019. DAPI (blue) was used as nuclear stain (right images of both A and B). Similar results were seen in five animals per group. ct, connective tissue; DAPI, 4′,6-diamidino-2-phenylindole; HRHF, high repetition high force [Color figure can be viewed at wileyonlinelibrary.com]

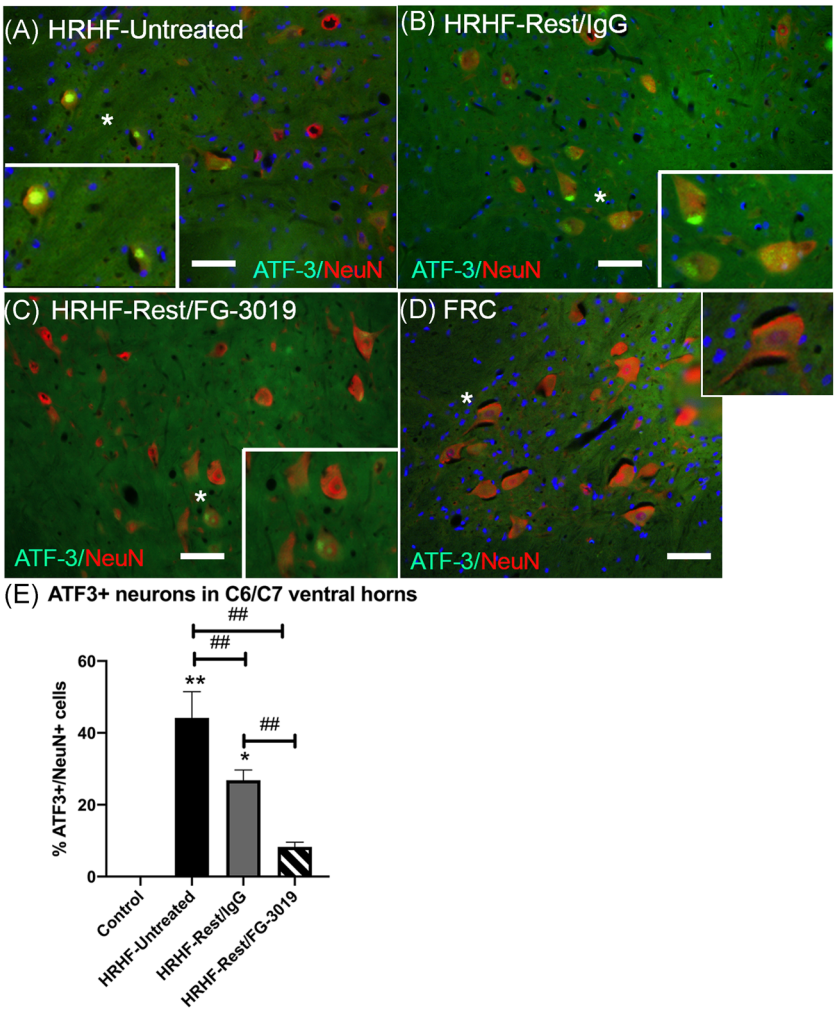

FIGURE 6 ATF3 immunostaining in nuclei of neurons in cervical level 7 (C7) spinal cord ventral horns, showing the highest presence in HRHF-untreated rats. A-D, ATF3 immunoexpression in motor neurons located in spinal cord ventral horns, at segmental level C7. NeuN was used as a neuronal counterstain and DAPI is used as a nuclear counterstain in panels A, B, and D. Asterisks in the panels indicate areas enlarged in their insets. Insets in panels A and B show the presence of ATF3 only in neuronal cell bodies of HRHF-untreated and HRHF-Rest/IgG rats. E, Quantification of the percent of neurons (NeuN+ cells) in cervical ventral horns that were also immunopositive for ATF3. Same number of limbs /gp as in the legend of Figure 3. $^{*}P < .05$ and $^{**}P < .01$ compared with FRC rats; $^{\#\#}P < .01$ compared with HRHF-untreated or HRHF-Rest/IgG rats as shown. Scale bar = 50 μm. FRC, food restricted control; HRHF, high repetition high force [Color figure can be viewed at wileyonlinelibrary.com]

Specifically, 6 weeks of rest, with the IgG as a vehicle control, was ineffective at restoring grip strength declines or reducing the established fibrosis. Silverstein et al[26] has shown in a study of human workers that more than half of workers (55.6%) with overuse-induced median mononeuropathy do not recover after 1 year, consistent with these data. In contrast, 6 weeks of rest combined with the FG-3019 treatment restored grip strength and reduced cold temperature hypersensitivity concomitantly with reduced indices of neuropathology, including: (a) increased perineural fibrosis thought to either compress axons or impede nerve gliding during movement[27]; (b) increased immunoexpression of degraded myelin indicative of nerve injury[28]; (c) reduced median nerve conduction velocity, a key sign of compressive median mononeuropathy[28]; (d) increased numbers of neurons with ATF3 nuclear staining in cervical level ventral horns indicative of injured yet regenerative competent motor neurons[24,29]; and (e) increased numbers of substance P expressing DRG neurons indicative of sensory nerve sensitization and suggestive of axon irritation.[30] The peripheral nerve demyelination and ventral horn motor neuronal cell body increased ATF3+ findings are suggestive of a chronic nerve constriction that is affecting nerve and hand function. Combined, our findings support the hypothesis that fibrotic processes underlie the observed sensorimotor declines by compressing or impeding the excursion of peripheral neural tissues during movement, and that reducing fibrotic processes can reverse these effects.

Past findings from our model of fibrotic median mononeuropathy and dysfunction after prolonged performance of a HRHF task for 12 to 18 weeks were replicated in this study, including perineural fibrosis and focal demyelination of the median nerve, reduced grip strength, and cold aversion.[15,16,22] Our new finding of lower sensory axon conduction velocities (assayed using an orthodromic fine wire type method) complement our prior finding that compound nerve conduction velocities were also lower in rats that performed high force tasks for 12 weeks (assayed using an open surgical method). In addition, here, lower conduction velocities correlated with perineural fibrosis. These together with our new findings of significantly improved median nerve dysfunction and perineural fibrosis with FG-3019 (ie, anti-CCN2) treatment offers new hope for patients with fibrotic compressive neuropathies, particularly patients for whom surgery has been recalcitrant or is contraindicated.[27]

The generalizability of use of the FG-3019 anti-CCN2 treatment in humans is strong, based on its success for reducing or reversing

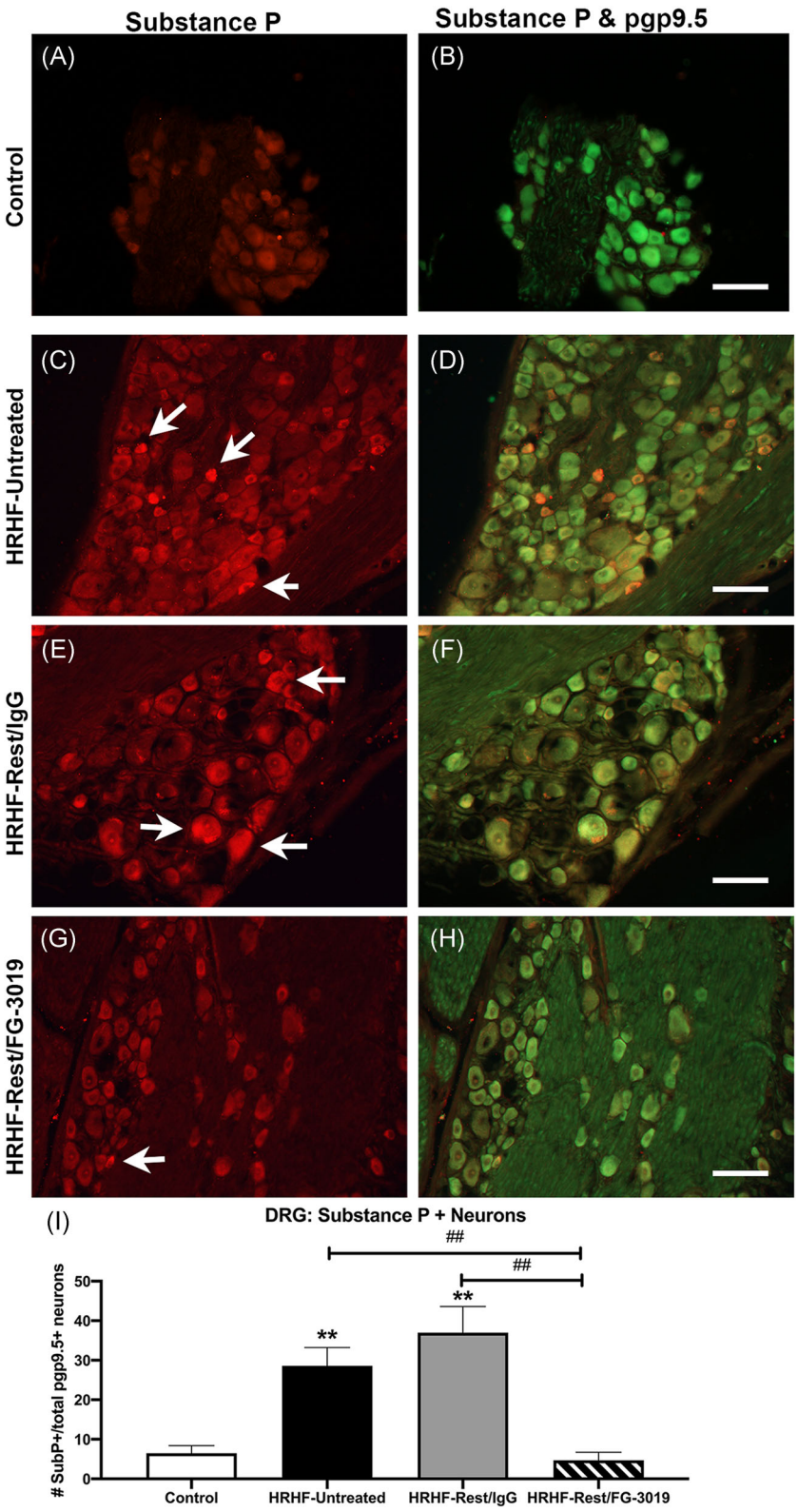

FIGURE 7 Substance P immunostaining in lower cervical level dorsal root ganglia (DRG), showing the highest presence in DRG of HRHF-untreated and HRHF-Rest/IgG treated rats. A-H, Substance P immunostaining (red) in sensory neurons located in cervical at segmental levels C7 or C8. PGP9.5 was used as a neuronal counterstain (green). Arrows indicate examples of substance P immunostained neuronal cell bodies. I, Percent of neurons (PGP 9.5+ neurons) in cervical DRG that were also immunopositive for substance P. Same number of limbs/gp as in Figure 3. $^{**}P < .01$ compared with FRC rats; $^{\#\#}P < .01$ compared with HRHF-untreated or HRHF-Rest/IgG rats as shown. Scale bar = 50 μm. FRC, food restricted control; HRHF, high repetition high force; PGP, protein gene product [Color figure can be viewed at wileyonlinelibrary.com]

idiopathic pulmonary fibrosis, pancreatic cancer, and Duchenne muscular dystrophy.[13,31,32] FG-3019 shows the ability to inhibit mesothelioma growth and cell proliferation in human mesothelioma cells lines and induces their apoptosis as well as fibroblast apoptosis.[33] This agent has successfully attenuated the progression of idiopathic pulmonary fibrosis in phase 2 trials[12] (ClinicalTrials.gov Identifier: NCT01890265) and is currently in phase 2 and 3 trials as a treatment for Duchenne muscular dystrophy (NCT02606136), and

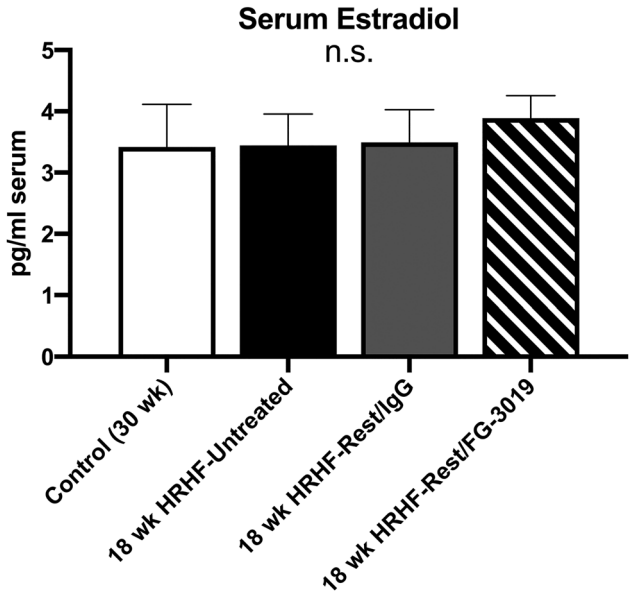

FIGURE 8 Serum estradiol levels assayed using ELISA at the time of final tissue collection. No significant differences (NS) were observed between the four groups. ELISA, enzyme-linked immunosorbent assay; HRHF, high repetition high force

idiopathic pulmonary fibrosis, and locally advanced, unresectable pancreatic cancer [13] (NCT02210559), respectively.

Furthermore, relevant to compressive median mononeuropathies, several groups have already identified the presence of CCN2 in fibrotic and thickened subsynovial specimens collected from patients with idiopathic median mononeuropathy during release surgeries.[1,34,35] Treatment of cultured subsynovial specimens collected from patients with idiopathic median mononeuropathy with TGFbeta receptor inhibitors has been shown to down regulate fibrotic gene expression in those ex vivo tissues, indicating that blocking TGFbeta signaling may be an important approach to the treatment of perineural fibrosis in this patient population. Given CCN2 is a downstream of TGFbeta-induced in the collagen production, one possible means to selectively block the profibrotic effects of TGFbeta signaling may be by blocking CCN2, as done in this study.

A key question is whether a local treatment for fibrotic compressive median mononeuropathy is sufficient. To date, FG-3019 has only been administered systemically for in vivo fibrotic disorders[13,31,32,36] and its effect as a local treatment in patients with a focal fibrotic median mononeuropathy is unknown. Unfortunately, upper extremity overuse injuries are not typically localized to a single region or structure. As demonstrated in our rat model, repeated and persistent inflammation and subsequent fibrogenic processes are present in all tissues across body regions (forepaws, cervical, and thoracic) involved in performing the task.[17,37] Widespread inflammatory processes are observed in nerves, tendons, muscles and bones of forepaws, forearms, and shoulder regions, as well as in distant regions, such as hindlimbs, due to systemic inflammatory responses.[15-17,37] Fibrogenic changes are observed within and around forepaw and forelimb nerves, muscles, tendons, and surrounding connective tissues.[5,14,21,22] Our lab and others have also reported that patients with upper extremity musculoskeletal disorders have increased frequency of pain and tenderness in multiple anatomical sites throughout their upper extremity.[38] Although future studies are needed to answer the local vs systemic administration question, we postulate that the multitissue involvement of these disorders will limit the effectiveness of local interventions.

With regard to duration of treatment needed to treat overuse-induced tissue fibrosis, a 6-week treatment was sufficient to reduce both established perineural and muscle fibrosis in 18-week HRHF rats, the latter of which we recently showed.[21] This time frame is similar to that seen in Mdx mice (an animal model of Duchenne muscular dystrophy) in which 2 months of treatment (3×/week, IP) with FG-3019 reduced muscle fibrosis, improved muscle strength, and improved locomotor performance.[39] We were able to halt the early progression of fibrogenic changes in perineural, tendon, and muscle tissues with only a 2-week treatment of FG-3019 (provided to 3-week HRHF rats during their last 2 weeks of task performance as a preventative treatment).[14] Significant reductions in skin fibrosis have also been observed in a mouse model of systemic sclerosis after only 2 weeks of treatment with FG-3019 (3×/week, IP).[40] Yet radiation-induced pulmonary fibrosis in mice shows significant reductions in collagen deposition and pulmonary function after 16 weeks of CCN2 blockade.[41] CCN2 levels and signaling responses are context dependent, and vary with cell type and matrix in which it is functioning, as well as with environmental stimulants, such as the cytokine profile.[42] Thus, there are likely disease specific differences in the time needed to observe significant reductions in tissue fibrosis and restoration of function when blocking CCN2 signaling with FG-3019. While further research is needed to fully answer this question, current literature on this drug favors limiting its duration over sustained use as a more effective treatment.

Some limitations of this study need to be considered. First, only female rats were included. The force transducer sensitivity of our model setup is currently tailored to the pulling strength of female rats, so inclusion of males would have reduced data quality and made the interpretation of findings more difficult. Furthermore, it is well known that sex is an important factor in the modulation of pain.[43] The inclusion of males would have introduced confound in the behavioral (and perhaps post-euthanasia) data that would have not been adequately controlled for due to our group sizes ($n \leq 10$). To control for the effect of female sex hormones on pain, which vary at different stages of the estrous cycle (the equivalent of menstrual cycle in humans),[44] we assessed serum estradiol levels at the time of euthanasia. We found no group differences and that estradiol levels did not correlate with grip strength and cold sensitivity outcomes. Future studies that include both sexes are encouraged to consider these factors when interpreting pain data. Limitations also include our inability to address rapid signaling changes (ie, within 10-30 minutes of cessation of a task session or treatment), because this is a prolonged in vivo animal model. We were unable to address rapid signaling changes as we also collected tissues at

36 hours after the last task session or after 6-week rest period in order to avoid acute activity-induced cytokine changes in the tissues. Finally, the results of this study cannot be directly interpreted to human outcomes. While we agree there are important physiological differences between rats and humans, we expect that the insights gained from this study will be relevant to humans. A key strength of our model is that it is an operant model in which rats develop structural and functional changes in the same manner as humans involved in performing prolonged repetitive reaching and grasping activities. Further studies are needed to address the usefulness of this agent in humans with neural fibrosis.

In conclusion, treatment with rest and human IgG proved ineffective in rats that performed a HRHF task for 18 weeks, suggesting that the incurred fibrotic changes were too advanced for rest alone to reverse. Conversely, rest in combination with systemically administered FG-3019 treatment to block CCN2 signaling proved successful, providing foundation for its use as a novel therapeutic strategy for reversing overuse injury induced neural fibrosis in humans.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

MFB and SNP were responsible for the research design; MFB, BAH, MA, MYH, LJH, GEC, DMK, and SNP and each contributed substantially to various aspects of the data acquisition, analysis, and interpretation; MFB, DMK, GEC, and SNP drafted and critically revised the paper; all authors gave approval of the final submitted version.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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