Increased CCN2, substance P and tissue fibrosis are associated with sensorimotor declines in a rat model of repetitive overuse injury

Paul W. Fisher · Yingjie Zhao · Mario C. Rico ·
Vicky S. Massicotte · Christine K. Wade · Judith Litvin ·
Geoffrey M. Bove · Steven N. Popoff · Mary F. Barbe

Received: 8 October 2014 / Accepted: 14 January 2015 / Published online: 24 January 2015
© The International CCN Society 2015

Abstract Key clinical features of cumulative trauma disorders include pain, muscle weakness, and tissue fibrosis, although the etiology is still under investigation. Here, we characterized the temporal pattern of altered sensorimotor behaviors and inflammatory and fibrogenic processes occurring in forearm muscles and serum of young adult, female rats performing an operant, high repetition high force (HRHF) reaching and grasping task for 6, 12, or 18 weeks. Palmar mechanical sensitivity, cold temperature avoidance and spontaneous behavioral changes increased, while grip strength declined, in 18-week HRHF rats, compared to controls. Flexor digitorum muscles had increased MCP-1 levels after training and increased TNF α in 6-week HRHF rats. Serum had increased IL-1 β , IL-10 and IP-10 after training. Yet both muscle and serum inflammation resolved by week 18. In contrast, IFN γ increased at week 18 in both muscle and serum. Given the anti-fibrotic role of IFN γ , and to identify a

mechanism for the continued grip strength losses and behavioral sensitivities, we evaluated the fibrogenic proteins CCN2, collagen type I and TGFB1, as well as the nociceptive/fibrogenic peptide substance P. Each increased in and around flexor digitorum muscles and extracellular matrix in the mid-forearm, and in nerves of the forepaw at 18 weeks. CCN2 was also increased in serum at week 18. At a time when inflammation had subsided, increases in fibrogenic proteins correlated with sensorimotor declines. Thus, muscle and nerve fibrosis may be critical components of chronic work-related musculoskeletal disorders. CCN2 and substance P may serve as potential targets for therapeutic intervention, and CCN2 as a serum biomarker of fibrosis progression.

Keywords Collagen type I · Cumulative trauma disorder · Fibrosis · IFN γ · Overuse injury · TGFB1

Electronic supplementary material The online version of this article (doi:10.1007/s12079-015-0263-0) contains supplementary material, which is available to authorized users.

P. W. Fisher · Y. Zhao · V. S. Massicotte · J. Litvin · S. N. Popoff ·
M. F. Barbe (✉)

Department of Anatomy and Cell Biology, Temple University School
of Medicine, 3500 North Broad St., Philadelphia, PA 19140, USA
e-mail: mbarbe@temple.edu

M. C. Rico
Sol Sherry Thrombosis Research Center, Temple University School
of Medicine, Philadelphia, PA, USA

C. K. Wade
Department of Physical Therapy, Thomas Jefferson University,
Philadelphia, PA, USA

G. M. Bove
Department of Biomedical Sciences, University of New England
COM, 11 Hills Beach Rd, Biddeford, ME 04005, USA

Abbreviations

CCN2	Connective tissue growth factor
ECM	Extracellular matrix
FRC	Food-restricted control
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
GRO/KC	Growth regulated oncogene alpha / keratinocyte-derived chemokine
HRHF	High repetition high force
IFN γ	Interferon gamma
IL-	Interleukin
IP-10	Interferon gamma-induced protein 10
MIP-	Macrophage inflammatory protein
MCP-1	Monocyte chemoattractant protein 1
NC	Normal control

OSHA	The United States Occupational Safety and Health Administration
RANTES	Regulated on activation normal T cell expressed and secreted
subP	Substance P
TGFB1	Transforming growth factor beta
TRHF	Trained to high force
TNFalpha	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor
WMSDs	Work-related musculoskeletal disorders

Introduction

The United States Occupational Safety and Health Administration (OSHA) estimates that work-related musculoskeletal disorders (WMSDs) account for over 600,000 injuries and illnesses (OSHA 2014). These disorders are estimated at \$20 billion a year in direct costs, and up to five times more in indirect costs for MSD-related workers' compensation, in addition to the substantial toll on affected workers who develop significant difficulties in performing simple upper extremity tasks (OSHA 2014). The incidence rate of occupation-related arm, wrist and hand injuries is 23 % of all workplace injuries/illnesses and requires a median of 12 days away from work (BLS 2013). Studies in humans with upper extremity WMSDs find evidence of inflammation, fibrosis and degeneration in tissues, changes thought to cause the concurrent sensorimotor dysfunction (Carp et al. 2007; Chikenji et al. 2014; Ettema et al. 2004; Rechart et al. 2011; Rempel and Diao 2004; Riondino et al. 2011). However, underlying pathophysiological responses are incompletely understood, particularly those associated with chronic fibrotic pathologies.

Several studies have detected serum biomarkers of inflammation in patients with short-term (<3 months) upper extremity WMSDs, including tumor necrosis factor alpha (TNF α) and interleukin 1 (IL-1) family members (Carp et al. 2007; Rechart et al. 2011; Riondino et al. 2011). These serum biomarkers of inflammation correlated with severity of patients' symptoms of pain and motor dysfunction. Patients with chronic (>3 months) WMSDs show continued symptoms of pain and motor dysfunction, yet an absence of inflammatory markers. Instead, these latter patients have increased tissue fibrosis and fibrogenic markers, such as transforming growth factor beta 1 (TGFB1) (Chikenji et al. 2014; Ettema et al. 2004; Freeland et al. 2002; Hirata et al. 2004), although serum biomarkers of fibrosis in relationship to WMSDs are still under investigation.

CCN2 (also known as connective tissue growth factor or CTGF) is a sensitive serum biomarker of cardiac fibroblast proliferation (Lipson et al. 2012) and idiopathic pulmonary

fibrosis, as is interferon gamma (IFN γ) (Tzortzaki et al. 2007). CCN2 is produced by multiple cell types and is a downstream mediator of transforming growth factor beta 1 (TGFB1) (Grotendorst 1997; Song et al. 2007). When increased, CCN2 induces fibroblast proliferation and extracellular matrix (ECM) deposition (Grotendorst 1997; Stratton et al. 2001). This is of interest since tissue CCN2 increases under conditions of muscle and tendon overload and/or injury (Heinemeier et al. 2007; Kjaer 2004; Smith et al. 2007), and has been linked to the pathogenesis of fibrosis (Hawinkels and Ten Dijke 2011; Ihn 2008; Seher et al. 2011; Tzortzaki et al. 2007). Since CCN2 is normally low or undetectable in sera of healthy individuals, it may serve as a predictive biomarker for patients in the fibrotic stage of WMSDs after exclusion of other possible health disorders, as shown recently in a study from our laboratory examining tendon fibrosis using a rat model of overuse (Gao et al. 2013).

The neuropeptide substance P has also been linked to tissue fibrosis. Substance P (subP) is an 11 amino acid neurotransmitter known to be involved in nociception in the peripheral and central nervous systems, yet has multiple effects on immune cells, endothelial cells, and fibroblasts. SubP has been reported to work in concert with TGFB1 in inflammation (Beinborn et al. 2010) and fibrotic responses (Dehlin et al. 2013; Koon et al. 2010) in various tissues, and is increased in ligaments and tendons of patients with carpal tunnel syndrome (Ozturk et al. 2010). In models of overloading in vitro and in vivo, increased SubP has been linked to increased tenocyte proliferation and tendinosis, respectively (Backman et al. 2011a, b; Fedorczyk et al. 2010), muscle derangement and myositis in a unilateral model of experimentally-induced muscle overuse (Song et al. 2013), and increased collagen type I expression by dermal fibroblasts during wound healing (Cheret et al. 2014). The importance of subP in scar formation is also well documented (Chen et al. 2006; Henderson et al. 2012; Henderson et al. 2006; Jing et al. 2010; Scott et al. 2005), indicating a link between increased subP and fibroblast proliferation and collagen production during wound healing. To our knowledge, no study has examined concomitant increases in subP and CCN2.

We have developed a unique rodent model of operant repetitive reaching and grasping in which the performance of a reaching and handle-pulling task causes injury and inflammation, followed by tissue fibrosis (Abdelmagid et al. 2012; Clark et al. 2004; Fedorczyk et al. 2010; Gao et al. 2013). We observed exposure-dependent declines in sensorimotor function after short-term performance (≤ 3 months) of these tasks, with a high repetition high force (HRHF) task inducing the greatest dysfunction (Barbe et al. 2008; Barbe et al. 2013; Fedorczyk et al. 2010). Short-term sensorimotor declines were associated with duration- and force-dependent increases in tissue inflammation and fibrosis (Abdelmagid et al. 2012; Barbe et al. 2008, 2013; Elliott et al. 2009a, b; Elliott et al.

2010; Fedorczyk et al. 2010; Gao et al. 2013; Kietrys et al. 2011, 2012). Unfortunately, we were not able to tease out if tissue inflammation or fibrosis (or both) were contributing to functional declines in these past studies of ≤ 3 months, due to concurrent inflammatory and fibrotic tissue responses.

Therefore, our goal here was to examine the effects of performing a high repetition high force (HRHF) handle-pulling task for up to 18 weeks on forearm grip strength, pain-related behaviors, inflammation, and fibrotic responses in forearm muscles, nerves and surrounding ECM. We hypothesized that inflammatory cytokine responses would resolve early, that fibrotic/nociceptor tissue responses (CCN2, TGFB1, collagen I, and subP) would increase significantly in flexor digitorum muscles, nerves and ECM with prolonged performance of a high demand repetitive task, in association with persistent sensorimotor declines. We further hypothesized that tissue increases in CCN2 would be matched by serum increases, supportive of it serving as a potential biomarker of fibrosis and therapeutic target to prevent the fibrosis and reduced function occurring as a consequence of repeated overuse.

Methods

Subjects

The Temple University Institutional Animal Care and Use Committee approved all experiments in compliance with NIH guidelines for the care and use of laboratory animals. Ninety-five young adult, female Sprague-Dawley rats (3 months of age at onset of experiments) were used. Adult female rats were used in this study because: (1) Human females have a higher incidence of work-related musculoskeletal disorders than males (Gerr et al. 2002); and (2) for comparison to data from our past studies on female rats using this model.

Rats were randomly divided into 4 groups: age-matched normal controls (NC, $n=15$); age- and weight-matched food restricted controls (FRC, $n=23$); age-matched trained-only rats that underwent an initial training (trained to high-force, TRHF, $n=15$) and then euthanized after training; and age-matched rats that were trained before performing the high-repetition, high-force task (HRHF) task for 3 weeks ($n=6$), 6 weeks ($n=8$), 12 weeks ($n=12$) or 18 weeks ($n=16$) before euthanasia (total number of HRHF rats=42). Rats were housed in a central animal facility in separate cages with a 12-h light:dark cycle and free access to water. Rats were weighed weekly and their food adjusted to maintain ± 95 % body weight of age-matched controls to avoid catabolic tissue changes that might occur with greater weight loss, and to avoid confounds of obesity (rats tend to work hard for the banana-flavored food pellets used as food reward). All rats

were inspected weekly and again post-mortem for presence of illness or tumors to reduce confounders for serum cytokine increases (none were observed). To further reduce illness-related confounders, additional sentinel rats were examined for presence of viral infections or other illnesses as part of regular veterinary care (none were detected).

Behavioral apparatuses, training and task regimen

Sixteen custom-designed behavioral apparatuses were used for these experiments, as previously described and depicted (Barbe et al. 2013). Briefly, animals reached through a shoulder height portal and isometrically pulled a force handle attached to a force transducer with a load cell (Futek Advanced Sensor Technology, Irvine, CA) located outside the chamber wall. The load cell was interfaced with custom Force-Lever software (version 1.03.02, Med Associates, St. Albans, VT). Auditory and light indicators cued the reaching rate (defined below). If reach and force criteria (defined below) were met within a 5 s cueing period, a 45 mg food pellet was dispensed into a food trough.

The trained-only (TRHF) and HRHF rats underwent an initial training period for 5 weeks in which they learned the task, as previously described (Barbe et al. 2013; Elliott et al. 2010). Briefly, all but NC rats were initially food-restricted for 7 days to no more than 10–15 % less than their naive weight, and the weight of age-matched rats with free access to food, to initiate interest in food reward pellets. After that week, they were given extra rat chow to gain weight quickly back to only 5 % less than age-matched normal control rats. Rats were weighed weekly, and allowed to gain weight during the study as they were young adult rats (Jain et al. 2014). The food-restricted rats trained to learn the HRHF reaching and handle-pulling tasks during a 5-week period of 10 min/day, 5 days/week, in which they ramped upwards from naive towards the HRHF task level (see below). Trained rats reached the HRHF level only during their last week of training. After training, rats were randomly divided into TRHF and HRHF rats. TRHF rats were euthanized at this point to determine the effect of training. The remaining trained rats went on to perform the HRHF task.

After the initial training period, a point equal to week 0 of the HRHF task, 30 rats began the HRHF task regimen for 2 h/day, 3 days/week for up to 18 weeks. The task was divided into 4, 0.5-h sessions separated by 1.5 h in order to avoid satiation. HRHF rats were cued to reach at a rate of 8 reaches/min and to grasp the force handle at a target force effort of 55 ± 5 % (which equals 120 to 128 g, and 1.02 to 1.12 N). HRHF rats had to grasp the force handle and exert an isometric pull at the target level for at least 50 ms to receive a food reward, as described previously (Barbe et al. 2013). Rats were allowed to use their preferred limb to reach (the “reach” limb), and data from this limb only is reported.

Spontaneous pain behaviors during reaching

Trained observers tracked changes in behaviors occurring during each period of task performance (30 min/task period; 4 times/day; 3 days/week). Data from the last day of task weeks 1, 6, 12 and 18 are reported. Bilateral pulling of the lever bar was recorded upon occurrence, as was incidence of a rat switching the forearm used to pull on the lever bar from their typical “preferred” reach limb to the contralateral limb. On occasion, a rat would twist its forelimb into a supinated position when pulling on the lever rather than the typical pronated pull; this was recorded as a “Supinated Pull”. Fumbling, guarding, or pulling with one digit rather than the typical four-digit grasp were recorded upon occurrence, as was refusal to participate (typically sitting in the corner of the chamber, and thus termed “sits in corner”). Spontaneous pain behavioral data is reported for HRHF rats only, since only this group performed the task.

Temperature sensitivity

Two choice temperature preference/aversion assays were used to determine hot or cold thermal thresholds, using methods similar to those previously described (Balayssac et al. 2014; Mishra and Hoon 2010; Noel et al. 2009). Rats were placed in an apparatus with two adjacent plates enclosed in a single chamber by Plexiglas: a reference plate at 22 °C (room temperature) and a test plate that was adjusted to specific temperatures of interest between 12 °C and 45 °C (T2CT, BioSeb, France). Rats were allowed to habituate to the chamber for 3 min, with each plate at room temperature. The variable temperature plate was then increased slowly or decreased slowly, with 3 min at each temperature. During this time period, the rats were free to choose their preferred position (comfort zone) on the two plates while their movement in three zones was recorded with a video tracking system: a zone that included only the room temperature plate, a zone that included only the varied temperature test plate, and a middle zone in which the rat had a portion of its body on each plate rather than just one. The number of crossings between the two plates was tracked by a motion sensitive camera and percent time spent in each zone calculated; data from the middle zone was not used. To ensure data were collected from trials in which rats were exposed to both test temperatures, only assays in which rats traveled between both plates in the acclimation period were counted. Preference/aversion to increasing temperature (22–45 °C) versus decreasing temperature (22–12 °C) were assayed on separate days. Temperature sensitivity was assayed at naïve, and after 18 weeks of HRHF task performance, and in age-matched FRC rats at matching the 18 week time point.

Forepaw mechanical sensitivity

Mechanical sensitivity was assayed as forepaw withdrawal behaviors to stimulation with calibrated von Frey filaments, at baseline (the naïve time point) and after 18 weeks of HRHF task performance. Aged-matched food restricted control rats (FRC) were tested at similar time points. Filaments were applied 10 times in the order of increasing stiffness, perpendicular to the plantar surface of the forepaw and pressed until they bent (5 filaments were used: sized 0.16, 0.4, 1.0, 6 and 26 g); the number of forelimb withdrawal responses to each was recorded.

Motor function assay

Reflexive grip strength (Elliott et al. 2010) of the forelimbs was measured in all animals, bilaterally, at baseline (the naïve time point), after training (the 0 week time point of the HRHF task), and every 6 weeks thereafter, using a rat grip strength recording unit (Stoelting, Wood Dale, IL). These assays were performed by an examiner naïve to group assignment. The test was repeated 3–5 times/limb/trial, and the maximum grip strength per trial is reported.

Serum biomarker assays

Following euthanasia (sodium pentobarbital, 120 mg/kg body weight), 36 h after completion of the final task session (to avoid exercise-induced serum cytokine changes), blood was collected by cardiac puncture using a 23-gauge needle and centrifuged immediately at $1000 \times g$ for 20 min at 4 °C. Serum was collected from: NC ($n=9$), FRC ($n=7$), TRHF ($n=4$), and HRHF rats that had performed the task for 18 weeks ($n=6$). Serum was collected and stored at -80 °C until analyzed using customized multiplex bead-based analysis (Millipore Corporation, Billerica, Massachusetts) for 13 cytokines and chemokines, including: 7 inflammatory cytokines and chemokines (interleukin-1 alpha (IL-1 α), IL-1 β , IL-6, IL-18, interferon gamma-induced protein 10 (IP-10/CXCL10), macrophage inflammatory protein 1 alpha (MIP-1 α /CCL3), and MIP-2/CXCL2), a key anti-inflammatory protein (IL-10), an anti-fibrogenic cytokine that also increases lysosomal activity of macrophages (IFN γ), an angiogenesis-related cytokine (vascular endothelial growth factor (VEGF)), and 3 chemotactic chemokines (eotaxin, growth-related protein/keratinocyte chemoattractant (GRO/KC, also known as CXCL1), and monocyte chemoattractant protein 1 (MCP-1/CCL2)). Serum from FRC ($n=14$), TRHF ($n=15$), and HRHF rats that had performed the task for 6 ($n=7$), 12 ($n=8$), and 18 weeks ($n=6$) was also assessed for tumor necrosis factor-alpha (TNF α) using singleplex ELISA (DY510, R&D Systems, Minneapolis, MN, as well as ThermoFisher Scientific Pierce Searchlight, Waltham, MA), CCN2 (22202,

BioMedical Assay, Beijing, China), IL-10 (KRC0102, Invitrogen Biosource, as well as ThermoFisher Scientific Pierce Searchlight), and IFN γ (ThermoFisher Scientific Pierce Searchlight).

Tissue biochemical assays

Following euthanasia and after serum collection, flexor digitorum muscles and tendons were collected from the distal forearm region of the preferred reach limb. Muscles were homogenized as previously described (Barbe et al. 2008) from: NC ($n=4$), FRC ($n=8$), TRHF ($n=4$), and HRHF rats that performed the task for 6 ($n=4$), 12 ($n=3$) or 18 weeks ($n=8$). Tendons were homogenized similarly for NC ($n=3$) and 18 week HRHF rats ($n=5$). Muscle and tendon homogenates were assayed by multiplex analysis using a LincoPlex rat 23-plex kit (Millipore Corporation, Billerica, MA) for cytokines and chemokines: 13 inflammatory cytokines and chemokines (IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-12, IL-13, IL-17, IL-18, interleukin 12 protein 70 (IL-12p70), MIP-1a, and TNF α), a key anti-inflammatory cytokine (IL-10), 7 chemotactic proteins (eotaxin, granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), GRO/KC, IP-10, MCP-1, and regulated on activation, normal T cell expressed and secreted (RANTES/CCL5)), an anti-fibrogenic and inflammatory cytokine (IFN γ), a adipocyte-derived cytokine involved in fatty acid metabolism in resting rodent skeletal muscle (leptin), and an angiogenesis-related cytokine (VEGF). Muscle homogenates were also assayed for CCN2 by ELISA (22202, BioMedical Assay, Beijing, China) and TGFB1 by ELISA (Aushon Biosystems). Results from tissue ELISAs were normalized to total protein concentration as measured by BCA assay (Pierce Biotechnology, Rockford, IL).

Muscle homogenates were also assayed via Western Blot for CCN2 (sc-14939, 1:400 dilution, Santa Cruz Biotechnology, Santa Cruz, CA), collagen type I (C2456, 1:500 dilution, Sigma-Aldrich, St. Louis, MO), TGFB1 (MAB240, 1:500, R&D Systems, Minneapolis, MN), and IFN γ (PA1-24782, 1:500, Thermo Scientific). Densitometry was performed using myImageAnalysis version 1.1 (Thermo Fisher Scientific). GAPDH or beta actin were used as a loading control (glyceraldehyde-3-phosphate dehydrogenase; AM4300, Invitrogen). Western blots were repeated at least three times for each analyte examined.

Immunohistochemical and histological analyses

Forelimb tissues that were not homogenized for biochemical analysis were used for histological analysis from: FRC ($n=12$) and HRHF rats that performed the task for 3 ($n=6$), 6 ($n=4$), 12 ($n=4$) or 18 weeks ($n=8$). Following euthanasia and after serum collection, animals were perfused transcardially with

4 % buffered paraformaldehyde. Forearm musculotendinous tissues were collected and sectioned longitudinally, as described previously (Barbe et al. 2003; Fedorczyk et al. 2010). Sections were immunostained in batched sets by the same individual for CCN2, collagen type I, IFN γ , protein gene product 9.5, smooth muscle alpha actin, subP, and TGFB1, using previously described methods (Abdelmagid et al. 2012; Fedorczyk et al. 2010), and the following antibodies: goat polyclonal anti-CCN2 at 1:400 dilution (sc-14939, Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal anti-Collagen type I at 1:500 dilution (C2456, Sigma, St. Louis, MO); goat polyclonal anti-IFN γ at 1:100 dilution (PA1-24782, Thermo Fisher Pierce, Rockford, IL); rabbit monoclonal anti-smooth muscle alpha actin at 1:1000 dilution (SMA; MABT381, Millipore, Billerica, Massachusetts); mouse monoclonal anti-protein gene product 9.5 (pgp9.5, a pan neuronal marker) at 1:250 dilution (ab8189; abcam, Cambridge, MA); rabbit polyclonal anti-subP at 1:100 dilution (AB1566, Millipore), and mouse monoclonal anti-TGFB1 at 1:500 dilution (MAB240, R&D Systems, Minneapolis, MN). Briefly, after a 0.5 % pepsin antigen retrieval step for 15 min at room temperature, sections were incubated for 20 min in the appropriate blocking serum for each antibody, and then were incubated with the primary antibody at the listed dilution for overnight at 4 °C. This was followed by incubation with appropriate secondary antibodies that were AffiniPure F(ab')₂ fragments, preabsorbed to reduce non-specific cross-reactivity with rat antigens, and conjugated to green or red fluorescent cyanine dyes (Cy2, DyLight 488, Cy3, or DyLight 594) (Jackson ImmunoResearch, West Grove, PA) at a dilution of 1:100 each for 2 h at room temperature.

The specificity of the TGFB1, collagen type I, CCN2 and IFN γ antibodies are shown in western blots that are part of this study. Preabsorption controls were also performed to demonstrate if the antibodies bound specifically to the antigen of interest using: CCN2 human recombinant protein produced in HEK293 cells (CXT-687, Prospec, East Brunswick, NJ), Collagen type 1 purified rat protein (C7661, Sigma), IFN γ rat recombinant protein (1857812, Thermo Scientific), TGFB1 human recombinant protein (GF111, Millipore), and subP full length 11 amino acid peptide (CAS 33507-63-0, catalog # 1156, Tocris Bioscience, R&D). A two to ten fold excess of purified protein or peptide was pre-incubated with the matching antibody overnight at 4 °C, the mixture centrifuged, and then the pre-absorbed antibody supernatant was incubated with the tissues (after pepsin and goat serum treatment) similarly to that described above before washing and incubation with secondary antibodies. No labeling was observed in the tissues for any pre-absorbed antibody, as shown in Supplemental Fig. 1. We also performed no primary antibody controls in which serum was substituted for the primary antibody, followed by secondary antibodies; no labeling was

Fig. 1 Sensorimotor behavioral changes in food restricted control rats (FRC) and in high repetition high force task (HRHF) rats. **a** Incidence of spontaneous pain behaviors in HRHF rats during task performance, from week 0 through week 18: bilateral pulling of lever bar (rather than with preferred reach limb), switching limb used to pull, supinated pulls (rather than typical pronated pull), and sitting in corner (rather than participating). *: $p < 0.05$ and **: $p < 0.01$, compared to week 0. **b** Temperature aversion compared to room temperature of 22 °C, assayed using cold and hot temperature place preference instrument in FRC rats, and HRHF rats at naïve and after 18 weeks of task. **c** Palmar mechanical sensitivity in age-matched FRC and 18-week HRHF rats, assayed using von Frey filaments of sizes indicated on x-axis. Number of responses out of 10 are reported. **d** Maximum grip strength in grams (g) in FRC and HRHF rats at naïve time point and across weeks of experiment. Symbols of panels **b–d**: *: $p < 0.05$ and **: $p < 0.01$, compared to naïve timepoint of HRHF rats; &: $p < 0.05$ and &&: $p < 0.01$, compared to age-matched FRC rats. Two-way ANOVA values are reported in individual panels

observed as a result of incubation of tissues with serum and then secondary antibodies alone (Supplemental Fig. 2).

The percent area of immunostaining for IFN γ , TGF β 1, CCN2, collagen 1 and subP was quantified in batched sets by the same individual (MB) in the epimysium region and in the ECM adjacent to the flexor digitorum muscles using an image analysis system (BioQuant Osteo, BioQuant, Nashville, TN), using previously described methods (Al-Shatti et al. 2005; Fedorczyk et al. 2010; Gao et al. 2013). Adjacent sections of the NC, FRC and 18-week HRHF rat muscles were stained with hematoxylin and eosin (H&E), and with CD11b (a macrophage marker), using previously described methods (Fedorczyk et al. 2010; Gao et al. 2013). These sections were examined for the presence of neutrophils, lymphocytes or macrophages.

Statistical analyses

GraphPad PRISM v.6.02 was used for the statistical analyses. All data are expressed as mean $\pm$ SEM. *P* values of <0.05 were considered significant for all comparisons. Behaviors were analyzed using two-way ANOVAs. Overall incidence of spontaneous behaviors occurring during the task was analyzed by two-way repeated measures ANOVA with the factors: observed behavior and week of task performance. Temperature sensitivity was analyzed using the factors: group and temperature; mechanical sensitivity was analyzed using the factors: group and filament size; grip strength was analyzed using the factors: group and week of task performance. One-way ANOVA was used to compare results from the muscle and serum multiplex cytokine analysis, and histology quantification. When only 18-week HRHF and TRHF or FRC rat tissues were analyzed for a particular cytokine (singleplex ELISAs, CCN2 western blot and immunohistochemical analyses), the results were compared using the Student's *t*-test. During the post-hoc ANOVA analyses for between group differences, the Bonferroni correction method was used to reduce the chances of obtaining false-positive results (type I errors) when multiple pair wise tests are performed on a single set of data. In accordance with this method, an adjustment was made to *p* values by the analysis program used (GraphPad PRISM) by dividing the critical *p* value ($\alpha=0.05$) by the number of comparisons made, thus increasing the stringency of the analysis. Pearson's correlation analyses (two-tailed) were used to determine if behavioral values correlated with serum or tissue protein levels, and if serum cytokines levels correlated with tissue cytokines levels, with *r* values greater than 0.75 defined as indicating good to excellent correlation and those between 0.5 and 0.75 as moderate correlation. For succinctness, except for Fig. 1, specifics of ANOVA and significant posthoc findings are reported in the figure panels.

Results

Spontaneous behavior changes with HRHF task performance

Two-way repeated measures ANOVA showed a significant ($p<0.05$) interaction between incidence of a particular behavior and week of task performance (Fig. 1a). Specifically, incidence of bilateral pulling (rather than with one limb) and switching limb used to pull the lever (from the preferred reach limb, as determined during training) increased significantly in HRHF task weeks 6, 12 and 18, compared to week 0 (onset of task performance). Incidence of sitting in the corner of task chamber rather than participating increased in task week 18, compared to week 0. Although observed, there was no significant increase in supinated pulls (rather than the typical pronated pulls; Fig. 1a), fumbling, guarding, or pulling with one digit only were not observed across weeks of task performance, compared to week 0.

Forepaw cold temperature aversion and mechanical sensitivity increased with HRHF task

Two-way ANOVA showed significant changes by both group ($p=0.008$) and temperature ($p=0.001$; Fig. 1b). Specifically, an aversion to cold temperatures was increased significantly in 18-week HRHF rats, compared to their naïve data and to age-matched FRC rats. The greatest aversion was observed at 12 °C, 14 °C and 16 °C temperatures, compared to the room temperature plate set at 22 °C. Two-way ANOVA showed a significant interaction between group and von Frey filament size ($p<0.0001$; Fig. 1c). Specifically, the number of forelimb withdrawals increased significantly in 18-week HRHF rats, compared to age-matched FRC rats, in response to mid-palmar forepaw probing with filaments with bending forces of 4 g or less.

Grip strength declines with HRHF task performance

Two-way ANOVA showed significant changes by both group ($p<0.001$) and week ($p<0.001$) (Fig. 1d). Specifically, forearm grip strength was decreased significantly in preferred reach limbs immediately after training (HRHF week 0), compared to naïve levels, and in HRHF rats at 6, 12 and 18 weeks, compared to age-matched FRC rats (Fig. 1d). No significant differences were detected between NC and FRC control groups (data not shown).

Muscle inflammatory cytokines increase early yet transiently, while muscle CCN2 increases in week 18 of HRHF task

Using multiplex ELISA, significant transient increases were observed in TRHF rat muscles for MCP-1 levels compared to NC, and 6-, 12- and 18-week HRHF rats (Fig. 2a), and for IL-

Fig. 2 Cytokines in flexor digitorum muscles, analyzed using ELISA. Data is shown for **a** MCP-1, **b** IL-18, **c** TNF α , **d** CCN2, **e** TGFB1, **f** Leptin, and **g** RANTES in normal control (NC), food-restricted controls (FRC), trained only (TRHF), and 6-, 12-, and 18-week high repetition high force (HRHF) rats. ANOVA values shown in individual panels. *: $p < 0.05$ compared to NC; &: $p < 0.05$ compared to FRC; #: $p < 0.05$ compared to TRHF rats; ϕ : $p < 0.05$ compared to 6-week HRHF rats

18 levels, compared to all other groups (Fig. 2b). In 6-week HRHF rat muscles, TNF α levels were increased compared to NC and 18-week HRHF rats (Fig. 2c). TGFB1 increased significantly in 12-week HRHF rat muscles, while CCN2 levels increased significantly in 18-week HRHF rat muscles, compared to NC (Fig. 2d,e). Leptin levels showed a transient but non-significant increase in TRHF rat muscles, yet a significant decrease

in 18-week HRHF rat muscles, compared to TRHF (Fig. 2f). RANTES levels showed a transient but non-significant increase in 6-week HRHF rat muscles, yet a significant decrease in 18-week HRHF rats, compared to 6-week HRHF (Fig. 2g). Muscle levels of the remaining cytokines tested (eotaxin, GM-CSF, GRO/KC, IL-1 β , IL-2, IL-4, IL-10 and VEGF) did not differ significantly between groups (data not shown).

Table 1 Serum Cytokines significant findings (ELISA; Mean $\pm$ SEM)

Serum Analyte	NC rats (n=9)	FRC rats (n=7)	TRHF rats (n=4)	18 weeks HRHF rats (n=6)
IL-1beta	4.84 $\pm$ 1.55	3.44 $\pm$ 0.80	101.7 $\pm$ 52.75**	5.02 $\pm$ 1.075&&
IL-10	6.34 $\pm$ 0.0 ^a	6.34 $\pm$ 0.0 ^a	91.93 $\pm$ 53.01*	6.34 $\pm$ 0.0 ^a &
IP-10	184.6 $\pm$ 16.21	183.4 $\pm$ 10.43	345.8 $\pm$ 65.80**	148.1 $\pm$ 7.102&&
Gro-KC	95.24 $\pm$ 19.96	156.8 $\pm$ 27.32	143.6 $\pm$ 30.11	50.12 $\pm$ 4.193*
IFN-gamma	34.95 $\pm$ 10.74	31.07 $\pm$ 9.02	14.60 $\pm$ 0.0 ^a	98.68 $\pm$ 19.93**&&
CCN2	1.73 $\pm$ 0.93	n.t.	n.t.	5.47 $\pm$ 1.48*

* and **: $p < 0.05$ and $p < 0.01$, compared to NC/FRC rats; & and &&: $p < 0.05$ and $p < 0.01$, compared to TRHF rats; n.t. not tested; ^a Undetectable values listed at LLOQ for statistical analysis

There were also no significant increases in inflammatory cytokines or chemokines in flexor digitorum tendons of 18-week HRHF rats, compared to FRC rats (data not shown). CCN2 was not tested using ELISA in tendons.

No inflammatory cells were observed in 18-week HRHF rat muscles or tendons

H&E and anti-CD11b stained sections of muscles and tendons collected for histomorphometry from NC, FRC and 18-week HRHF rats were examined for presence of neutrophils or macrophages. No inflammatory cells were observed in NC, FRC or 18-week HRHF rat muscles or tendons (data not shown). We have previously reported increased presence of inflammatory cells in the TRHF, 6- and 12-week HRHF rat muscles, concomitant with increased inflammatory cytokine levels (Barbe et al. 2013; Fedorczyk et al. 2010).

Serum inflammatory cytokines increase early yet transiently, while serum CCN2 and IFN γ increase in week 18 of HRHF task

To verify that inflammatory cytokine responses were resolved systemically, and not just in flexor digitorum muscles, we analyzed serum using multiplex and singleplex ELISAs

(Table 1). Serum levels of IL-1 β , IL-10 and IP-10 were elevated in TRHF rats, compared to FRC and NC rats, yet at baseline levels in 18-week HRHF rats. GRO/KC was not significantly increased in TRHF rat serum, yet significantly decreased in 18-week HRHF, compared to TRHF rats. IFN γ and CCN2 were the only tested cytokines increased in serum of 18-week HRHF rats, compared to control rats. Serum levels of eotaxin, IL-1 α , IL-6, IL-18, MIP-2, MCP-1, and VEGF did not differ significantly between groups (data not shown).

Fibrogenic proteins CCN2, collagen type I, IFN γ and TGFB1 increase in tissues with long-term HRHF task

Western blot analysis followed by densitometry showed significantly increased CCN2 protein in 12- and 18-week HRHF rat muscles, compared to TRHF rats (Fig. 3a, b). Western blot analysis followed by densitometry also showed presence of pro-collagen type I (approximately 150 kDa) in all groups with greater increases in 12-week HRHF rat muscles (Fig. 3c; densitometry not shown), and significantly increased mature collagen I (75 kDa) in 12-week and 18-week HRHF rat muscles, compared to TRHF rats (Fig. 3c, d).

Using cross-sections of mid-belly flexor digitorum muscles for immunohistochemistry, FRC and TRHF rats showed a thin epimysium with low CCN2 immunostaining, and low CCN2

Fig. 3 Western Blot analysis of CCN2 and collagen type I protein in flexor digitorum muscles. **a** A representative Western blot of muscle homogenates from trained only (TRHF), 12- and 18-week HRHF rats, probed with anti-CCN2 (at 37 kDa). GAPDH used as a loading control (at 40.2 kDa). **b** Densitometric analysis of three replicate Western Blots, showing ratio of CCN2 bands normalized GAPDH levels. **c** A representative Western blot of muscle homogenates from TRHF, 12-,

and 18-week HRHF rats, probed with anti-collagen type I, showing increased procollagen band (top procollagen band is approximately 150 kDa) in 12 week HRHF rats, and increased mature collagen type I band (75 kDa) in 12- and 18-week HRHF rats. **d** Densitometric analysis of three replicate Western blots, showing the ratio of mature collagen type I (75 kDa), normalized to GAPDH protein. [#] $p < 0.05$ and ^{##} $p < 0.01$, compared to TRHF rats

immunostaining in the endomysium and surrounding extracellular matrix (FRC only shown in Fig. 4a, d, g, i; Fig. 5a; TRHF reported previously (Abdelmagid et al. 2012)). The epimysium was increased in thickness in 18-week HRHF rat muscles (Fig. 4b double arrows). CCN2 immunostaining was increased in the epimysium (Fig. 4b), in extracellular spaces of the endomysium (Fig. 4e; Fig. 5b), as well as in small cells on the perimeter of myofibers (Fig. 4b and j; Fig. 5b), endothelium and surrounding extracellular matrix tissues (Fig. 4h), and in Schwann cells (Fig. 4h). Some of the small CCN2-positive cells were double-labeled with smooth muscle actin (SMA; Fig. 4j), indicating that this subset were myofibroblasts. Others were endothelial and Schwann cells.

Image analysis quantification confirmed a significant increase of CCN2 immunostaining in the epimysium and endomysium of 12- and 18-week HRHF rats, compared to FRC rats (Fig. 4c and Table 2). A fibrous muscle repair site is shown outlined in Fig. 4f, and also shows increased CCN2 and collagen type I deposition.

Collagen type I immunostaining was also increased in small cells on the perimeter of myofibers, endomysium and subsets of myofibers of 18-week HRHF rats (Fig. 4b, e, f; Fig. 6e). Fibrous bands immunostained with collagen type I were observed in the extracellular matrix around flexor digitorum muscles of 18-week HRHF rats (Fig. 4h; Fig. 6b), but not in FRC rats (Fig. 4g; Fig. 6a).

Fig. 4 (a–h) CCN2 (red) and collagen type I (green), and (i–j) CCN2 (red) and smooth muscle actin (SMA; green) immunostaining in mid-region of flexor digitorum muscles (epimysium, myofibers, endomysium, and surrounding extracellular matrix regions) of FRC and 18 week HRHF rats. Muscle cross-sections are from mid-muscle region. (a,b) CCN2 and collagen type I immunostaining in FRC and 18 week HRHF rats, with double-headed arrows indicating in epimysium (epim) thickness. Asterisks indicate myofibers shown magnified in insets. (c) Quantification of percent area with CCN2 immunostaining in epimysium. (d–f) CCN2 and collagen type I immunostaining in myofibers, perimysium (perim) and endomysium region of FRC (d)

and 18-week HRHF rats (e,f). Asterisks indicate myofibers shown enlarged in insets. (f) A fibrous scar-type region in the mid-muscle is outlined. (g,h) CCN2 and collagen type I immunostaining in extracellular matrix (ECM) around flexor digitorum muscle of FRC and 18 week HRHF rats. (i,j) CCN2 and SMA immunostaining in myofibers of FRC and 18-week HRHF rats, showing double-labeling of small cells surrounding subsets of myofibers, indicating subsets of small CCN2 stained cells are myofibroblasts. Art = artery, cap = capillary, ct = connective tissue, M = muscle, N = nerve. * $p < 0.05$, compared to NC. Scale bar=50 μ m

Fig. 5 IFN γ , CCN2, and TGFB1 immunostaining in flexor digitorum muscles and tendons of FRC and 18-week task rats. DAPI (blue) used as nuclear stain. (**a,b**) IFN γ (red) and CCN2 (green) immunostaining in muscle of FRC and 18 week HRHF rats. Inset in **b**: IFN γ immunostaining in small cells on myofiber perimeter. Arrows indicate examples of IFN γ stained cells; arrowheads indicate CCN2 deposition

around myofiber perimeter. (**c,d**) TGFB1 (green) immunostaining in muscle of FRC and 18 week HRHF rats. (**e,f**) TGFB1 in tenocytes of FRC and 18 week HRHF rat tendons (inset shows enlargement of area indicated with arrow). The Western blots for IFN γ and TGFB1 show the bands recognized by the antibodies used for IHC. Scale bar=50 μ m

Image analysis quantification confirmed a significant increase of collagen type I immunostaining in the endomysium of 12- and 18-week HRHF rat muscles, compared to FRC rats (Table 2).

Immunohistochemistry was used to determine if flexor digitorum muscles were one source of the serum IFN γ (Fig. 5a, b). IFN γ immunostaining was not evident in FRC rat muscles (Fig. 5a), yet was increased in small cells on the perimeter of myofibers in 18-week HRHF rats (Fig. 5b). Image analysis quantification confirmed this increase in 18-week HRHF rats, compared to FRC rats (Table 2).

TGFB1 was increased in small cells on the perimeter of myofibers and in flexor digitorum tendocytes of 18-week HRHF rats, compared to FRC rats (Fig. 5d and f, versus Fig. 5c and e). This endomysium differences were confirmed by quantification of TGFB1 staining (Table 2).

Substance P increases in tissues with long-term HRHF task

We used immunohistochemistry to determine if subP immunostaining increased in reach forelimb tissues of 18-week HRHF rats. We observed increased subP immunostained axons (determined by double-labeling with pgp9.5, a pan neuronal marker) in extracellular matrix tissues, and between or on the perimeter of myofibers of 18-week HRHF rats, compared to FRC rats (Fig. 6b–c versus 6A, and 6E–G versus 6days, respectively). Mast cells that were subP immunoreactive were also observed in the extracellular matrix. Since HRHF rats showed increased forepaw sensitivity, we examined subP immunostaining in the mid-palmar skin of reach limb forepaws. We observed increased subP immunoreactive axons (double-labeled with pgp9.5) in the dermis and epidermis of 18-week HRHF rats, compared to FRC rats (Fig. 6i–k

Table 2 Quantification of Immunofluorescence (% area with immunostaining; Mean±SEM)

Analyte	FRC rats (<i>n</i> =3-13)	3 weeks HRHF rats (<i>n</i> =4)	6 weeks HRHF rats (<i>n</i> =4)	12 weeks HRHF rats (<i>n</i> =4)	18 weeks HRHF rats (<i>n</i> =3-8)
Flexor Digitorum Muscle - Endomysium					
CCN2	0.18±0.13	2.32±0.48	12.42±1.49	33.05±14.71**	14.16±3.70*
Collagen I	0.35±0.18	0.96±0.36	4.48±1.37	8.83±1.99**	12.68±1.93**
IFN-gamma	0.75±0.48	n.t.	n.t.	n.t.	25.50±8.15*
TGFB1	1.04±0.33	2.21±0.71	6.28±0.92	21.79±1.59**	16.38±2.71**
Substance P	0.62±0.26	4.19±2.02	9.49±1.24**	10.31±2.00**	9.49±3.21**
Dermis of Mid-Forepaw – Extracellular Matrix					
CCN2	0.47±0.19	0.92±0.62	18.80±9.15*	22.89±2.90**	10.97±3.46
Collagen I	1.14±0.46	5.42±1.72	25.28±10.77**	25.28±2.97**	30.34±4.81**
TGFB1	0.70±0.29	n.t.	n.t.	n.t.	4.81±0.25**
Substance P	1.80±0.59	n.t.	n.t.	n.t.	6.75±0.48**

* and **: $p < 0.05$ and $p < 0.01$, compared to FRC rats; n.t. = not tested

Fig. 6 Substance P (SubP; red) and either collagen type 1 (COL1) or pgp9.5 (a pan neuronal marker) green immunostaining in FRC and 18 week HRHF tissues. DAPI (blue) used as nuclear stain in panels **a,b** and **d**. (**a,b**) SubP and COL1 immunostaining in extracellular matrix surrounding mid-forearm region of flexor digitorum muscle showing increased SubP axon profiles and COL1 in extracellular matrix of 18 week HRHF rat, compared to FRC rat. (**c**) SubP and pgp9.5 double-labeled axons (arrows) in extracellular matrix (ECM) and nerve bundle (N). (**d,e**) SubP (red) and COL1 (green) immunostaining in flexor digitorum muscle of FRC and 18 week HRHF rats. Arrow in panel E

indicates longitudinal profile of a SubP immunostained axon between myofibers. (**f**) Arrows indicate SubP immunostained axons around myofibers at myofiber tendinous (T) junction. (**g**) SubP and pgp9.5 double-labeled axon in muscle. (**h,i**) SubP and COL1 immunostaining in epidermis and dermis of mid-palmar forepaw of FRC and 18 week HRHF rat. Arrows indicate SubP immunostained axon profiles. Profiles indicated by arrow and asterisk are enlarged in inset in I. (**j,k**) SubP and pgp9.5 double-labeled axons in dermis (arrows indicate same axon regions that are double-labeled or pgp9.5 labeled). Scale bar=50 μm

versus h). These differences were confirmed by quantification of subP immunostaining in forearm muscles and dermis of forepaw regions (Table 2). These increases were accompanied by increased collagen type I (Fig. 6i versus h), CCN2 and TGFB1 immunostaining (Table 2).

Correlations between behavioral values and fibrogenic proteins, and between collagen type I and other fibrogenic proteins

As shown in Table 3, grip strength declines in the reach limbs correlated with increased CCN2, collagen type I, IFN γ and TGFB1 immunostaining, as well as CCN2 ELISA and collagen type I western blot findings in flexor digitorum muscle from these same limbs. The withdrawal response to stimulation of the reach limb's forepaw with a 1 g von Frey filament correlated with increased collagen type I, TGFB1 and subP

Table 3 Significant Pearson's Correlations. Only significant correlations ($p < 0.05$) with a correlation coefficient > 0.5 are reported

Correlations of Grip Strength to forearm Flexor Digitorum Muscle findings		
CCN2 IH	$r=-0.69; p<0.05$	
CCN2 ELISA	$r=-0.82, p<0.05$	
Collagen type I IH	$r=-0.63; p<0.05$	
Collagen type I western blot assay	$r=-0.78; p<0.05$	
IFN-gamma IH	$r=-0.79; p<0.05$	
TGFB1 IH	$r=-0.70; p<0.01$	
TGFB1 ELISA	$r=-0.63; p<0.05$	
Substance P IH	n.s.	
Correlations of withdrawal response to 1 g filament to Mid-Palmar Dermis findings		
CCN2 IH	n.s.	
Collagen type I IH	$r=0.94; p<0.01$	
TGFB1 IH	$r=0.98, p<0.01$	
Substance P IH	$r=0.82; p<0.01$	
Correlations with aversion to 16 °C temperature with Mid-Palmar Dermis findings		
CCN2 IH	$r=-0.70; p<0.05$	
Collagen type I IH	n.s.	
TGFB1 IH	n.s.	
Substance P IH	$r=-0.76; p<0.05$	
Correlations of Collagen Type I immunostaining to fibrogenic protein immunostaining		
	Flexor Digitorum Muscle	Mid-Palmar Dermis
IFN-gamma	$r=0.71; p<0.05$	n.t
CCN2	$r=0.52; p<0.01$	$r=0.59; p<0.01$
TGFB1	$r=0.76; p<0.001$	$r=0.94; p<0.01$
Substance P	$r=0.65; p<0.01$	$r=0.72; p<0.01$

IH immunohistochemistry quantification; n.s. not significant; n.t. not tested

immunostaining in the mid-palmar dermis. An aversion reaction to standing on a 16 °C plate correlated with increased CCN2 and subP immunostaining in the mid-palmar dermis. Lastly, collagen type I immunostaining correlated with IFN γ immunostaining in flexor digitorum muscles, and with CCN2, TGFB1 and subP immunostaining in flexor digitorum muscles and mid-palmar dermis (Table 3).

Discussion

Our hypotheses were upheld in that tissue inflammatory cytokine responses resolved early, and fibrotic tissue proteins (CCN2, TGFB1, collagen I, and subP) increased significantly in and around flexor digitorum muscles, extracellular matrix and nerves with prolonged performance of a HRHF task, and that the fibrotic tissue responses correlated with persistent sensorimotor declines. The observed increased serum CCN2 is supportive of it serving as a potential biomarker and therapeutic target to prevent the tissue fibrosis and reduced function that occurs as a consequence of overuse, once other potential sources of CCN2 are ruled out.

This is our first study examining sensorimotor declines in rats performing HRHF tasks for more than 12 weeks. The increased incidence of bilateral pulling, switching limb used to pull the lever, and sitting in the corner rather than participating in HRHF task rats are indicative of increased discomfort with continued task performance. The increased cold aversion and withdrawal response to mechanical stimulation are consistent with peripheral and/or central sensitization (Choi et al. 1994; Jorum et al. 2003) (Chaplan et al. 1994; Elliott et al. 2009b, 2010; Ma and Woolf 1995; Ren and Dubner 1999), secondary to neuropathies previously documented in this model (e.g., axonal inflammation and degeneration) (Clark et al. 2004, 2003; Elliott et al. 2009b, 2010, 2008). The grip strength declines match findings in our past studies examining 6- to 12-week HRHF rats (Abdelmagid et al. 2012; Barbe et al. 2013; Fedorczyk et al. 2010). In these shorter studies, forearm grip strength declines and other pain behaviors were linked to inflammation (Barbe et al. 2008; Elliott et al. 2009a, 2010; Fedorczyk et al. 2010). Others have reported inflammation-induced declines in forelimb grip force following intramuscular injection of carrageenan, an agent used to stimulate cutaneous inflammation and activate muscle nociceptors (Kehl and Fairbanks 2003), and declines in isometric force production after increased force loading (Baker et al. 2007). Our findings of persistent functional declines despite resolution of inflammation and concomitant with fibrotic responses, match findings from patients with chronic (> 3 months) WMSDs in which fibrotic responses are increased and inflammatory responses are not detected (Ettema et al. 2004; Freeland et al. 2002; Hirata et al. 2004). Driscoll and Blyum postulated that fibrosis in and around

muscles, tendons and nerves may distort dynamic biomechanical properties and increase tissue strain due to adherence to adjacent structures, reducing dynamic tissue function (Driscoll and Blyum 2011). Fibrosis in the connective tissue “container” surrounding nerves has been linked to chronic nerve compression (Bove et al. 2009; O’Brien et al. 1987), which is known to increase pain behaviors (as a consequence of compressive nerve irritation) and decrease grip strength (due to reduced nerve conduction). Similarly, we observed increased collagen deposition around myofibers, muscles and nerve processes, indicative of an increased fibrotic “container” with overuse. Our behavioral and correlative findings here support these hypotheses that fibrotic tissue changes are contributing to the observed sensorimotor behavioral declines.

We observed increases of several fibrogenic related proteins, including CCN2, TGFB1 and collagen type I in extracellular matrix, muscles and dermis of the forepaw at 18 weeks of task performance. Many CCN2-immunopositive cells appeared to be fibroblasts, although they may also be stem cells. Some of the small CCN2-immunopositive cells were smooth muscle actin immunopositive myofibroblasts, which is interesting since TGFB1 is an upstream modulator of CCN2 and collagen production in myofibroblasts (Cunningham et al. 2010; Garrett et al. 2004; Sonnylal et al. 2010). CCN2 was also expressed in Schwann cells (which we have previously confirmed using S100-beta double-labeling of cells surrounding nerve axons (Clark et al. 2003)) and endothelial cells (as shown by (Brigstock 2002; Guney et al. 2011)). We observed a small number of sites of fibrotic repair, with increased collagen type I and CCN2, within the muscles, suggestive of sites of focal muscle injury. TGFB1 immunostained cells in muscles were likely also fibroblasts, while those in tendons were tenocytes. Other groups have shown that fibrogenic proteins increase in fibroblasts and tenocytes under conditions of tissue overload or injury (Best et al. 2001; Heinemeier et al. 2007; Kjaer 2004; Nakama et al. 2006; Smith et al. 2007). Increased CCN2 has been linked to the pathogenesis of tissue fibrosis (Liu et al. 2013; Seher et al. 2011; Sonnylal et al. 2010; Sonnylal et al. 2013; Tzortzaki et al. 2007). It is the downstream mediator of TGFB1 (Clark et al. 2003; Grotendorst 1997; Song et al. 2007), and TGFB1-induced CCN2 expression leads to fibroblast proliferation and extracellular matrix deposition (Grotendorst 1997). With regard to repeated overloading, CCN2 immunopositive tenocytes increase in tendons in a rabbit model of cyclical loading (Nakama et al. 2006). Clinically, CCN2 is increased in tenosynovium and subsynovial connective tissue of carpal tunnel syndrome patients (Pierce et al. 2009) (Chikenji et al. 2014), as is TGFB1 (Chikenji et al. 2014). CCN2 has been suggested as a suitable target for prevention of fibrotic disorders (Daniels et al. 2003; van Nieuwenhoven et al. 2005).

Clinically, trials are underway using a fully human IgG1 monoclonal antibody that recognizes domain 2 of human and

rodent CCN2 (this monoclonal is called FG-3019 and was developed by Fibrogen, Inc., South San Francisco, CA) as a novel therapy to treat patients with pancreatic cancer, idiopathic pulmonary fibrosis, kidney disease (Adler et al. 2010; Gunther et al. 2006; Neesse et al. 2013), and liver fibrosis due to chronic hepatitis B infection (FibroGen, 2012). FG-3019 has also been used as a therapy in a mouse model of Duchenne muscular dystrophy, where it reversed muscle fibrosis and even improved motor function (Morales et al. 2013). These studies combined with our current and recent (Gao et al. 2013) findings of increased CCN2 in tissues and serum support the use of CCN2 as a biomarker of fibrosis in patients with WMSDs. The aggregate findings further suggest anti-CCN2 treatments be explored as potential therapeutics to reverse fibrosis and functional declines occurring as a consequence of overuse.

We also observed increased collagen immunostaining within subsets of myofibers. Increased collagen abundance within myofibers have been reported in a prior study using our model (Abdelmagid et al. 2012), in a rat model prenatal ischemia and immobilization in which muscles were affected negatively (Coq et al. 2008), in muscles collected from insulin resistant patients (Berria et al. 2006), and in myofibers undergoing repair (Alexakis et al. 2007). While we don’t fully understand the reason for this increase, its occurrence only in muscles after prolonged performance of a HRHF task and the absence of this type of staining in normal control or food restricted control animals, and after preabsorption control staining supports further investigation.

The anti-fibrotic agent, IFN γ (Diaz et al. 2012; Foster et al. 2003; Ziesche et al. 1999), was increased in serum and muscles at 18 weeks of HRHF task performance. IFN γ is an anti-fibrotic cytokine known to inhibit TGFB1 signaling (Leask and Abraham 2004). Foster et al. showed that the anti-fibrotic agents decorin and IFN γ can decrease fibrotic deposits in a mouse laceration injury model, and that IFN γ administration can improve muscle function (Foster et al. 2003; Fukushima et al. 2001). We suggest that the increased IFN γ was an attempt by HRHF rats to control a fibrotic tissue response.

SubP labeled axons and even mast cells increased in extracellular matrix, muscles, and forepaw dermis and epidermis at 18 weeks of task performance, and correlated with enhanced mechanical and thermal sensitivity. Past findings demonstrate increased nerve fibers in tissues as a consequence of repetitive tasks (Fedorczyk et al. 2010; Kadi et al. 1998; Lian et al. 2006). SubP is a nociceptor-related neuropeptide (Henry 1982; Jessell 1982) that increases in several painful conditions, including painful tendinopathies, rotator cuff tears, and arthritis (Dean et al. 2013; Gotoh et al. 1998; Lui et al. 2010; Munoz and Covenas 2011; Witonski et al. 2005). SubP participates in enhanced temperature hypersensitivity in dermatitis and nerve injury, mechanical hyperalgesia after persistent

inflammation, and painful peripheral neuropathy induced by paclitaxel (Murota et al. 2012; Rogoz et al. 2014; Tatsushima et al. 2011; Teodoro et al. 2013; Uematsu et al. 2011). However, subP is produced not only by neurons but also by mast cells, endothelial cells, fibroblasts and tenocytes (Andersson et al. 2011; Backman et al. 2011a, b; Fedorczyk et al. 2010; Katayama and Nishioka 1997), and stimulates fibroblast and tenocyte proliferation and collagen remodeling in vitro (Backman et al. 2011b; Fong et al. 2013; Katayama and Nishioka 1997). For example, subP, produced by tenocytes in vitro in response to mechanical loading, appears to regulate tenocyte proliferation through an autocrine loop involving its receptor, neurokinin 1 receptor (NK-1R) (Backman et al. 2011b). Exogenously administered SP accelerates tenocyte proliferation in the Achilles tendon during tendinosis development in a rabbit model (Andersson et al. 2011). At the systems level, Ozturk et al. found that patients with carpal tunnel syndrome have increased subP in the transverse carpal ligament and thickened synovial connective tissue of the flexor digitorum tendon (Ozturk et al. 2010). A link has been demonstrated between subP and dermal fibroblast proliferation and increased collagen type I during cutaneous wound healing (Cheret et al. 2014). Lastly, WT mice treated with the NK-1R antagonist as well as NK-1R knockout mice have reduced colonic fibrosis, fibroblast accumulation, and expression of fibrogenic factors in the colonic mucosa, and in vitro experiments with colonic fibroblasts show that SP stimulates fibroblast migration and, in the presence of TGF β 1 and IGF-1, increases collagen synthesis (Koon et al. 2010). These findings combined with our current findings suggest that subP plays a role in both increased pain sensitivity and fibrotic processes, although further studies in which subP signaling is blocked are needed to confirm this hypothesis.

In conclusion, these findings show that high demand tasks can induce tissue fibrotic changes if the work is performed for long periods of time. The temporal association of sensorimotor behavioral declines with increased tissue fibrotic proteins suggests that fibrosis contributes to functional declines occurring with overuse. The increased subP during the fibrotic process supports and extends findings from other groups linking its increase to morphological changes in tendons with overuse (Andersson et al. 2011; Backman et al. 2011a). These findings combined with similar finding in patients with chronic WMSDs (Ettema et al. 2004; Freeland et al. 2002; Hirata et al. 2004), indicate that therapies that prevent or treat fibrosis may be useful in preventing long-term functional motor declines occurring with long-term repetitive motion injuries. CCN2 may serve as a serum biomarker of fibrosis, while both CCN2 and subP deserve further exploration as targets for therapeutic intervention in fibrotic disorders with increased pain symptoms.

Acknowledgments Research reported in this publication was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Number AR056019 to MFB. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The authors would like to thank Michele Harris and Mamta Amin for their aid in the behavioral experiments, Mamta Amin for performing the immunohistochemistry, and Shreya Amin for sectioning the tissues.

Statement of financial disclosure and conflict of interest: None of the authors have any conflicts of interest issues to declare.

References

- Abdelmagid SM, Barr AE, Rico M, Amin M, Litvin J, Popoff SN, Safadi FF, Barbe MF (2012) Performance of repetitive tasks induces decreased grip strength and increased fibrogenic proteins in skeletal muscle: role of force and inflammation. *PLoS One* 7:e38359
- Adler SG, Schwartz S, Williams ME, Arauz-Pacheco C, Bolton WK, Lee T, Li D, Neff TB, Urquilla PR, Sewell KL (2010) Phase 1 study of anti-CTGF monoclonal antibody in patients with diabetes and microalbuminuria. *Clin J Am Soc Nephrol: CJASN* 5:1420–1428
- Alexakis C, Partridge T, Bou-Gharios G (2007) Implication of the satellite cell in dystrophic muscle fibrosis: a self-perpetuating mechanism of collagen overproduction. *Am J Physiol Cell Physiol* 293:C661–C669
- Al-Shatti T, Barr AE, Safadi FF, Amin M, Barbe MF (2005) Increase in inflammatory cytokines in median nerves in a rat model of repetitive motion injury. *J Neuroimmunol* 167:13–22
- Andersson G, Backman LJ, Scott A, Lorentzon R, Forsgren S, Danielson P (2011) Substance P accelerates hypercellularity and angiogenesis in tendon tissue and enhances paratendinitis in response to Achilles tendon overuse in a tendinopathy model. *Br J Sports Med* 45:1017–1022
- Backman LJ, Andersson G, Wennstig G, Forsgren S, Danielson P (2011a) Endogenous substance P production in the Achilles tendon increases with loading in an in vivo model of tendinopathy-peptidergic elevation preceding tendinosis-like tissue changes. *J Musculoskelet Neuronal Interact* 11:133–140
- Backman LJ, Fong G, Andersson G, Scott A, Danielson P (2011b) Substance P is a mechanoresponsive, autocrine regulator of human tenocyte proliferation. *PLoS One* 6:e27209
- Baker BA, Mercer RR, Geronilla KB, Kashon ML, Miller GR, Cutlip RG (2007) Impact of repetition number on muscle performance and histological response. *Med Sci Sports Exerc* 39:1275–1281
- Balayssac D, Ling B, Ferrier J, Pereira B, Eschaliere A, Authier N (2014) Assessment of thermal sensitivity in rats using the thermal place preference test: description and application in the study of oxaliplatin-induced acute thermal hypersensitivity and inflammatory pain models. *Behav Pharmacol* 25:99–111
- Barbe MF, Barr AE, Gorzelany I, Amin M, Gaughan JP, Safadi FF (2003) Chronic repetitive reaching and grasping results in decreased motor performance and widespread tissue responses in a rat model of MSD. *J Orthop Res* 21:167–176
- Barbe MF, Elliott MB, Abdelmagid SM, Amin M, Popoff SN, Safadi FF, Barr AE (2008) Serum and tissue cytokines and chemokines increase with repetitive upper extremity tasks. *J Orthop Res* 26:1320–1326
- Barbe MF, Gallagher S, Massicotte VS, Tytell M, Popoff SN, Barr-Gillespie AE (2013) The interaction of force and repetition on musculoskeletal and neural tissue responses and sensorimotor behavior in a rat model of work-related musculoskeletal disorders. *BMC Musculoskelet Disord* 14:303

- Beinborn M, Blum A, Hang L, Setiawan T, Schroeder JC, Stoyanoff K, Leung J, Weinstock JV (2010) TGF- β regulates T-cell neurokinin-1 receptor internalization and function. *Proc Natl Acad Sci U S A* 107:4293–4298
- Berria R, Wang L, Richardson DK, Finlayson J, Belfort R, Pratipanawatr T, De Filippis EA, Kashyap S, Mandarino LJ (2006) Increased collagen content in insulin-resistant skeletal muscle. *Am J Physiol Endocrinol Metab* 290:E560–E565
- Best TM, Shehadeh SE, Levenson G, Michel JT, Corr DT, Aeschlimann D (2001) Analysis of changes in mRNA levels of myoblast- and fibroblast-derived gene products in healing skeletal muscle using quantitative reverse transcription-polymerase chain reaction. *J Orthop Res* 19:565–572
- BLS (2013) Nonfatal occupational injuries and illnesses requiring days away from work, 2012. <http://www.bls.gov/news.release/pdf/osh2.pdf>. USDL-13-2257, November 26, 2013. Washington, D.C.
- Bove GM, Weissner W, Barbe MF (2009) Long lasting recruitment of immune cells and altered epi-perineurial thickness in focal nerve inflammation induced by complete Freund's adjuvant. *J Neuroimmunol* 213:26–30
- Brigstock DR (2002) Regulation of angiogenesis and endothelial cell function by connective tissue growth factor (CTGF) and cysteine-rich 61 (CYR61). *Angiogenesis* 5:153–165
- Carp SJ, Barbe MF, Winter KA, Amin M, Barr AE (2007) Inflammatory biomarkers increase with severity of upper-extremity overuse disorders. *Clin Sci (Lond)* 112:305–314
- Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL (1994) Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* 53:55–63
- Chen J, Wang JH, Zhuang HX (2006) Influence of substance P on the proliferation and apoptosis of fibroblasts of pathological scars. *Zhonghua shao shang za zhi = Zhonghua shaoshang zazhi = Chinese Journal of Burns* 22:277–280
- Cheret J, Lebonvallet N, Buhe V, Carre JL, Misery L, Le Gall-Ianotto C (2014) Influence of sensory neuropeptides on human cutaneous wound healing process. *J Dermatol Sci* 74:193–203
- Chikenji T, Gingery A, Zhao C, Passe SM, Ozasa Y, Larson D, An KN, Amadio PC (2014) Transforming growth factor- β (TGF- β) expression is increased in the subsynovial connective tissues of patients with idiopathic carpal tunnel syndrome. *J Orthop Res* 32:116–122
- Choi Y, Yoon YW, Na HS, Kim SH, Chung JM (1994) Behavioral signs of ongoing pain and cold allodynia in a rat model of neuropathic pain. *Pain* 59:369–376
- Clark BD, Barr AE, Safadi FF, Beitman L, Al-Shatti T, Amin M, Gaughan JP, Barbe MF (2003) Median nerve trauma in a rat model of work-related musculoskeletal disorder. *J Neurotrauma* 20:681–695
- Clark BD, Al-Shatti TA, Barr AE, Amin M, Barbe MF (2004) Performance of a high-repetition, high-force task induces carpal tunnel syndrome in rats. *J Orthop Sports Phys Ther* 34:244–253
- Coq JO, Strata F, Russier M, Safadi FF, Merzenich MM, Byl NN, Barbe MF (2008) Impact of neonatal asphyxia and hind limb immobilization on musculoskeletal tissues and S1 map organization: implications for cerebral palsy. *Exp Neurol* 210:95–108
- Cunningham JL, Tsolakis AV, Jacobson A, Janson ET (2010) Connective tissue growth factor expression in endocrine tumors is associated with high stromal expression of α -smooth muscle actin. *Eur J Endocrinol / Eur Fed Endocr Soc* 163:691–697
- Daniels JT, Schultz GS, Blalock TD, Garrett Q, Grotendorst GR, Dean NM, Khaw PT (2003) Mediation of transforming growth factor- β (1)-stimulated matrix contraction by fibroblasts: a role for connective tissue growth factor in contractile scarring. *Am J Pathol* 163:2043–2052
- Dean BJ, Franklin SL, Carr AJ (2013) The peripheral neuronal phenotype is important in the pathogenesis of painful human tendinopathy: a systematic review. *Clin Orthop Relat Res* 471:3036–3046
- Dehlin HM, Manteufel EJ, Monroe AL, Reimer MH Jr, Levick SP (2013) Substance P acting via the neurokinin-1 receptor regulates adverse myocardial remodeling in a rat model of hypertension. *Int J Cardiol* 168:4643–4651
- Diaz KT, Skaria S, Harris K, Solomita M, Lau S, Bauer K, Smaldone GC, Condos R (2012) Delivery and safety of inhaled interferon- γ in idiopathic pulmonary fibrosis. *J Aerosol Med Pulm Drug Deliv* 25:79–87
- Driscoll M, Blyum L (2011) The presence of physiological stress shielding in the degenerative cycle of musculoskeletal disorders. *J Bodyw Mov Ther* 15:335–342
- Elliott MB, Barr AE, Kietrys DM, Al-Shatti T, Amin M, Barbe MF (2008) Peripheral neuritis and increased spinal cord neurochemicals are induced in a model of repetitive motion injury with low force and repetition exposure. *Brain Res* 1218:103–113
- Elliott MB, Barr AE, Barbe MF (2009a) Spinal substance P and neurokinin-1 increase with high repetition reaching. *Neurosci Lett* 454:33–37
- Elliott MB, Barr AE, Clark BD, Amin M, Amin S, Barbe MF (2009b) High force reaching task induces widespread inflammation, increased spinal cord neurochemicals and neuropathic pain. *Neuroscience* 158:922–931
- Elliott MB, Barr AE, Clark BD, Wade CK, Barbe MF (2010) Performance of a repetitive task by aged rats leads to median neuropathy and spinal cord inflammation with associated sensorimotor declines. *Neuroscience* 170:929–941
- Ettema AM, Amadio PC, Zhao C, Wold LE, An KN (2004) A histological and immunohistochemical study of the subsynovial connective tissue in idiopathic carpal tunnel syndrome. *J Bone Joint Surg Am* 86-A:1458–1466
- Fedorczyk JM, Barr AE, Rani S, Gao HG, Amin M, Amin S, Litvin J, Barbe MF (2010) Exposure-dependent increases in IL-1 β , substance P, CTGF, and tendinosis in flexor digitorum tendons with upper extremity repetitive strain injury. *J Orthop Res* 28:298–307
- Fong G, Backman LJ, Hart DA, Danielson P, McCormack B, Scott A (2013) Substance P enhances collagen remodeling and MMP-3 expression by human tenocytes. *J Orthop Res* 31:91–98
- Foster W, Li Y, Usas A, Somogyi G, Huard J (2003) Gamma interferon as an antifibrosis agent in skeletal muscle. *J Orthop Res* 21:798–804
- Freeland AE, Tucci MA, Barbieri RA, Angel MF, Nick TG (2002) Biochemical evaluation of serum and flexor tenosynovium in carpal tunnel syndrome. *Microsurgery* 22:378–385
- Fukushima K, Badlani N, Usas A, Riano F, Fu F, Huard J (2001) The use of an antifibrosis agent to improve muscle recovery after laceration. *Am J Sports Med* 29:394–402
- Gao HG, Fisher PW, Lambi AG, Wade CK, Barr-Gillespie AE, Popoff SN, Barbe MF (2013) Increased serum and musculotendinous fibrogenic proteins following persistent low-grade inflammation in a rat model of long-term upper extremity overuse. *PLoS One* 8: e71875
- Garrett Q, Khaw PT, Blalock TD, Schultz GS, Grotendorst GR, Daniels JT (2004) Involvement of CTGF in TGF- β 1-stimulation of myofibroblast differentiation and collagen matrix contraction in the presence of mechanical stress. *Invest Ophthalmol Vis Sci* 45:1109–1116
- Gerr F, Marcus M, Ensor C, Kleinbaum D, Cohen S, Edwards A, Gentry E, Ortiz DJ, Monteilh C (2002) A prospective study of computer users: I. Study design and incidence of musculoskeletal symptoms and disorders. *Am J Ind Med* 41:221–235
- Gotoh M, Hamada K, Yamakawa H, Inoue A, Fukuda H (1998) Increased substance P in subacromial bursa and shoulder pain in rotator cuff diseases. *J Orthop Res* 16:618–621

- Grotendorst GR (1997) Connective tissue growth factor: a mediator of TGF-beta action on fibroblasts. *Cytokine Growth Factor Rev* 8:171–179
- Guney MA, Petersen CP, Boustani A, Duncan MR, Gunasekaran U, Menon R, Warfield C, Grotendorst GR, Means AL, Economides AN, Gannon M (2011) Connective tissue growth factor acts within both endothelial cells and beta cells to promote proliferation of developing beta cells. *Proc Natl Acad Sci U S A* 108:15242–15247
- Gunther A, Markart P, Eickelberg O, Seeger W (2006) Pulmonary fibrosis—a therapeutic dilemma? *Med Klin* 101:308–312
- Hawinkels LJ, Ten Dijke P (2011) Exploring anti-TGF-beta therapies in cancer and fibrosis. *Growth Factors* 29:140–152
- Heinemeier KM, Olesen JL, Haddad F, Langberg H, Kjaer M, Baldwin KM, Schjerling P (2007) Expression of collagen and related growth factors in rat tendon and skeletal muscle in response to specific contraction types. *J Physiol* 582:1303–1316
- Henderson J, Terenghi G, McGrouther DA, Ferguson MW (2006) The reinnervation pattern of wounds and scars may explain their sensory symptoms. *J Plast Reconstr Aesthet Surg : JPRAS* 59:942–950
- Henderson J, Ferguson MW, Terenghi G (2012) The reinnervation pattern of wounds and scars after treatment with transforming growth factor beta isoforms. *J Plast Reconstr Aesthet Surg : JPRAS* 65:e80–e86
- Henry JL (1982) Relation of substance P to pain transmission: neurophysiological evidence. *Ciba Foundation symposium*:206–224
- Hirata H, Nagakura T, Tsujii M, Morita A, Fujisawa K, Uchida A (2004) The relationship of VEGF and PGE2 expression to extracellular matrix remodelling of the tenosynovium in the carpal tunnel syndrome. *J Pathol* 204:605–612
- Ihn H (2008) Autocrine TGF-beta signaling in the pathogenesis of systemic sclerosis. *J Dermatol Sci* 49:103–113
- Jain NX, Barr-Gillespie AE, Clark BD, Kietrys DM, Wade CK, Litvin J, Popoff SN, Barbe MF (2014) Bone Loss from High Repetitive High Force Loading is Prevented by Ibuprofen Treatment. *J Musculoskelet Neuronal Interact* 14(1):78–94
- Jessell TM (1982) Substance P in nociceptive sensory neurons. *Ciba Foundation symposium*:225–248
- Jing C, Jia-Han W, Hong-Xing Z (2010) Double-edged effects of neuropeptide substance P on repair of cutaneous trauma. *Wound Repair Regen : official publication of the Wound Healing Society [and] the European Tissue Repair Society* 18:319–324
- Jorum E, Warncke T, Stubhaug A (2003) Cold allodynia and hyperalgesia in neuropathic pain: the effect of N-methyl-D-aspartate (NMDA) receptor antagonist ketamine—a double-blind, cross-over comparison with alfentanil and placebo. *Pain* 101:229–235
- Kadi F, Hagg G, Hakansson R, Holmner S, Butler-Browne GS, Thornell LE (1998) Structural changes in male trapezius muscle with work-related myalgia. *Acta Neuropathol* 95:352–360
- Katayama I, Nishioka K (1997) Substance P augments fibrogenic cytokine-induced fibroblast proliferation: possible involvement of neuropeptide in tissue fibrosis. *J Dermatol Sci* 15:201–206
- Kehl LJ, Fairbanks CA (2003) Experimental animal models of muscle pain and analgesia. *Exerc Sport Sci Rev* 31:188–194
- Kietrys DM, Barr AE, Barbe MF (2011) Exposure to repetitive tasks induces motor changes related to skill acquisition and inflammation in rats. *J Mot Behav* 43:465–476
- Kietrys DM, Barr-Gillespie AE, Amin M, Wade CK, Popoff SN, Barbe MF (2012) Aging contributes to inflammation in upper extremity tendons and declines in forelimb agility in a rat model of upper extremity overuse. *PLoS One* 7:e46954
- Kjaer M (2004) Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading. *Physiol Rev* 84:649–698
- Koon HW, Shih D, Karagiannides I, Zhao D, Fazelbhoz Y, Hing T, Xu H, Lu B, Gerard N, Pothoulakis C (2010) Substance P modulates colitis-associated fibrosis. *Am J Pathol* 177:2300–2309
- Leask A, Abraham DJ (2004) TGF-beta signaling and the fibrotic response. *FASEB J : official publication of the Federation of American Societies for Experimental Biology* 18:816–827
- Lian O, Dahl J, Ackermann PW, Frihagen F, Engebretsen L, Bahr R (2006) Pronociceptive and antinociceptive neuromediators in patellar tendinopathy. *Am J Sports Med* 34:1801–1808
- Lipson KE, Wong C, Teng Y, Spong S (2012) CTGF is a central mediator of tissue remodeling and fibrosis and its inhibition can reverse the process of fibrosis. *Fibrogenesis Tissue Repair* 5(Suppl 1):S24
- Liu S, Parapuram SK, Leask A (2013) Fibrosis caused by loss of PTEN expression in mouse fibroblasts is crucially dependent on CCN2. *Arthritis Rheum* 65:2940–2944
- Lui PP, Chan LS, Fu SC, Chan KM (2010) Expression of sensory neuropeptides in tendon is associated with failed healing and activity-related tendon pain in collagenase-induced tendon injury. *Am J Sports Med* 38:757–764
- Ma QP, Woolf CJ (1995) Noxious stimuli induce an N-methyl-D-aspartate receptor-dependent hypersensitivity of the flexion withdrawal reflex to touch: implications for the treatment of mechanical allodynia. *Pain* 61:383–390
- Mishra SK, Hoon MA (2010) Ablation of TrpV1 neurons reveals their selective role in thermal pain sensation. *Mol Cell Neurosci* 43:157–163
- Morales MG, Gutierrez J, Cabello-Verrugio C, Cabrera D, Lipson KE, Goldschmeding R, Brandan E (2013) Reducing CTGF/CCN2 slows down mdx muscle dystrophy and improves cell therapy. *Hum Mol Genet* 22:4938–4951
- Munoz M, Covenas R (2011) NK-1 receptor antagonists: a new paradigm in pharmacological therapy. *Curr Med Chem* 18:1820–1831
- Murota H, Izumi M, Abd El-Latif MI, Nishioka M, Terao M, Tani M, Matsui S, Sano S, Katayama I (2012) Artemin causes hypersensitivity to warm sensation, mimicking warmth-provoked pruritus in atopic dermatitis. *J Allergy Clin Immunol* 130(671–682):e674
- Nakama LH, King KB, Abrahamsson S, Rempel DM (2006) VEGF, VEGFR-1, and CTGF cell densities in tendon are increased with cyclical loading: An in vivo tendinopathy model. *J Orthop Res* 24:393–400
- Neesse A, Frese KK, Bapiro TE, Nakagawa T, Sternlicht MD, Seeley TW, Pilarsky C, Jodrell DI, Spong SM, Tuveson DA (2013) CTGF antagonism with mAb FG-3019 enhances chemotherapy response without increasing drug delivery in murine ductal pancreas cancer. *Proc Natl Acad Sci U S A* 110:12325–12330
- Noel J, Zimmermann K, Busserolles J, Deval E, Alloui A, Diochot S, Guy N, Borsotto M, Reeh P, Eschaler A, Lazdunski M (2009) The mechano-activated K⁺ channels TRAAK and TREK-1 control both warm and cold perception. *EMBO J* 28:1308–1318
- O'Brien JP, Mackinnon SE, MacLean AR, Hudson AR, Dellon AL, Hunter DA (1987) A model of chronic nerve compression in the rat. *Ann Plast Surg* 19:430–435
- Osha 2014 (2014) Prevention of work-related musculoskeletal disorders. Vol. 2014. U.S.D.o.L. Occupational Safety & Health Administration, editor
- Ozturk N, Erin N, Tuzuner S (2010) Changes in tissue substance P levels in patients with carpal tunnel syndrome. *Neurosurgery* 67:1655–1660, discussion 1660–1661
- Pierce CW, Tucci MA, Lindley S, Freeland A, Benghuzzi HA (2009) Connective tissue growth factor (ctgf) expression in the tenosynovium of patients with carpal tunnel syndrome - biomed 2009. *Biomed Sci Instrum* 45:30–35
- Rechardt M, Shiri R, Matikainen S, Viikari-Juntura E, Karppinen J, Alenius H (2011) Soluble IL-1RII and IL-18 are associated with incipient upper extremity soft tissue disorders. *Cytokine* 54:149–153
- Rempel DM, Diao E (2004) Entrapment neuropathies: pathophysiology and pathogenesis. *J Electromyogr Kinesiol : Off J Int Soc Electrophysiological Kinesiol* 14:71–75

- Ren K, Dubner R (1999) Inflammatory Models of Pain and Hyperalgesia. *ILAR J / National Research Council, Institute of Laboratory Animal Resources* 40:111–118
- Riondino S, La Farina F, Martini F, Guadagni F, Ferroni P (2011) Functional impairment in video terminal operators is related to low-grade inflammation. *Int Arch Occup Environ Health* 84:745–751
- Rogoz K, Andersen HH, Kullander K, Lagerstrom MC (2014) Glutamate, substance P, and calcitonin gene-related peptide cooperate in inflammation-induced heat hyperalgesia. *Mol Pharmacol* 85:322–334
- Scott JR, Muangman PR, Tamura RN, Zhu KQ, Liang Z, Anthony J, Engrav LH, Gibran NS (2005) Substance P levels and neutral endopeptidase activity in acute burn wounds and hypertrophic scar. *Plast Reconstr Surg* 115:1095–1102
- Seher A, Nickel J, Mueller TD, Kneitz S, Gebhardt S, ter Vehn TM, Schlunck G, Sebal W (2011) Gene expression profiling of connective tissue growth factor (CTGF) stimulated primary human tenon fibroblasts reveals an inflammatory and wound healing response in vitro. *Mol Vis* 17:53–62
- Smith CA, Stauber F, Waters C, Alway SE, Stauber WT (2007) Transforming growth factor-beta following skeletal muscle strain injury in rats. *J Appl Physiol* 102:755–761
- Song JJ, Aswad R, Kanaan RA, Rico MC, Owen TA, Barbe MF, Safadi FF, Popoff SN (2007) Connective tissue growth factor (CTGF) acts as a downstream mediator of TGF-beta1 to induce mesenchymal cell condensation. *J Cell Physiol* 210:398–410
- Song Y, Stal PS, Yu JG, Forsgren S (2013) Bilateral increase in expression and concentration of tachykinin in a unilateral rabbit muscle overuse model that leads to myositis. *BMC Musculoskelet Disord* 14:134
- Sonnlyal S, Shi-Wen X, Leoni P, Naff K, Van Pelt CS, Nakamura H, Leask A, Abraham D, Bou-Gharios G, de Crombrughe B (2010) Selective expression of connective tissue growth factor in fibroblasts in vivo promotes systemic tissue fibrosis. *Arthritis Rheum* 62:1523–1532
- Sonnlyal S, Xu S, Jones H, Tam A, Sreeram VR, Ponticos M, Norman J, Agrawal P, Abraham D, de Crombrughe B (2013) Connective tissue growth factor causes EMT-like cell fate changes in vivo and in vitro. *J Cell Sci* 126:2164–2175
- Stratton R, Shiwen X, Martini G, Holmes A, Leask A, Haberberger T, Martin GR, Black CM, Abraham D (2001) Iloprost suppresses connective tissue growth factor production in fibroblasts and in the skin of scleroderma patients. *J Clin Invest* 108:241–250
- Tatsushima Y, Egashira N, Kawashiri T, Mihara Y, Yano T, Mishima K, Oishi R (2011) Involvement of substance P in peripheral neuropathy induced by paclitaxel but not oxaliplatin. *J Pharmacol Exp Ther* 337:226–235
- Teodoro FC, Tronco Junior MF, Zampronio AR, Martini AC, Rae GA, Chichorro JG (2013) Peripheral substance P and neurokinin-1 receptors have a role in inflammatory and neuropathic orofacial pain models. *Neuropeptides* 47:199–206
- Tzortzaki EG, Antoniou KM, Zervou MI, Lambiri I, Koutsopoulos A, Tzanakis N, Plataki M, Maltezas G, Bouros D, Siafakas NM (2007) Effects of antifibrotic agents on TGF-beta1, CTGF and IFN-gamma expression in patients with idiopathic pulmonary fibrosis. *Respir Med* 101:1821–1829
- Uematsu T, Sakai A, Ito H, Suzuki H (2011) Intra-articular administration of tachykinin NK(1) receptor antagonists reduces hyperalgesia and cartilage destruction in the inflammatory joint in rats with adjuvant-induced arthritis. *Eur J Pharmacol* 668:163–168
- van Nieuwenhoven FA, Jensen LJ, Flyvbjerg A, Goldschmeding R (2005) Imbalance of growth factor signalling in diabetic kidney disease: is connective tissue growth factor (CTGF, CCN2) the perfect intervention point? *Nephrol Dial Transplant: official publication of the European Dialysis and Transplant Association - European Renal Association* 20:6–10
- Witonski D, Wagrowska-Danilewicz M, Raczynska-Witonska G (2005) Distribution of substance P nerve fibers in osteoarthritis knee joint. *Pol J Pathol : Off J Pol Soc Pathol* 56:203–206
- Ziesche R, Hofbauer E, Wittmann K, Petkov V, Block LH (1999) A preliminary study of long-term treatment with interferon gamma-1b and low-dose prednisolone in patients with idiopathic pulmonary fibrosis. *N Engl J Med* 341:1264–1269