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Manual therapy prevents onset of nociceptor activity, sensorimotor dysfunction, and neural fibrosis induced by a volitional repetitive task.

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Abstract

Painful and disabling musculoskeletal disorders remain prevalent. In rats trained to perform repetitive tasks leading to signs and dysfunction similar to those in humans, we tested whether manual therapy would prevent the development of the pathologies and symptoms. We collected behavioral, electrophysiological, and histological data from control rats, rats that trained for 5 weeks before performing a high repetition high force task (HRHF) for 3 weeks untreated, and trained rats that performed the task for 3 weeks while being treated 3x/week using modeled manual therapy (MMT) to the forearm (HRHF+MMT). The MMT included bilateral mobilization, skin rolling, and long axis stretching of the entire upper limb. HRHF rats showed decreased performance of the operant HRHF task and increased discomfort-related behaviors, starting after training. HRHF+MMT rats showed improved task performance and decreased discomfort related behaviors compared to untreated HRHF rats. Subsets of rats were assayed for presence or absence of ongoing activity in C- and slow A δ - neurons in their median nerves. Neurons from HRHF rats had a heightened proportion of ongoing activity and altered conduction velocities compared to control and MMT-treated rats. Median nerve branches in HRHF rats contained increased numbers of CD68+ macrophages and degraded myelin basic protein, and showed increased extraneural collagen deposition, compared to the other groups. We conclude that the performance of the task for 3 weeks leads to increased ongoing activity in nociceptors, in parallel with behavioral and

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histological signs of neuritis and nerve injury, and that these pathophysiologies are reduced by MMT

Introduction

Disorders attributed to chronic repetitive motion and overload comprise a large proportion of musculoskeletal and nerve disorders. These conditions are called repetitive motion disorders (RMDs) and include muscle strain injuries, carpal and cubital tunnel syndromes, myalgias, tendinoses, and epicondylopathies [23,28,36,55,56]. They are often associated with daily activities (both occupational and not), sports, or military activities [21,33,38,44], and are a leading cause of pain and physical disability [13,35,38,44]. Some cases become so severe that simple tasks, like buttoning a shirt, become difficult or impossible because of pain, discoordination, or sensory loss. Although acute trauma may be a causal factor in some RMDs, many result from cumulative small amplitude forces. There remains a call for effective, or ideally preventive, treatments for these often debilitating disorders [45,54,57].

Our group has published extensively on a rat model of RMD, where rats are trained to voluntarily perform a reaching and pulling task at levels that emulate conditions encountered in many work places and that leads to symptoms and pathologies similar to those expressed by humans [5,16,24,37,39]. Clinical studies examining biopsies and serum samples from patients with acute versus chronic RMDs, including several from our laboratories, support an early inflammatory response that is followed by development of degeneration and/or fibrosis with continued task performance [14,15,22,29,34]. Post-inflammatory fibrotic tissue pathology in the upper limb is hypothesized to be a key factor in the motor dysfunction, discomfort, pain, and paresthesia observed in subjects with chronic RMDs [4,15,27,36,53]. Attenuating the inflammatory responses in early stages of these disorders should prevent the development of fibrosis, and the symptoms associated with it.

Efforts to treat or prevent these changes have included ibuprofen, an anti-TNF α drug and task reduction [1,6,37,39,48,58], but none have fully prevented the progression of the disorders associated with task performance. We recently showed that modeled manual therapy (MMT) of the upper limb, initiated when rats start to show clinical signs, prevents symptoms and the fibrosis seen in the model [12]. Others have reported that mobilization of the skin prevents subcutaneous fibrosis in a mouse model [3,8]. Here, we tested the hypothesis that manual therapy, a commonly available and inexpensive treatment option, would impact the early inflammatory and fibrogenic phases of this model.

Patients with RMDs typically undergo clinical electrophysiology testing for nerve conduction velocities, which exclude nociceptors due to technical limitations. Assays of pain and nociception in animals typically rely on reflex withdrawal from an externally applied stimulus applied to the skin [9], and more recently, place preference testing, which requires a complex cognitive process to lead to altered behavior [25,41]. A more direct indication of pain is the presence or absence of nociceptor activity, not previously performed in this or other similar animal models of repetitive reaching in which electrophysiological testing of the median nerve was performed [16,17,19,20,26,37,52]. In this study, we tested the

prediction that the rats performing the task would exhibit increased activity in nociceptors innervating the forearm and hand.

Methods:

Subjects

The Temple University and the University of New England Institutional Animal Care and Use Committees approved these experiments in compliance with NIH guidelines for the care and use of laboratory animals. At Temple University, all rats were housed in AAALAC-accredited animal facility in separate cages with a 12-hour light:dark cycle, free access to water, and environmental enrichment in their home cages (chew toys and tunnels). All rats were gently handled extensively for 1 week prior to onset of experiments, and then 5 days/week thereafter. Young adult female Sprague-Dawley rats (3 months of age at onset of experiments) from Charles Rivers (Wilmington, MA) were used. Each rat was inspected weekly and again post-mortem for presence of illness to reduce potential confounders (none were observed). To further reduce illness-related confounders, additional sentinel rats were examined for presence of illnesses as part of regular veterinary care (none were detected).

A total of 34 rats were used. We collected data from 4 groups of rats: 1) four naïve rats (Controls); 2) ten rats that trained for 5 weeks before performing a High Repetition High Force task for 3 weeks with no treatment (HRHF); 3) ten trained rats that performed the HRHF task for 3 weeks while being treated 3x/week using modeled manual therapy to the forearm (HRHF+MMT; described further below), and 4) ten age-matched food-restricted controls (FRC). The naïve CON rats were given free access to food. In order to motivate interest in food reward pellets, the remaining rats were food-restricted to body weights of 5% less than age-matched normal controls (used only for weight comparison). In addition, all rats received Purina rat chow (Woodstock, Ontario, Canada) in their cages daily. The FRC and HRHF rats received the same food reward and chow rations daily. Each also received environmental toys daily until the end of the experiment. Five HRHF and five HRHF+MMRT rats were transferred to University of New England. Once transferred, the rats had free access to food and water, and were group housed.

Behavioral Apparatuses, Training, and Task Regimen

Sixteen custom-designed behavioral apparatuses were used in which 20 rats (the HRHF and HRHF+MMT rats) performed an operant reaching and lever pulling task, as previously described [5]. Briefly, animals reached through a shoulder height portal to pull on a force lever bar attached to a force transducer (Futek Advanced Sensor Technology, Irvine, CA) located outside the chamber wall and attached to a load cell. This bar was attached to a tension-compression load cell (LSB200, Futek) interfaced with a strain-gauge amplifier (CSG110, Futek). The load cell signal was sampled digitally at 100 Hz using custom-written Force Lever software (ENV-118M, Med Associates, St. Albans, VT).

Rats were randomized to be in either the FRC or HRHF group by one of the investigators (MA) who did not participate in the training or testing. All 20 rats chosen for the HRHF task group underwent a five-week training, before a subsequent three-week performance of a

standardized HRHF task, simulating occupational repetitive work. The five-week training steps were as previously described [5,7] and occurred for 15 min/day, 5 days/week. After this training period, a point equal to week 0 of the HRHF task, the 20 trained rats began the HRHF task regimen for 2 hrs/day, 3 days/wk for 3 weeks. The task was divided into 4, 0.5-hr sessions separated by 1.5 hrs in order to avoid satiation. HRHF rats were cued to reach at a rate of 4 reaches/min (the target rate) and to extend their forearm forward into a portal, grasp a force lever bar, and then exert a fairly isometric pull for at least 90 milliseconds (ms) at a target grasp force effort of 1.52N (55% of their maximum pulling force). The rats were allowed to pull within a range of 1.36 to 1.69N (50% to 60% of their maximum pulling force). If reach and force criteria were met within a 5 second cueing period, a food reward pellet (a mix of 45 mg purified chocolate flavored grain and banana flavored pellets; Bio-Serv, NJ, USA) was dispensed into a trough. Rats were allowed to self-regulate their participation, making this a voluntary task. The rats had a preferred reach limb that they used to pull on the lever bar, which was noted during the training sessions and during week 1. The 10 food-restricted control rats (FRC) remained in their cages with daily handling and environmental toys until the end of the experiment and received food reward pellets in troughs in their home cage.

Task performance data

Force lever data were recorded continuously by the computer and software linked to the force transducers. These data were used later for calculation of dependent variables: reach rate (i.e., all reaches/minute), grasp force, grasp duration (msec), reach impulse (Newtons x grasp duration), and number of successful reaches (i.e., reaches rewarded) using a custom-written executable automated script (MatLab, Mathworks, Natick, MA). These calculations were as previously defined [39,58]. Data for each task performance variable were calculated on the last day of week 1 and 3 from all task animals (HRHF and HRHF+MMT rats, n=10 per group). Task performance data could not be generated for the FRC rats because they did not perform the task.

Incidence of spontaneous behaviors suggestive of discomfort were tracked each Friday during the task performance sessions in the HRHF and HRHF+MMT rats, and included switching forelimb used to pull the lever bar during the work session, pulling on the lever bar with 1 or 2 fingers rather than a full grasp with all fingers, and guarding behaviors as previously described [12].

Modeled Manual Therapy (MMT)

The modeled manual therapy was performed by MYH, an experienced animal behaviorist who was trained by GMB and SLC in this technique and who performed the treatments for our previous project [12]. The treatment in these experiments included:

1. Using the thumb, index finger, and middle finger, the therapist gently compressed the flexor muscles as a whole, mobilizing them laterally over the radius and ulna (10 cycles).
2. The skin over the forearm was pinched together and rolled between the thumb and index finger (5 rolls), including all loose skin from the wrist to the elbow.

3. While the rat was stabilized at the shoulder, the treating thumb and fingers grasped the proximal upper limb and gently tractioned (a stroking traction) while sliding across the fur, rolling back and forth when reaching the forearm and continuing this to the rat's fingers until they slid out of the treating hand.

A video of the treatment is provided as supplemental data.

Treatment was performed for approximately 5 minutes per treatment period, on both arms. Untreated rats were lightly held on the same days and by the same person who performed the treatment for the same amount of time. Spontaneous behaviors suggestive of pain or discomfort during and following the 3x/week MMT treatment were also tracked, as described previously [12].

Other Sensorimotor Behavioral Tests

Ten animals per group (FRC, HRHF and HRHF+MMT rats) were used for the below-described sensorimotor behavioral assays. The individuals carrying out these behavioral tests were naïve to the group assignment of the rats being tested. Behavioral procedures were conducted at the same times per day to minimize effects related to diurnal factors.

Reflexive grip strength of both forelimbs was measured individually using a rodent grip strength meter (1027SR-D58, Grip Strength Meter with single sensor and a standard pull bar; Columbus Instruments, Columbus, Ohio) with sensor ranges from 0 to 5 Kg to best assay rat grip strengths. The test was repeated five times/limb/trial, and the maximum grip strength per trial reported in centinewtons (cN). This test was performed after food restriction, after the 5 week training period (HRHF week 0), and at the end of HRHF week 3. Forepaw sensitivity to mechanical probing was assayed at the end of the HRHF task week 3, bilaterally, using nylon monofilaments (Semmes-Weinstein monofilaments, Stoelting) and previously described methods [58]. This test was repeated 10 times/limb/trial, and the mean number of limb withdrawal responses out of 10 is reported for each monofilament used.

Electrophysiology

Five HRHF and five HRHF+MMT rats were randomly selected by one of the investigators (MA) to be sent to the University of New England for electrophysiological studies. They were used within one week of arrival to minimize possible recovery due to time. The four naïve control rats were studied using the same methods.

Rats were anesthetized using isoflurane in pure oxygen and maintained in an areflexic state for the duration of the experiment. The rat was placed supine on feedback-controlled heating pad (FHC-Inc, USA), and a water circulating heating pad was wrapped around the torso (Gaymar, USA), to maintain a core temperature of 37°C.

The fur on the axilla and posterior forearm was clipped closely. The abducted arm was then glued to a small metal platform for stability while also allowing full movement of the wrist. The skin was incised over the medial upper arm, and the median nerve was carefully dissected free of all other structures from the cubital fossa to its intersection with the ulnar nerve near the axilla (Fig. 1). The nerve was then covered with gauze soaked in buffered

saline to protect from drying, and the skin was glued using cyanoacrylate to a metal ring, forming a pool for recording. The gauze was removed and the pool filled with warmed light mineral oil. The median nerve was cut as proximally as possible and draped over a bipolar stimulating electrode positioned in the cubital fossa. The proximal part of the nerve was placed on a small glass plate used for recording. The epi-perineurium was removed from the proximal 1 mm of the nerve. Fine filaments (8–12 μ m) were teased from the nerve and draped over bipolar electrodes made from fine gold wire. The nerve was measured using dividers and was typically 11–13 mm between the stimulating and recording electrodes. This length is sufficient for the identification of C- and slow A δ - axons using electric stimulation. The nerve was electrically stimulated near the cubital tunnel using an isolated constant voltage stimulator (Grass), at intensities appropriate to elicit action potentials from these types of axons (up to 0.2ms and 40V). Because the axons were disconnected from their cell bodies for recordings, they are referred to as “units,” by convention. Only clearly identified units were recorded, usually one per filament. Action potentials were amplified, band-pass filtered (10–5,000 Hz), and monitored with an oscilloscope. Neuronal activity was digitized, monitored, and recorded with Spike 2 software (Cambridge Electronic Designs, Cambridge, United Kingdom). Units were classified as having either C- or A δ - fiber axons by their conduction velocity, determined by dividing the conduction distance by the response latency of individual units (Fig. 1). It was not possible to calculate the conduction velocities of axons with conduction velocities faster than 15 m/s, but these could usually be evaluated using their responses to innocuous mechanical stimulation and by the very different characteristics of their action potentials.

Ongoing activity, defined as activity present without applied stimulation, was the primary assay. To quantify ongoing activity, spontaneous activity was recorded for 3 minutes following electrical identification. To obtain the ongoing activity rate, data were analyzed offline using Spike 2. Mechanical responsiveness was evaluated using only innocuous stimulation, to avoid any possibility of sensitization of the receptive fields of the neurons under study as well as subsequent neurons [11]. This included gentle flexion and extension of the wrist, and light touching of the hand and forearm with fingers and blunt probes. The presence or absence of a mechanical response was recorded. To not over-represent one experiment, 12 or fewer slowly conducting neurons were recorded per arm.

Because rats show symptoms on both arms regardless of their preferred reaching limb, neurons innervating both arms were recorded in each experiment. Without receptive fields, there was no means in this experimental design to differentiate between afferent and sympathetic post-ganglionic axons, which constitute up to 1/3 the total population of unmyelinated axons in rat peripheral nerves [46,50], none of which would be expected to have ongoing activity in this experiment [10].

Histological and Immunohistochemical Analyses

The remaining FRC, HRHF and HRHF+MMT rats (n=5/group) were used for histological and immunohistochemical analyses. Rats were anesthetized and were then perfused transcardially with phosphate buffered saline followed by 4% buffered paraformaldehyde in PO₄ buffer (Pease’s fixative formula, pH 7.4). Forelimbs were post-fixed by immersion for

48 hours before incubating first in 10% sucrose in PO₄ buffer (pH 7.0) for 48 hours, and then 30% sucrose in PO₄ for 48 hours. Forearm musculotendinous tissues were dissected from forelimb bones, placed into mounting medium, frozen on dry ice, and then stored at -80°C until use. These tissues were cryosectioned into 18 micrometer longitudinal sections and placed on charged slides (Fisherbrand™ Tissue Path Superfrost™ Plus Gold Slides, Thermo Fisher Scientific). Subsets of sections were either stained with hematoxylin and eosin (Richard-Allan Scientific™ Modified Harris Hematoxylin, Thermo Fisher Scientific), Masson's trichrome, or immunostained in batched sets by the same individual for CD68 (a marker of phagocytic tissue macrophages), or degraded myelin protein, using previously described methods [2,16]. The specific antibodies included anti-CD68 (ab125212, 1:500 dilution in phosphate buffered saline (PBS), Abcam, Cambridge, MA), or anti-degraded myelin basic protein (DMBP, ab5864, 1:500 dilution in PBS, Millipore; antibody specifically recognizes only areas of myelin degeneration in lesioned peripheral nerves). Briefly, after a 0.5% pepsin antigen retrieval step for 15 minutes at room temperature, sections were incubated for 20 minutes in 4% goat serum in PBS, and then were incubated with the primary antibody at the listed dilution (diluted in PBS) 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 or Cy3; Jackson ImmunoResearch, West Grove, PA) at a dilution of 1:100 each for 2 hours at room temperature. DAPI was used as a nuclear counterstain following the immunostaining.

Quantification of macrophage immunostaining within the median nerve was performed in batched sets by individuals blinded to group assignment, using an epifluorescence microscope (E800, Nikon, Melville, NY) interfaced with a digital camera (Retiga 4000R QImaging Firewire Camera, Surry, BC Canada) and an image analysis system (Bioquant Image Analysis Corporation, Nashville, TN). The number of CD68 immunopositive cells in a region of interest that included only the median nerve was counted. Only regions inside the epineurium were assayed. Median nerve sections in three non-adjacent slides were counted per nerve, per animal, using a 20X objective. Degraded myelin basic protein was assayed in the same region using a thresholded pixel count and previously described methods (percentage of region) [2]. Masson's Trichrome-stained sections were examined for collagen deposition (blue) in the interstitial fascial region immediately surrounding median nerve branches, in a region of interest that was 20 µm external to the epineurium of the median nerve using a thresholded pixel count and an irregular region of interest cursor tool set to 20 µm. Hematoxylin and eosin-stained sections were examined qualitatively for the presence of neutrophils and axonal beading using a bright field microscope (E600, Nikon).

Statistics

Reach performance outcomes were compared between HRHF and HRHF+MMT rats using repeated-measures two-way ANOVAs, using the factors "week" and "treatment group," followed by Sidak's multiple comparisons posthoc tests. Sensorimotor assays were similarly compared between FRC, HRHF and HRHF+MMT rat groups using the factors "week" and "group" for grip strength and forelimb agility assays, and the factors "filament" and "group" for responses to nylon monofilament stimulation of the forepaws. The proportions of HRHF

and HRHF+MMT rats showing altered forelimb and forepaw movements suggestive of discomfort during the task performance were compared using Chi-square tests. One-way ANOVAs were used to compare the number of CD68 cells/mm², degraded myelin basic protein, and collagen staining around the median nerve between FRC, HRHF and HRHF+MMT rat groups, followed by Tukey's multiple comparisons posthoc tests. Electrophysiological data were analyzed and presented qualitatively and quantitatively. The proportions of slowly conducting units with ongoing activity were compared using Chi-square tests, with and without compensation for sympathetic recordings. Conduction velocity distributions were analyzed using one-way ANOVA. Adjusted p values of < 0.05 were considered significant for all comparisons and are reported, as are group means plus standard error of the mean (SEM) or standard error (SE). Results of ANOVAs are listed in Table 1, and results of posthoc tests are indicated in individual panels for succinctness.

Results

Task performance changes

Since we have observed declines in task performance in association with nerve injury and inflammation following performance of this HRHF task for longer time periods (6–12 weeks) [37,58], we examined 3-week HRHF and 3-week HRHF+MMT rats here to determine if these changes in task performance are also present at this earlier time point (Fig. 2). Mean reach rate (reaches/min) showed a significant interaction between week of task performance and treatment group (ANOVA $p=0.002$, Table 1), with the highest reach rate observed in 3-week HRHF+MMT rats, compared to 1-week HRHF+MMT and 3-week untreated HRHF rats (Fig. 2A, Table 1). Mean reach impulse showed a significant interaction between week and treatment (ANOVA $p=0.04$) with the highest reach impulse observed in 3-week HRHF+MMT rats, compared to 1-week HRHF+MMT and 3-week HRHF rats (Fig. 2B, Table 1). The number of successful reaches was low in all groups yet increased across weeks (ANOVA $p=0.009$, Table 1) in the 3-week HRHF+MMT rats, compared to 1-week HRHF+MMT and 3-week HRHF rats (Fig. 2C, Table 1).

Sensorimotor changes

Prior studies found declines in grip strength and forepaw mechanical hypersensitivity immediately following the 5 week training period that were only partially rescued by systemic anti-inflammatory treatments [1,39,48,58]. Here, reflexive grip strength in the preferred reach limbs showed a significant interaction between week of task performance and group (ANOVA $p=0.03$; Table 1). Grip strength declined in preferred reach limbs immediately after the training period in HRHF rats (Week 0, the time point immediately prior to the onset of MMT), compared to their naïve levels and FRC rats (Fig. 3A, Table 1). These declines were still evident in 3-week HRHF rats yet were ameliorated in 3-week HRHF+MMT rats, compared to untreated 3-week HRHF rats (Fig. 3A; Table 1). No significant group differences in grip strength were observed in contralateral non-preferred reach limbs (Fig. 3B, Table 1). Forepaw mechanical sensitivity (assayed using nylon monofilaments) showed significant group differences in a two-way ANOVA ($p<0.0001$). Posthoc analysis showed that 3-week untreated HRHF rats had more responses to 1g and 4g

sized filaments than FRC rats (Fig. 3C, Table 1); responses in HRHF+MMT rats were between those observed in HRHF and FRC rats.

Spontaneous behaviors suggestive of discomfort were observed in task animals during the task performance. These behaviors included increased proportions of untreated HRHF rats switching the limb used to pull on the lever bar during a work session, pulling with 1 or 2 digits rather than a full grasp, and forelimb guarding behaviors, with continued task performance across the three weeks (Fig. 3D). Chi Square analyses showed that these behaviors were reduced in HRHF+MMT rats, compared to untreated HRHF rats (Fig. 3D).

No spontaneous changes in behavior were observed during the MMT treatment periods (such as forelimb withdrawal or increased escape behaviors) or when home cage behaviors were tracked for one hour after the MMT treatments, matching previously reported findings with this model [12].

Electrophysiology

Early symptoms of sensorimotor discomfort detected in forelimbs and forepaws of HRHF rats in past studies [1,25,58], and above, prompted us to examine the median nerve for activity in neurons with slowly conducting axons (putative nociceptors). Extracellular recordings were made from slowly conducting units from naïve control rats (71 units from 7 arms), HRHF rats (79 units from 7 arms), and HRHF+MMT rats (40 units from 6 arms).

Ongoing activity.—The proportion of units with ongoing activity was statistically higher in the HRHF group (22%) than in the other groups (0% naïve control, 8% HRHF+MMT; Chi-square, $p=0.001$; Fig. 4A). The discharge was always irregular (Fig. 4B–C). The discharge rates ranged from 0.3 – 27 Hz and were skewed to the slower rates (median=1.3 Hz; skewness=1.3, kurtosis=2.6; Shapiro-Wilk $W=0.76$, $p=0.002$). There were too few units with ongoing activity in the HRHF+MMT group (only $n=3$ in this group showed ongoing activity) for statistical analysis and comparison of discharge rate with the HRHF group. Because up to 1/3 of the C-fibers recorded were possibly sympathetic axons, we performed a secondary analysis by reducing the denominator of the proportions by 33% and obtained the same results. There were not sufficient data to confidently compare dominant to non-dominant limb ongoing activity rates.

Conduction velocities.—The mean conduction velocity of the HRHF group (2.7 m/s) was significantly higher from the other groups (control = 2.0, HRHF+MMT = 1.8; $F_{(2, 189)} = 6.095$, $p = 0.0027$; Fig. 4E). However, since we have previously documented that extra- and intraneural fibrogenic changes, such as seen in this model by 10–12 weeks of task performance [16,17,37], leads to reduced conduction velocities of slowly conducting fibers, it is more likely that this currently 3-week observation represents the recruitment of faster A δ units into the “window” of conduction velocities that are possible to document in this experimental set-up (see other results below and Discussion). In support of this was the appearance of two units that were characterized by response to mechanical stimulation as muscle spindles but presented with slow conduction velocities (Fig. 4D), suggesting that their axons were demyelinated. There were not sufficient data to confidentially compare dominant to non-dominant limb conduction velocities.

Faster-conducting mechanically responsive units.—A variety of other receptor types with axons of faster conduction velocity than could be characterized (>15 m/sec) were recorded. These included muscle spindles and low threshold mechanoreceptors (rapidly and slowly adapting). The low threshold mechanoreceptors responded to light brushing or other touching of the skin of the forearm or hand, or light movement of the joints or muscles. In control recordings, 4 of 37 units had ongoing activity, compared to 0 of 30 in HRHF and 3 of 40 HRHF+MMT (Chi-square not significant). Little else can be stated about these recordings, because we were not able to obtain the conduction velocities or evaluate the receptive fields in full in the experimental paradigm. Their presence was useful to confirm the continued health of the nerves while recording.

Histological signs of median nerve inflammation and injury

Since inflammation can induce ongoing activity in nociceptor axons [10,49], branches of the median nerve at the level of the wrist were examined for increased CD68 immunopositive macrophages (Fig. 5 and Fig. 7A). Significant increases were observed in the nerve within the boundaries of the epineurium only in untreated HRHF rats (Fig. 5B), compared to HRHF+MMT and FRC rats (Fig. 5A and C; Fig. 7A, Table 1). Evaluation of hematoxylin and eosin-stained nerves also revealed low increases in neutrophils in untreated HRHF rats (Fig. 6A middle panel), a cell type not observed in the median nerves from the other groups.

Because the electrophysiological investigations suggested demyelination, the median nerve was also examined for changes in immunoexpression of DMBP (Figs. 6B and 7B). Significant increases were observed in the median nerve within the boundaries of the epineurium only in the untreated HRHF rats (Fig. 6B middle panel), compared to FRC and HRHF+MMT rats (Fig. 6B left and right panels; Fig. 7B, Table 1).

Lastly, since, collagen and other connective tissue components increase around nerves and within their epineurium after different types of nerve injury [17,18,43], we also quantified collagen deposition around nerves. We observed increased collagen deposition around median nerve branches both at the level of the wrist (Fig. 6C) and in the forepaw immediately distal to the wrist in untreated HRHF rats and in a few HRHF+MMT rats (Fig. 6D). This was quantified using an irregular region of interest tool with a 20 μ m cursor in the fascia immediately surrounding median nerve branches (Fig. 7C); significant increases were observed in untreated HRHF rats, compared to FRC and HRHF+MMT rats (Fig. 7D, Table 1).

Discussion

Consistent with our previous report, we show that an intervention that emulates a type of manual therapy as practiced by numerous professions (e.g. massage therapy, physical therapy, chiropractic) prevents functional declines, improves task performance, prevents behavioral changes indicative of discomfort, and reduces neural inflammation, myelin degradation, and extraneural fibrosis. Compared to several of our previous experiments examining untreated HRHF rats, which evaluated tissues of rats after 6 or 12 weeks of performing the task, we chose to evaluate the rats at three weeks into the task performance to capture early behavioral and pathophysiological effects. Importantly, we added a new assay,

single unit recordings, which gives a direct indication of putative nociceptor discharge, considered to be directly associated with pain.

The electrophysiological recordings performed for this study provide direct evidence for increased primary afferent nociceptor discharge, consistent with ongoing pain, at this early time point in this model. We have previously reported decreased compound action potentials, which exclude unmyelinated axons, in the median nerve in this model after 12 weeks of performance of this HRHF task [16,37]. Here, we show that a proportion of unmyelinated axons in 3-week HRHF rats displayed irregular ongoing activity, as well as altered conduction velocities, consistent with the effect of inflammation on nociceptor axons within peripheral nerves [10,49]. Other sources of nociception in this model include inflammatory responses in surrounding musculotendinous structures. There is also increased fibrosis within and around these musculotendinous structures and median nerve branches. We hypothesize that this increased collagen around and between structures [16,24,25,37], which likely impedes normal sliding of the structures, may become a source of inflammation and nociception as tethered tissues begin to tear during the excursion required to perform the task. It has been suggested that reduced inflammation and neural fibrosis after massage therapy may be underlying reasons for improved functional status and reduced symptom severity in patients receiving massage therapy for carpal tunnel syndrome [42,47], although neural tissues were not collected and examined in these nonsurgical patient studies. Our observed increases in CD68 macrophages and degraded myelin in parallel with increased extraneural collagen in untreated HRHF rats support our hypothesis, especially in light of the reductions in nerve inflammatory, injury, and fibrosis in the HRHF+MMT rats. As the pathological changes in the upper limb worsen with continued HRHF task performance, it is predicted that ongoing nerve activity will be present and perhaps increased when assayed at later time points in this model. It is unfortunate that we were not able to characterize the receptive fields of the recorded neurons. However, the search strategy required to identify nociceptors is by definition noxious and has to be repeated for each recording. This would cause acute cumulative trauma, leading to increased ongoing activity, as we have previously shown [11], and thus would be a confound to the experiment.

We have previously reported improvement of task performance declines, and the prevention of detrimental histological changes and sensory testing parameters in rats receiving more comprehensive and daily MMT treatment lasting 12 weeks [12]. In this report, we show that a more limited treatment provided for 5 minutes/day, 3 days/week for 3 weeks had a similar effect, improving task performance and reducing several of the sensorimotor indices of discomfort observed in untreated 3-week HRHF rats, decreasing pathological histological findings, and, importantly, preventing the development of ongoing activity in putative nociceptors. Studies using instrumented massage of skeletal muscle in rabbits (using an instrument that provides compressive loading to muscles to mimic deep massage) for 15 or 30 minutes per day for four days after a single bout of nerve stimulation-induced maximum eccentric exercise show acute and cumulative (over four days) increases in muscle viscosity and tibiotarsal joint torque output, as well as reduced muscle inflammatory responses, compared to that in untreated exercised animals [30–32]. A 30-minute sports massage protocol administered two hours after eccentric exercise in humans further suggests that sports massage can reduce muscle soreness and neutrophilia [51]. Our results here are

similar in that we see improved muscle function (grip strength in our case) in HRHF+MMT rats, compared to untreated HRHF rats. We also show reduced nerve inflammation and injury responses in animals who received manual therapy treatment concomitant with continued task performance.

We have not previously examined histological changes in peripheral nerves at 3 weeks into performance of this HRHF task. As the task creates progressive declines in reaching and lever pulling performance and increased tissue pathologies, it is not surprising that the effects at this early time point are less severe than we have previously published at 6 or 12 weeks of task performance [5,19,37]. However, the number of CD68 immunopositive macrophages in the median nerve were less here than the number of ED1 immunopositive macrophages previously identified in rats trained to perform the high force lever pulling task (i.e., immediately following training to the high force reach level) [5,19,37]. The epitopes targeted by the CD68 antibody used here differ from the ED1 antibody used in those past studies, making it hard to make direct comparisons. Yet, similar to those past studies, the number of activated macrophages in the median nerve of HRHF rats was significantly higher than in control rats, indicating that the task performance is an inducer of this neuroinflammation. Likewise, several sensorimotor behavioral declines were evident in the 3-week HRHF rats, compared to controls, although less severe than previously observed at later time points [1,5,19,37].

With disorders that are due to the performance of a repetitive task, structures can become overloaded and injured. If the task is repeated without enough time for healing, the inflammation becomes persistent as it is being reinforced during each task session. The normal response of the body is to heal the injury, which starts with inflammation. Inflammation is typically painful, reflected in our experiments in untreated HRHF rats by irregular and aberrant low rate nociceptor discharge. The inflammatory and healing responses lead to all the other observations and dysfunctions in this model. Following the idiom “an ounce of prevention is worth a pound of cure,” we initiated an intervention before the rats developed severe problems, and maintained the treatment until the end of the experiment. The data support that MMT prevents and rescues most if not all metrics of deleterious changes in sensory function, task performance, and tissue integrity. Further, our observations suggest that MMT prevents the inflammation and thus all the subsequent changes in function and tissue pathology, possibly by reducing collagenous tethers between tissues and maintaining mobility between the forearm structures.

The methods we used were translated from the clinic to the laboratory, and therefore we already know that translating this work to our population is likely to have substantial preventive effects in people whose work requires repetitive tasks. However, although adopted by and a benefit of some companies, manual therapy is not included or suggested in most if not any documents as a preventive for these overwhelmingly prevalent disorders. Persons experiencing repetitive strain injury often wait for changes to occur that cause pain during their task and/or at rest. By this time though, it seems likely that changes have occurred that might be difficult to reverse. We suggest that if a patient could be referred for manual therapy early before pathological changes occur, that medical expenses for treatments that are often ineffective might be avoided. Combined with our studies, clinical

trials using manual therapy as a preventive measure for repetitive motion disorders are supported.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations:

cN	centiNewton
FRC	food-restricted control
DMBP	degraded myelin basic protein
HRHF	high repetition high force
ms	milliseconds
MMT	modeled manual therapy
RMDs	repetitive motion disorders

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Figure 1. Electrophysiological methods.

The median nerve was dissected from the cubital tunnel to the axilla. Classical teased fiber techniques were used to isolate slowly conducting axons innervating the front paw and forearm muscles. Electric stimulation was delivered via a stimulating electrode (a), activating fine filaments from the nerve resting on a recording platform (2), with the differential potentials picked up by the recording electrode (3). Inset depicts the recording of a slowly conducting axon, identified by the latency between the stimulation and the beginning of the action potential (9.8 ms), over 13 mm of nerve, giving a conduction velocity of 1.33 m/s.

Performance of Operant Reaching Task

Figure 2. Operant task performance.

Performance of the operant reach task for 3 weeks by HRHF and HRHF+MMT rats (n=10 each). A: The mean number of reaches per minute in HRHF and HRHF+MMT rats. B: Mean reach impulse (Newtons of force times the grasp duration in milliseconds). C: Mean successful reaches, defined as reaches for which a food reward was received. Post-hoc analyses at each week * and **: $p < 0.05$ and $p < 0.01$, compared to 3-week HRHF rats, and ##: $p < 0.01$, compared to 1-week HRHF+MMT rats, as shown in figure panels.

Figure 3. Sensorimotor assays.

A and B: Maximum reflexive grip strength (cN), tested using a rat grip strength meter, in the preferred reach limb and non-preferred reach limb (panels A and B, respectively), at the naïve time point, after food restriction (–5 weeks), after training (week 0), and task week 3.

C: Number of responses out of ten during monofilament testing task week 3, in which a response is defined as a withdrawal of the forelimb to a monofilament sized as shown on the x axis. D: Percent of rats showing spontaneous alterations in forelimb and forepaw use during task performance by HRHF and HRHF+MMT rats in task weeks 1 through 3.

Analyzed using Chi-Square tests. *:p<0.05 and **:p<0.01, compared to naïve levels, or † and ††:p<0.05 and **:p<0.01, compared to age-matched FRC rats.

Figure 4. Electrophysiology results.

A: Ongoing activity was not present in naive control rats' recordings, present in 8% of recordings from treated rats, but was present in 22% of recordings from HRHF rats. *:p = 0.001. B and C: Examples of ongoing discharge of two neurons in HRHF rats, 0.35 Hz and 3.35 Hz, respectively (raw data; same time scale). D: The recording from the same neuron as shown in panel C also exhibited mechanical sensitivity from wrist extension (bars above trace). The responses resembled a Group II muscle spindle but had a conduction velocity of 2.4 m/s (raw data). E: Conduction velocities were significantly different between groups. *:p < 0.05 compared to control and HRHF+MMT data, respectively.

Figure 5. CD68 immunostaining for macrophages in the median nerve.

A: CD68 (green fluorescence) and DAPI (blue fluorescence) staining in a representative FRC median nerve. B: CD68 and DAPI staining in a representative untreated HRHF median nerve. C: CD68 and DAPI staining in a representative HRHF+MMT median nerve. Insets show higher power examples of CD68 immunoreactive macrophages within the nerves. Location of the epineurium versus nerve, as shown. Scale bars = 50 μ m.

Figure 6. Hematoxylin and eosin (H&E), degraded myelin basic protein (DMBP), and Masson's trichrome staining of the median nerve.

A: H&E staining of the median nerve at the level of the wrist of each group as shown. Upper inset in middle panel shows an enlarged inflammatory cell. Lower inset of this same panel shows similar enlarged inflammatory cells as well as a neutrophil (with a polymorphonucleus). Inset in right panel of a representative HRHF+MMT rat also shows an enlarged inflammatory cell, which were present at low levels in nerves of this group. B: DMBP (red fluorescence) and DAPI staining (blue) of the median nerve at the level of the wrist of each group. C and D: Masson's trichrome staining of median nerve branches and

surrounding tissues in the forepaw and at the level of the wrist of each group, respectively. Collagen is stained blue. Scale bar = 50 μm in panel A and 100 μm in panels B-D.

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Figure 7. Quantification of CD68 immunopositive macrophages, DMBP, and collagen deposition.

A: Quantification of the mean number of CD68 immunopositive macrophages in median nerve. B: Quantification of the mean percentage of DMBP immunoreexpression in median nerve. C: Image of a HRHF+MMT rat's median nerve branches, taken immediately distal to the wrist), showing the quantification method used. The median nerve was encircled (green line) 20 μ m external to the epineurium (small white double arrow; i.e., in the fascia immediately surrounding median nerve branches) using an irregular region of interest tool with a 20 μ m cursor. D: Quantification of percent blue-stained fibrous collagen around longitudinally sectioned median nerves, at the level of the wrist and immediately distal to the wrist (data averaged per rat). * and **: $p < 0.05$ and $p < 0.01$, compared to HRHF rats; $\dagger$ and $\dagger\dagger$: $p < 0.05$ and $p < 0.01$, compared to FRC rats, as shown in the figure panels.

Table 1.

Mean, SEM and ANOVA Results for Behavioral and Histological Changes

	FRC ¹	HRHF ²	HRHF+MMT ³	ANOVA Results	F(DFn, DFd)	P value
Reaches/Minute (expected target reach rate of 4 reaches/min)						
HRHF Week 1	N/A ¹	1.62 ± 0.44	0.62 ± 0.20 ^{##}	Interaction	F (1, 18) = 12.78	P=0.002
HRHF Week 3	N/A ¹	0.5 ± 0.18	2.68 ± 0.74 ^{**}	Treatment Group	F (1, 18) = 0.99	P=0.33
				Week	F (1, 18) = 1.75	P=0.20
				Subjects matching	F (18, 18) = 1.09	P=0.43
Reach Impulse (Newtons of force x msec of pull on lever bar)						
HRHF Week 1	N/A ¹	227.90 ± 24.56	269.95 ± 5.29 ^{##}	Interaction	F (1, 18) = 3.71	P=0.04
HRHF Week 3	N/A ¹	226.56 ± 18.84	474.32 ± 5.70 [*]	Treatment Group	F (1, 18) = 2.92	P=0.10
				Week	F (1, 18) = 7.36	P=0.01
				Subjects matching	F (18, 18) = 1.24	P=0.33
Successful reaches (expected target of 120 per session)						
HRHF Week 1	N/A ¹	2.15 ± 0.76	4.1 ± 0.70 ^{##}	Interaction	F (1, 18) = 3.22	P=0.09
HRHF Week 3	N/A ¹	1.61 ± 1.08	10.1 ± 2.94 [*]	Treatment Group	F (1, 18) = 2.49	P=0.13
				Week	F (1, 18) = 8.49	P=0.009
				Subjects matching	F (18, 18) = 0.88	P=0.60
Reflexive Grip Strength: Preferred Reach Limb						
Naïve (~5 wks)	739 ± 82.42	738.6 ± 54.60	702.3 ± 50.09	Interaction	F (4, 54) = 2.86	P=0.03
Week 0 (post training)	769.7 ± 5.34	578.9 ± 36.66 ^{*,†}	605.8 ± 53.02 ^{*,†}	Week	F (2, 54) = 2.21	P=0.12
HRHF Week 3	817.8 ± 4.88	534.8 ± 24.26 ^{*,††}	681.3 ± 38.29 [†]	Group	F (2, 27) = 5.08	P=0.01
				Subjects matching	F (27, 54) = 1.98	P=0.02
Reflexive Grip Strength: Contralateral Non-Preferred Reach Limb						
Naïve (~5 wks)	739 ± 82.42	693.3 ± 17.22	647.8 ± 51.96	Interaction	F (4, 54) = 1.15	P=0.34
Week 0 (post training)	769.7 ± 45.34	700.9 ± 40.05	608.6 ± 47.17	Week	F (2, 54) = 0.85	P=0.43
HRHF Week 3	817.8 ± 54.88	655.1 ± 47.26	723.1 ± 51.46	Group	F (2, 27) = 2.60	P=0.09
				Subjects matching	F (27, 54) = 2.45	P=0.003

	FRC ¹	HRHF ²	HRHF+MMT ³	ANOVA Results	F(DFn, DFd)	P value
Von Frey Filament Testing: Forepaws, Week 3 (# Responses out of 10)						
0.4 gram filament	0.2 ± 0.133	0.7 ± 0.70	0.00 ± 0.00	Interaction	F (6, 81) = 1.21	P=0.31
1 gram filament	1.4 ± 0.54	3.7 ± 0.87 [†]	2.6 ± 0.81	Filament Size	F (6, 81) = 1.21	P=0.0001
4 gram filament	4.9 ± 1.10	7.3 ± 0.78 [†]	5.7 ± 1.29	Group	F (2, 27) = 5.033	P=0.03
				Subjects matching	F (27, 81) = 2.73	P=0.0003
CD68 cells/mm² in Median Nerve						
	0.73 ± 0.21	18.96 ± 3.51 ^{††}	3.69 ± 0.98 ^{**}	Treatment	F (2, 12) = 21.6	P=0.0001
% degraded myelin basic protein in Median Nerve						
	0.16 ± 0.07	12.19 ± 3.78 [†]	2.59 ± 1.94 [*]	Treatment	F (2, 12) = 6.70	P=0.01
% collagen staining in Median Nerve						
	4.34 ± 0.62	19.32 ± 4.62 [†]	5.72 ± 2.48 [*]	Treatment	F (2, 12) = 7.39	P=0.008

¹ N/A = not applicable; FRC (food restricted control rats) were unexposed rats and did not perform the operant task.

² HRHF = high repetition high force task rats.

³ HRHF+MMT = HRHF rats that received modeled manual therapy (MMT) during task weeks 1 through 3.

^{*} p<0.05

^{**} p<0.01, compared to age-matched untreated HRHF rats

^{##} p<0.01, compared to week 1 HRHF+MMT rats

[†] p<0.05

^{††} p<0.01, compared to age-matched FRC rats